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DNA-protein, DNA interstrand cross-links induced by camphoramine chloroacetic platinum *in vitro*

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Abstract Effects of camphoramine chloroacetic platinum (CCP) on DNA migration and transcription, DNA-protein and DNA interstrand cross-links induced by CCP were investigated by using agarose gel electrophopresis, alkaline elution and enzymatic techniques, respectively. Chromosome break down and migration alteration of DNA modified by CCP were observed. Plasmid pAR 436 DNA transcription was also blocked when the DNA was treated with CCP. The cross-links took place 8 h after HeLa cells were exposed to CCP 10 μmol/L and higher number of cross-links were obtained after treatment with the agent 20 μmol/L. The number of cross-links was also found to be decreased when the cell lysis was digested with proteinase K. These results suggest that CCP can also induce DNA-protein cross links. Enzymatic studies indicated that CCP preferentially attacks guanine in DNA and restriction enzymes are unable to cleave G-platinated at interval of one base to the restriction sequence.

Key words camphoramine chloroacetic platinum; alkaline elution; DNA; HeLa cells; cultured cells; cross-linking reagents

Cisplatin is an important drug used alone or in combination in the treatment of several types of human cancer, but with serious adverse reactions. Camphoramine chloroacetic platinum (CCP) is a new potential antimumor agent with a structure in its ligand different from that of cisplatin[1]. We have reported that the antitumor action of CCP is similar to that of cisplatin in several experimental animal tumors and its toxicity is lower[2]. CCP was shown to inhibit DNA replication, bind with DNA covalently and noncovalently and change the secondary structure of DNA[3]. According to these facts we suppose that like bifunctional alkylating agent, CCP may produce DNA-protein and DNA interstrand cross-linking. In this work alkaline elution and enzymological techniques were used to test our hypothesis in order to understand the mechanism of antitumor action of CCP.

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Materials and methods

CCP, white crystal powder, was synthesized by Nanjing University. The water solution of CCP in low TE buffer (Tris-HCl 0.01 mol/L and EDTA 0.01 mmol/L, pH 7.4) was prepared immediately before use. [^{14}C] Thymidine, proteinase K and other chemicals were obtained from Sigma Co. Restriction enzymes were purchased from New England Biolabs Inc. pAR 436 DNA and T7 RNA polymerase were kindly provided by Professor John DUNN.

Preparation of modified DNA pAR 436 DNA was dissolved in low TE buffer and CCP solution was then added to the DNA solution to get final concentrations of CCP 1, 5, 15 and 30 μmol/L. Final DNA concentration was 50 μg/ml. These solutions were kept in the dark at 20℃ for 2 d and then stored at −35℃ until use.

HeLa, HeLa SMR and CHO cells were grown in HAM's and in F12 medium. The mediums were complemented with FBS 10%, glutamine 3 mmol/L, penicillin 10 IU and streptomycin 10 μg/ml. Cells were incubated at 37℃ in humidified atmosphere containing 5% CO_2.

Survival test Cells were plated on duplicate 60 mm dishes with 10^2, 10^3

and 10^4 cells/dish containing 5 ml medium. After 6 h, the cells were treated with CCP. After 10 d, the culture medium was removed, the cells were washed twice with PBS, fixed in Cernoy's solution (methanol: glacier acetic acid, 3:1) for 1 h and stained with freshly prepared Giemsa solution (3% Giemsa in PBS 0.01 mol/L, pH 6.8) for 25 min. The stain was washed off with tap water. The dishes were dried in the air and the colonies counted.

Determination of transcription Ten μl modified DNA solution and 1 U of T7 RNA polymerase were added to 5 × transcription buffer (100 mmol/L of NaH_2PO_4: Na_2HPO_4, 1:3; DDT 50 mmol/L; $MgCl_2$ 40 mmol/L; spermidine 20 mmol/L and NTP 5 mmol/L). The solution was incubated at 37℃ for 30 min. The reaction was stopped by adding 9 μl TCCB and 1 μl 10% SDS and heated at 100℃ for 2 min, then running 1% agarose gel electrophoresis.

Alkaline elution analysis Alkaline elution was performed as previously described [4]. HeLa cells were seeded at the density of 2.5×10^5 cells/ml for 24 h and then reseeded at the same density for another 6 h. [^{14}C] Thymidine 0.1 kBq/ml was added to the cultures to label the cells. After 24 h the medium containing radioactivity was removed, fresh medium was added and incubated at the same conditions as before for another 12 h, then treated with CCP for 8 h. The concentration of CCP were 10 μmol/L and 20 μmol/L respectively. The medium was removed and the cells were rinsed twice with ice-cold PBS. Two ml cold PBS was added to the cultures and the cells were irradiated with X-ray at 5 Gy. A 2-μ polyvinyl chloride filter was dipped edge first into EDTA 0.02 mol/L (pH 10) solution to wet the filter uniformly without leaving any air pockets. The filter was then mounted in a funnel. About 20 ml of ice-cold PBS was added to the funnel and the vacuum was turned on to produce a rapid flow rate which expels the air from the filter holder. The vacuum was then adjusted to a slow flow rate, the cell suspension was added to the funnel and the cells were washed with 20 ml of ice-cold PBS twice. Two ml of SDS-EDTA lysis solution with or without 0.5 mg/ml proteinase K was carefully added to the filter holder.

The lysis solution was allowed to drop by gravity into a scintillation bottle. This procedure lasted 1 h, then the filter was washed with 20 ml PBS and 40 ml of Pr_4 NOH-EDTA solution was added (Pr_4 NOH was added to EDTA to get final pH 12.1, EDTA 0.02 mol/L). Alkaline elution was then proceeded in the standard fashion and the data were analysed by IBM personal computer.

Digestion of modified DNA with restriction enzymes One μl of normal or modified plasmid pAR 436 DNA (50 μg/ml) and 1 μl (1 U) of different restriction enzyme were added to a suitable restriction buffer as described in the Biolabs Catalog 1986/1987. The final volume of the reaction mixture was 10 μl. The mixtures were incubated at 37℃ for 2 h. The products of digestion were then measured by 1% agarose gel electrophoresis and the gels were stained with EB (1 μg/ml) solution.

Results

Cytotoxicity studies The killing ability of CCP was estimated by using HeLa, HeLa SMR and CHO cells. The survival curves for these cell lines treated with different concentrations of CCP are shown in Fig 1. The D_{10} value (increase in dose resulting in a decrease in survival to 10% of the inital value) of HeLa, HeLa SMR and CHO for CCP were 2.5, 6.5 and 11.3 μmol/L, respectively. The results indicate that the cytotoxicities of CCP for

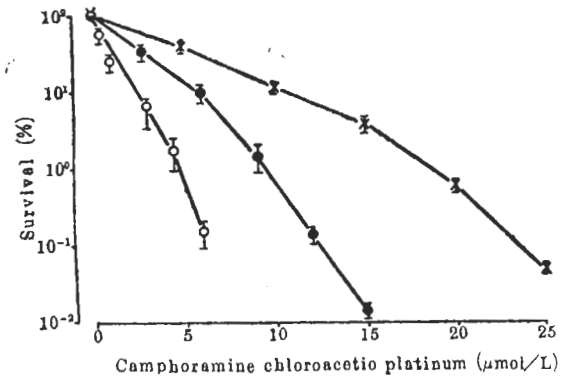

Fig 1. Cytotoxicities of CCP on HeLa (○), HeLa SMR (●) and CHO (×) cells. Exponentially growing cells were exposed to varying concentration of CCP under normal culture conditions and cytotoxicity was estimated by the ability of cells to form colonies.

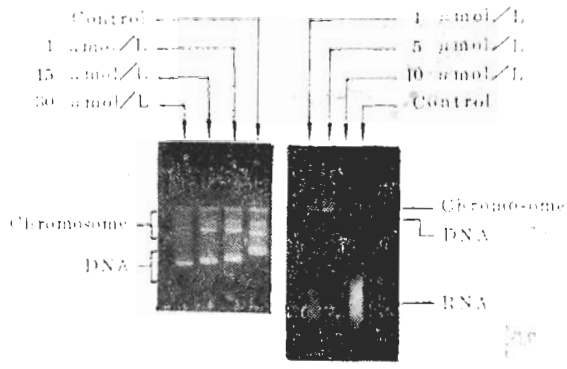

Fig 2. Effects of CCP on pAR 436 DNA mobility (A) and transcription (B) were determined using 1% agarose gel electrophoresis.

these cell lines are lower than those of cisplatin.

Effects of CCP on DNA transcription As shown on Fig 2 b, the transcription function of pAR 436 DNA was affected by CCP. RNA synthesis was inhibited when the DNA was treated with CCP 1 μmol/L and the reaction was almost stopped after treatment with high concentration of CCP.

Alteration of DNA migration The mobility changes of plasmid DNA modified by CCP was analysed by using 1% agarose gel electrophoresis. Ten μl of modified DNA was put on the gel and subjected to electrophoresis. After the electrophoresis, the gel was stained with EB solution 0.5 μg/ml. As shown in Fig 2 a, comparing the modified DNA with the control, one more band appeared on the chromosome band area. This may come from the CCP induced breaking of chromosome. Alteration of migration of the modified DNA was also observed after electrophoresis. The platinated DNA shifted faster than normal DNA in the electric field, the higher dose, the stronger action. This result is another evidence for our provious report that CCP can bind to DNA and induce changes of the DNA secondary structure.

DNA–protein and DNA interstrand cross–linking The occurrence of interstrand cross–links in the HeLa cells treated with CCP was determined at 8 h after the treatment. Cell lysis was performed both with (Fig 3 b) and without (Fig 3 a) proteinase K. The results showed that the cross–links took place in the HeLa cells when treated with CCP 10 μmol/L and a sharp increase in the number of cross–links was observed when the HeLa cells were exposed to CCP 20 μmol/L. Less number of cross–links was obtained when the cell lysis was digested with proteinase K. This result suggests that CCP not only induces DNA interstrand cross–linking, but also induces DNA–protein cross–links.

Enzymatic studies pAR 436 DNA can be cut by several restriction enzymes and is easily separated by agarose gel electrophoresis. We used various restriction endonucleases to check the sensitive reaction sites of CCP. Selective inhibition of cleavage was observed when the DNA modified by CCP was digested with different enzymes. Tab 1 illustrates the result of selective cleavage. At low level of CCP binding, no effect of CCP on cleavage for some enzymes, such as Bgl Ⅱ, Hpa Ⅰ, Pvu Ⅱ

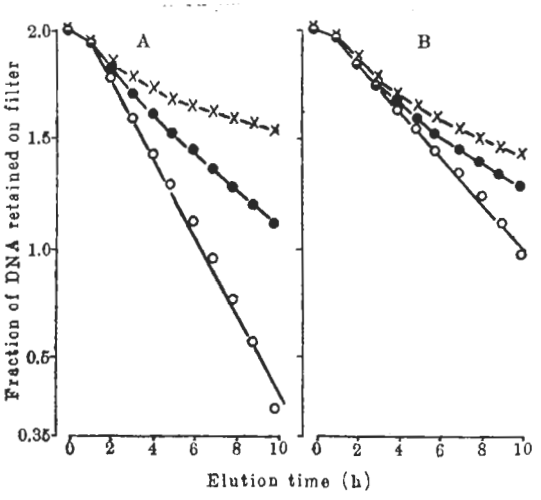

Fig 3. Analysis of DNA-protein and DNA interstrand cross-links induced by CCP. HeLa cells were treated with CCP 0 (○), 10(●), and 20 (×) μmol/L for 8 h at 37℃, washed, exposed to X-ray, 500 rad at room temperature and digested without (A) or with (B) proteinase K.

Alu I and Hha I was observed, but the digestions of some enzymes, such as Ava II, were inhibited. At high level of CCP binding, the digestion of some enzymes was compeletly stopped, such as Acc I, but for others, in spite of partial inhibition, continued. When we summerized the results (as shown in Tab 1) we found that (A): enzyme digestion was stopped preferentially at di(dG) sequences; and (B): enzyme was unable cleave the restriction site with G-platinated at interval of one base.

Discussion

Similar to cisplatin, the cytotoxic properties of CCP are most likely a consequence of interaction with its major intracellular target, DNA, despite their different ligand structure. In the current work although different concentrations of CCP were used in different experimental system, the same conclusions as our previous studies were obtained in that CCP showed strong antitumor action, bind to DNA

Tab 1. Action of restriction enzyme on pAR 436 DNA modified by different concentration of CCP (final concentration of DNA in this experment was 50 μg/ml). *: Normal action; **: No action.

Enzyme	Cut site	Action of the enzyme 1	5	15 μmol/L
Bgl II	A↓GATCT	+ + +*	+ +	−**
Hpa I	GTT↓AAC	+ + +	+	+
Pvu II	CAG↓CTG	+ + +	+	+
Alu I	AG↓CT	+ + +	+	+
Hha I	GCG↓C	+ + +	+ +	+ +
Acc I	GT↓$\frac{AG}{CT}$AC	+ +		−
Ava II	G↓G$\frac{A}{T}$CC	+ +	+	−
FnuD II	CG↓CG	+ +	+	−
Hind III	A↓AGCTT	+ +	+	−
Apa I	G↓TGCAC	+ +	+	−

covalently or noncovalently and resulted in alteration of DNA secondary structure. The alkaline elution technique is based on two phenomena: (A) dependence of DNA alkaline elution rate on single-strand size; and (B) tendency of protein molecules to adhere to the filters[5]. Both interstrand cross-links and DNA-protein cross-links reduce the rate of elution and DNA-protein cross-linking can be reduced or eliminated by proteolytic digestion. It has been reported that cisplatin, like bifunctional chloroethylnitrosoureas, produce DNA-protein and DNA interstrand cross-links and this kind of interaction is considered to be correlated with cytotoxicity, mutagenicity and autitumor activity of cisplatin[6-8]. We had postulated in previous report that this kind of damage to DNA may also be induced by CCP. Our present results support the original hypothesis.

Enzymatic studies show that cisplatin is an electrophilic agent which attacks the N^7 position preferentially and other nucleophilic sites of guanine less frequenctly, this includes the N^3, O^6, 2 NH^2 and C^8 positions[9]. Our results show that guanine in DNA is the easiest to be attacked

by CCP and several restriction enzymes, such as Acc Ⅰ, Ava Ⅱ, FunD Ⅱ, Hind Ⅲ and Apa Ⅰ are unable to cleave G-platinated at interval of one base to the restriction sequence. These findings indicate that CCP has similar action on the DNA template as cisplatin. Our studies also support the previous hypothesis that platinum bound at or near the recognition sequence would unwind the duplex and most likely would result in destruction of the requisite two-fold symmetry for cleavage[10,11].

References

1 唐雯霞、曲 筠、胡宏纹、戴安邦、籍秀娟、张福荣. 一类低毒高活性铂配合物. 自然杂志 1985; 8: 764

2 Wang LG, Liu XM, Ji XJ. Effect of camphoramine chloroacetic platinum (CCP) on biomacromolecules in L_{1210} leukemic cells. *Acta Pharm Sin* 1987; 22: 87

3 Wang LG, Liu XM, Ji XJ. Effect of camphoramine chloroacetic platinum (CCP) on DNA template. *Ibid* 1987; 22: 6

4 Ewig RAG, Kohn KW. DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L_{1210} cells. *Cancer Res* 1978; 38: 3197

5 Ewig RAG, Kohn KW. DNA damage and repair in mouse leukemia L_{1210} cells treated with nitrogen mustard, 1,3-bis (2-chloroethyl) -1-nitrosourea, and other nitrosoureas. *Cancer Res* 1977; 37: 2114

6 Plooy ACM, van Dijk M, Lohman PHM. Induction and repair of DNA cross-links in chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA cytotoxicity, mutagenicity, and antitumor activity. *Ibid* 1984; 44: 2043

7 Ciccarelli RB, Solomon MJ, Varshavsky A, Lippard SJ. *In vivo* effect of *cis*- and *trans*-diamminedichloroplatinum (Ⅱ) on SV 40 chromosomes: Differential repair, DNA-protein cross-linking, and inhibition of replication. *Biochemistry* 1985; 24: 7533

8 Sariban E, Kohn KW, Zlotogorski C, *et al.* DNA cross-linking responses of human malignant glioma cell strains to chloroethylnitrosureas cisplatin, and diaziquone *Cancer Res* 1987; 47: 3988

9 Rosenberg B. Fundamental studies with cisplatin. *Cancer* 1985; 55: 2303

10 Cohen GL, Bauer WR, Barton JK, Lippard SJ. Binding of *cis*- and *trans*-dichlorodiammineplatinum (Ⅱ) to DNA: Evidence for unwinding and shortening of the double helix. *Science* 1979; 203: 1014

11 Smith HO. Nucleotide sequence specificity of restriction endonucleases. *Ibid* 1979; 205: 455

樟脑胺氯乙酸铂引起的体外DNA-蛋白，DNA链间交联

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提要 采用琼脂糖凝胶电泳、碱洗脱及酶学等方法观察了樟脑胺氯乙酸铂(CCP)引起的DNA结构变化及DNA-蛋白、DNA链间交联。实验表明CCP能引起染色体断裂、改变DNA迁移率、阻断pAR 436 DNA转录，并引起HeLa细胞DNA-蛋白及DNA链间交联。CCP首先进攻DNA分子中的鸟嘌呤，当与限制性位点相隔一个碱基的鸟嘌呤铂化后，内切酶失去作用。

关键词 樟脑胺氯乙酸铂；碱洗脱；脱氧核糖核酸；HeLa细胞；培养的细胞；交联试剂

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