Experimental determination and calculations of redox potential descriptors of compounds directed against retroviral zinc fingers: Implications for rational drug design

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Abstract

A diverse set of electrophilic compounds that react with cysteine thiolates in retroviral nucleocapsid (NC) proteins and abolish virus infectivity has been identified. Although different in chemical composition, these compounds are all oxidizing agents that lead to the ejection of Zn(II) ions bound to conserved structural motifs (zinc fingers) present in retroviral NC proteins. The reactivity of a congeneric series of aromatic disulfides toward the NC protein of the human immunodeficiency virus type 1 (HIV-1), NCp7, has been characterized by HPLC separation of starting reagents from reaction products. We calculated the absolute redox potentials of these compounds in the gas phase and in aqueous solvent, using a density functional theory method and a continuum solvation model. Pulsed polarography experiments were performed and showed a direct correlation between calculated and experimentally determined redox propensities. A dependence between protein reactivity and redox potential for a specific compound was shown: Reaction with NCp7 did not take place below a threshold value of redox potential. This relationship permits the distinction between active and nonactive compounds targeted against NCp7, and provides a theoretical basis for a scale of reactivity with retroviral zinc fingers. Our results indicate that electrophilic agents with adequate thiophilicity to react with retroviral NC fingers can now be designed using known or calculated electrochemical properties. This may assist in the design of antiretroviral compounds with greater specificity for NC protein. Such electrophilic agents can be used in retrovirus inactivation with the intent of preparing a whole-killed virus vaccine formulation that exhibits unaffected surface antigenic properties.

Keywords: Nucleocapsid; antiretroviral compounds; pulsed polarography; zinc ejection; redox potential; viral inactivation; zinc fingers; density functional theory methods

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Abbreviations: AT-2, aldrithiol-2 (2,2'-dithiodipyridine); BEM, boundary element method; BLYP, Becke-Lee-Yang-Parr exchange correlation method; DFT, density functional theory; DMSO, dimethyl sulfoxide; DZ-VPD, double zeta-split valence potential plus diffuse d-functions; EA,

electron affinity; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; NC, nucleocapsid; NCp7, Zn(II)-bound HIV-1 nucleocapsid protein; NEM, *N*-ethylmaleimide; SCF, self-consistent field; QSAR, quantitative structure-activity relationship; TFA, trifluoroacetic acid.

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The nucleocapsid (NC) protein is a constituent of retroviruses that, as a domain in the polypeptide Gag precursor, is responsible for the recognition and packaging of the genomic RNA (Gorelick et al. 1988, 1990, 1993; Aldovini and Young 1990; Dupraz et al. 1990; Zhang and Barklis 1995; Schwartz et al. 1997). After release by the viral protease, NC proteins participate also in auxiliary functions in the early stages of infection (Gorelick et al. 1993; Zhang and Barklis 1995). NC proteins of all lentiviruses and oncornaviruses carry one or two copies of a short, highly conserved sequence (zinc finger) that has been shown to chelate Zn(II) ions in vivo and in vitro (Chance et al. 1992; Summers et al. 1992), and is required for efficient binding of NC protein to nucleic acids (Urbaneja et al. 1999). Mutations that disrupt or abolish the coordination of divalent ions in NC protein drastically impair the encapsidation of viral RNA (Gorelick et al. 1988; Rein et al. 1994). Mutations that result in coordination of zinc ions by metal cluster environments other than the retroviral type (involving three cysteine thiolates and one histidine imidazole in an absolutely conserved spacing, C-X₂-C-X₄-H-X₄-C) were recently phenotypically characterized (Gorelick et al. 1999), and were shown to result in noninfectious virions while preserving packaging of viral genomic RNA at levels comparable to those of wild-type virus (Rossio et al. 1998). Ongoing research suggests that NC protein plays a vital role in some (as yet poorly understood) steps of the viral life cycle that precede integration of the viral RNA into the host's genome.

The strict conservation of zinc fingers (a structural motif required during viral RNA recognition, packaging, viral assembly, maturation, preintegration and reverse transcription) in all retroviruses (except the *spumaviridae*) makes them an attractive target for therapeutic intervention in retroviral infection (McDonnell et al. 1997). A number of chemical compounds have been identified that are capable of reaction with retroviral NC proteins (Rice et al. 1993, 1995; Yu et al. 1995; Hathout et al. 1996; Loo et al. 1996; Maynard et al. 1998) and result in the ejection of bound Zn(II) (Rice et al. 1993). Most such reagents attack the cysteine side chain in the metal binding site(s) of NC protein. Cysteine thiols are the most nucleophilic groups among all twenty naturally occurring amino acids, and can be chemically modified with a large library of compounds through a variety of reaction mechanisms (Lundblad 1991). In general, oxidation of the zinc-coordinating thiolates in NC proteins ensues upon exposure to suitable electrophilic reagents. Unfolding of the zinc finger domain in apo NC proteins severely decreases their affinity for nucleic acid lattices (Urbaneja et al. 1998, 1999). Reaction with susceptible cysteines has been achieved in viral suspensions exposed to disulfides and maleimides (Rice et al. 1995). Functional inactivation of NC protein is manifested by the appearance of a ladder of NC oligomers resulting from intermolecular disulfide formation in the virion (Rice et al.

1995; Arthur et al. 1998; Rossio et al. 1998). Loss of all detectable infectivity following exposure of HIV-1 virions to 2,2'-dithiodipyridine (aldrithiol-2) has been achieved while preserving the conformational and functional integrity of proteins in the virion surface (Arthur et al. 1998; Rossio et al. 1998). Although retroviral inactivation by electrophilic compounds is of obvious interest in the medical setting for the treatment of laboratory and biological samples, as well as in the prophylactic development of vaccines based in whole-killed viral particles, the direct use of electrophilic compounds as therapeutic agents appears more distant. Indeed, some of the compounds tested in vitro in the inactivation of retroviral NC proteins are very active chemically, and react indiscriminately with many chemical moieties present in biological systems. Cysteine-specific agents may conceivably be capable of reacting with crucial host's proteins in the absence of additional selectivity. Yet, disulfide compounds of low toxicity and with minor side effects in humans (e.g., Antabuse) have been known for decades (Gallant 1991; Borup et al. 1992). In small-scale experiments, disulfide compounds have been administered to retroviral-infected mice with a beneficial decrease in viral load, and a delay of disease onset was observed (Ott et al. 1998). Thus, electrophilic compounds can be effective in retroviral inactivation in the absence of specificity for their target (Rein et al. 1996, 1997).

In vitro reactions with HIV-1 NC protein (NCp7) have been carried out with N-ethylmaleimide (NEM) (Chertova et al. 1998), a widely used cysteine modification agent that generates stable alkyl-cysteine derivatives, and the disulfide bridge-forming aldrithiol-2 (AT-2) (Chertova et al. 1997). A discrete order of target reactivity among the six thiol groups present in NCp7 was observed with both compounds (Chertova et al. 1997, 1998). AT-2 induced first the formation of a disulfide linkage between Cys-39 and Cys-49 in NCp7 (Chertova et al. 1997), whereas in reaction with N-ethylmaleimide (NEM), Cys-49 was identified as the first site of chemical attack (Chertova et al. 1998). In these studies, the solvent exposure largely determined the order of Cys target reaction: different reagents all initiated reaction at Cys-49 and followed similar reaction pathways. Cysteine accessibility and microenvironment are expected to be significant factors in the competition of various targets with a reagent. Indeed, isolated ZF peptides lacked the preferential reactivity seen with full-length NC protein sequences (Chertova et al. 1997, 1998). In an HPLC-based screening of about 160 electrophilic compounds available from chemical libraries, their intrinsic reactivity against NCp7 was found to vary widely (E. Chertova, unpubl.). A small subset of these chemicals including compounds of high, low and null reactivity toward HIV-1 NC protein was selected for a more detailed investigation, presented in this manuscript. These agents—phenyl, tolyl, or pyridyl disulfides (Fig. 1)—are structurally related and can be treated as a congeneric series

COMPOUND	CODE	R1	R2	R3	R4	X1	X2
phenyl disulfide	I	H	H	H	H	CH	CH
aldriethiol-2	II	H	H	H	H	N	CH
p-tolyl disulfide	III	H	H	CH ₃	CH ₃	CH	CH
4-nitrophenyl p-tolyl disulfide	IV	H	H	CH ₃	NO ₂	CH	CH
2-nitrophenyl disulfide	V	NO ₂	H	H	H	CH	CH
3-nitrophenyl disulfide	VI	H	NO ₂	H	H	CH	CH
4-nitrophenyl disulfide	VII	H	H	NO ₂	NO ₂	CH	CH
2-amino-4-chlorophenyl disulfide	VIII	NH ₂	H	Cl	Cl	CH	CH
4-chloro-2-nitrophenyl disulfide	IX	NO ₂	H	Cl	Cl	CH	CH
2-aminophenyl disulfide	X	NH ₂	H	H	H	CH	CH
4-aminophenyl disulfide	XI	H	H	NH ₂	NH ₂	CH	CH
aldriethiol-4	XII	H	H	-	-	CH	N

Fig. 1. Name, chemical structure, and code number for the compounds used.

to study the dependence of thiol reactivity on ring substituents. They are also molecules of tractable size for the application of quantum chemical techniques at the ab initio level. Finally, they were subjected to electrochemical assessment of their relative redox propensity and, in some instances, their redox potential. The goal of this study was to seek a correlation between the redox potential (either calculated or experimentally determined) for a given compound and its reactivity toward NC proteins as a tool in the rational design of electrophilic drugs directed against retroviral zinc fingers.

Results

Retroviral NC proteins have been proposed as an attractive target for viral inactivation and therapy (Rice et al. 1995; Rein et al. 1996, 1997) due to the presence of a highly conserved motif (zinc fingers) that is required for their activity. Chemical reactivity toward these sequences has been demonstrated with compounds bearing different active groups, which constitute a virtual library numbering in the thousands. It is apparent from a comparison of the structures of these compounds and their reaction products that their redox characteristics are relevant in this reaction. Electron affinity relates directly to the redox potential and can be

empirically derived or calculated ab initio. Disulfides are attractive from a number of chemical and biochemical considerations that include a relatively straightforward synthesis, mild chemical reactivity, and low toxicity. We have selected a congeneric series of available disulfides among the 160 compounds used in our initial screen to investigate the use of quantum-chemical calculations in the screening and rational design of compounds targeted against retroviral NC proteins, and to determine the factors that influence activity by comparing the NCp7 reactivity of members of this series that carry different chemical substitutions.

Nucleocapsid reactions with disulfides

The reaction of HIV-1 NCp7 with disulfide reagents selected from our initial screen of ~160 compounds and used in this study (Fig. 1) was monitored by reverse phase HPLC. Recombinant NCp7 was incubated with a given compound at a 1 : 6 molar ratio (protein : reagent) at pH 7.0 and 37°C for varying times (1 min, 10 min or 60 min) and then injected onto the HPLC column. The amount of unreacted NCp7 was determined by integration of the elution peak area (Fig. 2). In the absence of redox reaction, the height, shape, and elution time of the protein did not change compared to a reference standard. Compounds were scored "un-

Fig. 2. HPLC elution profile for HIV-1 NCp7 (*top trace*) and the products of its reaction with compound II.

reactive” if they failed to modify the NC protein after a 60-min reaction time. For reactive compounds, the extent of reaction was scored (as an “NC value”) based on the fraction of unreacted NCp7. For example, if 80% of the protein was recovered, then 20% of the starting NCp7 underwent reaction, and the NC value for this particular compound was recorded as NC = 2 for the specified reaction time. A reaction time of 10 min was used for the initial screening of all compounds in this study; however, some were too reactive (NC = 10, 10 min) for comparison by this parameter. Therefore, a reaction time of 1 min was used to compare a series of compounds that included highly reactive members. Applying this single-parameter metric to rank and quantify the kinetics of oxidation reaction with NCp7 of the various compounds used in this study, we observed that their reaction rates varied significantly (Table 1) within a fairly narrow congeneric series. A cursory examination of the NC numbers (Table 1) that were measured for twelve closely related chemical compounds (Fig. 1) shows significant positional and chemical group effects on reactivity.

Generally, the observed effects can be accounted for by the electron-withdrawing and electron-releasing properties of the chemical substituents around the aromatic ring. Thus, the strongly electron-withdrawing NO₂ group imparts an inductive effect to the aromatic ring due to the partial charge of the nitrogen atom; the polarized N-C (ring) bond induces a positive charge in the ring that can be palliated by molecular orbital mixing with nonbonding *d* electrons of the disulfide sulfur atoms. The delocalization of disulfide electron density renders nitrophenyl disulfides more electrophilic and enhances their NC reactivity (compare the NC values for the unsubstituted compound I with those of the NO₂-derivatized compounds V, VI, and VII; Table 1), whereas neither a mildly electron-donating methyl group (compound III) nor a strongly electron-donating amine group (compound XI) in the *para* positions showed an effect (the unsubstituted compound I is already unreactive against NCp7). This general ranking is expected from the well-known values of the Hammett σ descriptor, defined as

$$\sigma = \log K_X - \log K_H$$

where K_H is the ionization constant for benzoic acid in water at 25°C and K_X is the ionization constant for a *meta* or *para* derivative under the same experimental conditions (Hansch and Leo 1979). Positive values of σ denote electron withdrawal by a substituent group from the aromatic ring, whereas negative σ values represent electron release to the

ring (Hansch and Leo 1979). The values for nitro, methyl and amino substitution in *para* are 0.78, -0.17, and -0.66, respectively (Hansch and Leo 1979). It is generally recognized that electronic effects of substituents on the ionization of benzoic acids constitute a model system with predictive value that extends to other reaction centers attached to aromatic systems. This analysis applies even when the “substituent” on a benzene ring may actually be a ring heteroatom, as in the case of the pyridine derivatives studied in this work.

It is worth noting that sigma constants are position-dependent, with the largest effects usually observed at positions *ortho* and *para*, due to the possibility of accepting/donating electrons via resonance interactions (*ortho* substituents may exert steric effects on the reaction center, as well). Resonance interactions do not play a significant role for substituents in *meta* positions. The sigma values for nitro substitution in *o*-, *m*- and *p*- are 0.95, 0.71, and 0.78, respectively (Hansch and Leo 1979). Since NO₂ substitution in *meta* (compound VI) resulted indeed in lower NC values than the *para* analog (compound VII) (6.1 vs. 8.1, respectively; Table 1), resonance processes that would involve ring stabilization of a 4-substituted benzenesulphenyl ion may be implicated. However, separation of inductive and resonance effects is complex and was not attempted in our analysis. Nitro substitution in the *ortho* position (compound V) resulted in less activation (NC value 2.4; Table 1) than expected or seen at the other positions. This effect could result from a stabilizing intramolecular H bond of the 2-nitro group that renders the thiophenol species less acidic, or from an inhibition of the approach to a negatively charged thiolate sulfur due to the partial negative charge on the nitro oxygens. It is worth noting that the aforementioned two effects involve different reaction mechanisms (with dissimilar charges in the leaving group); only an RS⁻ species is consistent with the anionic state suggested by the *o*-amino results and findings using an asymmetric phenyl disulfide reagent (see below). Density functional theory (DFT) calculations performed on the energy-minimized *o*- and *m*-nitrophenyl disulfide structures suggest a more stabilized ion for the *meta* position, with the S-S bond in the *o*-compound partially dissociated (possessing radical character). Thus, the possibility of a zinc finger reaction by the *o*-nitro compound by a mechanism different from that of the other positional substituents is not ruled out. The possibility of decreased NC scoring with the *o*-NO₂ analog due to disappearance of the highly activated compound by reaction with other nucleophile targets (e.g., water) was tested chromatographically. No evidence for instability of compound V was uncovered.

Interestingly, the *ortho*-substituted diamine (compound X) exhibited anomalous reactivity, as well (Table 1). The sigma values for *o*- and *p*-amine substituents are -0.35 and -0.66, respectively. Deactivation of the unreactive phenyl

Table 1. Comparison of NC reactivity values from HPLC assays, $E_{1/2}$ values from polarographic experiments, and redox potential (RP) values obtained from density functional theory calculations^a

Name and code no.	Structure	RP kcal mole	$E_{1/2}$ mV	NC 1 min
Phenyl disulfide (I)		85.61	-782	0.00
2,2'-dipyridyl disulfide aldrithiol-2 (II)		91.08	-762	5.80
p-Tolyl disulfide (III)		85.24	-875	0.00
4-Nitrophenyl p-tolyl disulfide (IV)		95.51	-626	6.40
2-Nitrophenyl disulfide (V)		92.43	-670	2.40
3-Nitrophenyl disulfide (VI)		95.06	N.D.	6.12
4-Nitrophenyl disulfide (VII)		99.57	-550	8.10
2-Amino-4-chlorophenyl disulfide (VIII)		90.90	-714	6.25
4-Chloro-2-nitrophenyl disulfide (IX)		83.24	N.D.	0.00
2-Aminophenyl disulfide (X)		87.53	N.D.	2.79
4-Aminophenyl disulfide (XI)		83.64	-910	0.00
4,4'-Dipyridyl disulfide aldrithiol-4 (XII)		95.10	N.D.	4.50

^a Ab initio calculations of electron affinity, electrochemical and reactivity measurements were carried out as described in Materials and Methods. The polarographic half-wave values quoted in the table are from the following: Benaichouche et al. (1990) (for compounds V and VII); and Latypova et al. (1989) (for compound XI).

^b (N.D.) Not determined.

disulfide (compound I) by amino substitution would be expected in both *para* (compound XI) and *ortho* (compound X) analogs. Yet, a reactivity enhancement was seen for compound X (Table 1), and is attributed to an intramolecular H bond that stabilizes the resulting thiophenol anion, making it more acidic and a better leaving group. DFT geometry optimization predicts a structure consistent with this model, as reflected in a 3.1 Å S-H distance and a planar disposition of the N, H and S atoms. This behavior was not limited to the phenyl disulfides, because the *o*-amine substituted naphthyl disulfide 2,2'-dithiobis(1-naphtylamine)

showed an NC value somewhat higher than the phenyl analog, whereas the unsubstituted 2-naphtyl disulfide was unreactive. An alternative possibility for such enhancement in reactivity, namely that the protonated amine is undergoing reaction, would not explain our findings with a chloro analog that are discussed below. Note that the hypothesis of a negative charge on the leaving group species was also invoked as a possible explanation of the depressed reactivity of the *o*-nitro compounds, and would provide a unifying hypothesis for the anomalous reactivity of *o*-substituted nitro and amino phenyl disulfide derivatives.

The *p*-chloro derivative of the *o*-NH₂ substituted phenyl disulfide (compound VIII) enhanced the reactivity of compound X (Table 1), as expected from the high electronegativity of halogen substituents (sigma values for Cl or Br in the *para* positions are 0.23). Halogen derivatization in *para* of the unsubstituted phenyl disulfide was not sufficient to render the resulting compounds (not listed in Table 1) reactive, suggesting that more powerful electron-withdrawing substituents are required for activation of compound I. A trichlorophenyl disulfide, however, reacted readily with NC protein.

We have found that a number of 2-nitro and 2-amino substituted phenyl disulfide behave anomalously in screening assays based on NC reactivity (Fig. 3), with most *ortho*-nitro compounds exhibiting diminished reactivity, whereas many *ortho* amino or amido analogs showed enhanced reactivity. Thus, the anomalous reactivity of the *ortho*-substituted analogs listed in Table 1 is not an isolated incident, but rather a general feature. Most notably, when the anomalous reactivity of the above compounds is accounted for, a sharp transition from unreactive to essentially complete reaction after 10 min was seen to occur for compounds with sigma values exceeding approximately 0.4 (Fig. 3). It is also noteworthy that the *ortho* analogs used in our study (compounds V and X) fall farthest off of the correlation line between NC reactivity and calculated redox potential.

Competition experiments in which a large excess of unreactive compound was added to an NC-reactive agent did not cause a decrease in apparent NC number (not shown), suggesting that the oxidation reaction occurs through a collisional random process, rather than through a pre-reaction complex; alternatively, unreactive complexes might be un-

able to form such a pre-reaction species. To our knowledge, no experimental evidence currently supports the formation of a stable complex between NCp7 and any of the reactive compounds used in this and other studies.

The first Cys-Cys linkage to appear in p7 upon reaction with aldrithiol-2 (compound II) has been identified (Hathout et al. 1996). Reactions with apo-p7 exhibit a dissimilar reaction pattern and kinetic evolution, in that fast reaction with all reagents assayed was observed (including those found to be unreactive with full-length, zinc-bound NCp7) at all cysteine positions (Chertova et al. 1997). This finding is in agreement with the profoundly different conformational and electronic properties of the cysteine targets in the unfolded, metal-free peptide.

Electrochemical investigation

Electrochemical protocols, including polarographic, voltammetric, and coulometric methods, have been applied to a number of thiol and disulfide compounds in the characterization of their redox properties (Prue et al. 1972; Mairesse-Ducarmois et al. 1975, 1976a,b). Polarographic experiments (Fig. 4) were carried out to directly determine half-wave potential ($E_{1/2}$) values that validate our ab initio computer calculations. Table 1 lists the $E_{1/2}$ measurements obtained via polarographic methods. The half-wave potential is an empirical value that is defined as the midpoint of the rise in current of a polarographic wave and, as such, it differs from the standard reduction potential (E°) of the compound. The empirical nature of $E_{1/2}$ values is noted by their dependence on the choice of pH and solvent system. We would like to stress, however, that the magnitude of observed shifts in the

Fig. 3. Plot of reactivity (NC values rescaled for 10-min reaction) versus sigma values for 50 aromatic disulfides (see text). 2-amino and 2-amido phenyl disulfides (solid triangles) exhibit enhanced reactivity, whereas 2-nitro and 2-halogen analogs (solid squares) show low reactivity. For the rest of the compounds in the congeneric series (circles), there is a sharp transition from unreactive (NC value = 0–2) to reactive NC value = 8–10) as the Hammett σ parameter increases.

Fig. 4. Polarogram of compound VIII, dissolved in DMSO containing 100 mM tetrabutylammonium fluoroborate.

$E_{1/2}$ values when using solvents of different polarity was similar for several compounds investigated (all compounds were measured using similar conditions). Hence, the relative nature of the $E_{1/2}$ values presented in this study is deemed to be a relevant descriptor of the redox potential of these agents.

As noted by the comparison of compounds III, IV, and VII (Table 1), the $E_{1/2}$ values are strongly dependent on substituents (Fig. 1), demonstrating that the resulting changes in the electronic density distribution of the aromatic ring affect the reduction propensity of the disulfide bond. Moreover, the values of the half-wave potential for this family of compounds correspond well with calculated redox potential values (Fig. 5 and Table 1). It is worth mentioning, as noted in the Materials and Methods section, that various exchange-correlation density functionals yielded calculated redox potentials that ranked identically for the compounds used in this study (Fig. 6). Furthermore, the energies calculated with different DFT approximations could be made to collapse into single values for each compound by using a scaling (baseline) factor that was nearly identical for all reagents (as can be inferred from the parallel traces in Fig. 6).

Most significantly, the existence of a threshold in the reactivity of aromatic disulfides with HIV-1 p7 is apparent (Fig. 7). This behavior strongly suggests that oxidation of the thiolate groups in HIV-1 NC protein can occur only if the electrophilic reagent exceeds a redox potential (or electron affinity) preset by the metal cluster target. Since metal clusters with different thiolate/imidazole compositions exhibit different electronic distributions in their HOMO and LUMO (Topol et al. 1998a,b), dissimilar reactivity with electrophilic agents would be expected for proteins carrying various metal cluster types. Reagents capable of inducing chemical attack of the more oxidation-susceptible CCHC (but not CCHH) zinc fingers are thus conceivable, based on available evidence (Maynard et al. 1998; Topol et al. 1998a,b). Preliminary $E_{1/2}$ measurements carried out at various pH values support the involvement of an ionization

process in the reaction with NCp7 of certain electrophilic compounds, and suggest that disulfides with enhanced reactivity in the low pH range assumed in the intravirion environment may be used for further enhancement of reaction selectivity between retroviral and cellular zinc fingers.

Discussion

Quantitative structure-activity relationship (QSAR)

A simple statistical analysis demonstrated with high confidence a significant correlation between the calculated electron affinity values and the experimental NC reactivity numbers (Fig. 7). This finding supports the assumption that the redox potential of a given compound is an important determinant in its reaction with NCp7. In this regard, the Hammett σ descriptor used in the analysis of aromatic disulfide reactivity can be mirrored by consideration of the target's thiophilicity, described as the ability of a nucleophile to attack a sulfur atom. It has been demonstrated that the nucleophilicity (thiophilicity) of a thiolate correlates with the oxidation potential of the RS^- ion (Foss 1961). Thus, if the thiophilicities of thioanions for various targets differ, their reaction rates with a given electrophile will also be different. The pH of the reaction conditions would also modulate greatly the reaction rate, as it is affected by the pK_a of the conjugate acid. Therefore, it appears relevant to also characterize by electrochemical methods the oxidation properties of zinc fingers carrying various divalent ion chelation centers.

Additional effects that may influence the reaction of NCp7 with electrophiles can be considered. Given that the oxidation process appears to be of a collisional nature, reaction rates would be expected to depend on the rate of productive encounters between the disulfides and NCp7. However, differences in diffusion rates due to dissimilar molecular weights would be limited in the narrow congeneric series chosen, and were not analyzed further in the present study. We also found little dependence of the reaction rate on the ionic strength or buffer type. On the other hand, significant effects of solvent polarity and pH on the NCp7 reactivity of phenyl disulfides were observed (not shown), and are currently the subject of a more detailed investigation.

The relatively small congeneric series used in this study shows that the structural determinants of NCp7 reactivity in a chemical compound can be elucidated. Additional insight can be obtained from the use of a larger, systematically constructed series. For instance, all of the compounds used in this study are symmetrically substituted phenyl disulfides, with the exception of compound IV, which has two dissimilar substituents in the *para* positions: a methyl and a nitro group. The magnitude of the values of electron affinity calculations, electrochemical measurements, and NC reac-

Fig. 5. Plot of $E_{1/2}$ versus electron affinity calculated as in equation 2 in Materials and Methods.

Fig. 6. Calculated gas phase electron affinities for nine disulfide compounds assayed, based on various DFT approximations.

tivity measurements (Table 1 and Fig. 7) all show that for compound IV there are higher than the arithmetic mean of the symmetric methyl and nitro *p*-substituted species (compounds III and VIII). This result may be related to the fact that in a homolytic S-S bond cleavage of a symmetric disulfide, either moiety can be the leaving group; in an asymmetric disulfide, however, the choice of leaving group will be skewed toward the more stable moiety. Further modulation of the reaction properties of a given disulfide may be achieved by synthesis of asymmetric compounds that combine favorable oxidation potential at one sulfur center with improved leaving ability of the second moiety. In particular, the ionizing power of the solvent or the dielectric character in the microenvironment of the reactive thiolate may affect very sensitively the nucleophilic reaction under conditions where the initial attack is rate-determining, suggesting that further examination of the reaction's transition state is warranted.

Biological implications of the results

Disulfide compounds have been tested in antiviral assays in vitro as well as in vivo in virus-infected rodents, and their use brought about delayed disease onset and an improved outcome for the infected animals (Ott et al. 1998). In particular, aldrithiol-2 has shown promising NCp7 and HIV-1 inactivation properties, and has been utilized to generate conformationally competent, noninfectious SIV preparations for use in whole-killed viral vaccine strategies (Arthur et al. 1998; Rossio et al. 1998). Our current results set the stage for a more detailed computational, chemical, and bio-

physical study of aldrithiol-2 (compound II) and related compounds to better understand their reaction mechanism with retroviral NC proteins. The predictive power of redox potential measurements or calculations may be an important tool in the design and synthesis of small organic compounds for which there is preliminary evidence of favorable biological properties: selectivity for CCHC zinc fingers, low toxicity, low molecular weight, long shelf life, and low cost.

Conclusion

Our investigation of the redox properties of electrophilic compounds shows that the rate of reaction with HIV-1 nucleocapsid protein increases in a linear manner with oxidative power. The correlation between reactivity (measured as

Fig. 7. Plot of NC reactivity values versus calculated electron affinities. A linear dependence and the presence of a threshold point are apparent. Vertical dashed line is arbitrarily placed to segregate reactive from unreactive compounds.

NC value) and calculated redox potential (Fig. 7), although impressive, is obviously not simple. It is worth noting that, rather than a reflection on the metric used for quantifying the reactivity or inaccuracies in the redox potential calculations, such complex behavior probably stems from steric and chemical details of the interaction of the various disulfide compounds with the retroviral zinc finger. Our finding that a significant correlation can be established between the redox potential of an electrophilic compound and its reactivity toward NCp7 strongly suggest that redox potential is the single most relevant (albeit not the sole) parameter that determines the reactivity of an electrophilic agent with a retroviral metal cluster. Moreover, a threshold exists below which no reaction with HIV-1 p7 can be detected in our standard assay. Since DFT analysis of these electrophilic reagents yields a ranking of redox propensities analogous to that found in polarographic experiments, we propose that the redox properties of compounds untested experimentally can be predicted *ab initio*. Conversely, knowledge of the oxidation potential of a compound (which has been determined for a large number of chemical species) would permit an initial prediction of its reactivity against NC proteins. For instance, if the redox potential of an untested compound was below that of compounds known to be below the reactivity threshold uncovered in our study, they would be predicted to be unreactive and prescreened out of a test series. This may prove useful for compounds available in amounts insufficient for application of electrochemical techniques or other *in vitro* assays, or unsuitable due to their solubility or chemical properties. Most important, however, may be the capability of predicting the reactivity of agents not yet synthesized and thus directing a drug design effort in a rational manner. Note that the confidence in the predictions of DFT calculations is supported internally by the similarity of the results obtained using various computer protocols, as well as by the most relevant test: agreement of their forecast with experimental results. As a large number of compounds for which the reduction potential has been determined exists, it should be straightforward to extract a threshold E° for NC targets from reference handbook data. Actual E° values (as opposed to $E_{1/2}$ measurements that are influenced by kinetic effects) may improve the observed correlation, and are currently being obtained from cyclic voltammetric experiments to allow such reactivity forecasting. The reliability of QSAR predictions is expected to increase as additional compounds are entered into the database. In addition, the behavior of chemical entities described here, as "outliers" from the main trend, may be further characterized. Our current data strongly indicate that leaving groups in the *ortho* position has a strong effect on the initial oxidation event. We suggest that steric influences on the leaving thiolate may contribute to the observed anomalies. Compounds with enhanced reactivity (e.g., the 2-amino substituted compounds in this study), if better understood, may prove advantageous in

regard to their activity or specificity toward a particular target.

Our results also indicate that the good correlation found in most cases between the reaction rate and a drug's redox activity is due to the fact that the oxidation reaction takes place after a random collisional event. As such, a dependence of reactivity on diffusion rates would be predicted. We have not attempted to analyze our data in this regard; however, correction factors that account for diffusion can be introduced in future studies. For two compounds that have been investigated in detail from a mechanistic standpoint (the thiol alkylating agent NEM and the disulfide bridge-forming aldrothiol-2), the rate of reaction and the initial site of attack with retroviral zinc finger peptides were found to depend on the flanking sequences of the zinc finger target. We also observed previously differences in reactivity between peptides carrying retroviral (CCHC) metal cluster sequences versus transcription factor (CCHH) or steroid receptor (CCCC) chelation spheres. Thus, our findings indicate that compounds that are able to attack HIV-1 NCp7 with enhanced reactivity may possess weak activity against other cellular targets, either as a result of steric protection of their cysteine groups or their intrinsically lower reactivity compared to that of the retroviral metal cluster environment. In addition, preliminary results suggest that the particular pH and dielectric environment of the NC Cys thiolate target within the virion may further amplify its selective reaction with electrophilic agents.

We have shown that aromatic disulfides are part of a general class of electrophilic chemical agents that can be used in HIV inactivation, and in this work we obtained evidence of the theoretical predictability of their reactive properties. In addition to their therapeutic potential, the chemical selectivity of these agents renders them advantageous in the generation of candidate antigens to be used in whole-killed virus vaccine strategies (Rossio et al. 1998).

Materials and methods

Chemicals

Disulfide reagents (purchased from Aldrich Chemical Co.) were dissolved as 1 mmol stocks in spectral-grade dimethyl sulfoxide (DMSO; B&J Brand; UV grade, Burdick & Jackson), and were stored at -20°C . Acetonitrile and H_2O (UV grade) were obtained from EM Science. Trifluoroacetic acid (TFA, HPLC/Spectra grade) was from Perkin Elmer (Applied Biosystems Div.). Tris (ULTROL^R Grade) was from Calbiochem.

HIV-1 recombinant NCp7

The coding sequence of the NC domain of HIV-1 was cloned into the pMal-c vector, and expressed as a fusion protein in *E. coli* (Wu et al. 1996). After factor Xa cleavage, the 55-residue NC protein

was purified by HPLC (Wu et al. 1996), complexed with two equivalents of Zn^{+2} , and stored as a lyophilized powder at -70°C .

Reaction of NCp7 with disulfide compounds

HIV-1 NCp7 protein (7.5 μM), either containing 15 μM ZnCl_2 or as an apo-peptide, was reacted with a 6-fold excess of a given disulfide reagent (*R-S-S-R*) in 20 mM Tris-HCl, pH 7.0 at 37°C for 1, 10 or 60 min. Reactions were stopped by injection of the reagent mixtures into the HPLC apparatus. The reactants and reaction products were separated by reverse-phase HPLC (Fig. 2) on a Vydac 5C₁₈-300 column (2.1×150 mm) at 0.3 mL/min using a Shimadzu HPLC system equipped with LC-10AD pumps, an SCL-10A system controller, a CTO-10AC oven, an FRC-10A fraction collector, and an SPD-M10AV diode array detector. The elution gradient evolved from Buffer A (0.1% TFA in water) to Buffer B (0.1% TFA in acetonitrile) as follows: 0–16%, 5 min; 16–25%, 20 min; 25–80%, 10 min; 80%, 5 min. Elution peaks were detected in a flow cell at 206 and 280 nm. Quantification for calculations of NC values was based on the integrated area of absorption peaks monitored at 206 nm, and was determined at least in duplicate. Results for initial reaction rates from HPLC reactivity assays are listed in Table 1 for a reaction time of 1 min.

Electrochemical characterization

Polarography experiments were performed on a subset of the congeneric disulfide compounds described above, determined by the availability and sufficient solubility of the reagent. The first series of experiments were done using 100 mM tetrabutylammonium fluoroborate (Aldrich Chemical) as the supporting electrolyte in acetonitrile (Fisher Scientific) or methanol (Fisher Scientific). Subsequently, DMSO (Aldrich Chemical) was used for all compounds due to its better solubilization properties. Analytes (from Aldrich) that had sufficient solubility in all three solvents were used to assess the occurrence of solvent effects. All materials were used without further modification.

Blanks were run and subtracted from the analyte signal; the background-subtracted signal was used to determine the $E_{1/2}$ values reported in this work. The system used to perform the experiments consisted of a BAS-CV-50W electrochemical analyzer (Bioanalytical Systems) with a controlled-growth mercury electrode cell. The auxiliary electrode was a Pt wire, and the reference electrode was Ag/AgCl. The disulfide samples were dissolved in the supporting electrolyte, and deoxygenated with filtered N_2 for 10 min prior to analysis. The experiments were carried out using normal pulse polarography (NPP) with a sample width of 17 msec, a scan rate of 4 mV/sec, a dropping time of 1000 msec, and a pulse width of 50 msec. A dropping mercury electrode was used, and the current was sampled at a time such that each drop was of the same size. This protocol results in a smooth sigmoidal-shaped wave. Values of the half-wave potential, or $E_{1/2}$, were calculated using the first derivative of the resulting curve. Each sample was run in triplicate.

Density functional theory calculations

Formally, the reduction of a molecule A in solution can be represented by the following equation:

The free energy of this reaction [$\Delta E_\text{s}(\text{A} \rightarrow \text{A}^-)$] corresponds to the absolute redox energy for the above process. The free energy of an electron (e^-) at rest in the gas phase is set to zero (Zhang and Friesner 1995). Then, it is possible to calculate the redox energy of reaction (1) using the thermodynamic cycle presented in Figure 8. In this cycle, $\Delta G_\text{s}(\text{A})$ and $\Delta G_\text{s}(\text{A}^-)$ are the solvation energies of molecule A and its anion A^- , respectively, and $\Delta E_\text{g}(\text{A} \rightarrow \text{A}^-)$ is the energy difference between molecule A and its anion (which is defined as the redox energy in the gas phase). On the basis of this thermodynamic cycle, one can obtain $\Delta E_\text{s}(\text{A} \rightarrow \text{A}^-)$, the absolute redox energy, as

$$\Delta E_\text{s}(\text{A} \rightarrow \text{A}^-) = \Delta E_\text{g}(\text{A} \rightarrow \text{A}^-) + \Delta G_\text{s}(\text{A}) - \Delta G_\text{s}(\text{A}^-) \quad (2)$$

Thus, by calculating the gas phase energies and solvation energies of molecule A and its anion A^- , one can derive the absolute redox potential (scaled) of molecule A in solution. A scaling coefficient that translates electron affinity to standard redox potentials can be thus extracted.

The calculations for all molecules (and their anions) considered in this work were performed using DFT methods as implemented in the UniChem 3.0 (Dgauss 3.1) (Andzelm and Wimmer 1992) and Gaussian 94 (Frisch et al. 1995) program packages. In the initial stage of the DFT calculations, the Dgauss program was employed for geometry optimizations of all systems using BLYP exchange-correlation functional (Lee et al. 1988; Becke 1993) and DZVPD basis set (Rashin et al. 1994a,b). The calculations of the gas phase energies, and of their differences $\Delta E_\text{g}(\text{A} \rightarrow \text{A}^-)$ as introduced in equation (2), were performed at optimized geometries using Gaussian 94 with the hybrid exchange-correlation potentials B3LYP (Lee et al. 1988; Becke 1993) and B3P86 (Perdew 1986; Becke 1993) and the DZVPD and 6-311+G(d,p) gaussian basis sets.

The free energies of solvation, $\Delta G_\text{s}(\text{A})$ and $\Delta G_\text{s}(\text{A}^-)$ in equation 2, were calculated using a reaction field approach based on the boundary element method (BEM) (Rashin 1993). The effective atomic charges used in the BEM calculation of solvation energies were obtained from fitting to the B3P86/DZVPD electrostatic potentials for neutral and anionic systems at the optimized gas phase geometries. We showed that a combined B3P86/DZVPD-BEM approach provides agreement between experimental and theoretical solvation energies to within 1.5 kcal/mol for 50 small molecules (Rashin et al. 1994a,b).

In the present study, we considered different DFT functionals to investigate the dependence of calculated redox potentials on the particular type of exchange and correlation functionals used. Although it is known that the calculated proton affinities of molecules show only a minor dependence on the form of the DFT functional (Schmiedekamp et al. 1994; Topol et al. 1998a,b), this

Fig. 8. Thermodynamic reaction cycle used in the theoretical calculation of redox potentials.

could not be assumed for calculations of negatively charged radicals, as there is scant information about the dependence of anion-radical energies on the exchange-correlation functional used in DFT calculations. Figure 6 presents the gas phase redox energies for the set of disulfide molecules examined in the present study calculated with BLYP, B3LYP, or B3P86 functionals while using the same DZVPD basis set. It is obvious that there is a strong dependence of the value of the redox potential in the gas phase on the approximations used in the construction of exchange and correlation functionals (Fig. 6). This dependence is mainly due to differences in the anion-radical description by different correlation potentials; variations in exchange energies (e.g., BLYP vs. B3LYP) have a weak influence on the calculated energies, yielding gas phase redox potentials within 2 kcal/mol of each other. It is worth noting that relative changes in the redox potential among compounds are similar for all DFT functionals used (Fig. 6). Thus, any of the aforementioned functionals can be used to investigate the correlation between calculated redox energies and experimental redox potentials measured in different solvents; the differences between sets simply reflect a baseline shift in the calculated energies, which retain the same rank order. The data listed in Table 1 and depicted in Figures 5 and 7 were obtained using a B3P86 functional. The calculated values of redox potential in aqueous solvent for a given compound were subsequently compared with its reactivity toward HIV-1 nucleocapsid protein.

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