Delocalized Electronic Structure of the Thiol Sulfur Substantially Prevents Nucleic Acid Damage Induced by Neocarzinostatin†

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ABSTRACT: Neocarzinostatin is a potent antitumor antibiotic and is a prodrug, which induces genome damage after activation by a thiol. The prodrug is stored as a protein-bound chromophore that contains an enediyne nucleus. A thiolate attack on the chromophore cyclizes the nucleus and produces radicals that abstract hydrogen from DNA. Because thiol is the only cofactor in the vital activation process, the structure of the thiol plays an important role in the activity of the drug. Here we systematically examine the effect of the electronic structure of some thiols on the efficiency of the drug, and compare particularly aromatic with aliphatic thiols. The values of drug-induced base release from DNA are remarkably different between thiophenol (3.6%) and benzyl mercaptan (12.5%), the activity of which is comparable with those of aliphatic thiols. Cleavage results determined by DNA electrophoresis are consistent with the results of base release; they show that the total number of DNA lesions is more than 3-fold lower for thiophenol than for aliphatic thiols or benzyl mercaptan. We conclude that among aromatic thiols, only those that have delocalized thiol sulfur electrons can substantially reduce the DNA cleavage activity. This result suggests that the effect of an aromatic ring arises from an inductive effect imposed on the thiol sulfur electron through π-resonance rather than through effects such as aromatic stacking, steric hindrance, or hydrophobic interaction. Replacing thiophenol with substituted derivatives with electron-releasing or -withdrawing groups changes the drug activity and supports the important role of the electronic structure of the thiol sulfur in determining the drug activity.

Neocarzinostatin (NCS)[1] (*1*−*4*) is a natural prodrug that contains an enediyne warhead, which is responsible for its very potent cytotoxic activities. Like radiotherapy, NCS exerts its antineoplastic effects through damage to the cellular genome (*5*). To trigger the DNA cleaving activity, the enediyne needs to be converted to a lethal radical bullet by a thiol activator. This feature is essential for controlling and minimizing toxic side effects of the resulting antitumor compounds. Because thiol is the only cofactor in this vital activation process, the structure of the thiol can play an important role in the activity of the drug. Consequently, studying the influence of the thiol structure not only is of biochemical interest but also is important for drug development.

It is known that both thiol and oxygen (*3*, *6*) are vital for the reaction of NCS with DNA in general, although recent studies indicate that cleavage of certain bulged conformations of DNA is thiol-independent (*7*, *8*). Because molecular oxygen is involved in DNA cleavage only after hydrogen abstraction has occurred (*3*), thiol becomes the sole cofactor in the important initial action of the drug in causing DNA damage. Scheme 1 shows how a thiol is involved in the mechanism of activation for this prodrug. NCS is the first enediyne chromoprotein whose chromophore (NCS-C), with an unusual bicyclic dienediyne moiety (*9*), is the key center in causing DNA damage. A nucleophilic attack by an activating thiol at C-12 of NCS-C results in carbanion **1**, which generates cumulene **2** (*10*, *11*) and subsequently cyclizes to intermediate **3** with active radical centers at C-2 and C-6. Both centers abstract hydrogen from DNA (*12*) or other sources (*13*) to form an inactive thiol adduct **4**. The NCS-induced hydrogen abstraction from DNA, which causes oxidative strand scission (*14*), occurs primarily at the sugar moiety of T and A residues (*3*). Dioxygen can add to the carbon-centered radical of DNA to form a peroxy radical (*15*). Because ∼2 mole of sulfhydryl groups are consumed

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[1] Abbreviations: NCS, neocarzinostatin; NCS-C, neocarzinostatin chromophore; apoNCS, apoprotein component of neocarzinostatin; holoNCS, chromoprotein of neocarzinostatin; AET, *S*-(2-aminoethyl)-isothiouronium bromide; BSH, benzyl mercaptan; PhSH, thiophenol; HTP, 4-hydroxythiophenol; BME, β-mercaptoethanol; DTT, dithiothreitol; CYS, cysteine; GSH, glutathione; Tris, tris(hydroxymethyl)-aminomethane; P:D, molar ratio of DNA nucleotide phosphorus to drug; SS, single-strand (breaks or lesions of DNA); DS, double-strand (breaks or lesions of DNA); TS, total strand (breaks or lesions of DNA).

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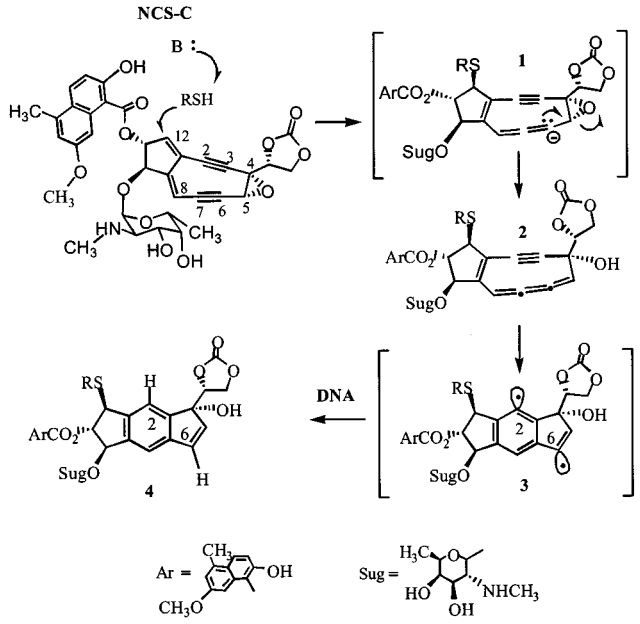
Scheme 1: Thiol-Induced Activation Process of NCS-C



per mole of NCS-C in the presence of DNA (*16*), the peroxy radical degrades and causes final DNA damage presumably following a reduction by thiol. Thus, the role of thiol seems to be multifunctional and is not limited to drug activation. Experimental results show that preincubation with a thiol, or reaction with DNA in excess thiol, results in rapid inactivation of the drug (*17*). It has long been recognized that the extent of DNA damage depends somewhat on the type of thiol, although reported variation in the total number of DNA lesions has not exceeded 1.6-fold (*17*, *18*). The ratio of single-strand (SS) to double-strand (DS) lesions induced by NCS is also found to be thiol-dependent (*18*). In addition, the structure and charge of the activating thiol also influence kinetic partitioning between various sites of attack on deoxyribose (*3*, *19*). Apparently, although the mechanism is not fully understood, the thiol structure plays very important roles in both activation and execution of the DNA cleavage induced by NCS. For these reasons, it is essential to correlate systematically the thiol structure to the activity of the antitumor warhead.

In addition to important amino acids or peptides, such as cysteine (CYS) and glutathione (GSH), many other thiols are abundant in nature. Most of the earlier studies have focused on aliphatic thiols. We also consider here the effect of an aromatic ring, which has a very different geometric and electronic structure. We systematically compare the effects of aliphatic and aromatic thiols and use a series of aromatic thiols that vary in their functional substituents. For example, thiophenol (PhSH) is compared with benzyl mercaptan (BSH), and electron-releasing thiols are compared with electron-withdrawing ones, etc. We aim to study whether and why the aromatic structure of the thiol can affect the DNA lesions caused by NCS.

MATERIALS AND METHODS

NCS Chromophore (NCS-C). NCS-C is extracted from holoNCS that is a gift from Kayaku Co., Ltd. (Itabashi-Ku, Tokyo, Japan). The fluffy powder, which contains a 1:1 apoNCS:NCS-C ratio, is dissolved in water and stored in the dark at −80 °C. The concentration of the chromoprotein stock (0.5−1.5 mM) is determined from A_{340} (ϵ = 10 800 M^{-1} cm^{-1}). The chromophore extraction procedure is modified from the method for a clinical ampule (*20*). Before lyophilization, 1 M sodium citrate buffer (pH 4.0) is added to holoNCS stock to a level of 20 mM in the final extracted solution. After lyophilization, methanol is introduced and the mixture is incubated at 0 °C for 8−15 h before the protein is spun at 8000 rpm for 30 min at 0 °C. The supernatant methanol solution of NCS-C is stored in an amber glass vial at −80 °C. The integrity and concentration of NCS-C are checked by A_{340} using excess apoNCS to reconstitute NCS-C. By repetitively treating the remaining protein pellet four or five times with citrate-acidified methanol, we can increase the yield of NCS-C up to 80%. The diluted mixtures can be incubated at 0 °C for more than 2 days without noticeable decomposition, probably because the very high level of apoNCS stabilizes NCS-C.

DNA. Calf thymus DNA, which is from Sigma (St. Louis, MO), is sonicated (∼400 bp) before purification. After S_1 nuclease treatment, repetitive phenol extractions, precipitation, and dialyzation [against 0.1 M EDTA (pH 7)], purified DNA is dialyzed against 20 mM sodium citrate buffer (pH 4). [*methyl*-^{3}H]Thymidine-labeled λ DNA (specific activity ≈ 1.8×10^4 cpm/μg) is prepared from tritium-labeled thymidine, and the final DNA solution is also stored in 20 mM sodium citrate buffer (pH 4). The supercoiled ϕX 174 RF DNA (90% form I) is obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Base Release. The NCS-induced DNA damage activated by a thiol is determined by measuring the total level of thymine release. All thiols (best grade available) are from Aldrich, Fluka, or Sigma, of Sigma-Aldrich, Inc. An aliquot of the DNA mixture [total molar ratio of nucleotide phosphorus to drug (P:D) equal to 10], 15 μM NCS-C (final concentration), a thiol at different concentrations, and 3 mM diethylenetriaminepentaacetic acid buffer (pH 4) are mixed at 0 °C. The DNA mixture consists of a 2.5:1 molar ratio of nucleotide of calf thymus DNA to [*methyl*-^{3}H]thymidine-labeled λ DNA. The total methanol content of the reaction mixture is ≤20% v/v. The NCS−DNA reaction is triggered by adding 62.5 mM (final concentration) Tris-HCl (pH 8) to increase the pH of the solution. After incubation for 30 min at 25 °C, a 10 μL aliquot (10%, v/v) of the solution is used to determine the total amount of radioactivity in the sample. The rest of the solution is adjusted to pH 13 with 1 M NaOH and is heated at 90 °C for 30 min. After neutralization with 1 N HCl, it is subjected to analysis via determination of the total level of thymine release by paper chromatography. The running solvent consists of 0.3 M sodium acetate in 70% ethanol, and the running period is approximately 5 h. The paper is cut into 0.5 in. long sections and mixed with 0.3 mL of water. After the mixture sits for 15 min at room temperature to ensure complete dissolution, hydrofluor, a scintillation fluid from National Diagnostics (Atlanta, GA), is added. The amount of radioactivity in each segment is counted a minimum of three times by a Searle Delta 300 liquid scintillation counter.

Agarose Gel Electrophoresis. The extent of DNA strand damage induced by NCS-C is monitored by following the conversion of supercoiled DNA (form I) to its relaxed circular (form II) and linear duplex form (form III). The

chemical reaction is initiated by a pH change upon addition of 50 mM (final concentration) Tris-HCl buffer (pH 7.4) to a mixture of ϕX 174 RF DNA (50 ng), 5 mM EDTA, 5−10 nM NCS-C (final concentrations), and one of the following thiols: *S*-(2-aminoethyl)isothiouronium bromide (AET), BSH, or PhSH. Except for thiol concentration dependence experiments, the optimum concentration of the thiol, i.e., 0.4 mM AET, 0.8 mM BSH, or 0.15 mM PhSH, is applied to achieve maximum levels of cleavage. The total methanol content of the reaction mixture is ≤20% (v/v). The concentration of NCS-C is well below that of plasmid (P:D ratio of 940−1880) to avoid production of DNA fragments. To measure the number of direct breaks, reaction mixtures (total volume of 16 μL) are incubated at 0 °C for 30 min. To convert abasic site lesions to strand breaks, either hydrazine (1 M, pH 8) or putrescine (1 M, pH 8) is added to a final concentration of 100 mM. The solution is then incubated further at room temperature for 1 h. After incubation, each sample, untreated or treated, is added to 4 μL of a gel loading solution that contains bromophenol blue before it is loaded into a 1% (w/v) 12-well agarose horizontal slab gel. Following electrophoresis at a constant voltage, the gels are immersed in a running buffer with 0.5 μg/mL ethidium bromide for 15−30 min for analysis under UV light.

Quantitation of SS, DS, and TS Breaks Produced by NCS. The topological forms in the drug-treated ϕX 174 RF DNA are analyzed by an Alpha Imager 2000 from Alpha Innotech Corp. using a fluorescence photographic technique. Fractions of forms I, II, and III are determined by integration of each band. Because of the reduction in the level of ethidium staining in form I, a correction is made to the form I signal given its 70% fluorescence intensity compared with forms II and III. The number of total strand (TS) breaks per molecule of DNA induced by the thiol-activated drug (N_{TS}) is determined by assuming a Poisson distribution (*18*, *21*):

$$N_{TS} = -\ln(f_I/[f_1]_o) \quad (1)$$

where f_I is the fraction of form I DNA remaining after cleavage and $[f_1]_o$ is the fraction of form I in the control sample. The control experiment is carried out side by side with the sample under the same condition except that an applied buffer is added to replace thiol. The quantity of DS breaks per molecule of DNA (N_{DS}) is calculated from the fraction of form III that is induced specifically by NCS:

$$\ln(N_{DS}) - N_{DS} = \ln[(f_{III} - [f_{III}]_o)/[f_1]_o] \quad (2)$$

where f_{III} is the observed fraction of form III from the sample and $[f_{III}]_o$ is the fraction of form III in the control sample. Because only forms I and II are detectable in most controls, eq 2 can be simplified to

$$\ln(N_{DS}) - N_{DS} = \ln(f_{III}/[f_1]_o) \quad (3)$$

The quantity of TS breaks is the sum of the observed number of SS and DS breaks, and the number of SS breaks (N_{SS}) can thus be estimated by

$$N_{SS} = N_{TS} - N_{DS} \quad (4)$$

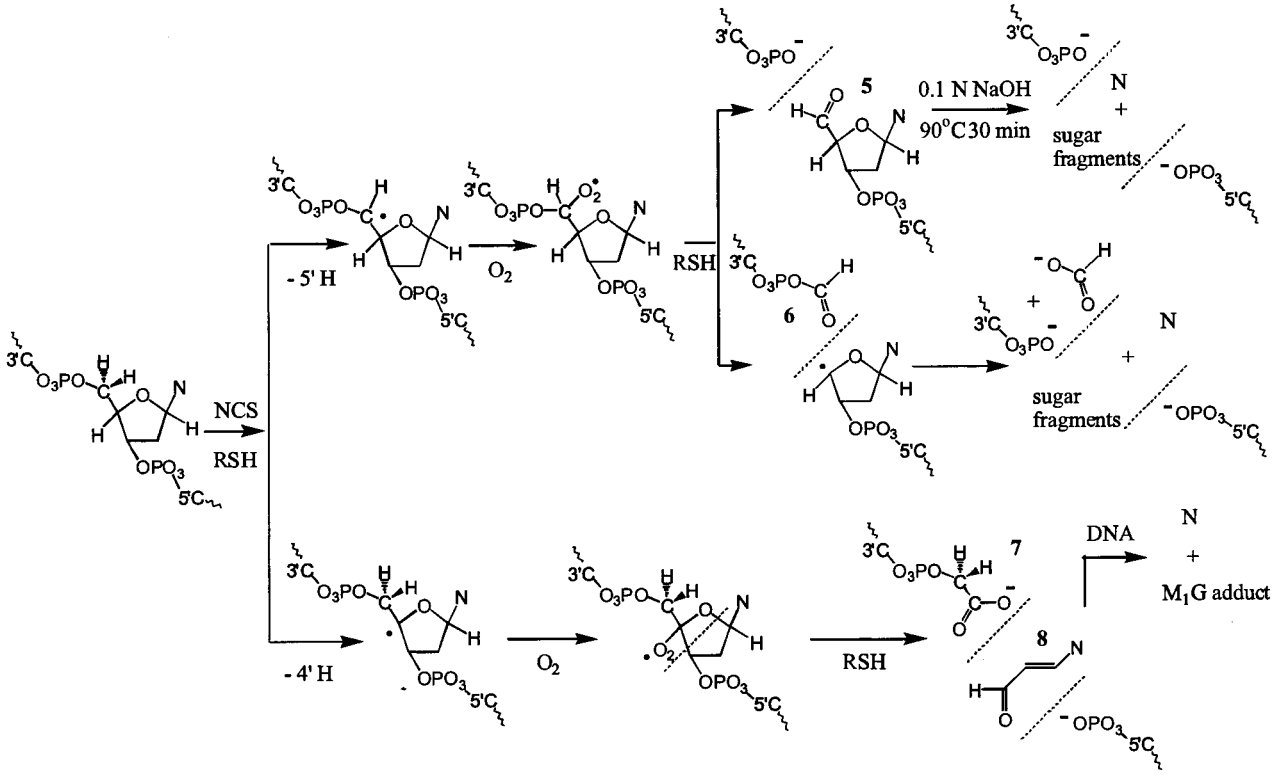
RESULTS

Assessing base release from intact DNA has long been used as a tool for estimating the efficiency of genome damaging agents such as bleomycin (*22*), or radiation (*23*). Because 75% of the NCS-induced lesions occur at T residues (*3*), we synthesize radioactive [*methyl*-^{3}H]thymidine-labeled λ DNA to quantify the release of free base caused by the drug action so that we have a sensitive probe for studying the effect of thiol structure. The total level of drug-induced thymine release represents satisfactorily the extent of total DNA damage induced by the drug. As a different approach to studying the effect of thiol structure, the DNA strand breaks induced by NCS-C and mediated by different thiols can be monitored by following the conversion of supercoiled DNA (form I) to its relaxed circular (form II) and linear duplex form (form III). Analysis of these topological forms, whose cleavage follows the Poisson distribution (*21*), allows the total strand (TS) breaks to be further quantified into SS and DS breaks. Such information is useful because the number of DS breaks is considered to correlate with the level of cell killing in human tumors (*24*). In addition to the major lesion produced by direct strand breaks, abasic sites without strand breaks can be induced by NCS as well. Various abasic site lesions can be converted to strand breaks with differing levels of sensitivity by alkaline treatment. Here we use hydrazine, which is known to react predominantly with abasic sites derived from 4′-H abstraction (*25*), and putrescine, which cleaves virtually all abasic sites (*26*), to cleave abasic site lesions. The results from both assays are presented as follows.

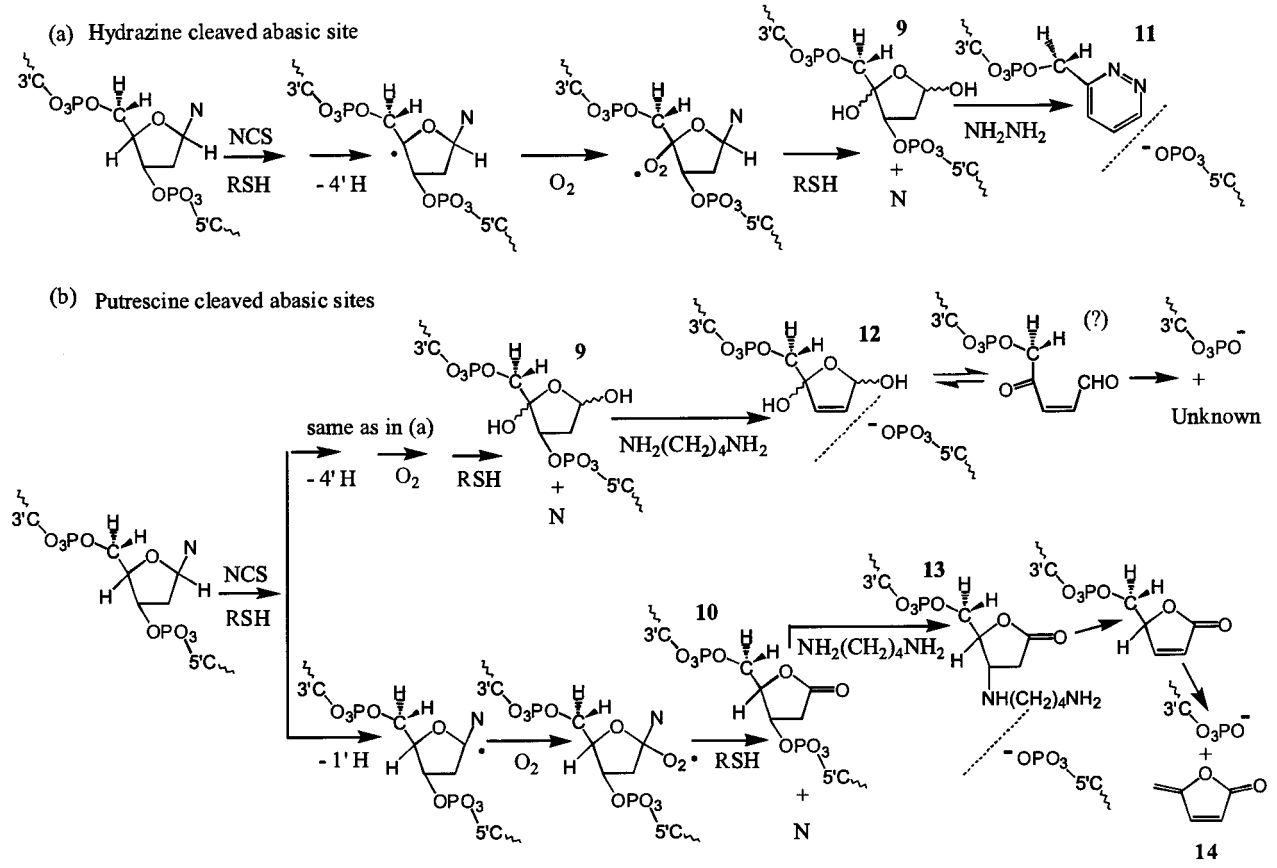
Base Release. Both Schemes 2 and 3 show the mechanism of NCS-induced DNA damage. Spontaneous thymine release is mainly produced from fewer than 20% of the direct strand breaks via 5′-H abstraction; these produce gaps with phosphate at each end (Scheme 2). Other spontaneous base release results from 1′- and 4′-chemistry via either direct strand breaks (Scheme 2) or abasic site lesions (Scheme 3). Major thymine release occurs after strong alkaline treatment, mainly from the breakdown of 5′-thymidine 5′-aldehyde **5**, which is produced by more than 80% of the direct strand breaks via 5′-H abstraction (Scheme 2). Treatment with 0.1 N NaOH at 90 °C probably causes all DNA lesions to result in base release. Using [*methyl*-^{3}H]thymidine-labeled DNA as a probe, the total base release can be quantitatively analyzed by chromatography. The results, expressed as a percentage of the level of thymine release on the basis of the total amount of thymine in DNA, are summarized in Table 1.

Base Release from Aliphatic Thiols. In general, the extent of the drug-induced DNA damage is dependent on the thiol concentration (*17*, *18*). Most aliphatic thiols show an obvious optimum thiol concentration, at which maximum DNA damage is produced. Below and above this level there is a sharp decrease in the drug activity. AET is one of the most efficient aliphatic thiols, because a very low optimum concentration (0.4−0.8 mM) is required (Table 1C). When the DNA:drug ratio (P:D) = 10, the total level of AET-mediated release of thymine is 12.5%, which is consistent with a value of 11.5% obtained earlier at the same P:D level (*20*). At a higher NCS level (1.5 P:D ratio), the level of DNA damage, estimated by the level of acid-soluble radioactivity, is 50% higher (*17*). It is conceivable that the actual percentage of DNA damage depends on the P:D ratio and other variables. However, the effect of thiol structure seems to remain proportionally constant from one set of conditions to another. For instance, the ratio of total DNA lesions for

Scheme 2: Major Pathways of NCS-Induced DNA Direct Strand Breaks under Aerobic Conditions



Scheme 3: Pathways of NCS-Induced DNA Abasic Site Lesions under Aerobic Conditions[a]



[a] The lesions become strand breaks by the action of (a) hydrazine and (b) putrescine.

β-mercaptoethanol (BME) to dithiothreitol (DTT) remains 1.3:1, regardless of whether it is found from measuring acid-solubilized DNA fragments when the P:D ratio = 1.5 (*17*) or from analyzing strand breaks when the P:D ratio is more than 1000-fold higher (*18*). On the basis of this property, we estimated the total level of base release for several

Table 1: Effect of the Sulfur Electronic Structure of a Thiol on the NCS-Induced DNA Damage As Determined by Base Release

thiol	DNA damage	
	this study[a]	other studies[c,d,e]
(A) aromatic thiol with delocalized sulfur electrons		
(i) conjugated with electron-releasing substituents		
2-aminothiophenol ($NH_2C_6H_4SH$)	7.5% (0.50 mM)[b]	
o-thiocresol ($CH_3C_6H_4SH$)	6.6% (0.15−0.5 mM)	
thiosalicyclic acid[f] (*o*-$HOOCC_6H_4SH$)	4.4% (0.50 mM)	
2-methoxybenzenethiol ($CH_3OC_6H_4SH$)	4.3% (0.15 mM)	
4-hydroxythiophenol (HOC_6H_4SH) (HTP)		6.0%[c]
average	5.8% (0.33 mM)	
(ii) conjugated with electron-withdrawing substituents		
2,3,5,6-tetrafluorothiophenol (HC_6F_4SH)	3.0% (0.06−1.0 mM)	
pentafluorothiophenol (C_6F_5SH)	2.5% (0.06−1.0 mM)	
average	2.8% (0.06 mM)	
(iii) Thiophenol and others		
thiophenol (C_6H_5SH) (PhSH)	3.6% (0.15 mM)	
4-chloro-*m*-benzenedithiol [$ClC_6H_3(SH)_2$]	5.5% (0.06−0.15 mM)	
2-mercaptobenzimidazole [$C_6H_4N_2HC(SH)$]	1.0% (no maximum point)	
overall average of parts i−iii	4.4% (0.19 mM)	
(B) aromatic thiol without delocalized sulfur electrons		
benzyl mercaptan ($C_6H_5CH_2SH$) (BSH)	12.5% (0.8 mM)	
(C) aliphatic thiol		
S-(2-aminoethyl)isothiouronium bromide	12.5% (0.4−0.8 mM)	11.5%[d]
[$H_2NC(SCH_2CH_2NH_2)$═NH] (AET)		19% (1.0 mM)[e]
cysteine [$HSCH_2CH(NH_2)COOH$] (CYS)	14.8%[g]	22.5% (20 mM)[e]
β-mercaptoethanol ($HSCH_2CH_2OH$) (BME)	11.2%[g]	17% (10 mM)[e]
dithiothreitol {[$HSCH_2CH(OH)]_2$} (DTT)	8.6%[g]	13% (5−7 mM)[e]
average	11.8%	

[a] The NCS-induced DNA damage is estimated in this study by the total level of thymine release after alkaline treatment on the basis of the percentage of total thymine in DNA. The P:D ratio is maintained at 10 for each reaction. [b] The optimum concentration of the thiol, which provides maximum DNA damage, is shown in parentheses. [c] Average from two studies. One is calculated as 3.3% from the reported fraction of fragments after strand cleavage of oligomer $d(GCATGC)_2$ induced by NCS that is activated by HTP (*37*). The applied concentration of HTP is 1 mM, and the P:D ratio is 6.7. The other is estimated to be 8.7% for HTP by normalization with the values obtained for BME and DTT from the same study (*18*). The total strand breaks from the NCS-mediated pBR322 is quantified from gel electrophoresis after putrescine treatment. The applied concentration of HTP is 0.2 mM. [d] From ref *20*. The NCS-induced DNA damage is calculated by the total level of thymine release after alkaline treatment. The applied concentration of AET is 0.4 mM, and the P:D ratio is 10. [e] From ref *17*. The NCS-induced DNA damage is measured from the amount of trichloroacetic acid-soluble radioactivity after NCS treatment on the basis of the percentage of total radioactive labeled λ DNA. The optimum concentration of the applied thiol is shown in parentheses. The P:D ratio is maintained at 1.5 for each reaction. [f] Under the aqueous and slightly alkaline conditions used in our study, the electron-withdrawing substituent COOH with low pK_a is mostly converted to electron-releasing carboxylate anion COO^-. [g] The calculated base release values of this study, based on normalization by experimental values obtained from AET under similar conditions.

commonly used aliphatic thiols, namely, CYS, BME, and DTT (*17*), etc., and compared the results with the value found by using AET. The results are listed in Table 1C; an average of ∼12% DNA damage for aliphatic thiols is obtained.

Base Release from Thiophenol Type Derivatives. The most prominent result in Table 1 is the fact that PhSH and its derivatives are extremely inefficient in causing total DNA damage (average of 4.4%) compared to aliphatic thiols (Table 1A). The difference in activity is far beyond expectation and is much larger than the variation among aliphatic thiols, indicating that the structural effect of an aromatic ring is extremely large compared to those of additional functional groups (−COOH, −OH, etc.) in the thiol compound. The optimum thiol concentrations of PhSH and its derivatives are in general very low, probably because the aromatic ring can intercalate into the DNA helix. Unlike the case for aliphatic thiols, no sharp drop in activity is observed by increasing the concentration above the optimum point in most cases, and the optimum concentration generally lies on a plateau. For example, *o*-thiocresol shows a plateau in drug activity from 0.15 to 0.5 mM [Table 1A (i)] and 4-chloro-*m*-benzenedithiol from 0.06 to 0.15 mM [Table 1A (iii)]. For both 2,3,5,6-tetrafluorothiophenol and pentafluorothiophenol, the drug activity reaches a maximum at 0.06 mM and does not drop even at a level of 1 mM [Table 1A (ii)].

The aromatic thiols that contain delocalized sulfur electrons are grouped in Table 1A according to whether substituting groups are electron-releasing or -withdrawing. NCS seems to exhibit a much lower activity (average of 2.8%) for thiol compounds that contain electron-withdrawing groups and a higher activity for thiols with electron-releasing groups. This correlation suggests that higher electron density on the sulfur atom of the sulfhydryl group can help the drug perform better.

Base Release from Benzyl Mercaptan and Its Implication for an Inductive Effect. By replacement of an aliphatic thiol with PhSH, a 3.5-fold lower drug activity is observed [Table 1A (iii)]. Because the effect of an aromatic ring structure on activity is very evident compared to that using aliphatic thiols, the role of an aromatic ring needs to be further clarified. NCS-C binds to the minor groove of DNA through intercalation of the naphthoate group (*3*). The aromatic ring of PhSH, which is present as a partial structure of the radical intermediate **3** via thiolate addition (Scheme 1), has the potential to change the affinity of the drug for DNA. On the other hand, the bulky or inflexible planar aromatic ring may alter the geometry of the drug's binding conformation in the closely packed DNA helix. The radical **3** is highly reactive but poorly selective (*10*). Slight changes in conformation upon binding can alter the preferred distance for H abstrac-

tion between the deoxyribose hydrogen and the C-2 and C-6 radical centers, and this can lower the rate of DNA cleavage.

To test these hypotheses, we examined BSH, which also contains a phenyl ring, for comparison with PhSH. BSH and PhSH have similar hydrophobicity and geometric structures. The major difference between BSH and PhSH is whether sulfur electrons can be freely delocalized into the aromatic π-system through bond conjugation. Surprisingly, Table 1B clearly shows that the drug activity is fully restored when BSH replaces PhSH. This result demonstrates that neither an aromatic stacking interaction nor steric hindrance of the phenyl ring plays an essential role in decreasing the activity of the drug. Because polarity is approximately the same for BSH and PhSH, the main factor that contributes to the activity of the drug has to be the inductive effect through adjacent bonds. Because the electron-releasing or -withdrawing groups obviously affect the drug activity, decreasing the electron density on the sulfur atom via aromatic π-resonance must play an important role. The more the sulfur electron is delocalized, the lower the observed drug activity. Part iii of Table 1A shows that 2-mercaptobenzimidazole, which contains a fused imidazole−phenyl ring that gives a higher degree of electron delocalization than the phenyl ring alone, almost totally quenches the drug activity.

Strand Breaks. DNA strand breaks induced by NCS are monitored by following the decrease in the level of supercoiled DNA (form I). Among all the thiols we studied, three were chosen for detailed strand break analyses. PhSH, which is neutral (without electron-releasing or -withdrawing substituents to influence the sulfhydryl functional group), is chosen to represent aromatic thiols whose sulfur electron can be delocalized into the π-ring system. BSH, whose structure is similar to that of PhSH, is chosen to represent aromatic thiols whose sulfur electron is not delocalized. AET is chosen to represent aliphatic thiols, because the drug activity found with AET is close to the average of aliphatic thiols and its optimum concentration is similar to that of aromatic thiols.

Direct Strand Breaks. Scheme 2 shows that NCS-induced direct strand breaks can result from 5′- and 4′-chemistry. From 5′-H abstraction, either an aldehyde **5** at the 5′-end (*27*) or a high-energy 3′-formyl phosphate **6**, which breaks down to formate and sugar fragment (*28*), is produced. Similarly, a 3′-phosphoglycolate residue **7** and a base propenal **8** (*29*, *30*) are produced from 4′-chemistry. A recent study (*31*) indicates that base propenal **8** can further react with DNA to form an M_1G adduct, which is known to be mutagenic.

Using an analysis based on the Poisson distribution, the total strand breaks are further borken down into SS and DS breaks. Figure 1 shows the quantities of direct DS, SS, and TS breaks when NCS is mediated with AET, BSH, or PhSH. The results show that SS cleavage is the major lesion induced by NCS for all thiols and the DS lesion is relatively minor. This conclusion is in good agreement with other studies using aliphatic thiols (*3*), which suggest that SS lesions occur mainly at C-5′ of thymidine (Scheme 2). Compared to AET and BSH, it is clear that PhSH is quite inefficient in producing all types of direct breaks, including DS, SS, and TS ones. The amount of TS breaks for PhSH shown in Figure 1 is only 20 or 27% of those found for AET or BSH, respectively. The results support those found from base release.

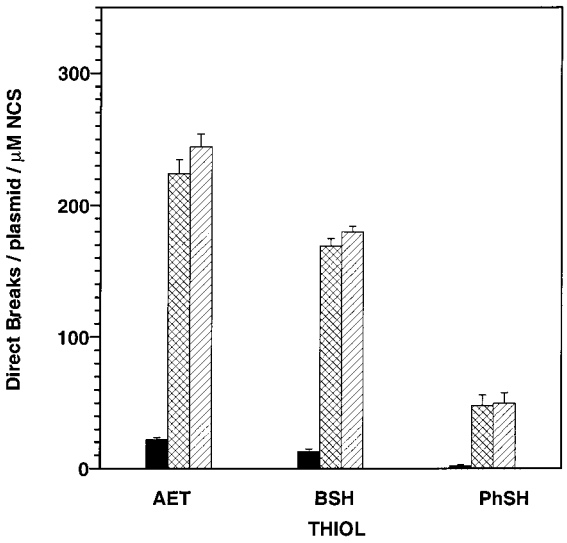


FIGURE 1: Direct strand breaks after the DNA−drug reaction without further treatment. The number of strand breaks is expressed as DS breaks (black bars), SS breaks (cross-hatched bars), and total strand (TS) breaks (striped bars). All values are averages from three to seven repeated experiments. A cap on the top of each bar represents the standard error.

Abasic Site Lesions. Scheme 3 shows the abasic site chemistry induced by NCS. Through 4′-H abstraction, breakdown of the peroxy radical results in a 4′-hydroxylated abasic site **9** (*3*). Abstraction of 1′-H, with subsequent formation of the peroxy radical, results in a 2′-deoxyribonolactone abasic site **10** (Scheme 3b) (*29*, *30*). Unlike 5′-H chemistry, where the carbonyl oxygen of 5′-aldehyde **5** arises exclusively from dioxygen (*15*), the carbonyl oxygen of lactone **10** is substantially taken up from water (*32*, *33*), indicating that a different mechanism is involved in the oxidative route at the C-1′ center.

Hydrazine, which is known to react predominantly with the 4′-hydroxylated abasic site **9** (*25*), can generate a strand break by formation of a 3′-phosphopyridazine derivative **11** (*34*) (Scheme 3a). On the other hand, the reaction of abasic site **9** with putrescine involves breakdown of a putative 3′-dihydrofurfuryl derivative **12** (*25*) which generates a gap between 3′- and 5′-phosphate termini (Scheme 3b). Putrescine can also cleave NCS-induced abasic site **10** resulting from 1′-H chemistry to generate an adduct **13**, the presence of which was recently identified by ESI-MS (*35*). A strand break can occur by breakdown of adduct **13** via elimination (*36*), which generates a gap and a furanone **14** as a fragment from the sugar moiety (*32*, *33*).

Figure 2 shows the number of DS, SS, and TS breaks from direct strand breaks and also the abasic sites that become strand breaks by the action of hydrazine. Compared to direct strand breaks, the increase in the level of TS breaks, which correspond to total lesions from 4′-hydroxylated abasic site **9**, ranges from 8 to 39% for all thiols, a result which is comparable to the values of 5−28% reported from an earlier study (*18*). The TS breaks of PhSH again are only 23 and 24% as large as those for AET and BSH, respectively; this observation is similar to the results depicted in Figure 1. Figure 3 shows the total lesions, including both direct strand breaks and all abasic site lesions that become strand breaks by the action of putrescine. Compared to Figure 2, the increase in the level of TS breaks, which presumably

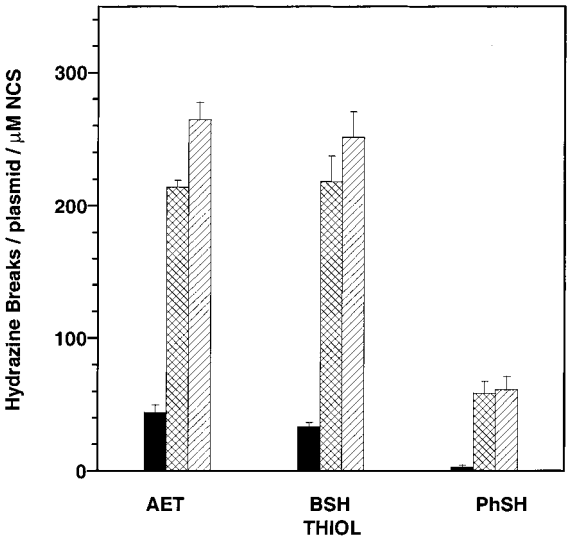


FIGURE 2: Direct strand breaks and 4′-hydroxylated abasic sites that become strand breaks by the action of hydrazine. The number of strand breaks is expressed as DS breaks (black bars), SS breaks (cross-hatched bars), and total strand (TS) breaks (striped bars). All values are averages from three to seven repeated experiments. A cap on the top of each bar represents the standard error.

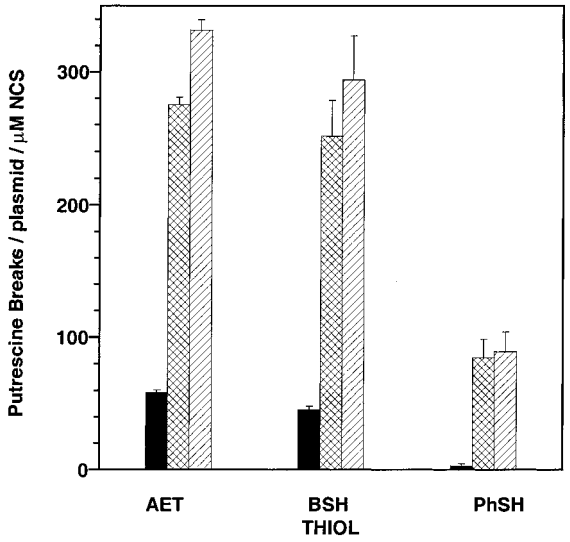


FIGURE 3: Direct strand breaks and all abasic sites that become strand breaks by the action of putrescine. The number of strand breaks is expressed as DS breaks (black bars), SS breaks (cross-hatched bars), and total strand (TS) breaks (striped bars). All values are averages from three to seven repeated experiments. A cap on the top of each bar represents the standard error.

corresponds to 2′-deoxyribonolactone abasic site **10**, ranges from 17 to 46%, suggesting that NCS-induced abasic site lesions via 1′-H abstraction or other abasic site chemistry probably are significant for all thiols. More importantly, the level of the lesions found by the action of putrescine is 3.2-fold lower for PhSH than for AET, which is in good agreement with data for base release. Figure 3 clearly shows that the extent of DNA lesions found when BSH activates NCS is comparable to that found with an aliphatic thiol but not with PhSH. This result supports the hypothesis that the large reduction in drug activity by PhSH is caused by an inductive effect, resulting from delocalization of sulfur electrons through π-resonance.

Distribution of SS and DS Breaks. The strand break analyses consistently show that SS breaks are dominant in all thiol-mediated reactions, regardless of whether the breaks are direct or latent and induced by hydrazine or putrescine treatment (Figures 1−3). Although DS breaks occur less often, they are more lethal than SS breaks. PhSH is not only inefficient in causing total DNA lesions, but also is very prominent in reducing the number of DS breaks. When putrescine is used to induce all lesions to become strand breaks, the percentage of DS breaks out of the total breaks is ∼3.1% for PhSH, which is significantly lower than the value found for BSH (15.4% DS) or for AET (17.5% DS) (Figure 3). A similar result was also obtained earlier with 4-hydroxythiophenol (HTP) (*18*), which shows a high SS:DS ratio of breaks (3.2% DS), in contrast to low SS:DS ratios (11.5−31% DS) for 10 aliphatic thiols studied. A novel cyclization product of NCS-C was reported for its reaction with HTP (*37*), which suggests that the C-2 radical of **3** is able to undergo an intramolecular addition to the aromatic ring of the thiolate adduct. The intramolecular trapping of the C-2 radical possibly accounts for the low rate of DS breaks from HTP. The low quantity of DS breaks from PhSH presumably is also caused by intramolecular quenching of the C-2 radical center. The NCS-induced DS lesions are sequence specific and frequently occur at an AGC·GCT or AGT·ACT sequence (*3*). The AGC·GCT sequence involves 1′-chemistry that can be expressed as DS breaks by putrescine. Modeling and deuterium labeling support the idea that the C-6 radical of **3** is near the C-5′ H of the T of GCT and the C-2 radical near C-1′ of the C of AGC (*3*). If the majority of the C-2 radical is quenched by the neighboring aromatic ring, the level of C-1′ abasic site lesions should be reduced significantly and very few additional strand breaks could be expressed by putrescine treatment. However, this seems not to be the case because putrescine treatment shows a significant increase in the level of strand breaks for all thiols.

Table 2: Comparison of the Base Release Assay with the DNA Cleavage Assay on the Drug Activity of NCS

	drug activity[a]			
	DNA cleavage			base
thiol activator	DS	SS	TS	release
aliphatic thiol (6.0 mM)[b]	1	1	1	0.94
aromatic thiol (0.26 mM)[c]				
thiyl electron localized	0.8	0.99	0.96	1
thiyl electron delocalized	0.075	0.35	0.31	0.35
conjugates with an electron-releasing group				0.46
conjugates with an electron-withdrawing group				0.22

[a] All numbers are normalized with 1 as the observed maximum activity in each column. [b] The optimum thiol concentration, averaged from all available data for aliphatic thiols reported in this study, in ref *17*, and in ref *18*. [c] The optimum thiol concentration, averaged from the data in this study for all aromatic thiols and a single value for HTP (0.2 mM) from ref *18*.

DISCUSSION

The Effect of the Aromatic Structure of a Thiol Is Electronic and Not Steric. Table 2 summarizes results from a comparison of the base release assay with the DNA cleavage assay for the drug activity. The data show that measurements of the levels of base release and DNA strand

breaks are consistent with each other. The average optimum thiol concentration for aliphatic thiols (6 mM) is more than 20 times higher than for aromatic thiols (0.26 mM). Aromatic structure is likely to be effective in intercalating thiol molecules into the DNA helix. However, the thiol aromatic structure by itself does not cause NCS to perform better. The aromatic thiol BSH, whose electronic structure of the thiol sulfur is close to that of aliphatic thiols, performs in a manner equivalent to that of aliphatic thiols, whereas PhSH, whose sulfur electrons are delocalized, lowers the drug activity to about one-third of its original value. Electron-withdrawing substituents further reduce the drug activity to less than one-fourth of its original value.

Because replacement of PhSH with BSH restores the full strength of the drug found with an aliphatic thiol, the drug activity is highly influenced by the electronic structure of the thiol sulfur. The effect of the electronic structure is much stronger than the effects of other functional groups in a thiol compound. Earlier studies show that the range of the total level of NCS-induced DNA lesions found among four (*17*) or 10 (*18*) aliphatic thiols with various functional groups is between 100 and 60%, whereas our data show a range as large as 100−22% for thiol sulfurs with differing electronic structures. The results suggest that any effects of the phenyl ring, such as aromatic stacking, steric hindrance, hydrophobic interaction, etc., do not participate in the mechanism of reducing the drug activity. Instead, an inductive effect on the polarization of the sulfur electron through delocalized π-bonding resonance must be responsible for the prominent influence of some aromatic thiols in preventing NCS-induced DNA damage. The strong influence of electron-withdrawing or -releasing substituents supports such an inductive effect.

Role of the Thiol Structure. Aliphatic thiols are present in vegetables, whereas aromatic ones, such as derivatives of PhSH and furan-3-thiol, are present in both meat and fish (*38*). Thiols, including aromatic ones such as methimazole (*39*), are active metabolites, and they are widely distributed in biological systems as effective cancer-protective factors (*40*, *41*). Because they are chemically reactive, thiols are among the most important pharmacological compounds. In addition to activation of a number of drugs, the role of a thiol in antitumor action can be complex. Our results show that the electronic structure of the thiol activator plays an important role in preventing DNA damage in vitro. By using a thiol with a proper electronic structure, we demonstrate here that the drug activity can be changed by as much as 4-fold. However, because of the complex chemistry found in a cell, the application of this approach in vivo is not likely to be as facile.

To understand the effect of the thiol structure on drug action, studying NCS may offer a good model for fully elucidating the mechanism. Thiol has an initial role in activating the prodrug to the active form. We analyzed the final drug product from the thiol-induced activation of NCS-C in the absence of DNA by established chromatographic and spectroscopic methods (*42*). We found that the postactivated thiol−drug adduct **4** is the major product whether the reaction is induced by AET, BSH (*42*), or PhSH (unpublished data). These results indicate that PhSH is equally able to induce the active drug radical **3** even though the resulting level of DNA lesion is reduced by 3-fold. The function of the thiol is not only to activate NCS. Thiol is known as a good electron donor and is able to quench the active drug radical **3**. The intramolecular quenching of the C-2 radical of **3** by PhSH may be responsible for causing the rate of lethal DS breaks to be reduced by more than 10-fold (Table 2). Thiol is also a good reducing agent; it is involved in fixing the nascent DNA damage by reducing the peroxy radical of DNA (Schemes 2 and 3). In addition, thiol is known to be efficient in DNA repair by donating its sulfur electron to the carbon-centered DNA radical. Epstein et al. (*43*) recently demonstrated that the efficiency of repair of DNA lesions by the esperamicins is dependent on the structural features of the thiol reductant. Thus, the thiol has several functional roles in determining the activity of this drug. In basic research on development of this drug, it is necessary to determine at which steps its potency can be controlled by the thiol structure. Further study on this problem is in progress in this laboratory.

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