DNA CLEAVAGE ACTIVITY OF LIBLOMYCIN (NK313), A NOVEL ANALOG OF BLEOMYCIN

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Liblomycin (NK313), a novel analog of bleomycin and peplomycin (PEP), produced acid soluble DNA, base propenals and nucleo bases from isolated DNA. This was similar to the action of PEP. However, the DNA cleavage activity of NK313 was 1/2~1/10 of that of PEP in the absence of reducing agents. In the presence of reducing agents such as 2-mercaptoethanol and ascorbic acid, the activity of NK313 was stimulated more strongly than PEP. NK313 was also different from PEP in the formation and decomposition of active intermediates. This result suggested that differences in DNA cleavage activity between NK313 and PEP may be due to the different properties of their active intermediates. NK313 released preferentially pyrimidine bases from DNA, and the molar ratio of the released pyrimidine bases to the total of the released bases was little affected by the concentration of NK313 relative to DNA. In contrast, the ratio of the released purine bases by PEP increased with the concentration of PEP relative to DNA. NK313 induced double strand cleavage on pBR322 DNA as efficiently as did PEP. The major cleavage sites of NK313 on pBR322 DNA were similar to those of PEP. However, certain minor cleavage sites of NK313 were specific for NK313. Increase of PEP concentration led to increased degradation of DNA fragments; this was not the case with NK313. These results indicate that the cleavage sites of NK313 were similar to, but more limited than those for PEP.

Bleomycin (BLM) is a chemotherapeutic agent for human cancers and useful in combination chemotherapy because of its lack of myelosuppressive properties. However, its use is limited by pulmonary toxicity. Peplomycin (PEP), an analogue of BLM, has been used clinically since 1981[1]. PEP has a lower pulmonary toxicity in animals, but this toxicity remains dose-limiting. Liblomycin (NK313) is an analog of BLM selected after an intensive screening program to find analogs with a stronger antitumor activity and reduced pulmonary toxicity[2]. NK313 has a bulky lipophilic substituent on terminal amine[2]; it exhibits activity against PEP-unresponsive tumors and has only slight or no pulmonary toxicity in mice and dogs, respectivity[2,3].

In the present study, in order to understand the mode of action of NK313, we compared NK313 and PEP in their DNA cleavage activity, the specificity of their DNA cleavage sites and the properties of active intermediates formed with ferrous ion and oxygen.

Materials and Methods

Chemicals

NK313 and PEP were products of Nippon Kayaku Co., Ltd., Tokyo. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2-mercaptoethanol and L-ascorbic acid were purchased from Kanto Chemical Co., Inc., Tokyo, Tokyo Kasei Industries, Tokyo and Daiichi Pure Chemicals Co., Ltd., Tokyo, respectively. Calf thymus DNA was obtained from Sigma Chemical Co., St. Louis. Fe(II) complexes of NK313 and PEP

were prepared by mixing equimolar aqueous solutions of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and the drugs. [^{3}H]-DNA was prepared from rat hepatoma AH66F cells cultured with [6-^{3}H]thymidine (5 Ci/mmol Amersham International plc. Amersham) according to MARMUR[4]. Plasmid pBR322 DNA was isolated from *Escherichia coli* strain HB101 accoding to RADLOFF *et al.*[5].

Determination of Acid-solubilization of DNA

The reaction mixture (0.25 ml) consisted of 0.05 M potassium phosphate buffer, pH 7.4, 2 mM KCl, various concentrations of the Fe(II) complex of drug and 0.25 μg of [^{3}H]DNA. The mixture was incubated at 37°C for 10 minutes. To terminate the reaction, 0.05 ml of an aqueous solution of bovine serum albumin (10 mg/ml) and 0.2 ml of 25% trichloroacetic acid - 12.5 mM sodium pyrophosphate solution were successively added at 0°C. After centrifugation at 3,000 rpm for 5 minutes, the radioactivity of acid-soluble DNA contained in 0.4 ml of the supernatant was counted by liquid scintillation counter.

Determination of Base Propenal from DNA

The reaction mixture (0.60 ml) consisted of 0.05 M Tris-HCl buffer, pH 7.8, various concentrations of the Fe(II) complex of drug and 2 mM calf thymus DNA. The mixture was incubated at 37°C for 10 minutes. Two ml of 0.6% 2-thiobarbituric acid solution containing 1% SDS and 1.25 mM EDTA was added to the reaction mixture. The solution was then heated for 30 minutes at 90°C. After cooling to room temperature, absorbance at 532 nm was measured. Base propenal produced by the reaction was estimated using a molar absorption coefficient determined for a standard solution of malondialdehyde.

Determination of Bases Released from DNA

The reaction mixture consisted of 0.05 M Tris-HCl buffer, pH 7.8, various concentrations of the Fe(II) complex of drug and calf thymus DNA. The reaction was conducted for 10 minutes at 37°C. The concentration of nucleo bases released from DNA was measured by HPLC as described previously[6].

Cleavage of Plasmid DNA pBR322

The reaction mixture (0.05 ml) consisted of 0.05 M Tris-HCl buffer, pH 7.8, various concentrations of the Fe(II) complex of drug and 50 μM of form I pBR322 DNA. The mixture was incubated at 37°C for 10 minutes. The reaction was stopped by addition of a solution containing 75 mM EDTA, 75% glycerol and 0.075% bromophenol blue. The different forms of DNA were electrophoretically separated on an agarose gel (1.1%) at 30 V for 18 hours. The gel was stained with ethidium bromide and photographed under a UV light. The negative film was scanned with a Gelman DCD-16 Densitometer to determine the relative amounts of the various forms of plasmid DNA.

Specificity of Cleavage Sites on Plasmid DNA pBR322

The reaction mixture (0.05 ml) of 0.05 M Tris-HCl buffer, pH 7.8, various concentrations of the Fe(II) complex of drug and 60 μM pBR322 DNA was incubated for 10 minutes at 37°C. It was then adjusted to 0.04 M Tris-HCl buffer, pH 7.8, 30 mM NaCl and 2 mM $MgCl_2$ and incubated with *Pst* I for 120 minutes at 37°C. The reaction was terminated by the addition of 5 μl of 75 mM EDTA, 75% glycerol and 0.075% bromophenol blue and incubated for 10 minutes at 65°C. Samples were electrophoresed on 0.9% agarose gels at 30 V for 18 hours.

After photographing, the negative film was scanned by densitometer.

Determination of Oxygen Consumption

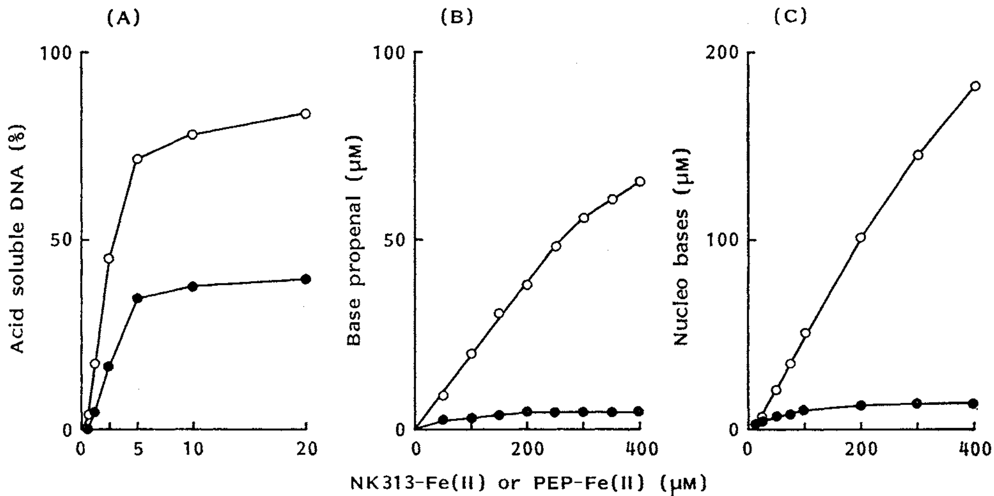
Oxygen consumption of the Fe(II) complex of drug was determined in a Gilson Model K-ICT-O oxygraph with a Clark electrode[7]. Fifty μl of ferrous ion solution was added to an electrode cell containing 1.3 ml of drug in 50 mM Tris-HCl, pH 7.8, and oxygen uptake was recorded.

Results

DNA Cleavage in the Absence of Reducing Agents

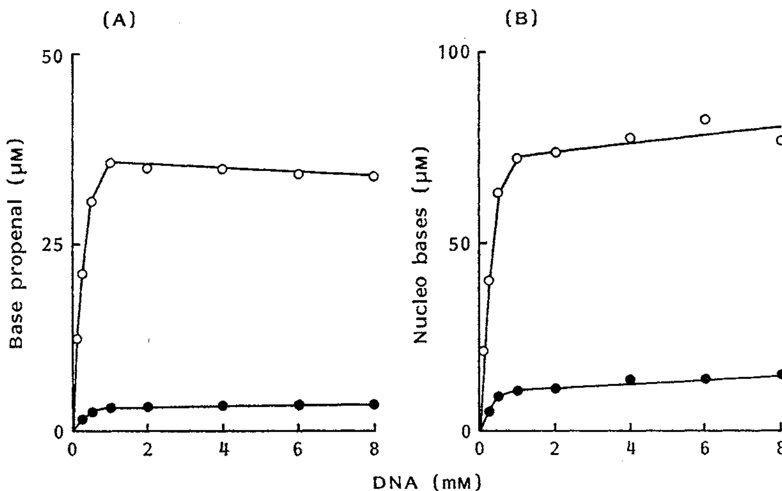
Both NK313 and PEP produced acid soluble DNA, base propenals and nucleo bases (Fig. 1).

Fig. 1. DNA cleavage activity of NK313-Fe(II) and PEP-Fe(II).
● NK313, ○ PEP.



Experimental procedures are described in Materials and Methods. (A) Acid-solubilization of DNA. (B) Production of base propenals. (C) Release of nucleo bases.

Fig. 2. DNA concentration dependency of DNA cleavage reaction.



The mixtures containing 0.2 mM NK313-Fe(II) (●) or PEP-Fe(II) (○), various concentrations of calf thymus DNA and 0.05 M Tris-HCl buffer, pH 7.8, were incubated for 10 minutes at 37°C. Base propenal production (A) and base release (B) was determined as described in Materials and Methods.

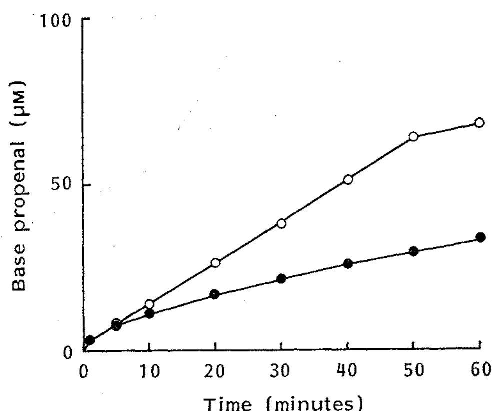
However, the activity of NK313 was less than that of PEP. NK313 reached a plateau level at lower concentrations than PEP and the plateau levels were 1/2~1/10 of these of PEP. The DNA saturation levels for NK313 were 1/5~1/10 of those for PEP (Fig. 2).

DNA Cleavage in the Presence of Reducing Agents

Activity of both NK313 and PEP was stimulated by 2-mercaptoethanol in a biphasic reaction. As reported previously, the first reaction is due to residual PEP-Fe(II) and the second is due to PEP-

Fe(II) produced by the reduction of PEP-Fe(III) with 2-mercaptoethanol[8]. The amounts of base propenal production and base release by NK313 after a 60-minute incubation were 3.1- and 4.5-fold of those after a 1-minute incubation, respectively. On the other hand, those by PEP after 60 minutes incubation were 1.6- and 2.8-fold of those after 1 minute incubation, respectively. In the presence of 1 mM ascorbic acid NK313 produced almost as much base propenal as PEP for the first 10 minutes (Fig. 3).

Fig. 3. Stimulation of DNA cleavage reaction by ascorbic acid.



The mixtures containing 5 μM NK313-Fe(II) (●) or PEP-Fe(II) (○), 2 mM calf thymus DNA, 1 mM ascorbic acid and 0.05 M Tris-HCl buffer, pH 7.8, were incubated at 37°C. At various incubation times, base propenal production was determined as described in Materials and Methods.

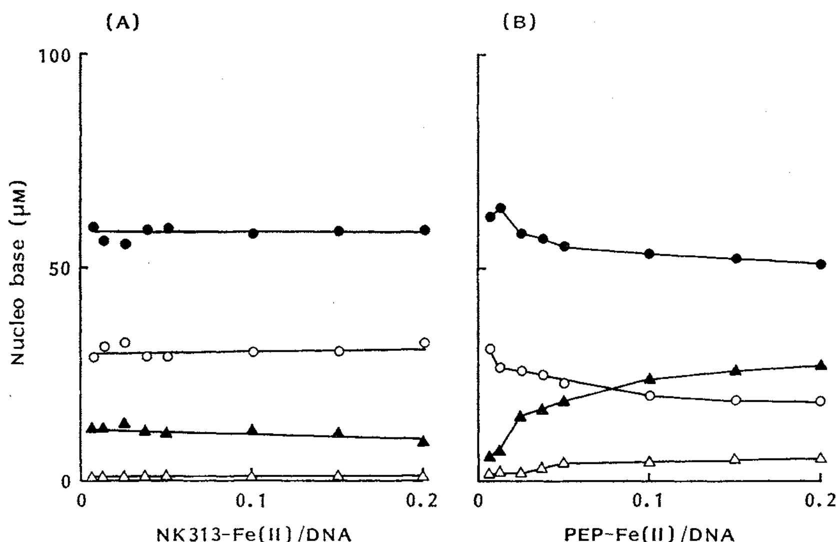
Specificity of Base Release from Calf Thymus DNA

Thymine was most readily released from DNA by NK313, and the molar ratio of the released pyrimidine bases to the total released bases was over 0.8 (Fig. 4). The ratio was little affected by the ratio of NK313 to DNA. On the other hand, although PEP released pyrimidine bases preferentially at low ratios of PEP to DNA, the ratio of the released purine bases increased with increasing PEP to DNA ratios.

Single and Double Strand Cleavage of Plasmid DNA pBR322

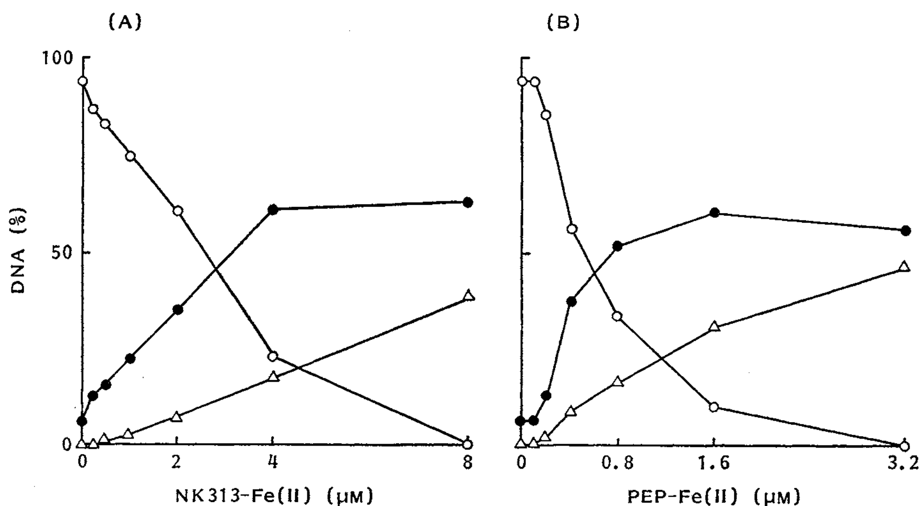
NK313 transformed form I of pBR322 DNA to form II and from III, but the activity was

Fig. 4. Specificity of base release from calf thymus DNA by NK313-Fe(II) (A) or PEP-Fe(II) (B).



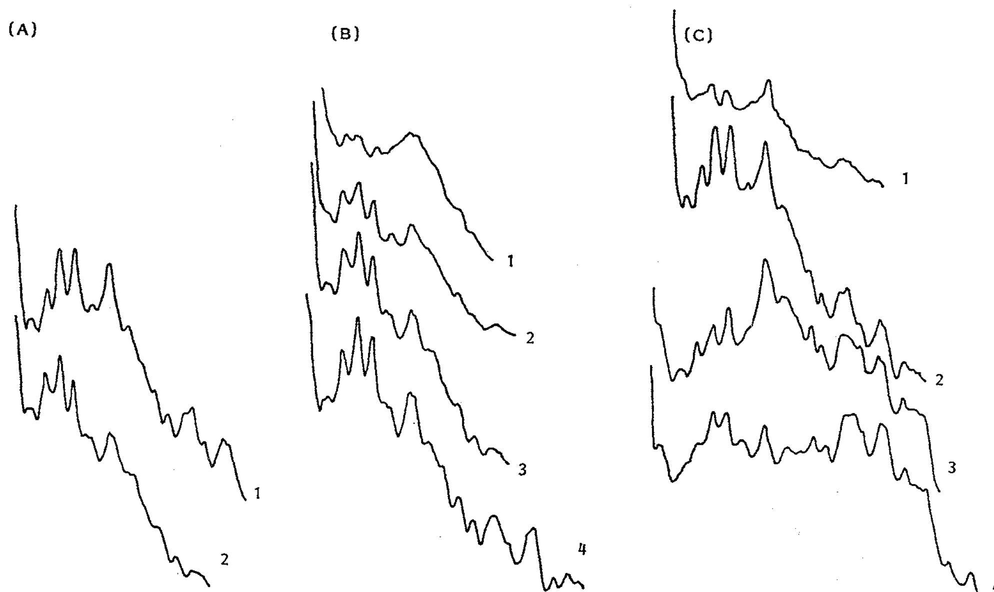
The mixtures containing various concentrations of NK313-Fe(II) or PEP-Fe(II), 2 mM calf thymus DNA and 0.05 M Tris-HCl buffer, pH 7.8, were incubated for 10 minutes at 37°C. Release of thymine (●), cytosine (○), adenine (▲) and guanine (△) was determined as described in Materials and Methods.

Fig. 5. DNA cleavage activity of NK313-Fe(II) (A) and PEP-Fe(II) (B) on plasmid DNA pBR322.
○ Form I, ● form II, △ form III.



Experimental procedures are described in Materials and Methods.

Fig. 6. Specificity of cleavage sites on plasmid DNA pBR322.



Experimental procedures are described in Materials and Methods.

Figures show densitometric scans of NK313 or PEP treated pBR322 DNA digested with *Pst* I restriction enzyme. DNA fragments were electrophoresed from left to right.

(A) 1, PEP 0.30 μM; 2, NK313 1.00 μM; (B) NK313: 1, 0.50 μM; 2, 0.75 μM; 3, 1.00 μM; 4, 1.25 μM; (C) PEP: 1, 0.20 μM; 2, 0.30 μM; 3, 0.40 μM; 4, 0.50 μM.

about one-fourth of that of PEP (Fig. 5). As indicated by the transformation to form III, NK313 induced efficient double strand cleavage.

Specificity of Cleavage Sites on Plasmid DNA pBR322

Double strand cleavage of pBR322 DNA by NK313 and PEP was examined by comparison of

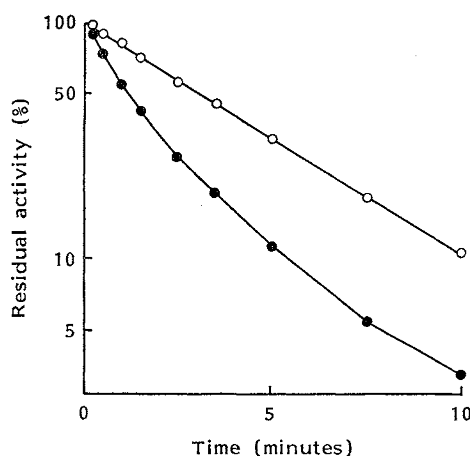
Table 1. Oxygen consumption of NK313-Fe(II) and PEP-Fe(II).

DNA (2 mM)	−	−	−	+	+	+
2-ME (10 mM)	−	+	+	−	+	+
	Consumed O_2 (μM)	Consumed O_2 (μM)	O_2 consumption rate (μM O_2/minute)	Consumed O_2 (μM)	Consumed O_2 (μM)	O_2 consumption rate (μM O_2/minute)
NK313-Fe(II) (100 μM)	38.8	79.8	9.55	45.0	62.2	1.03
PEP-Fe(II) (100 μM)	43.6	89.9	14.1	82.1	88.5	0.30

In the absence or presence of 2-mercaptoethanol (2-ME) and/or DNA, oxygen consumption of NK313-Fe(II) and PEP-Fe(II) was determined as described in Materials and Methods.

DNA fragments produced by treatment with drug and *Pst* I restriction enzyme which cleaved single site on pBR322 DNA. Fig. 6(A) shows densitometric scans of agarose gel electrophoresis of DNA fragments induced by NK313 or PEP on pBR322 DNA followed by *Pst* I digestion. The major fragments produced by treatment with NK313 were qualitatively similar to those of PEP. Certain minor peaks of NK313 were specific for NK313. The length of the DNA fragments produced by PEP was reduced as the concentration of PEP increased (Fig. 6(C)); NK313 did not further degrade DNA fragments at higher concentration (Fig. 6(B)).

Fig. 7. Inactivation rate of base propenal production activity of activated NK313 and PEP at 0°C.



The mixture containing 0.05 M Tris-HCl buffer, pH 7.8, 0.24 mM NK313 (●) or PEP (○) and 0.20 mM Fe(II) was incubated at 0°C under aerobic condition. Ferrous ion was added last. At the indicated incubation time, aliquot of the mixture was added to DNA solution to measure the remaining base propenal production activity by the method described in Materials and Methods.

Oxygen Consumption of the Fe(II) Complex

The active intermediate of BLM (activated BLM) is formed from the Fe(II) complex with oxygen[6]. We examined oxygen consumption of the Fe(II) complexes of the two analogues. Oxygen consumption by 100 μM NK313-Fe(II) complex was 38.8 μM, and slightly lower than that of PEP (Table 1). In the presence of 2-mercaptoethanol, oxygen consumption proceeded in a biphasic manner. In the second phase, the rates for NK313 and PEP were 9.55 and 14.1 μM O_2/minute, respectively. In the presence of DNA, the oxygen consumption of NK313 was about half that of PEP. However, in the presence of both 2-mercaptoethanol and DNA the consumption rate with NK313 was 1.03 μM O_2/minute, over three times that of PEP (0.30 μM O_2/minute).

Decomposition of Activated NK313 and PEP

The decomposition of activated PEP by Fe(II) and oxygen displayed first-order kinetics at 0°C, whereas the decomposition of activated NK313 did not (Fig. 7). The half times of decomposition of activated NK313 and PEP were 2.2 and 5.7 minutes, respectively.

Discussion

BLMs possess two sites each having a different role in drug activity. One site is an active site in which ferrous ion is chelated and oxygen is activated[9,10]; the other is a DNA binding site containing the bithiazole group and terminal amine region[10~12]. The active site of NK313 is identical with that of PEP, but the DNA binding site differs in its terminal amine region[2]. Therefore, we expected NK313 would have a different DNA cleavage site specificity compared to PEP. However, only slight differences in specificity of the bases released from calf thymus DNA and cleavage of pBR322 DNA were seen. Base release by NK313 showed a strong preference for pyrimidine bases and the ratio of pyrimidine to purine was constant, independent of the ratio of NK313 to DNA. Furthermore total DNA cleavage by NK313 was less than that by PEP and was not concentration dependent. These results indicate that the DNA cleavage sites of NK313 are more limited than those of PEP. The major pBR322 DNA cleavage sites by NK313 corresponded with those at low concentrations of PEP. This suggests that the sites on superhelical DNA most susceptible to double strand cleavage by NK313 are identical to those of PEP (Fig. 6(A)). However, minor cleavage sites by NK313 were different from those of PEP; NK313 sites were more limited than those of PEP (Figs. 6(B) and 6(C)).

DNA cleavage by NK313 is assumed to be identical with that of PEP; however, the activity of NK313 was lower than that of PEP in all assay systems. One cause of this lower activity is that NK313 attacks fewer cleavage sites on DNA than PEP. In addition, NK313 is activated less efficiently than PEP and the active intermediate is less stable, as shown by the finding that the NK313-Fe(II) complex consumed less oxygen than the PEP-Fe(II) complex and that the activity of activated NK313 decreased faster than that of activated PEP. Interestingly, the decreasing rate of activity of NK313-Fe(II) was not first-order, in contrast to that of PEP. This suggests that the lipophilic substitution of NK313 interacts with the active site and influences oxygen activation and stability.

In the absence of reducing agents the activity of NK313 was much lower than that of PEP. However, in the presence of reducing agents NK313 activity was stimulated more efficiently than PEP. This is consistent with the observation that 2-mercaptoethanol stimulated oxygen consumption of NK313 more greatly than PEP. NK313 induced almost as much DNA cleavage as PEP in the presence of 1 mM ascorbic acid. The difference in turnover rate caused by reducing agents between NK313 and PEP may be due to the difference in reduction rate between NK313-Fe(III) and PEP-Fe(III). This also implies an effect on the active site by the terminal amine substitution.

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