Experimental verifications on chemical carcinogenesis, a bifunctional alkylation between DNA interstrands

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Abstract It is evidenced by the filter elution method that two carcinogenic aromatic hydrocarbons, benzo[a]pyrene and dibenzo[a,h]anthracene, two carcinogenic metal salts, beryllium chloride and cadmium chloride, four carcinogenic aromatic amines, 2-aminofluorene, β-naphthylamine, 4-aminobiphenyl and benzidine, can all induce DNA interstrand and DNA-protein cross-link in L_{1210} culture. However, under the same condition, the corresponding non-carcinogenic compounds, including benzo[k]fluorancene, anthracene, magnesium chloride, zinc chloride, α-naphthylamine, 2-aminobiphenyl and *m*-toluidine, cannot produce any cross-link adducts. All these results are consistent with the di-region theory that carcinogens are bio-bifunctional alkylation agents. This method can also be used to discriminate carcinogens and non-carcinogens.

Keywords: mechanism of chemical carcinogenesis, di-region theory, DNA interstrand cross-link, aromatic amines, polycyclic aromatic hydrocarbons, beryllium salt , cadmium salt.

Based upon the research on the relationship between molecular structure and carcinogenic activity of 49 polycyclic aromatic hydrocarbons (PAHs), Dai Qianhuan put forward[1] the di-region theory on the mechanism of chemical carcinogenesis. He proposed that most environmental carcinogens can be metabolized into bi-functional alkylation agents that can induce DNA interstrand cross-link, the key step of chemical carcinogenesis is the mutagenesis of the gene for the control of transcription through the cross-link between DNA complementary bases. It has been evidenced that the di-region theory possesses general significance for most carcinogen series[2]. Further, in this work we verified with the filter elution method[3] that two carcinogenic aromatic hydrocarbons, benzo[a]pyrene and dibenzo[a,h]anthracene, two carcinogenic metal salts, beryllium chloride and cadmium chloride, four carcinogenic aromatic amines, 2-aminofluorene, β-naphthylamine, 4-aminobiphenyl and benzidine, can all induce DNA interstrand and DNA-protein cross-link in L_{1210} culture. However, under the same condition, the corresponding non-carcinogenic compounds, including benzo[k]fluorancene, anthracene, magnesium chloride, zinc chloride, α-naphthylamine, 2-aminobiphenyl and *m*-toluidine, cannot produce any cross-link adducts. All these results are consistent with the di-region theory that carcinogens are bio-bifunctional alkylation agents. This method can also be used to discriminate carcinogens and non-carcinogens.

1 Experimental method

The filter elution method proposed by Kohn[3] was used to examine the DNA interstrand and

DNA-protein cross-link products in L_{1210} culture. All the tested organic compounds were cultivated with rat liver homogenate S_9 activated by polychlorobiphenyl. DNA double-strand was denaturated by diluted alkaline solution, then broken into fragments by the ^{60}Co γ-ray irradiation of 10 GY. The cross-link products remained on the filter after passing through the 1.25 μm microfilter. The quantity of DNA was determined by fluorescence with calibration line. The relative cross-link rate R was calculated according to the formula $R=(R_1-R_2)/R_1 \times 100\%$, in which R_1 is the residue of DNA cross-link products on the filter, and R_2 is the residue of the control cell culture on the filter.

Calf thymus DNA, dimethylaminobenzoic acid and proteinase K were the products of Sigma Company; the purities of all the other agents were above 99% by GLC analysis. Benzo[k]fluorancene, which has a GLC purity, was synthesized by a new method developed in our laboratory[4].

2 Experimental results

Carcinogenic benzo[a]pyrene (++++) and dibenzo[a,h]anthracene (++) can all induce DNA interstrand and DNA-protein cross-link in L_{1210} culture after being metabolized by S_9, and the cross-link rate is in positive relation to the concentration of carcinogens. After the cross-link products were hydrolyzed with proteinase K, about 95% of the cross-link products were DNA interstrand cross-link products. The corresponding linear equation can be expressed as $\lg C=a+bR$, in which the logarithm of the concentration C (μmol/L) is abscissa, the relative cross-link rate R is ordinate. The linear relation between $\lg C$ and R is illustrated in fig. 1, the value of a, b and correlation coefficient γ and the standard deviation S are listed in table 1. From fig. 1 we can see that the cross-link rate of PAHs is in very good linear relation to the concentration, and the value of the correlation coefficient is over 0.99. However, under the same condition the non-carcinogens, benzo[k]fluorancene and anthracene, cannot induce any cross-link.

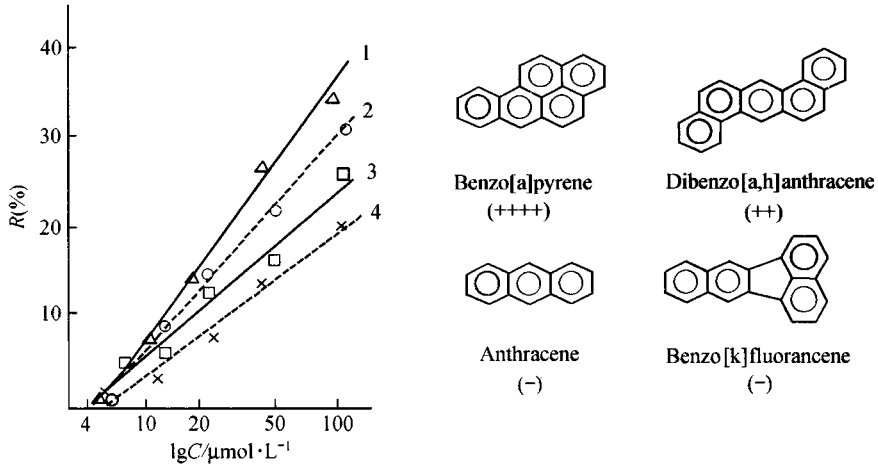

Fig. 1. Linear relation between cross-link rate and concentration of the carcinogenic polycyclic aromatic hydrocarbons. 1, Benzo[a]pyrene without proteinase K; 2, benzo[a]pyrene with proteinase K; 3, dibenzo[a,h]-anthracene without proteinase K; 4, dibenzo[a, h]anthracene with proteinase K.

The structural formulae and carcinogenicities of the above four PAHs are illustrated on the right of fig. 1. The potent, marked, certain, slight and non-carcinogenic activities are expressed as (++++), (+++), (++), (+) and (−), respectively.

It has been evidenced by epidemiological investigation and animal tests that beryllium and cadmium salts are certain (++) and slight (+) carcinogens, respectively, but their salts of the same family elements, magnesium and zinc salts, are non-carcinogens. In our opinion, beryllium and cadmium ions could complex tightly with the negative atoms in minor groove of DNA, while magnesium and zinc ions may be short of such complexing abilities[5,6]. Now, our prediction has been verified in this laboratory by the filter elution method. Beryllium chloride and cadmium chloride induced DNA interstand and DNA-protein cross-link in L_{1210} culture in quantity dependence. The

Table 1 Results of filter elution of carcinogenic polycyclic aromatic hydrocarbons as well as carcinogenic beryllium and cadmium salts

	Experimental results					Linear equation ($\lg C=a+bR$)			
Benzo[a]pyrene (C)	6.2	12.4	24.8	49.6	99.2	a	b	γ	S
R (with PK [#])	4.1	7.9	17.0	21.5	29.8	14.049 1	21.591 9	0.992 5	1.130 5
R (without PK)	0.7	10.4	18.4	26.5	34.8	20.889 1	28.003 1	0.999 4	0.429 6
Dibenzo[a,h]AC (C)	5.6	11.2	22.5	45.0	90.0	a	b	γ	S
R (with PK)	1.8	4.4	11.1	18.2	25.2	15.072 9	20.130 9	0.989 3	1.261 4
R (without PK)	0.8	9.4	17.9	29.1	35.1	21.192 8	29.332 7	0.997 0	0.967 7
$BeCl_2$ (C)		0.56	1.39	2.78	8.33	a	b	γ	S
R (with PK)		8.35	11.59	12.34	17.03	10.090 9	7.123 7	0.984 1	0.550 9
R (without PK)		0.75	4.97	5.31	8.97	2.226 1	6.616 5	0.973 5	0.666 0
$CdCl_2$ (C)		13.14	26.28	59.12	78.83	a	b	γ	S
R (with PK)		1.68	6.2	12.22	13.14	15.339 6	15.241 5	0.997 1	0.354 7
R (without PK)		3.19	4.03	8.5	9.49	7.159 4	8.675 8	0.969 3	0.672 0

A, Anthracene; R, cross-link rate; PK, proteinase K; C, concentration in μmol/L.

corresponding linear equation, analogous with that of polycyclic aromatic hydrocarbons, can be expressed as $\lg C=a + bR$, the correlation line is illustrated in fig. 2, in which $\lg C$ is abscissa and cross-link rate R is ordinate. The value of a, b, correlation coefficients γ and standard deviation S are listed in table 1. From fig. 2, it is interesting to see that the addition of proteinase K increased the cross-link rate for beryllium salt; but for the circumstance of cadmium salt, after the addition of proteinase K the cross-link rate showed decreasing in low concentration, and increasing at high concentration. According to the recent research[7], proteinase K has two active centers in which two calcium cations are situated, it would lose its activity after calcium ions, with low complex ability, were substituted by beryllium or cadmium ions. However, the magnesium or zinc ions cannot induce cross-link even in high concentration.

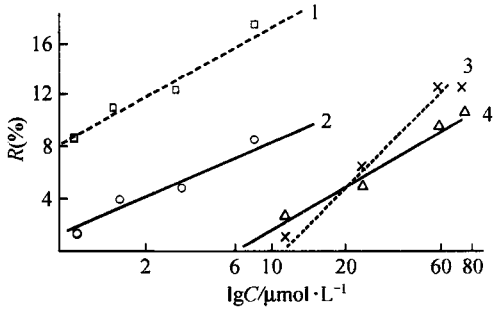

Fig. 2. Linear correlation between cross-link ratio and logarithms of concentration of beryllium and cadmium salts. 1, Beryllium chloride with proteinase K; 2, beryllium chloride without proteinase K; 3, cadminm chloride with proteinase K; 4, cadminum chloride without proteinase K.

By the DNA filter elution method we evidenced that four carcinogenic aromatic amines, 2-aminofluorene (+++), β-naphthyl- amine (++), 4-amino-biphenyl (++) and benzidine (+), can all induce DNA interstrand and DNA-protein cross-link. There is a fair linear correlation between the logarithm of the relative cross-link ratio and the concentration of the carcinogen. But three non-carcinogenic amines, α-naphthylamine, 2-amino-biphenyl and *m*- toluidine, cannot induce any cross-link product even at a high concentration of 100 μmol/L for the former two or 1 000 μmol/L for *m*-toluidine. Different from carcinogenic aromatic hydro- carbons, the linear correlation for carcinogenic aromatic amines is $\lg R = a + bC$, where R is the relative cross-link ratio in percent and C is concentration in μmol/L. It is consistent with the prediction of the di-region theory that all the above-mentioned four carcinogens induced DNA interstrand and DNA-protein cross-link products in quantity dependence. The correlation lines are illustrated in fig. 3, in which the logarithm of cross-link rate is the ordinate and C, the concentration in μmol/L, is abscissa. The constant a, the slope b, correlation coefficients γ and standard error S of the linear equations, are listed in table 2. The structural formulae of the seven tested aromatic amines are illustrated on the right side of fig. 3.

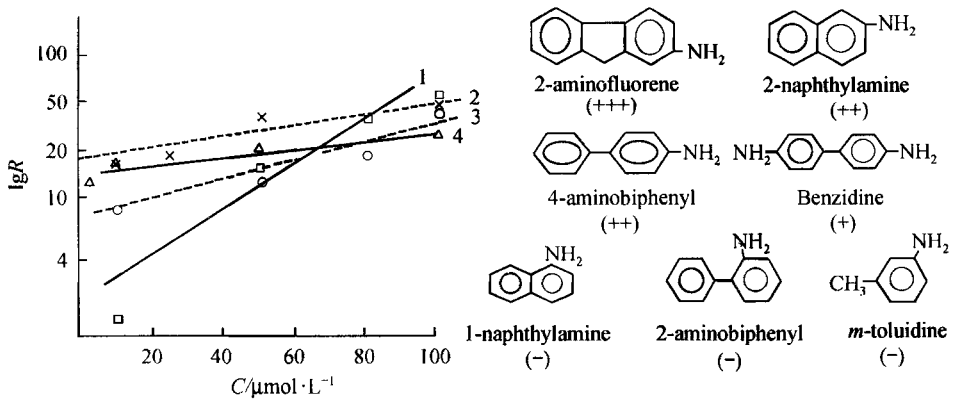

Fig. 3. The cross-link between DNA interstrands and DNA-protein induced by four carcinogenic aromatics amines. 1, 2-naphthylamine; 2, benzidine; 3, 2-aminofluorence; 4, 4-aminobiphenyl.

Table 2 Relative cross-link rate of the adducts induced by carcinogenic aromatic amines

	Experimental results						Linear correlation ($C = a + b\lg R$)			
C/μmol · L^{-1}	5	10	25	50	80	100	a	b	γ	S
2-AF		10.74		15.80	21.40	45.93	0.920 0	0.006 4	0.940 8	0.078 5
β-NA		2.37		19.76	42.29	50.20	0.351 2	0.015 0	0.962 4	0.143 3
4-ABP	13.44	18.75		23.20		31.38	1.179 8	0.003 3	0.936 8	0.047 0
Aniline		18.71	20.21	40.20		48.80	1.240 6	0.004 9	0.923 3	0.069 8

2-AF, 2-aminofluorene; β-NA, β-naphthylamine; 4-ABF, 4-aminobiphenyl.

3 Discussion

Most previous investigators considered that the carcinogens are mono-functional alkylating agents, thus the research on the cross-link between DNA interstrands was only limited in the field of anticancer agents. By the di-region theory, it is discovered for the first time that chemical carcinogens always metabolized into bi-functional alkylating agents and induce cancer through DNA interstrand cross-link between complementary bases. It is consistent completely with the di-region theory that the above-mentioned eight carcinogens cause DNA interstrand and DNA-protein cross-link, while the seven non-carcinogens cannot offer any cross-link. At present, however, the filter elution method could not identify if the cross-link occurs between the complementary bases or not. In 1978, Kohn et al. discovered that the cross-link induced by nitrogen mustard can be repaired within 24 h, the cross-link induced by β-chloroethyl-nitrosourea can hardly be repaired within the same period of time, yet they could not explain this phenomenon. In the period of 1980—1983, in terms of the di-region theory Dai et al[9]. predicted that the cross-link of nitrogen mustard must occur between the non-complementary bases because the distance of its two reactive centers is far larger than 2.80 Å, the average distance between the pair-wise negative atoms in DNA complementary bases, but the cross-link induced by β-chloroethyl-nitrosourea must occur between the complementary bases because the distance between its reactive centers is just 2.80 Å. Therefore, the former cross-link is very easy to be repaired based upon the templates of the complementary bases in opposite DNA strand, yet the latter one is hard to be repaired because there is no template to be found. Later experiments showed complete consistence with the prediction of the di-region theory that the cross-link induced by nitrogen mustard occurs on DNA interstrands, or more exact, on the N^7 of two guanines[10, 11] separated by one base pair. But the cross-link induced by β-chloroethyl-nitrosourea[12] just occurs on the G-C complementary bases. Hence, we propose the 24-hour-repair method to determine if the cross-link occurs between complementary or non-complementary bases. Details will rely on our future work. The cover-figure is the conformation of G-C bases cross-linked by β-naphthylamine calculated by AM1 quantum chemical calculation, in which the 1-position of β-naphthylamine links with G-N^2 and 7-position combines with C-O^2. It will be

very convincing if we can separate and determine the structure of the cross-link product induced by carcinogen. At present the corresponding work is undertaken in this laboratory.

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