Vol. 206, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 5, 1995 Pages 133-137

MECHANISM FOR A NEW ANTITUMOR VANADIUM COMPLEX: HYDROXYL RADICAL-DEPENDENT DNA CLEAVAGE BY 1,10-PHENANTHROLINE-VANADYL COMPLEX IN THE PRESENCE OF HYDROGEN PEROXIDE

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SUMMARY: Among vanadium complexes which show inhibition of cell growth for human nasopharyngeal carcinoma KB cell, a newly synthesized 1:1 vanadyl-1,10-phenanthroline complex, $VO(phen)^{2+}$, was found to cleave supercoiled plasmid Col E1 DNA effectively when hydrogen peroxide was added. But VO^{2+} ion was less effective. Lineweaver-Burk plots of the complex binding to calf thymus DNA indicated that $VO(phen)^{2+}$ complex has a high affinity to DNA, as supported by CD spectral measurements. To examine the active species for DNA cleavage by the complex, ESR spin trapping was performed and was found that hydroxyl radicals are generated in a pH-dependent manner in the $VO(phen)^{2+}$ -H_2O_2 system, the optimal pH region being 8.5-9.5. In contrast, no optimum pH was observed in VO^{2+}-H_2O_2 system. Thus, the $VO(phen)^{2+}$ complex is proposed to bind DNA and cleave it when hydrogen peroxide is present.

The success of cis-diamminedichloroplatinum(II) complex (CDDP) in clinical use for human malignancies (1) has stimulated researches in the field of new platinum complexes (2) and other metal-containing antitumor complexes such as titanium (3), gold (4), germanium (5), copper (6), iron (7), ruthenium (8), cobalt (7) and vanadium (9). Our strategy to find new antineoplastic metal complexes is based on vanadium complexes. Among numerous vanadium complexes tested, we found strong antitumor activities in the vanadyl complexes of 1,10-phenanthroline (phen) and the derivatives (10). In the 50% inhibition concentration (IC_{50}) of cell growth test, using the human nasopharyngeal carcinoma, KB cell line, the cytotoxic effects (16-22 ng/mL) of the vanadium complexes were superior to that (30 ng/mL) of the therapeutic drug, CDDP (10). During the attempts to gain the mechanism for the antineoplastic activities of vanadium complexes, we found that a newly synthesized 1:1 vanadyl-phen complex, $VO(phen)^{2+}$ induces DNA cleavage in the presence of

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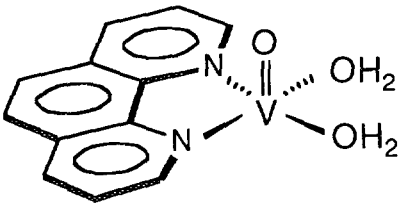
0006-291X/95 $5.00

hydrogen peroxide H_2O_2, in which hydroxyl radicals · OH are formed. We report here the hydroxyl radical-dependent DNA cleavage by the $VO(phen)^{2+}$-H_2O_2 system, comparing with the results of vanadyl ion ($VOSO_4$)-H_2O_2 system.



MATERIALS AND METHODS

1. Preparation of $VO(phen)^{2+}$ complex : $VO(phen)SO_4 \cdot 3.5H_2O$ was prepared by mixing equivalent concentration of $VOSO_4$ and 1,10-phenanthroline in ethanol at 50℃ for 15 min under argon atmosphere. The precipitate was filtered, washed well with ethanol and dried *in vacuo* over night in silica-gel desicator. Analytical data : Calcd: C, 35.48; H. 3.72; N, 6.90. Found: C, 35.28; H, 3.55; N, 6.92. The structure of the complex was estimated by ESR spectrum with typical 8 line signals due to VO^{2+} ($g_{\parallel}$ =1.93, $A_{\parallel}$ =183.8x10^{-4} cm^{-1} at 77K) and IR spectrum ($\nu_{V=O}$ = 970 cm^{-1}; KBr disk).

2. DNA-cleaving activity : Supercoiled plasmid Col E1 DNA (0.2 μg) was incubated in a reaction mixture (10μl) containing various concentrations of vanadyl compound (0~ 20 μM) and H_2O_2 (0~ 20 μM) in 20 mM Tris-acetate buffer, pH 7.8 at 37℃. Reactions were started by addition of H_2O_2 and stopped after 60 min by addition of 2.5μl of a terminating agent containing 20 mM Tris-acetate buffer, 50% glycerol, 3 mM EDTA and 0.1 % bromophenol blue. The samples were loaded on 1 % neutral agarose gel containing 20 mM Tris-acetate buffer and were subjected to electrophoresis in a horizontal slab gel apparatus (50 V for 8 ~ 10hr). The gel was stained with a solution of 0.5μg/ml ethidium bromide for 20-30 min. Bands of DNA were detected and photographed (Polaroid 600SE camera with an orange filter) under UV light (235.7 nm) in a dark room. Determination of form I (supercoiled), form II (nicked) and form III (linear) was performed with a dual-wavelength flying spot scanner (Shimadzu CG-9000, Kyoto, Japan). DNA cleaving activity of the complex was estimated by the following equation: DNA cleaving activity (%)=[(forms II + III)$_{complex}$-(forms II + III)$_{V(IV)}$]/(forms I + II + III)x100.

3. ESR spin-trapping method : A mixture (60μl) of 50μM $VO(phen)^{2+}$ and 50μM H_2O_2 or 100μM $VOSO_4$ and 100μM H_2O_2 and 0.4M 5,5-dimethyl-1-pyroline-N-oxide (DMPO) in 20mM Tris-acetate buffer, pH 7.8, was trasferred into a quarz ESR tube, which was fixed in the cavity of an ESR spectrometer. ESR spectra were recorded with a JEOL RE1X ESR spectrometer at a modulation frequency of 100 KHz, modulation amplitude of 0.63 mT and microwave power of 8 mW. The spectrometer was calibrated with an ADVANTEST microwave counter, R5372. Recording of the spectra was started 60 sec after the addition of H_2O_2 at 21℃, and each scan took 2 min. Other instrument settings were as follows: Magnetic field 337 ± 5 mT, amplitude 630 and response 0.1 sec.

RESULTS AND DISCUSSION

Figure 1 shows the results of cleavage of supercoiled plasmid Col E1 DNA by various amounts of $VO(phen)^{2+}$ and $VOSO_4$ in the presence of H_2O_2. After 1hr reaction at pH 7.8 and 37 ℃ with $VO(phen)^{2+}$-H_2O_2 system, dose-dependent

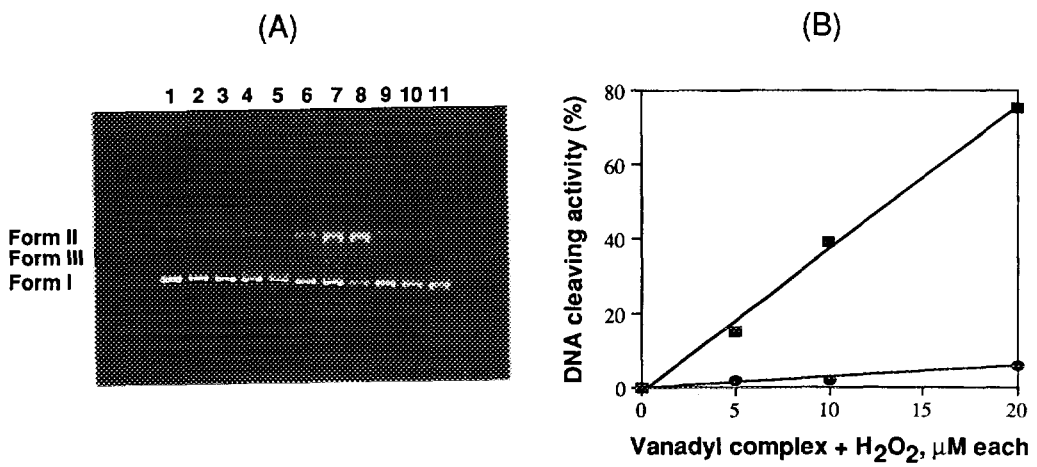


Figure 1. Agarose gel electrophoretic patterns (A) and the plots of DNA cleaving activities (B) for plasmid DNA cleavages by $VO(phen)^{2+}$-H_2O_2 and $VOSO_4$-H_2O_2 systems. Col E1 DNA (0.2 μg) was incubated with 0-20 μM $VOSO_4$ or 0-20 μM $VO(phen)^{2+}$ and 0-20 μM H_2O_2 under air for 60 min at 37℃.
(A) Lanes 1 and 11, DNA alone; lanes 2-4, DNA + 5, 10 and 20 μM $VOSO_4$ + 5, 10 and 20 μM H_2O_2, respectively; lane 5, DNA + 20 μM $VOSO_4$; lanes 6-8, DNA + 5, 10 and 20 μM $VO(phen)^{2+}$ + 5, 10 and 20 μM H_2O_2, respectively; lane 9, DNA + 20 μM $VO(phen)^{2+}$; lane 10, DNA + 20 μM H_2O_2.
(B) ■: $VO(phen)^{2+}$-H_2O_2 system, ●: $VOSO_4$-H_2O_2 system

conversions of form I (supercoiled) to form II (nicked) DNA were apparent and superior to those with $VOSO_4$-H_2O_2 system (11), suggesting the binding of $VO(phen)^{2+}$ to DNA. In fitting Lineweaver-Burk plots of vanadyl complex binding to calf thymus DNA as monitored at 260 nm, the binding parameter, Km was 1.6 μM for $VO(phen)^{2+}$, while that for $VOSO_4$ was 4.8 μM. This was supported by the fact that CD possitive absorption band at around 270 nm due to calf thymus DNA increases by addition of $VO(phen)^{2+}$, while no increase in the band is observed by addtion of $VOSO_4$.

ESR spin trapping was performed by using DMPO (5,5-dimethyl-1-pyroline-N-oxide) to examine the true active species for inducing DNA cleavage by vanadyl complex-H_2O_2 systems. Hydroxyl radicals were detectable during the reaction as a strong spin adduct (DMPO-OH) ESR spetrum which consisted of a 1:2:2:1 quartet with a splitting of $a_\alpha^N = a_\beta^H = 1.49$ mT, in which a_α^N and a_β^H represent the hyperfine splitting of the nitrosyl nitrogen and β-hydrogen atoms, respectively (11, 12). Generations of · OH radical adduct signals by vanadyl-H_2O_2 system which resemble the Fenton reaction (Fe^{2+}-H_2O_2) (13) were reported previously (14) , but we found the structure- and concentration -dependent generation of hydroxyl radicals. Two-fold signal intensity in $VO(phen)^{2+}$- H_2O_2 system compared to the intensity in $VOSO_4$- H_2O_2 system was observable. Optimum reaction ratio was found to be 1:1 of vanadyl : H_2O_2 in both systems. Formation of · OH radicals was remarkably pH-dependent, the optimum pH region being at around pH 8.5-9.5 in $VO(phen)^{2+}$ system, while that in $VOSO_4$ system is not observed (Figure 2). The result supports the difference in DNA strand scission by the two compounds as shown in Figure 1. Thus

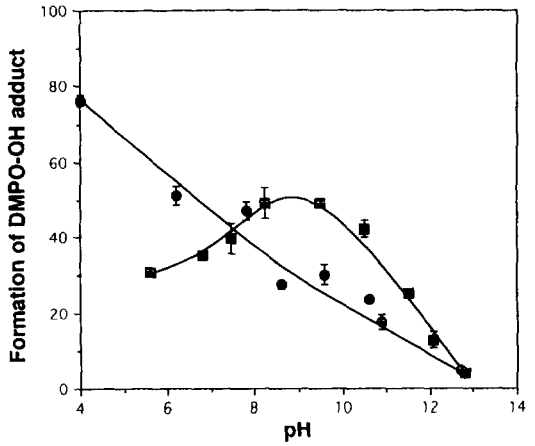


Figure 2. pH-Dependent formation of DMPO-OH adduct in $VO(phen)^{2+}$-H_2O_2 and $VOSO_4$-H_2O_2 systems. The ESR spectra of DMPO-OH adducts were identified by the hyperfine parameters ($a_\alpha^N = a_\beta^H = 1.49$ mT) and computer simulation.
▩: $VO(phen)^{2+}$-H_2O_2 system, ●: $VOSO_4$-H_2O_2 system

the effective hydroxyl radical-dependent DNA cleavage is apparent in the $VO(phen)^{2+}$-H_2O_2 system.

Based on these results, $VO(phen)^{2+}$ complex is presumed to bind DNA by intercalation, where the planar phenanthroline ring stacks between adjacent base pairs of the duplex (15). It is known that intercalative drugs can be strongly mutagenic as adriamycin and daunomycin and can serve as potent chemotherapeutic agents (16).

In living systems, H_2O_2 is formed by dismutation of superoxide anions, which are generated in various systems such as xanthine-oxidase, NADPH oxidase and NADH-dependent cytochrome P-450 and neutrophiles (17). Thus H_2O_2 is thought to react with $VO(phen)^{2+}$ bound with DNA to generate active oxygen species like · OH radicals. Further studies on site-specific binding of the complex to DNA is necessary to prove the effective DNA cleavage by the complex.

In conclusion, the new complex $VO(phen)^{2+}$ showed high antitumor activity for the human nasopharyngeal carcinomas cancer KB cell. $VO(phen)^{2+}$ is suggested to bind DNA and cleave it with the formation of · OH radicals when H_2O_2 is present in the system.

Acknowledgments. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and the Kyoto Pharmaceutical University Foundation to H. S.

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