

NMR Studies of metal complexes and DNA binding of the quinone-containing antibiotic streptonigrin

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Optical and ¹H NMR techniques have been applied to the study of a few metal complexes (Co²⁺, Fe²⁺, and Yb³⁺) of the antitumor antibiotic streptonigrin (SN) produced by *Streptomyces flocculus* to elucidate the structure of the complexes. The hyperfine-shifted ¹H NMR signals of these paramagnetic complexes were fully assigned by means of relaxation and two-dimensional NMR techniques. These studies revealed that SN binds transition metal and lanthanide ions and forms stable metal–drug complexes, with the metal located at the quinolinequinone–picolinate site. This configuration requires a ≈180° twist of the C2–C2' bond in the crystal structure of the drug. The hyperfine-shifted ¹H NMR signals of the Co²⁺–SN complex are significantly changed upon addition of calf thymus DNA or poly[dA–dT], indicating direct binding of Co²⁺–SN complex with DNA.

Streptonigrin (SN, also known as rufochromomycin and bruneomycin) is a metal-dependent quinone-containing antibiotic produced by *Streptomyces flocculus*¹ (Fig. 1²). This antibiotic has been shown to exhibit active inhibition toward several tumors and cancers (e.g. lymphoma, melanoma, and breast and cervix cancers) as well as viruses in some early *in vitro* and clinical observations.^{3,4} However, the high toxicity and serious side effects of this drug reduce its clinical value, and limit its use only as an experimental antitumor agent.^{3,4} Nevertheless, because of its antitumor potency and unique structure, SN has served as a lead drug molecule for chemical modification and synthesis in order to correlate specific structure features with the biological activity of the molecule.⁵ Since SN contains a quinone moiety, it may share some common mechanistic characteristics with other quinone-containing antibiotics such as the anthracyclines in inhibition of cancer growth. Two mechanisms for this action have been proposed:⁶ (1) by way of interference with cell respiration and (2) through disruption of cell replication and transcription. A key step in this action is reflected by the induction of severe irreversible damage to DNA and RNA *in vitro* and *in vivo* in the presence of reducing agents.^{6,7}

Streptonigrin is able to bind several different metal ions, and requires metal binding for full antibiotic and antitumor activity.^{6,8} The transition metal ions Cu²⁺ and Fe²⁺ have been known to accelerate SN-mediated DNA scission in the presence of NADH (reduced nicotinamide adenine dinucleotide), thus enhancing the antitumor activity of this antibiotic.^{9,10} This antibiotic also exhibits a strong EPR signal upon reduction in the presence of a bound metal ion, indicating the formation of a metal–semiquinone form of this drug.¹¹ These results indicate that metal ions are directly involved in the action of SN. Metal–SN complexes can be reduced to their semiquinone forms by NADH to induce cleavage of DNA. This reduction process is inhibited by superoxide dismutase and catalase, indicating the involvement of superoxide and peroxide.^{6,9d} Moreover, the interaction of metal–SN complexes with DNA has also been proposed on the basis of some optical studies.¹² However, the role of metal ion in the action of SN has not yet been fully defined, and the metal binding mode and structure of these metal complexes could not be definitely determined in previous studies. Particularly, two different configurations of the metal–SN complexes have been proposed (Fig. 1).⁶ with the metal

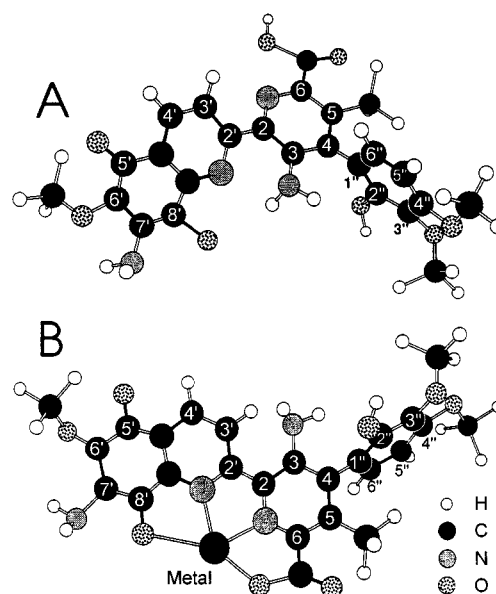


Fig. 1 (A) The molecular structure of streptonigrin based on the crystallographic study.² (B) The molecular structure of a metal complex of streptonigrin based on the NMR studies discussed in this report. The metal is put in the quinolinequinone–picolinate site according to the results from the NMR studies. This structure requires ≈180° rotation of the bipyridine C2–C2' bond in the crystal structure of the drug (A). The numbering of SN follows the nomenclature: 3-amino-2-(7'-amino-6-methoxy-5',8'-dioxoquinolin-2'-yl)-6-carboxy-4-(2'-hydroxy-3'',4''-dimethoxyphenyl)-5-methylpyridine

bound through the quinolinequinone–amine functionalities based on the crystal structure;² and *via* the quinolinequinone–picolinate functionalities which requires a significant twist of the crystal structure.

We report here a study of the binding of SN with paramagnetic metal ions, including the transition metal ions Co²⁺ and Fe²⁺, and the lanthanide Yb³⁺. Since the chemical shift and the relaxation times of paramagnetic molecules are very sensitive to structural changes,¹³ they can be utilized as very sensitive ‘probes’ for the studies of molecular structures and interactions. The paramagnetically shifted ¹H NMR signals of the metal–SN complexes have been fully assigned and their relaxation times measured, which afford an accurate determination of their structures in solution. The interaction of the

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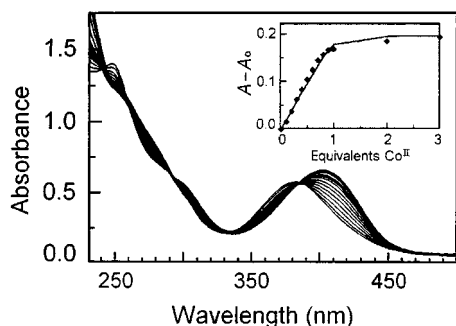


Fig. 2 Electronic spectra of SN and its binding with Co^{2+} in methanol. The formation of the 1:1 complex is clearly shown in a titration of Co^{2+} into a 0.033 mM drug solution (inset). A fitting of the change of the absorption at 404 nm against $[\text{Co}^{2+}]$ using the simple equilibrium $\text{Co}^{2+} + \text{SN} \rightleftharpoons \text{Co}^{2+}\text{-SN}$ gives an affinity constant $3.30 \times 10^6 \text{ M}^{-1}$. Similarly, the binding of the Fe^{2+} and Yb^{3+} to SN shifts λ_{max} to 400 and 420 nm with affinity constants 5.43×10^6 (CH_3OH) and $1.58 \times 10^6 \text{ M}^{-1}$ (CH_3CN), respectively

Co^{2+} -SN complex with DNA has also been monitored by the use of optical and NMR spectroscopies. A direct interaction was observed, where a significant change of the hyperfine-shifted ^1H NMR signals of the complex was detected in the presence of DNA. These paramagnetic metal-SN complexes can serve as prototypical model systems for future investigation of other paramagnetic metal-drug complexes and their binding with DNA.

Results and Discussion

Titration of streptonigrin with metal ions

A freshly prepared methanol solution of SN gives a deep brown solution with $\lambda_{\text{max}} = 392 \text{ nm}$. An optical titration shows Co^{2+} ion can bind SN tightly in methanol to form a very stable 1:1 Co^{2+} -SN complex ($\lambda_{\text{max}} = 404 \text{ nm}$, Fig. 2). A fitting of the change of the absorption at 404 nm of SN with respect to the amount of Co^{2+} gives an affinity constant of $3.30 \times 10^6 \text{ M}^{-1}$ for the simple equilibrium $\text{Co}^{2+} + \text{SN} \rightleftharpoons \text{Co}^{2+}\text{-SN}$ (inset, Fig. 2). Similarly, the addition of Fe^{2+} to SN in methanol under argon shifts the electronic absorption of the drug to 400 nm upon the formation of a 1:1 Fe^{2+} -SN complex with an affinity constant of $5.43 \times 10^6 \text{ M}^{-1}$. Upon the addition of Yb^{3+} to SN in CH_3CN the λ_{max} shifts to 410 nm (greenish yellow) with an affinity constant $1.58 \times 10^6 \text{ M}^{-1}$ for the formation of a 1:1 Yb^{3+} -SN complex. The change of the electronic transition in SN upon the binding of these three metal ions (*cf.* Fig. 2) is similar to that observed previously for Cu^{2+} and Zn^{2+} binding to the drug.⁶

^1H NMR of ytterbium(III)-streptonigrin complex

The ^1H NMR spectrum of a freshly prepared 1:1 Yb^{3+} -SN complex in methanol is shown in Fig. 3 (spectrum B), in which the signals due to the drug are paramagnetically shifted to the region of δ 5 to -10 . Since the Yb^{3+} -bound SN is undergoing chemical exchange with the free drug, signal assignment of the Yb^{3+} -SN complex can be achieved by the use of saturation transfer two-dimensional EXchange Spectroscopy (EXSY) on a sample with both the free drug and the complex present (Fig. 4).¹⁴ The paramagnetically shifted signals in an EXSY spectrum can thus show cross-peaks with their diamagnetic counterparts of the free drug, which can easily be assigned on the basis of chemical shift and COSY (Fig. 3A). For example, the signal at $\delta -8.9$ (which integrates to 3 protons with $T_1 = 114.5 \text{ ms}$) is assigned to 5- CH_3 on the picolinate ring (Fig. 1), and the signals at $\delta -5.5$ (73.7) and -1.8 (167.9 ms) are assigned to quinolinequinone 3'-H and 4'-H protons, respectively (Fig. 4).

Since the relaxation time T_1 of a proton in paramagnetic molecules is proportional to the sixth power of the proton-

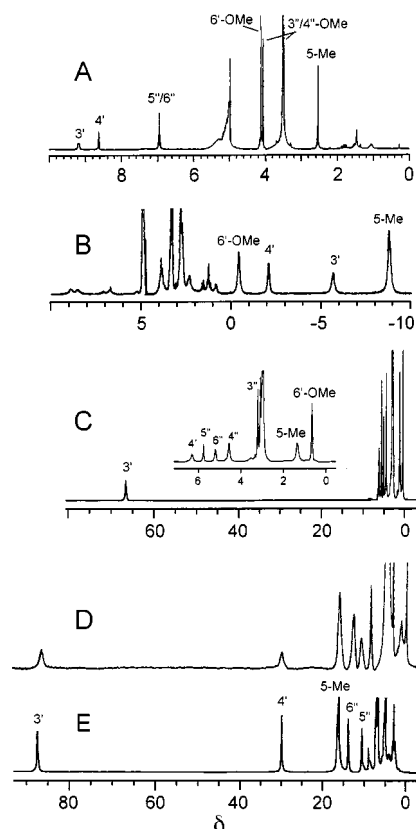


Fig. 3 Proton NMR spectra (360.13 MHz, 298 K, 90° pulse $\approx 7 \mu\text{s}$) of (A) free drug and the 1:1 complexes ($\approx 4 \text{ mM}$) Yb^{3+} -SN (B), Fe^{2+} -SN (C), and Co^{2+} -SN (E) in CD_3OD , and (D) Co^{2+} -SN ($\approx 2 \text{ mM}$) in borate- D_2O buffer at pD 8.0. The signals are assigned based on their T_1 values and EXSY studies (Figs. 4–6)

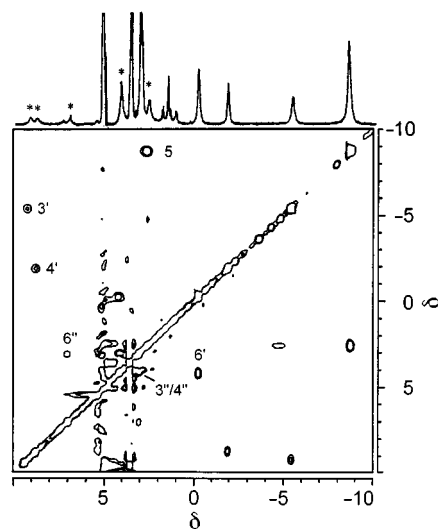


Fig. 4 The ^1H EXSY spectrum (360.13 MHz, 298 K, mixing time 20 ms) of the complex Yb^{3+} -SN in the presence of residual free drug (asterisked) in CD_3OD . The numbers show the assignment of the signals to the structure in Fig. 1

metal distance (*i.e.* $T_1 \propto r_{\text{M-H}}^{-6}$),¹⁵ it is therefore extremely sensitive to structural changes. Thus, it can be taken as a 'ruler' for the measurement of the proton-metal distances in paramagnetic molecules. The three most upfield shifted signals in the spectrum of the complex with the shortest relaxation times are attributable to the protons closest to the paramagnetic Yb^{3+} center. The large paramagnetic shift and short T_1 value of the 5- CH_3 protons suggest that they are close to the bound Yb^{3+} . This T_1 value is shorter than that of the 6''-H protons (Table 1), indicating that the 5- CH_3 protons are closer to the metal. This is

Table 1 Proton NMR chemical shifts and T_1 values and metal–proton distances of metal–SN complexes in CD_3OD

Signal assignment	Yb^{3+} –SN			Fe^{2+} –SN			Co^{2+} –SN			M–H/Å	
	δ	T_1/ms	Yb^{3+} –H $^a/\text{\AA}$	δ	T_1/ms	Fe^{2+} –H $^a/\text{\AA}$	δ	T_1/ms	Co^{2+} –H $^a/\text{\AA}$	Model I b	Model II b
3'-H	-5.5	73.7	5.16	65.6	7.5	5.14	87.0	13.8	4.97	5.06	5.06
4'-H	-1.8	167.9	5.97 c	6.37	18.1	5.97 c	29.6	40.8	5.97 c	5.97	5.97
6'-OCH $_3$	-0.4	521.0	7.47	0.70	90.2	7.85	3.75	d	—	6.77 e	6.77 e
5-CH $_3$	-8.9	114.5	5.58	1.44	15.7	5.82	16.2	38.8	5.92	6.08 e	6.19 e
3''-OCH $_3$	d	d	—	3.11	328.7	9.95	8.72	291.5	8.48	9.95 e,f	7.66 e,f
4''-OCH $_3$	≈ 5.0	d	—	3.24	349.7	10.08	7.02	d	—	11.24 e,f	8.44 e,f
5''-H	d	d	—	5.78	158.6	8.68	10.2	291.4	8.48	9.05 f	6.41 f
6''-H	2.93	361	6.90	5.22	35.6	6.69	13.4	87.0	6.80	6.73 f	4.57 f

a A 0.5 s^{-1} diamagnetic contribution has been added to the relaxation in the calculation of the distance, *i.e.* $T_1'^{-1} + 0.5 = T_1^{-1}$. b Model I is shown in Fig. 1B with the metal bound to the drug through the bipyridyl moiety (quinolinequinone–picolinate) and the fourth ring perpendicular to the bipyridine moiety. The alternative configuration, Model II, is based on the crystal structure of the free drug (Fig. 1A) in which the metal is bound through the quinolinequinone–amine functionalities. The M–N distances are set to be 2.1 Å in these models. c This distance is used as the reference distance. The other metal–proton distances are calculated as $(T_1'/T_1'(\text{M–H}))^{1/6} \times 5.97 \text{ \AA}$. 15,16 d Not resolved or measured. e Average with the assumption of free rotation of the methyl group. f Average of two distances with the ring rotated by 180° .

consistent with binding of the Yb^{3+} at the quinolinequinone–picolinate site as shown in Fig. 1B. The T_1 values of other signals are also consistent with this binding mode for this complex (Table 1).

Since Yb^{3+} , like alkaline earth metal ions, 17 prefers an oxygen-rich ligand binding environment with little covalency, the ethylenediamine diacetate-like binding mode shown in Fig. 1B is presumably the preferred binding mode for the biologically relevant Ca^{2+} and Mg^{2+} ions as well. As transition metal ions have been proposed to be involved in the binding of SN to DNA and cleavage of DNA by the drug, the study of metal–SN complexes is important to provide further mechanistic information about SN action. 6,8 However, because the ligand binding preferences between transition metal ions and the lanthanides (and the alkaline earth metals) are very different, whether or not this Yb^{3+} binding mode is applicable to transition metal–SN complexes cannot be answered at this stage.

^1H NMR of iron(II)–streptonigrin complex

The redox-active Fe^{2+} ion has been shown to enhance the activity of SN. 9 Hence, it is important to reveal the exact binding mode of Fe^{2+} with this antibiotic and solve the structure of the Fe^{2+} –SN complex in order to gain further insight into the mechanism of SN action and the role of metal ion in the action. Since Fe^{2+} can afford relatively sharp hyperfine-shifted ^1H NMR signals, 13 the Fe^{2+} complex of SN can be thoroughly analysed by means of NMR techniques. The ^1H NMR spectrum of a 1:1 Fe^{2+} –SN complex shows several well defined hyperfine-shifted signals (Fig. 3C). The 'clean' spectrum indicates that there is only one Fe^{2+} –SN complex formed under the experimental conditions. The binding mode of Fe^{2+} ion can be determined when signal assignment is achieved, as discussed below.

All the ^1H NMR signals of the Fe^{2+} –SN complex can be assigned by means of two-dimensional NMR techniques (COSY and EXSY, Fig. 5) and T_1 measurement (Table 1). The most downfield-shifted signal at δ 65.6 (7.5 ms) can be assigned to the 3'-H proton, which is four bonds away from the bound Fe^{2+} and is the closest to the metal. Therefore, it should gain the largest through-bond contact shift and shortest relaxation time compared to all other protons. The rest of the hyperfine-shifted signals can be assigned based on their correlations with their diamagnetic counterparts of the free drug in the EXSY spectrum. The only COSY cross-peaks of the complex (inset, Fig. 5) are associated with the phenyl ring protons 5''- and 6''-H at δ 5.78 and 5.22, respectively. A complete signal assignment is shown in Table 1. The 15.7 ms T_1 value of the 5-CH $_3$ signal at δ 1.44 is shorter than that of the 6''-H signal at δ 5.22 (35.6 ms). This indicates that Fe^{2+} is bound to SN at the

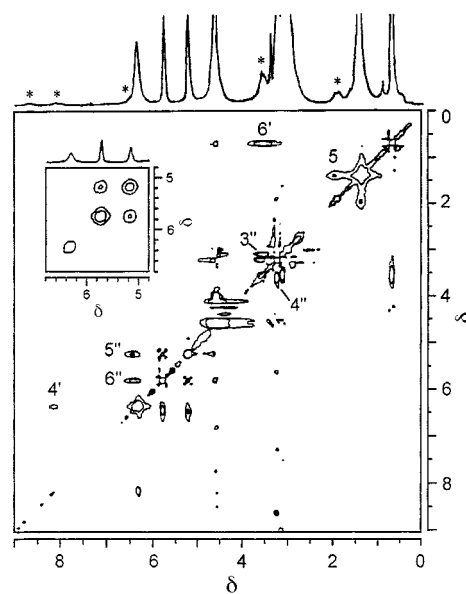


Fig. 5 The ^1H EXSY spectrum (360.13 MHz, 298 K, mixing time 20 ms) of the complex Fe^{2+} –SN in the presence of residual free drug (asterisked) in CD_3OD . The inset is a COSY spectrum showing the $\text{H}5''$ – $\text{H}6''$ through-bond coupling. The numbers indicate the assignment of the signals to the structure shown in Fig. 1

quinolinequinone–picolinate site (Fig. 1B, Model I in Table 1), similar to that in Yb^{3+} –SN. This binding mode requires a $\approx 180^\circ$ rotation of the bipyridine C2–C2' bond in the crystal structure (Fig. 1A).

Another configuration with the metal bound through the 3-NH $_2$ nitrogen of SN has been proposed in previous studies 6d based on the crystal structure of the free drug 2 (Model II, Table 1). This alternative would afford a Fe–H (5-CH $_3$) distance much longer than the Fe–H6'' distance, thus a longer T_1 value for the 5-CH $_3$ protons than the 6''-H proton. This configuration can be discarded based solely on the T_1 values reported in our study (Table 1). The Fe^{2+} –SN complex is presumably the iron-bound form of the drug under the reduction conditions in the cells. The unambiguous assignment of the ^1H NMR signals and the determination of the structure of this complex described here provide an important step for further study of the interaction of this complex with biomolecules and cell components.

The NMR results also indicate that the formation of a Fe^{3+} –SN (semiquinone) complex *via* electron transfer from Fe^{2+} to SN is not likely to occur. This is because: (1) there is no indication of a high unpaired electron density on the quinone ring (as a result of the free radical on a semiquinone moiety),

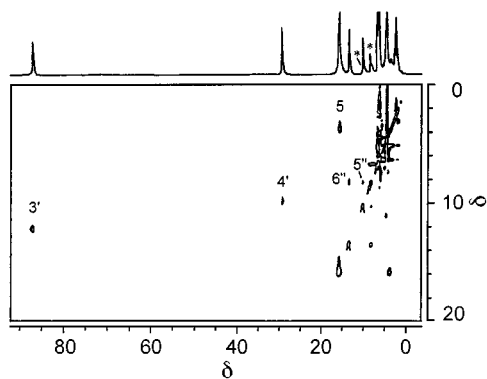


Fig. 6 The ^1H EXSY spectrum (360.13 MHz, 298 K, mixing time 20 ms) of the complex Co^{2+} -SN in the presence of residual free drug (asterisk) in CD_3OD . The numbers indicate the assignment of the signals according to the structure shown in Fig. 1

which would afford a large contact shift and an even shorter relaxation time on the $6'$ - OCH_3 protons; and (2) there is no sign of a larger magnetic moment and longer electronic relaxation time due to the $S = \frac{5}{2}$ Fe^{3+} (relative to the $S = 2$ Fe^{2+}) in a Fe^{3+} -SN (semiquinone) complex, which would afford faster relaxing and broader hyperfine-shifted ^1H NMR signals.

^1H NMR of cobalt(II)-streptonigrin complex

A 1:1 Co^{2+} -SN complex is formed upon the addition of 1 equivalent Co^{2+} to SN in methanol as shown by its electronic and ^1H NMR spectra (Figs. 2 and 3E). The hyperfine-shifted ^1H NMR signal at δ 87 ($T_1 = 13.8$ ms) can be assigned to the $3'$ -H proton which is four bonds away from the metal and is the proton closest to the metal. The signal at δ 29.6 (40.8 ms) can be assigned to $4'$ -H five bonds away from the metal that gains significant contact shift *via* the aromatic pyridine ring. The assignment of most hyperfine-shifted signals can be achieved by the use of the EXSY technique to reveal saturation transfer between the complex and free drug (Fig. 6, Table 1). For example, the 5-CH_3 , $5''\text{-H}$, and $6''\text{-H}$ are found at δ 16.2 (38.8), 10.24 (291.4), and 13.41 (87.0 ms), respectively. The shortest T_1 value of the 5-CH_3 protons among all protons reflects that these protons have the shortest distance to the Fe^{2+} . The signal assignment and the T_1 values of the hyperfine-shifted signals of Co^{2+} -SN (Table 1) are consistent with the structure shown in Fig. 1B, with the Co^{2+} bound to the quinolinequinone-picolinate function groups (Model I, Table 1) rather than to the quinolinequinone and the 3-NH_2 groups (Model II, Table 1). This binding mode is similar to that found in the Fe^{2+} -SN complex. Again, this indicates that the bipyridine C2-C2' bond of the free drug in the crystal structure² has to rotate by $\approx 180^\circ$ upon metal binding.

The electronic ($\lambda_{\text{max}} = 370$ nm) and NMR (Fig. 3D) spectra of the Co^{2+} -SN complex observed in borate buffer solution at pH 8 are similar to those acquired in methanol solution. The acquisition of the NMR spectrum of a metal-SN complex in aqueous solution is important for further study of its interaction with DNA (see below). This complex shows broader isotropically shifted ^1H NMR signals in water than in methanol, possibly attributable to a coagulation of this hydrophobic drug in aqueous solution. The broadness of ^1H NMR signals in aqueous solution has also been observed for metal-anthracene complexes which also contain an extended hydrophobic ring system.¹⁸ The virtually identical spectral features of the Co^{2+} -SN complex in water and methanol, however, indicate the formation of the same complex in these two solutions. This suggests that the structural information acquired in methanol can assist the assignment of the structure and better understanding of the action of metal-SN complexes in aqueous solutions under physiological conditions.

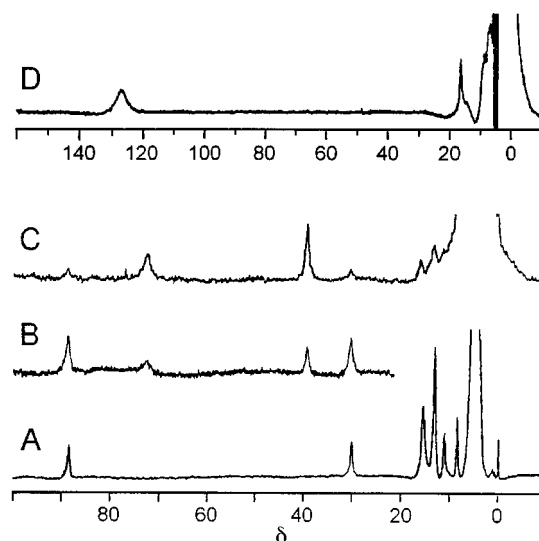


Fig. 7 Proton NMR spectra (360.13 MHz and 298 K) of (A) Co^{2+} -SN (200 μl at ≈ 2 mM) and this sample with the addition of 220 (B) and 380 μl (C) calf thymus DNA (1 mg mL^{-1}), and the spectrum of the complex in the presence of 10 units poly[dA-dT] (D). All the samples were in borate- D_2O buffer at pD 8.0

Interaction of Co^{2+} -SN complex with poly[dA-dT]

The air-sensitive Fe^{2+} -SN complex is difficult to handle when sample transfer is necessary during experiments. The binding mode of Co^{2+} with this drug is similar to that of Fe^{2+} , suggesting that the more air-resistant Co^{2+} -SN complex can serve as a substitute for Fe^{2+} -SN, and as a good model system to provide molecular information and DNA-binding property of metal-SN complexes. Moreover, the sensitivity of hyperfine-shifted signals toward subtle structural changes¹³ also suggests that the paramagnetic Co^{2+} -SN complex can serve as a good probe for monitoring the binding of metal-SN complexes with DNA. Previous studies showed that SN exhibited a preferred cleavage site at cytosine bases adjacent to purine bases in DNA.^{10b} Moreover, addition of poly[dA-dT] to the complex Cu^+ -SN was previously observed to cause small perturbation of the drug signals (0.22 to 0.31 ppm), which was suggested to be due to the binding of this complex to poly[dA-dT].^{10b}

Upon addition of 10 units of poly[dA-dT] to Co^{2+} -SN in borate buffer D_2O solution at pD 8.0 three new ^1H NMR signals appear, one sharp peak at δ 16.8 and two broad peaks at $\delta \approx 15$ (overlapped) and 127 (Fig. 7D), with concomitant disappearance of the downfield hyperfine-shifted signals of the Co^{2+} -SN complex (Fig. 7A). This significant change of the paramagnetically shifted signals suggests that Co^{2+} -SN complex is bound to poly[dA-dT], forming a ternary Co^{2+} -SN-poly[dA-dT] complex.

Binding of Co^{2+} -SN complex with calf thymus DNA

The addition of a soluble form of calf thymus DNA to Co^{2+} -SN complex in 10 mM Tris buffer [tris(hydroxymethyl)methylamine] at pH 7.5 causes a shift of the electronic transition of the complex from 370 to 385 nm with a slight decrease in intensity and an isosbestic point at ≈ 415 nm. This result indicates that the Co^{2+} -SN complex can also bind to naturally occurring DNA. This red-shift of the optical absorption is similar to that of the Zn^{2+} -SN complex upon the addition of calf thymus DNA.¹²

Upon the addition of calf thymus DNA to ≈ 3 mM Co^{2+} -SN in borate buffer at pD 8.0 the ^1H NMR signals of the complex at δ 84 and 30.5 decrease in intensity, and two new signals appear at δ 73 and 40 that are presumably due to the $3'$ -H and $4'$ -H protons, respectively (Fig. 7, A through C). These two new signals are not observed when ≈ 5 mM Co^{2+} is present in the DNA solution under the same conditions, suggesting that the complex is bound to the DNA (or that the drug assists the

binding of metal ion to DNA). This observation clearly indicates that Co^{2+} remains bound to SN upon binding of the Co^{2+} -SN complex to large pieces of native calf thymus DNA. Since irradiation of these signals does not reveal any noticeable saturation transfer, the two new signals may not be in fast exchange with the two hyperfine-shifted 3'- and 4'-H signals of the Co^{2+} -SN complex under the experimental conditions. This indicates that this complex binds calf thymus DNA to form a kinetically inert Co^{2+} -SN-DNA ternary complex. The results presented here clearly reveal the binding of the complex Co^{2+} -SN with poly[dA-dT]₂ and native calf thymus DNA.

Conclusion

Optical titration and one- and two-dimensional NMR techniques have been applied to the study of the few metal complexes (Co^{2+} , Fe^{2+} , and Yb^{3+}) of the antitumor antibiotic streptonigrin. These studies reveal that SN binds transition metal and lanthanide ions with the metal located in the quinolinequinone-picolinate site, which affords a configuration that requires a $\approx 180^\circ$ rotation of the bipyridine C2-C2' bond in the crystal structure. The Co^{2+} -SN complex shows different isotropically shifted ^1H NMR signals upon addition of calf thymus DNA and poly[dA-dT], indicating direct binding of the complex with DNA. These studies provide the foundation for future investigation of the interactions between metal-SN complexes and different oligonucleotide sequences to reveal detailed information about the mechanism of SN action and the structures of metal-SN-DNA ternary complexes. This report also demonstrates that NMR can be a versatile tool for the study of paramagnetic metal-DNA complexes.

Experimental

Chemicals and sample preparations

Streptonigrin was purchased from Sigma Co., and was also supplied as a gift by Rhône-Poulenc Rorer, Recherche-Développement Laboratories (Pairs) and by the National Cancer Institute (Drug Synthesis & Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment). The drug is soluble in some organic solvents such as 1,4-dioxane, pyridine, dmf, dmsO, and slightly soluble in alcohol and CHCl_3 . It is barely soluble in aqueous solution at $\text{pH} < 7$, but is slightly soluble at higher pHs to low mM levels. However, it is unstable and photosensitive at $\text{pH} > 8$.^{6d} This low solubility in water causes difficulty for NMR studies. To overcome this problem, SN was first dissolved in aqueous solution at high pH and then adjusted to the desired pH value. The drug solution was prepared just before the experiments, and the concentration of the drug was determined by using $\epsilon_{365} = 14\,200\ \text{M}^{-1}\ \text{cm}^{-1}$ at $\text{pH}\ 7.2$.^{6d} The metal complexes of SN were prepared by direct addition of stoichiometric amount of metal ions to the SN solutions.

All metal salts were obtained as the highest grade. Metal ion concentrations were determined by edta titration with xylenol orange as indicator. All the organic solvents used in the experiments were HPLC grade. The DNA solutions were prepared by dissolving, respectively, 1 mg soluble calf thymus DNA (Sigma Chemical Co.) and 10 units polydeoxy(adenylic acid-thymidylic acid) (poly[dA-dT], Sigma) in borate buffer D_2O solution at $\text{pD}\ 8.0$ and stored at 4°C . One unit of poly[dA-dT] yields $A_{280} = 1.0$ in 1.0 mL water (at 1 cm path length). The Co^{2+} and Fe^{2+} samples were prepared under anaerobic conditions, and transferred to an optical cell or an NMR tube under argon using a gas-tight syringe.

The electronic spectra were acquired on a Hewlett Packard 8452A diode array spectrophotometer using a quartz cell of 1 cm path length. Metal titrations were performed by continuous addition of metal ions to SN solutions (e.g. 0.033 mM in the case

of Co^{2+} titration shown in Fig. 2). The spectra were recorded and calibrated against dilution factors. The affinity constant can be obtained by fitting the change in the absorptions (i.e. $\Delta A = A_{\text{M-SN}} - A_{\text{SN}}$) with respect to the metal concentration according to the equilibrium $\text{M} + \text{SN} \rightleftharpoons \text{M-SN}$.

Nuclear magnetic resonance experiments

The metal complex concentrations in organic solvents for NMR studies were about 4 mM, whereas those in aqueous solutions were about 2 mM. All ^1H NMR spectra were acquired on a Bruker AMX360 spectrometer at 360.13 MHz. The ^1H chemical shift was referenced to external tetramethylsilane to avoid the effect on the chemical shift of an internal reference by the paramagnetism of the metal complexes. A 90° pulse with pre-saturation for solvent suppression was used for the acquisition of one-dimensional ^1H NMR spectra (8K data points). A line-broadening factor of 10–30 Hz was introduced to the spectra via exponential multiplication prior to Fourier transformation to enhance the signal-to-noise ratio.

In the presence of chemical exchange (such as an equilibrium $\text{M} + \text{L} \rightleftharpoons \text{M-L}$), saturation transfer can occur between counterparts, such as between the paramagnetically shifted signals in M-L and their diamagnetic counterparts in L in NMR experiments. This can be conveniently studied by the saturation transfer techniques used for detection of the nuclear Overhauser effect (NOE), such as one-dimensional difference spectroscopy with the decoupler set on and off the signal of interest and the two-dimensional EXSY pulse sequence ($D_1-90^\circ-t_1-90^\circ-\tau_{\text{mixing}}$ -free induction decay). Owing to the fast nuclear relaxation rates and the fast molecular rotational correlation time, NOE cannot be detected in small paramagnetic complexes. The cross-peaks observed in the EXSY spectra of the M-SN complexes are thus due to chemical exchange of the drug between its free and complexed forms. The EXSY spectra were acquired with pre-saturation for solvent suppression and 1024×512 data points. A $45-60^\circ$ shifted sine-squared-bell window function was applied in both dimensions prior to Fourier transformation in phase sensitive EXSY spectra. Magnitude-COSY spectra of the complexes were acquired for the elucidation of through-bond proton couplings as shown in Fig. 5. The spectra were acquired with 1024×256 data points, and then a 0° -shifted sine-squared-bell window function was applied to both dimensions and processed in magnitude mode.

Proton spin-lattice relaxation times (T_1) for all the metal complexes were determined by the use of the inversion-recovery method ($D_1-180^\circ-\tau-90^\circ$ -free induction decay) with 16 different τ values and a recycle time of $\approx 5T_1$. The peak intensities were fitted against the τ values by a three-parameter fitting program on the spectrometer to give the T_1 values. Since nuclear relaxation in paramagnetic molecules is dependent upon the metal-nucleus distance, relative distances can be obtained with respect to a reference nucleus [i.e. $r_{\text{M-H}} = (T_{1\text{M}}/T_{1\text{Mref}})^{1/6} r_{\text{M-Href}}$]. The proton 4'-H ($r_{\text{M-4'H}} = 5.97\ \text{\AA}$) was chosen as the reference proton. Since dipolar relaxation in paramagnetic metal-pyridine complexes has been demonstrated to be the predominant contribution to nuclear relaxation,¹⁶ the contact contribution to the nuclear relaxation was not taken into consideration in this study. In most cases, paramagnetic relaxation is the predominant contribution to nuclear relaxation. To demonstrate this, a $0.5\ \text{s}^{-1}$ diamagnetic contribution was considered in the calculation of the distance $r_{\text{M-H}}$. There is no significant difference in the calculated $r_{\text{M-H}}$ with or without considering the diamagnetic contribution.

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