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The Human Immunodeficiency Virus Type 1 (HIV-1) Nucleocapsid Protein Zinc Ejection Activity of Disulfide Benzamides and Benzisothiazolones: Correlation with Anti-HIV and Virucidal Activities

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It has been shown previously by our group and others that a series of four disulfide benzamides with cellular anti-human immunodeficiency virus (HIV) activity can eject zinc from HIV type 1 nucleocapsid protein (NCp7) in vitro while analogs without antiviral activity do not. We also found that the zinc ejection activity correlates with the loss of the ability of NCp7 to bind to HIV Ψ RNA in vitro. These observations indicate that the antiviral disulfide benzamides may act at a novel retroviral target of action, i.e., the nucleocapsid protein. The present studies examine the relationship among disulfide benzamide structure, in vitro NCp7 zinc ejection activity, and antiviral activity for a larger series of compounds. All of the antiviral disulfide benzamides were found to eject NCp7 zinc, while some disulfide benzamides with zinc ejection activity are not antiviral. Utilizing the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid), it was determined that the *o*-amido-phenyl disulfides being studied cyclize in aqueous solution to form benzisothiazolones. A series of benzisothiazolones, which are stable in solution in the absence of dithiothreitol, were found to eject NCp7 zinc at a rate similar to that of their disulfide benzamide analogs and to possess similar antiviral activity. It was also found that the relative rates of HIV inactivation by various disulfide benzamides and benzisothiazolones correlate with their relative kinetic rates of NCp7 zinc ejection, which is consistent with the nucleocapsid protein being the target of action of these compounds.

Recent clinical studies for the treatment of AIDS have demonstrated the effectiveness of combination therapy in improving clinical markers and delaying the emergence of resistance (4, 5, 10, 14, 18, 19). These studies point to the importance of identifying anti-human immunodeficiency virus (HIV) compounds with novel retroviral targets of action as a means of developing new drug combinations.

One potential novel HIV target is the nucleocapsid protein (NCp7). HIV NCp7, tightly associated with the viral genomic RNA dimer and $tRNA^{Lys,3}$ primer in the core of mature virions (6), is necessary for proper viral RNA packaging and virion budding (2) and stimulates reverse transcription in vitro (16, 23, 25). NCp7 is a small, basic protein with two $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ (CCHC) sequences that each tetrahedrally coordinate a zinc ion (13, 27). Mutagenesis of any of the cysteines or histidines in the zinc fingers of HIV type 1 (HIV-1) NCp7 yields virions with defective RNA encapsidation and noninfectious particles (9, 12). Therefore, compounds which specifically disrupt zinc coordination to NCp7 may have an antiviral effect.

A series of four nontoxic disulfide benzamides from Parke-Davis Pharmaceutical Division of Warner-Lambert Company were shown to possess low micromolar anti-HIV activity in both acutely and chronically infected cells. These compounds were shown to inhibit infection by laboratory viral strains in lymphocyte and monocyte-macrophage cultures, by clinical isolates in fresh human peripheral blood lymphocytes, and by HIV-2 and simian immunodeficiency virus in lymphocyte cultures (24). The antiviral disulfide benzamides were found to eject zinc coordinated to NCp7 in vitro, while structural analogs without antiviral activity did not (24, 28). We also found that the zinc ejection activity of the disulfide benzamides correlated with the loss of NCp7 binding to HIV Ψ RNA in vitro. Loo et al. have demonstrated that NCp7 zinc ejection is accompanied by formation of covalent complexes between disulfide benzamide monomers and cysteine residues of the zinc-depleted viral protein (20). A study of the structure activity relationships for this series of compounds demonstrates that only a small subset of the disulfide benzamides possess substantial antiviral activity (7, 8). Sharmeen and coworkers have performed viral time of addition studies with these compounds as a means of determining when these compounds are active in the viral life cycle (26). The results indicate that the disulfide benzamides act after virus-cell fusion and before late-stage reverse transcription. While all of the functions of the nucleocapsid protein in the viral life cycle are not fully understood as yet, this data is consistent with the proposal that the antiretroviral disulfide benzamides act at this novel target of action.

The purpose of the studies described here was to determine the structural requirements of the disulfide benzamides for NCp7 zinc ejection in vitro and the relationship among compound structure, zinc ejection activity, and cellular anti-HIV activity. This work also led to the findings that the disulfide benzamides cyclize to form benzisothiazolones under our assay conditions and that the benzisothiazolones possess in vitro and cellular activity similar to their disulfide benzamide counterparts. The kinetics of in vitro NCp7 zinc ejection, as well as the time dependence of HIV inactivation, for these compounds was also studied and compared.

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MATERIALS AND METHODS

Materials. The disulfide benzamides were synthesized as described by Domagala and coworkers (7), and the benzisothiazolones were prepared by general methods (11, 22). TSQ [N-(6-methoxy-8-quinolyl)-*p*-toluene sulfonamide] was purchased from Molecular Probes, Eugene, Oreg. A.C.S. spectrophotometric-grade methyl sulfoxide, purchased from Aldrich, Milwaukee, Wis., was used to dissolve the disulfide benzamides and benzisothiazolones. The CEM-SS line of T cells and the HeLa CD4 LTR-*lacZ* cell line (MAGI) were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md.

Cloning, expression, and isolation of NCp7. A synthetic gene encoding a variant of the 55-amino-acid NCp7 protein from pNL4-3 (1), which contains a mutation of the first residue (His to Met), was designed and constructed by using published methods (15). The NCp7 protein was expressed in *Escherichia coli* and purified by ammonium sulfate precipitation followed by anion exchange chromatography and high-performance liquid chromatography, as previously described (28). The protein was found to be essentially pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and zinc chloride was added in a 2:1 molar ratio to NCp7. The concentration of NCp7 was determined by using an extinction coefficient of $\varepsilon_{280} = 6{,}050\ M^{-1}\ cm^{-1}$.

HIV NCp7 zinc ejection assay monitored by TSQ fluorescence. The zinc ejection assay buffer used was 10% glycerol–50 mM Tris-HCl buffer, pH 7.6, at 24°C. The ejection of zinc from the protein was monitored by the change in fluorescence of the zinc-selective fluorophore TSQ in the assay buffer. The zinc ejection assay was initiated by the addition of 10 μM (final concentration) disulfide benzamide or benzisothiazolone to 2.0 μM (final concentration) NCp7 (with a Zn/protein ratio of 2:1)–50 μM (final concentration) TSQ in assay buffer to a total volume of 200 μl in a 96-well plate at room temperature. Beginning immediately after reaction initiation, TSQ fluorescence was monitored at several time points over 90 min (excitation filter, 355 nm; emission filter, 460 nm) by using a Fluoroskan II 96-well plate fluorescence reader (Labsystems, Needham Heights, Mass.). A zinc chloride standard curve was generated from the same 96-well plate under the same conditions in the absence of NCp7 and disulfide benzamide. To control for time-dependent fluorescence changes in the assay not due to the ejection of zinc, the above experiment was run with each compound using apo-NCp7, and no significant fluorescence changes were observed over the 90-min time course for any of the compounds.

The zinc standard curve (28) was used to determine the amount of zinc ejected from the NCp7 after 90 min. Since it was known that 4.0 μM zinc was bound to 2.0 μM NCp7 in the assay, the percent zinc ejected could be calculated for each compound and the value used to rank the compounds. The rankings for NCp7 zinc ejection are as follows: I (inactive), <25% ejected; MA (moderately active), 25 to 75% ejected; A (active), >75% ejected; and AF (active fast), >75% ejected after 2 min of NCp7-compound incubation.

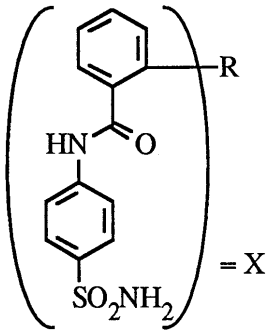
The kinetics of zinc ejection by disulfide benzamides and benzisothiazolones was measured on an LS 50 luminescence spectrometer (Perkin-Elmer, Beaconsfield, United Kingdom) with excitation = 355 nm and emission = 490 nm. Other experimental conditions were the same as described above, except that 16 μM compound was used in each assay.

Determination of extent of disulfide benzamide cyclization with DTNB. The extent of disulfide benzamide cyclization to form benzisothiazolone in the assay buffer was determined by measuring the amount of free thiol formed, detected with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (see Fig. 1). Disulfide benzamide compounds were dissolved in methyl sulfoxide to a stock concentration of 1.6 mM. The disulfide benzamide was then diluted into zinc ejection assay buffer, 16 μM final concentration, and incubated at room temperature for 1 to 5 min followed by addition of 40 μM (final concentration) DTNB (from a 4.0 mM stock in the same buffer). DTNB was allowed to react with free thiol for 30 s (found to be an adequate time for complete reaction), and A_{406} was then measured. The values measured were corrected for background absorbance due to DTNB in the absence of free thiol and due to disulfide benzamide.

Cellular antiviral assays. The antiviral activity of the compounds was determined in an HIV-1_{RF}-infected CEM-SS line of human T4-lymphoblastoid cells (21) by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] cytoprotection assay (3). The cytotoxicity of the compounds was determined in uninfected CEM-SS cells with the same XTT assay. The assay quantitates the compound-induced protection from the cytopathic effects of HIV-1. All measurements were performed in triplicate. The studies were performed at the Southern Research Institute, Birmingham, Ala.

Inactivation of HIV studies. The time-dependent inactivation of HIV by disulfide benzamides and benzisothiazolones was determined by measuring the reduction of infectious titer of purified virions over time. Purified HIV-1_{IIIb} virions were suspended in medium at a concentration of 7.5×10^4 infectious units/ml, and 25-μl aliquots were distributed into 1-ml polypropylene tubes. Test compounds were dissolved in methyl sulfoxide at a concentration of 50 mM and diluted by mixing 2 μl of disulfide benzamide or 4 μl of benzisothiazolone per ml of medium, and the assay was initiated by the addition of 25 μl of the drug solution to the virus sample. The final concentration of 50 μM disulfide benzamide or 100 μM benzisothiazolone maintained equivalent masses of compound. At several time points, the drug exposure was terminated, and the virus titer was determined by diluting the samples with 500 μl of medium and transferring 20 μl into the HeLa CD4 LTR-*lacZ* (MAGI) cell cultures. The cultures had been prepared the previous day by inoculating 180 μl of medium containing 5×10^3 MAGI cells per well in a 96-well plate. This dilution effectively reduced the drug concentration 100-fold and was below the effective antiviral concentrations. Virus titer determination by the MAGI cell assay was performed in triplicate. After 48 h, the cells were fixed and stained for beta-galactosidase as previously described (17). The number of blue-stained syncytia was determined under a light microscope to determine the surviving viral titer in the samples. The normal reduction of titer due to the instability of the virus over time was accounted for by using the comparable methyl sulfoxide time point as the standard. The percent inactivation of HIV was calculated as a percentage of syncytia formed compared to the methyl sulfoxide control at the same time point.

TABLE 1. NCp7 zinc ejection and anti-HIV activities of compound 1 analogs



Compound	R	Rank (Zn ejection)[a]	CIC_{50} (μM)	TC_{50} (μM)
1	S-S-X	MA	3.4	>100
			5.4	>163
2	SH	I	6.2	>100
3	SO_2H	I	>100	>100
4	OH	I	>100	>100
5	SCH_2-X	I	>100	>100
6	S-$SCH(CH_3)CO_2H$	A	4.9	>100 (2)[b]

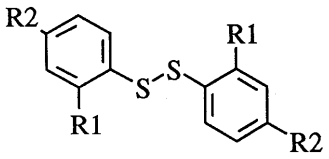
[a] The rankings for NCp7 zinc ejection from a 90-min assay are as follows: I (inactive), <25% ejected; MA (moderately active), 25 to 75% ejected; A (active), >75% ejected; AF (active fast), >75% ejected after a 2-min incubation.
[b] Determination performed twice.

RESULTS

Structure-activity relationship for phenyl disulfides. The necessity of the disulfide bond for the NCp7 zinc ejection and cellular anti-HIV activity by disulfide benzamides was examined, and the results are shown in Table 1. While the disulfide compound 1 has moderate zinc ejection activity and low micromolar antiviral activity, its free thiol form, compound 2, does not eject zinc but possesses similar antiviral activity. Replacement of the free thiol with a sulfinic acid (compound 3) or an alcohol (compound 4) abolishes both in vitro and cellular activities. If the disulfide of compound 1 is replaced with a methylene sulfide (compound 5), both in vitro and cellular activities are again lost. A mixed disulfide between group X of compound 1 and sulfanyl propionic acid (compound 6) ejects zinc and is antiviral. Thus, neither in vitro nor cellular activity requires two identical groups bound through a disulfide, but the disulfide itself is necessary. One explanation for the antiviral activity observed with the free thiol, compound 2, is that it oxidizes and then forms a mixed disulfide with a thiol (e.g., glutathione) in the cellular assay.

With the phenyl disulfide group held constant, substituents were studied to determine structural requirements for NCp7 zinc ejection and anti-HIV activity (Table 2). On the phenyl disulfide, incorporation of an *o*-amino (compound 7) or an

TABLE 2. NCp7 zinc ejection and anti-HIV activities of disulfide compounds



Compound	R1	R2	Rank (Zn ejection)[a]	CIC_{50} (μM)	TC_{50} (μM)
7	NH_2	H	MA	>53	53
8	CO_2H	H	MA	>100	>100
9	$CONH_2$	H	A	>21	21
10	$CONH_2$	OCH_3	A	>13	13
11	$CONH_2$	NH_2	MA	5.9	21
12	$CONH_2$	$NHCOCH_3$	A	6.0 (2)[b]	>100 (2)[b]

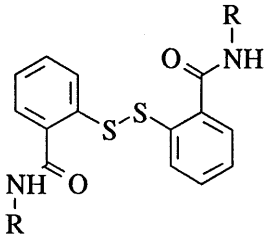
[a] For abbreviations, see Table 1, footnote *a*.
[b] Determination performed twice.

o-carboxylate (compound 8) results in a compound with moderate in vitro activity and no antiviral activity. The *o*-amido compound 9 is more active in vitro yet also possesses no cellular activity. Compound 9 is the core structure of all the antiviral disulfide benzamides described here. It has been shown that the ortho orientation of the amide is important for both zinc ejection and antiviral activity, since analogs of compound 1 with the amido group *meta* and *para* to the disulfide (otherwise identical structures) are inactive in both assays (data not shown). While the incorporation of a *p*-methoxy group into compound 9 does not result in antiviral activity (compound 10), addition of a *p*-amino group (compound 11) or a *p*-acetylamino group (compound 12) does. Thus, the simple incorporations of an *o*-amide and a *p*-acetylamino on the phenyl disulfide leads to a compound that ejects NCp7 zinc in vitro and possesses low micromolar antiviral activity with no measured toxicity.

Structure-activity relationship for *o*-amido-phenyl disulfides. Table 3 shows in vitro and cellular data correlated with incorporations on the amide group of the core structure *o*-amido-phenyl disulfide (compound 9). While the core structure (compound 9) is not antiviral, addition of an aliphatic carboxylate (compound 13) has cellular activity. The antiviral activity is related to the carboxylate group, since the addition of a branched aliphatic (compound 14) is inactive in vitro and in cells, while the corresponding branched aliphatic carboxylate (compound 15) addition is active in both assays. A close structural analog, compound 16, is being evaluated in advanced preclinical studies as a potential treatment for AIDS. It was found that both pure enantiomers and the racemate of compound 16 are all equally active in both assays (data not shown). Different aromatic substituents on compound 9 can have dramatic effects on zinc ejection activity, since the compound with the addition of a phenyl (compound 18) does not eject NCp7 zinc while the 2,6-pyrimidine (compound 17) ejects zinc at a very fast rate. As observed above, addition of an aliphatic carboxylate to compound 18 yields a compound (no. 19) that is active in both assays (as does the addition of a *para* or *ortho* sulfonamide [compounds 1 and 20]). Compounds 21, 22, and 23 are analogs of compound 1 with various in vitro and cellular activities. The kinetics of zinc ejection by disulfide benzamides that are active fast are discussed below.

Disulfide benzamide cyclization to benzisothiazolones. It was found that the disulfide benzamides being studied were stable in spectrophotometric-grade methyl sulfoxide but would

TABLE 3. NCp7 zinc ejection and anti-HIV activities of disulfide benzamides



Compound	R	Rank (Zn ejection)[a]	CIC_{50} (μM)	TC_{50} (μM)
9	H	MA	>21	21
13	$(CH_2)_2CO_2H$	A	6.2	>100
14	$CH(CH_3)$-isobutyl[b]	I	>35	35
15	$CH(CO_2H)$-isobutyl[b]	MA	5.7	92
16	$CH(CO_2H)$-sec-butyl[c]	A	9	>100
17	2,6-Pyrimidine	AF	>6.6	6.6
18	Ph	I	>6.6	6.6
19	*p*-(CH_2CO_2H)-Ph	A	5.7 (2)[d]	65 (2)[d]
1	*p*-(SO_2NH_2)Ph	MA	4.4 (2)[d]	>100 (2)[d]
20	*o*-(SO_2NH_2)Ph	MA	2.9 (2)[d]	67 (2)[d]
21	*p*-$(SO_2NHCOCH_3)$Ph	AF	26 (2)[d]	54 (2)[d]
22	*p*-[SO_2NH(2,6-pyrimidine)]Ph	A	3.9	32
23	*p*-[$SO_2NHCH(CH_3)CO_2H$]Ph	AF	>26 (2)[d]	26 (2)[d]

[a] For abbreviations, see Table 1, footnote *a*.
[b] Racemic mixture.
[c] L Enantiomer.
[d] Determination performed twice.

cyclize to a benzisothiazolone in aqueous buffer at neutral pH (Fig. 1). This was determined by comparing the analytic high-performance liquid chromatography retention time for the disulfide benzamide in methyl sulfoxide or in aqueous buffer with the retention time for the benzisothiazolone form of a disulfide benzamide which was independently synthesized (11a). In order to estimate the extent of cyclization of these compounds under our in vitro assay conditions, we monitored for the formation of 1 equivalent of free thiol (detected by reaction with DTNB) upon cyclization of 1 equivalent of disulfide benzamide. Figure 2 shows data for the release of free thiol versus time for compound 16 compared with the control of the free thiol of compound 16. The data indicates that cyclization occurs very quickly and is complete within 90 s. It also demonstrates that all of the disulfide benzamide has cyclized, since the disulfide benzamide values are actually slightly higher than those of the free thiol (it is unclear why there is this small difference in values). The benzisothiazolone form of compound 16 (compound 25) releases no free thiol at all, as expected.

The cyclization was found to be very fast for several disulfide

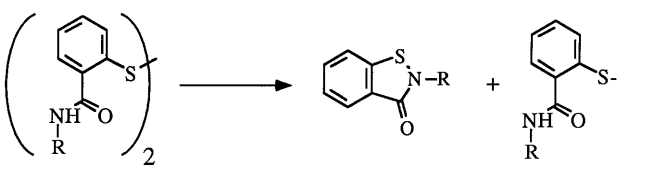


FIG. 1. Cyclization of *o*-amido-phenyl disulfide to benzisothiazolone.

TABLE 4. Formation of benzisothiazolones from disulfide benzamides determined by DTNB[a]

Compound	A_{406}	
	Free thiol form	Disulfide form
9		0.404
9a		0.024
1	0.047	0.029
12	0.389	0.421
16	0.316	0.352
21	0.379	0.336

[a] Compounds were incubated in buffer for 2 min; this was followed by a 30-s incubation with 40 μM DTNB. Compound 9 is *o*-amido-phenyl disulfide; compound 9a is *m*-amido-phenyl disulfide.

benzamides in the assay buffer. The extent of cyclization for several compounds was determined in the same manner, by measuring absorbance after the disulfide benzamide had been incubated in assay buffer for 2.5 min, and the results are summarized in Table 4. Compound 9, *o*-amido-phenyl disulfide, was found to cyclize to a significant degree as indicated by the absorbance at 406 nm. Compound 9a differs from compound 9 only by having the amido group *meta* to the disulfide instead of *ortho*. This orientation does not allow for benzisothiazolone formation, and no cyclization is detected by absorbance. This indicates that the DTNB does not react with these disulfides directly but only with the free thiol formed upon cyclization. The compounds 1, 12, 16, and 21, with diverse substituents, were mostly or entirely in the cyclic form. The unusually low values for compound 1 and its free thiol appear to be due to poor solubility. Thus, the disulfide benzamides studied in the in vitro assay have mostly or entirely cyclized to benzisothiazolone.

Structure-activity relationship for benzisothiazolones. Because of the cyclization of disulfide benzamides in the NCp7 assay, a series of benzisothiazolones were synthesized and studied directly (data summarized in Table 5). All of the benzisothiazolones shown have NCp7 zinc ejection activities, antiviral activities, and cellular toxicities equivalent to those of their disulfide benzamide counterparts. This result is consistent with the finding that the disulfide benzamides cyclize quickly. The only exception is compound 28 (relative to compound 1), where the cyclic form is more active in vitro and more toxic to cells. Compound 26 is not a benzisothiazolone, since the sulfur has been replaced with a methylene group, and it is not active in vitro or in cells. We have proposed that the disulfide benzamides eject NCp7 zinc by forming a disulfide bond with one of the cysteines formerly chelated to zinc, thus releasing the metal ion (28). Benzisothiazolones can react with cysteines to form a disulfide, and thus their in vitro activity is consistent with the proposed mechanism of action. Also consistent is the observation that removal of the sulfur abolishes in vitro activity.

Titration of in vitro NCp7 zinc ejection versus time by a benzisothiazolone (compound 28) was performed, and the results are shown in Fig. 3. As indicated by the fluorescence values, titration after a 1-min reaction is incomplete and then approaches completion at reaction times of 19 min and longer. The data is fit to the equation $Y = A/\{1 + ([\text{Compound}]/EC_{50})^B\}$ where A is the maximal fluorescence value, EC_{50} is the compound concentration at which 50% of the fluorescence increase is achieved, and B is the slope of the titration curve. The EC_{50} average value for the four curves with reaction times of 19 min or greater is 3.73 ± 0.25 μM. Since 2.8 μM NCp7 was used in the assay and there are two zinc fingers per NCp7, it requires 1.3 equivalents of benzisothiazolone to eject one NCp7 zinc. It is probable that this value would approach 1.0 if the reaction were taken out to very long reaction times. The average slope of the four titration curves equals 2.00 ± 0.49. Similar results were obtained when the titration was performed with a disulfide benzamide.

Kinetics of NCp7 zinc ejection. The kinetics of in vitro NCp7 zinc ejection was studied for several disulfide benzamides and benzisothiazolones using 16 μM compound. Typical results are shown in Fig. 4. The time-dependent fluorescence change for zinc ejection by compound 28 is fit to the biexponential equation $y = 29 + A[1 - \exp(-k1^*x)] + B[1 - \exp(-k2^*x)]$, where the fluorescence increase associated with each kinetic constant (A and B) is approximately equal and $k1 = 1.705 \pm 0.033$ min^{-1} and $k2 = 0.2063 \pm 0.0035$ min^{-1}. The fluorescence value before addition of compound was 29. The eightfold difference in the values of the two constants is similar to the kinetics of zinc ejection for compound 21 that we reported previously (28).

Kinetic data was obtained and analyzed in a similar manner for a total of 10 disulfide benzamides and benzisothiazolones, and the kinetic constants derived are shown in Table 6. In fitting the data, it became clear that the fit for $k1$ was very good while $k2$ values could vary greatly through different iterative fits depending on the magnitude of the estimated total fluorescence increase. Therefore, $k1$ is a better quantitation of the rate of NCp7 zinc ejection. Compounds similar in structure were found to differ in their rate of zinc ejection by two orders of magnitude, as measured by $k1$. For example, replacement of the *p*-aminoacetyl group of compound 12 with a *p*-methoxy group (compound 10) increases $k1$ 75-fold, yet this substitution greatly increases cellular toxicity and abolishes measurable antiviral activity (possibly due to the high compound toxicity).

From the data in Table 6, there is no relationship between $k1$ and CIC_{50} (compound concentration that yields 50% inhi-

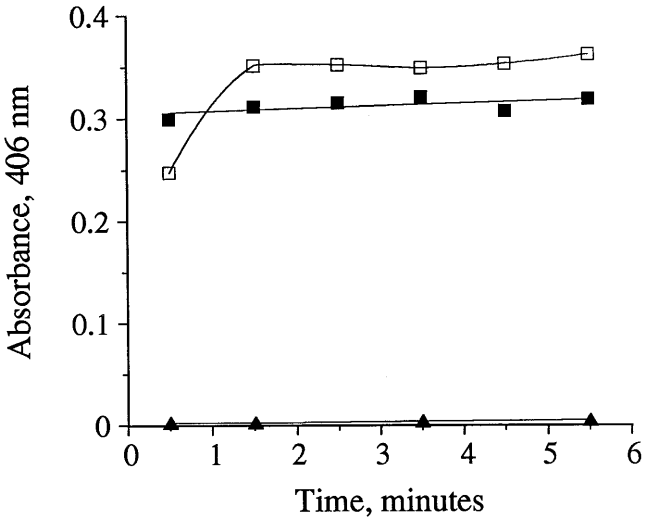
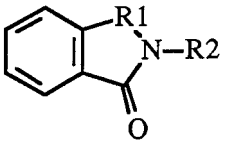


FIG. 2. DTNB detection of free thiol formation versus time upon cyclization of a disulfide benzamide (compound 16) to a benzisothiazolone. As shown in Fig. 1, cyclization of an *o*-amido-phenyl disulfide to form a benzisothiazolone yields 1 equivalent free thiol which can react with DTNB in the assay. 16 μM (final concentration) of compound was added to zinc ejection assay buffer from a methyl sulfoxide stock solution and incubated for various times, followed by addition of 40 μM (final concentration) DTNB and a 30-s incubation before measurement of DTNB A_{406}. The x axis refers to total incubation time for the compound in solution. □, compound 16, disulfide benzamide; ■, free thiol form of compound 16; ▲, compound 25, benzisothiazolone form of compound 16.

TABLE 5. NCp7 zinc ejection and anti-HIV activities of benzisothiazolones and an analog



Compound	Disulfide form	R1	R2	Rank (Zn ejection)[a]	CIC_{50} (μM)	TC_{50} (μM)
24		S	$(CH_2)_3CO_2H$	A	4.8 (2)[c]	>100 (2)[c]
25	16	S	$CH(CO_2H)$-sec-butyl[b]	MA	11 (2)[c]	>100 (2)[c]
26		CH_2	$CH(CO_2H)$-sec-butyl[b]	I	>100	>100
27	19	S	*p*-(CH_2CO_2H)Ph	A	5.8	69
28	1	S	*p*-(SO_2NH_2)Ph	A	5.5 (2)[c]	21 (2)[c]
29	21	S	*p*-$(SO_2NHCOCH_3)$Ph	A	24	67

[a] For abbreviations, see Table 1, footnote *a*.
[b] L enantiomer.
[c] Determination performed twice.

bition of viral replication) values. But there appears to be a qualitative relationship between higher $k1$ values, or faster zinc ejection, and lower TC_{50} values, or increased toxicity. The three compounds with the lowest $k1$ values (12, 16, and 25) are the only three with TC_{50} values above 100 μM. The three compounds with the highest $k1$ values (17, 23, and 10) are the among the most toxic to cells.

Time dependence of HIV inactivation. The inactivation of HIV by exposure to disulfide benzamides and benzisothiazolones occurs in a time-dependent manner. The initial titer of the methyl sulfoxide control as determined by the MAGI cell assay was approximately 80 syncytia produced per well (Fig. 5A), and declined to about 50 within 30 min and to 25 by the end of 3 h due to the instability of the virus. In comparison, the samples treated with compounds 1, 28, and 21 contained 18, 7, and 25 surviving virions, respectively, after only a 5-min exposure (at least a 50% inhibition compared to the methyl sulfoxide control). This increased to 81, 95, and >75% inhibition within 30 min. At 90 min, a >95% inactivation had occurred for all three compounds. Compounds 1 and 28 are the disulfide benzamide and benzisothiazolone forms of the same compound (both of which were found to rapidly equilibrate in aqueous buffer). The two compounds rapidly inactivate virus in comparison to other compounds which act more slowly (Fig. 5B). Samples treated with compounds 16, 25, and 12 exhibited only 12, 19, and 41% inhibition, respectively, after 30 min and required 90 min or more to achieve a 50% reduction in HIV titer. At the end of 3 h, the percent inactivation had reached 89, 72, and 84%, respectively. Again, compounds 16 and 25, with similar rates of virus inactivation, are the disulfide benzamide and benzisothiazolone forms of the same compound.

DISCUSSION

The disulfide group of disulfide benzamides is necessary for in vitro NCp7 zinc ejection activity and may be necessary for antiviral activity. The free thiol compound 2 does not eject zinc

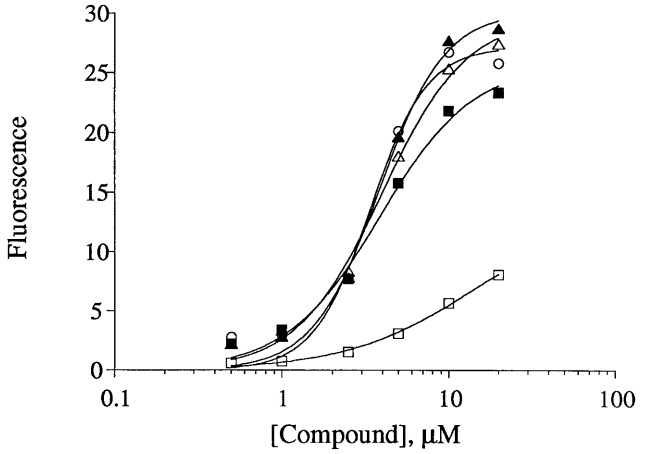


FIG. 3. Titration of NCp7 zinc ejection by compound 28 versus time. Reaction was initiated by the addition of compound 28 to 2.8 μM HIV-1 NCp7, and TSQ fluorescence was measured versus time. Reaction times were 1 min (□), 19 min (■), 30 min (△), 60 min (▲), and 90 min (○). The data were fit to the equation $Y = A/\{1+([\text{Compound}]/EC_{50})^B\}$, where A is the maximal fluorescence value, EC_{50} is the compound concentration at which 50% of the fluorescence increase is achieved, and B is the slope of the titration curve. Excluding the titration at 1 min, the average EC_{50} value was 3.73 ± 0.25 μM and the average B value was 2.00 ± 0.49.

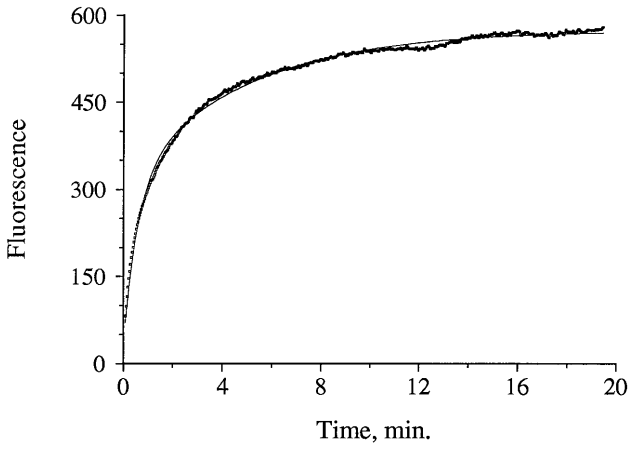


FIG. 4. The kinetics of HIV NCp7 zinc ejection by compound 28. Compound 28 (16 μM final concentration) was added to 2.0 μM NCp7–50 μM TSQ in zinc ejection assay buffer, and TSQ fluorescence was measured immediately. The data is shown fit to the double exponential equation $y = 29 + A(1 - \exp(-k1^*x) + B(1 - \exp(-k2^*x)$, since the fluorescence value before addition of compound was 29. (Addition of 16 μM compound was found to not affect TSQ-zinc fluorescence.) $A = 282.5 \pm 3.2$; $k1 = 1.705 \pm 0.033$ min^{-1}; $B = 262.3 \pm 2.8$; $k2 = 0.2063 \pm 0.0035$ min^{-1}; $R = 0.998$.

TABLE 6. Kinetic constants of HIV NCp7 zinc ejection and anti-HIV activities for disulfide benzamides and benzisothiazolones

Compound (16 μM)[a]	k1 (min^{-1})	k2 (min^{-1})	CIC_{50} (μM)	TC_{50} (μM)
12	0.145	0.0158	6.0 (2)[b]	>100 (2)[b]
16	0.276	0.0192	9	>100
25	0.372	0.0199	11 (2)[b]	>100 (2)[b]
22	0.446	0.0262	3.9	32
20	1.413	0.101	2.9 (2)[b]	67
28	1.70	0.206	5.5 (2)[b]	21 (2)[b]
21	1.92	0.342	26 (2)[b]	54 (2)[b]
17	4.65	0.33	>6.6	6.6
23	7.61	0.832	>26 (2)[b]	26 (2)[b]
10[a]	10.8	0.128	>13	13

[a] Better fit obtained with three exponentials.
[b] Determination performed twice.

but has antiviral activity equivalent to that of its disulfide form. While the in vitro NCp7 zinc ejection assay is performed in the absence of a reducing agent, compounds in a cellular assay can form mixed disulfides with a thiol compound in the serum (e.g., glutathione). Therefore, the antiviral activity associated with the free thiol, compound 2, may be due to a mixed disulfide it forms with a thiol in the cell assay. Substitutions of the thiol group of compound 2 which abolish its ability to form a mixed disulfide (compounds 3 and 4) result in loss of antiviral activity, while a mixed disulfide of compound 2 is active both in vitro and in cells. It cannot be ruled out, though, that the antiviral activity of the free thiol compound 2 is due to its action at a retroviral target other than nucleocapsid protein. The activities in both assays of the mixed disulfide compound 6 demonstrate that the homodimeric nature of the disulfide benzamides is not a requirement.

Disulfide benzamide added to cells may equilibrate to several different forms. Because of the intracellular reducing environment, disulfide benzamides may exist as a free thiol or as a mixed disulfide between compound and glutathione. The disulfide benzamide under in vitro assay conditions mostly or entirely cyclizes to form benzisothiazolone and free thiol, and it probably does so to some degree in cells. Thus, there are four potential forms of the compound (disulfide, mixed disulfide, free thiol, and benzisothiazolone), and any of the four forms added to cells is antiviral with similar potency. It was found that the benzisothiazolones and a mixed disulfide possess NCp7 zinc ejection activities similar to those of their disulfide benzamide counterparts. Also, disulfide benzamides have been studied that cannot cyclize and are active in vitro and in cells (8). It is unclear from the current studies what their relative amounts in equilibrium in a cell assay are, but the in vitro data suggests that the disulfide, mixed disulfide, and benzisothiazolone forms may all eject NCp7 zinc and be antiviral.

Consistent with the model that the disulfide benzamide target of antiviral action is nucleocapsid protein, it was found that all of the antiviral compounds in this study eject NCp7 zinc in vitro while some of the compounds that eject zinc are not antiviral. It is not uncommon that within a series of compounds that are active in vitro, some do not inhibit the virus (this has been reported with numerous series of HIV protease inhibitors). The lack of antiviral activity may be due to the poor cell penetration of some compounds. With the exception of one disulfide benzamide and its cyclic form, all of the antiviral compounds have approximately equivalent antiviral potency, with CIC_{50} values of 3 to 9 μM. We have proposed that the mechanism of NCp7 zinc ejection involves formation of a disulfide between the compound and an NCp7 cysteine formerly chelated to zinc. If this mechanism accounts for the antiviral activity, a certain minimal concentration of compound would be required to inactivate enough NCp7 to inhibit viral replication. That minimal concentration for this cellular assay may have been achieved with several of these antiviral compounds.

The structural component common to all the antiviral compounds described in these studies is the *o*-amido-phenyl disulfide, which itself ejects zinc but is not antiviral. Both aromatic and aliphatic substitutions on the *o*-amido group of the phenyldisulfide lead to antiviral compounds. It was generally observed that terminal carboxylates improve, or are necessary for, zinc ejection and antiviral activity. While kinetic studies provided no evidence for a compound-NCp7 binding step prior to zinc ejection (28), the NCp7 zinc fingers are adjacent to many basic residues which may have a favorable interaction with the terminal carboxylate groups of these compounds.

Several conclusions can be drawn from the studies on inactivation of HIV by the disulfide benzamides and benzisothia-

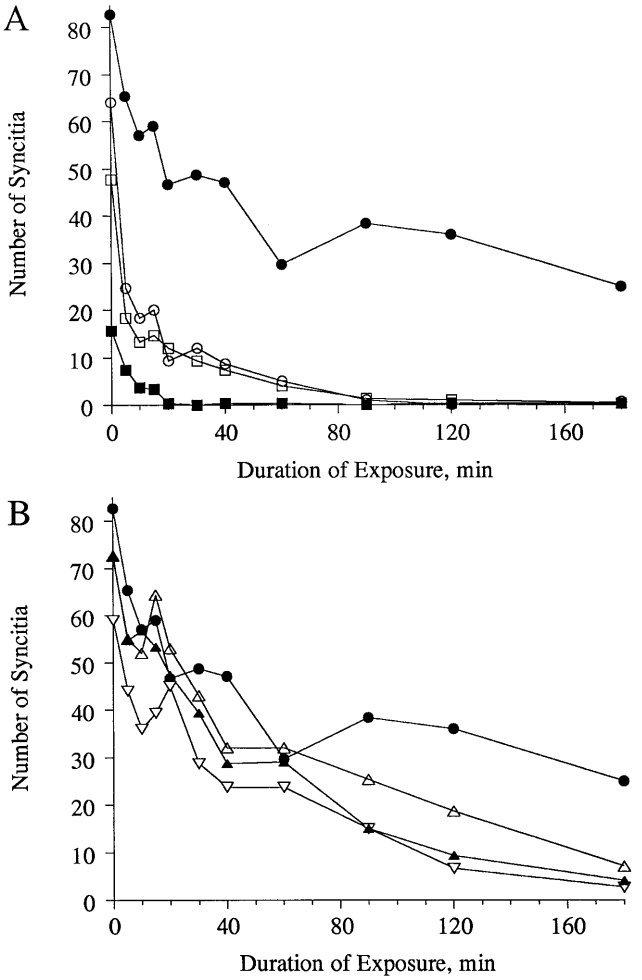


FIG. 5. The time dependence of HIV inactivation by exposure to disulfide benzamides and benzisothiazolones. Test compound or a 0.2% methyl sulfoxide control was incubated with purified virions suspended in medium for various amounts of time (expressed as duration of exposure, *x* axis), and then the mixture was diluted and transferred to MAGI cell cultures (100-fold effective dilution). After 48 h, the syncitia were counted (*y* axis) by using beta-galactosidase staining. (A) □, 50 μM compound 1; ■, 100 μM compound 28; ○, 50 μM compound 21. (B) △, 50 μM compound 16; ▲, 100 μM compound 25; ▽, 50 μM compound 12; ●, 0.2% methyl sulfoxide control.

zolones. First, these compounds act in a time-dependent manner since the inactivation of HIV was a progressive, continuous process which required up to 3 h to achieve 80% inhibition. Similarly, the in vitro NCp7 zinc ejection described here and the inhibition of NCp7-Ψ RNA binding (28) by these compounds are also time dependent. Secondly, there is a correlation between the relative rates of virus inactivation and the kinetic rates of in vitro NCp7 zinc ejection. The disulfide benzamides compounds 1 and 21 and the benzisothiazolone compound 28 all exhibited markedly faster inactivation of HIV, and compounds 21 and 28 possess faster rates of NCp7 zinc ejection (the rate of zinc ejection by compound 1 was difficult to determine due to compound insolubility). Conversely, the disulfide benzamides compound 12 and compound 16 and the benzisothiazolone compound 25 all inactivated HIV at a much slower rate, and all three possess slower rates of NCp7 zinc ejection. The correlation of the in vitro and virucidal relative rates is consistent with the nucleocapsid protein being the target of action of these compounds.

Comparison of the kinetics of NCp7 zinc ejection with that of cellular antiviral activity qualitatively indicates that faster rates of in vitro zinc ejection correlate with increased cellular toxicity and not with antiviral potency. If the disulfide benzamides react with NCp7 cysteines to form a disulfide and eject zinc, then disulfide benzamides that react faster may be expected to be more reactive to cysteines in other proteins. An increased nonspecific reactivity could account for increased toxicity. Conversely, disulfide benzamides such as compound 16 and its benzisothiazolone form, compound 25, which eject zinc much more slowly, are novel low micromolar antiviral agents that exhibit no toxicity and provide promising compounds for further development.

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