RESEARCH PAPERS

Carcinogenesis switched on by DNA cross-link between complementary bases aroused by aflatoxin and N-nitroso compounds*

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Abstract The di-region theory put forward by Dai Qianhuan, a molecular mechanism of chemical carcinogenesis, concluded that the carcinogenesis induced by most of the environmental carcinogens is switched on by the cross-linking between DNA complementary bases aroused by the bifunctional alkylation of their metabolic intermediates. It was evidenced in this paper with DNA filter elution method that one carcinogenic mycotoxin, aflatoxin G_1, four carcinogenic N-nitroso compounds, N-nitrosodiethyl-amine, N-nitrosodibutyl-amine, N-nitrosomorpholine and N-nitrosopyrrolidine, one carcinogenic diazo color, 4-dimethylaminodiazobenzene and one carcinogenic nitrogen-containing heterocyclic compound, quinoline, all induced DNA interstrands cross-linking with dosage correlation after metabolic activation. However, the non-carcinogens in corresponding series for control, aflatoxin B_2, N-nitroso-diphenylamine, 4′-bromo-4-dimethylamino-diazobenzene and isoquinoline, cannot induce DNA interstrands cross-linking at all in the same condition. A method for the determination of cross-linking ratio between DNA complementary bases in total DNA interstrands cross-linking, which has no monitoring measure as yet, has been established for the first time based upon a 24 hour repairing experiment. The DNA complementary pair cross-linking ratio induced by a metabolized carcinogen is correlated with its carcinogenic potential. It may be concluded that the mutations including point and frameshift mutagenesis induced by aflatoxin and other carcinogens are switched on by their corresponding cross-linking base pair between complementary bases. Therefore, the di-region theory is a reasonable molecular mechanism for chemical, endogenous and physical carcinogenesis.

Keywords: cross-linking between DNA complementary bases, di-region theory, mycotoxin, N-nitroso compounds, mechanism of carcinogenesis, mutational spectrum.

Before the end of the 1970s, the explanation of the molecular mechanism for the carcinogenesis of the chemical carcinogens in our environment has been a challenging and pending scientific problem. This puzzling and difficult problem has been resolved through a systematic quantum chemical calculation on a large data set of structure-carcinogenic activity relationship by the first author of this paper in 1979[1]. This reasonable mechanism for chemical carcinogenesis, which has been verified by later experimental facts and got the recognition from the international community, is named di-region theory by referring to the names of previous conceptions about chemical carcinogenesis[2,3]. Di-region theory discovered that most carcinogens in the environment always become a bifunctional alkylation agent after metabolism, which arouses the covalent cross-link between DNA complementary pair bases, an error very difficult to repair, would transform the transcriptase gene to a reverse transcriptase gene by remote chance. Thus, the reverse transcriptional mechanism would transform the whole normal cell to a cancer cell after a long time of incubation period[1]. In this paper, by using an improved Kohn's DNA filter elution method modified in this laboratory, all the tested carcinogens, aflatoxin G_1 of a mycotoxin, N-nitrosodiethylamine, N-nitrosodibutylamine, N-nitrosomorpholine and N-nitrosopyrrolidine of N-nitroso compounds, 4-dimethylaminodiazobenzene of a diazo color and quinoline of a heterocyclic aromatic hydrocarbon, induced cross-links between DNA interstrands and DNA-protein with a dosage correlation. In contrary, all the tested non-carcinogens in corresponding series, aflatoxin B_2, N- nitrosodiphenylamine, 4′-bromo-4-dimethylaminodiazobenzene and isoquinoline, cannot induce the corresponding cross-linking. It has been evidenced in

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our previous papers[4,5] that the carcinogenic polycyclic aromatic compounds, benzo [a] pyrene and dibenzo [a, h] anthracene, carcinogenic aromatic amines, 2-aminofluorene, 2- naphthylamine, 4-aminobiphenyl and benzidine, and carcinogenic metallic ions, beryllium and cadmium ion, all induced the cross-linking between DNA interstrands or DNA-protein. However, The non-carcinogens in corresponding tested series, benzo [k] fluoranthrene, anthracene, 1-naphthylamine, 2-aminobiphenyl, m-toluidine, magnesium ion and zinc ion, cannot induce these sorts of cross-linking. Recently, it was discovered in our laboratory through a DNA filter elution method that the endogenous carcinogens[6], estrodiol and diethylstilbestrol, and the physical carcinogenic factor[7], asbestos, can induce the cross-link between DNA interstrands with a dosage correla- tion. Therefore, it is consistent with the conclusion of di-region theory that the carcinogens of various types in the environment after metabolism all induce the cross-link between DNA interstrands or DNA-protein. Recently, Dai indicated in his book[3] and a recent paper[7] that the point and frameshift mutations are guided almost only by the cross-linking between DNA complementary bases but are not guided, or only to a very small extent, by the mono-functional alkylation on DNA. These mutations will switch on the carcinogenesis of the cell through the deep mutation of the chromosome by undergoing reverse transcription. A method for the determination of DNA complementary bases cross-link ratio over total DNA interstrands cross-links, has been used for the first time based upon Dai's design[8] by using a post 24 hours repairing experiment.

1 Material and method

The Kohn's DNA filter elution method[9] for determination of DNA cross-link ratio was adopted with some modifications. We used an improved fluorescent analysis after the standardization by adding a DNA fluorescence synergist that has a high sensitivity but does not need the using of troublesome isotope labeling. Furthermore, in our experiments, the tested compounds were activated first[4~7] before analysis through metabolism with rat liver S_9 fraction after the stimulation by polychlorobiphenyl administration to the animals. The determination of cross-link ratios between DNA interstrands or DNA-protein was realized with the culture of a mouse leukemia cell line, L_{1210}. DNA double strands were separated after the treatment with alkaline; then the DNA single strand was broken to pieces after the irradiation with 1000 rad γ ray of ^{60}Co. The crosslinked products between DNA interstrands and DNA-protein remained on the film after the filtration of the filtrate through a filter film with 1. 25 μm aperture. DNA contents were quantified based upon the correction curve. The relative cross-link ratio was calculated according to the following equation: $R = ((R_1 - R_2)/R_2) \times 100\%$. R_1 and R_2 are the cross-link ratios after addition of the tested compound and the control, respectively. Calf thymus DNA, dimethylaminobenzoic acid, proteinase K and the carcinogens, aflatoxin G_1, N-nitrosodiethylamine, N-nitrosodibutylamine, N-nitroso-morpholine, N-nitrosopyrrolidine and quinoline as well as the non-carcinogens for control, aflatoxin B_2, N-nitroso-diphenyl and isoquinoline, were all the products of Sigma Company. 4-Di-methylamino- diazobenzene and 4′-bromo-4-dimethylamino-diazobenzene with TLC pure grade were synthesized in this laboratory by referring to the related method in Organic Synthesis and Miller's papers[10,11]. Both diazoaminobenzenes were recrystallized twice and their structures were verified with element analysis and spectroscopic methods. As for equipments and other materials please refer to our previous papers[4~7].

2 Results

2.1 Tests of cellular toxicity

The dosage for half L_{1210} cell inhibition, ID_{50}, was determined by dyestuff exclusion method. The damaged cells dyed by trypan blue were measured in percent over total cell number, and ID_{50} was calculated by least square method. The ID_{50} of carcinogenic samples and non-carcinogenic samples for control of 13 compounds were determined and the results are listed in Table 1. The various carcinogenic activity levels, non-, slight, certain, marked and potent carcinogenic potentials, are shown as (−), (+), (+ +), (+ + +) and (+ + + +), respectively. The toxicities of the drugs are correlated with the mono-alkylating abilities of the metabolized drugs by cell itself but have no correlation with the carcinogenicities of these compounds.

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Table 1. Half inhibition dosages to L_{1210} cell of the tested compounds

Compound	2-NA	1-NA	Q	IQ	DAB	BDAB	
Carcinogenicity	(++)	(−)	(+++)	(−)	(++)	(−)	
ID_{50}(μmol/L)	1926.54	1876.81	2645.88	2867.71	2833.33	2917.24	
Compound	G_1	B_2	NDBA	NDEA	NMOR	NPRD	NDPA
Carcinogenicity	(++++)	(−)	(++)	(+++++)	(+++)	(++)	(−)
ID_{50}(μmol/L)	1.10	1.8	5.74	576.8	615.3	1388	2559.5

NA, naphthylamine; Q, quinoline; IQ, isoquinoline; DAB, 4-N, N-dimethylaminodiazobenzene; BDBA, 4′-bromo-4-N, N-dimethylaminodiazobenzene; G_1, aflatoxin G_1; B_2, aflatoxin B_2; NDBA, N-nitrosodibutylamine; NDEA, N-nitrosodiethylamine; NMOR, N-nitrosomorpholine; NPRD, N-nitrosopyrrolidine; NDPA, N-nitrosodiphenylamine

2.2 Cross-links between DNA interstrands and DNA-protein induced by various carcinogens

The cross-link ratios without or with the treatment of proteinase K of various tested compounds in different concentrations after culturing with rat liver S_9 were determined. All the carcinogens, 2-naphthylamine, quinoline and 4-N, N-dimethylaminodiazobenzene (DAB), induced cross links in dosage correlation without or with the treatment of proteinase K. The corresponding data are listed in Table 2, but the related correlation figures are omitted here. However, it may be noted from the correlation figures that DNA interstrands cross-links over total cross-link for 2-naphthylamine, DAB and quinoline approach 75%, 75% and 65%, respectively. In contrast, none of the non-carcinogens of control, 1-naphthylamine, isoquinoline and 4′-bromo-4-N, N-dimethylaminodiazobenzene (BDAB), can induce the cross-links under the same condition whether with or without the addition of proteinase K; the data not listed in the table fluctuated only slightly along the base line and did not show a linear correlation with the dosage. Three carcinogenic nitrosoamines, N-nitrosodiethylamine (NDEA), N-nitrosomorpholine (NMOR) and N-nitrosopyrrolidine (NPRD), induced also the cross-links whether without or with the addition of proteinase K. The corresponding data are listed in Table 3, and correlation figures are omitted too. The DNA interstrand cross-link ratios over total cross-link induced by NDEA, NMOR and NPRD approached 95%, 80% and 75%, respectively. The non-carcinogen, N-nitrosodiphenylamine, cannot induce the cross-links whether with or without the addition of proteinase K; the data that fluctuated slightly along the base line and without dosage correlation are omitted. All the linear equations are shown as: $Y = AX + B$. The corresponding correlation coefficients γ, coefficients A and constants B, are listed in the last three columns of Tables 2 and 3.

Table 2. Cross-link ratios between DNA interstrands and DNA-protein induced by aromatic amine, diazodye and quinoline

	Concentration (μmol/L)					Coefficient		
	10	20	50	80	100	γ	A	B
2-NA	2.49	5.79	15.67	21.50	28.87	0.9958	0.2834	0.1257
2-NA+K	0.84	3.66	11.37	17.30	21.09	0.9973	0.2248	−0.8354
Q	0.22	7.30	18.63	23.78	24.79	0.9541	0.2680	1.0096
Q+K	0.14	2.49	12.52	14.17	16.22	0.9507	0.1713	0.5615
DAB	1.02	1.62	13.65	17.18	22.27	0.9802	0.2428	−1.4372
DAB+K	0.88	1.90	10.00	12.13	18.78	0.9822	0.1910	−1.1961

K, proteinase K; the abbreviations of drug names are the same as in Table 1

Table 3. Cross-link ratios between DNA interstrands and DNA-protein induced by N-nitrosamines

	Concentration (μmol/L)					Coefficient		
	20	40	60	80	100	γ	A	B
NDEA	11.35	13.97	20.74	23.58	29.48	0.9910	0.2293	6.0630
NDEA+K	10.14	13.68	19.34	22.41	27.36	0.9968	0.2159	5.6350
NMOR	13.30	18.30	20.90	23.75	29.69	0.9888	0.1912	9.7190
NMOR+K	9.60	15.22	17.33	18.97	24.12	0.9772	0.1640	7.2110
NPRD	10.44	13.57	16.28	21.09	28.39	0.9777	0.2171	4.9280
NPRD+K	7.53	12.47	14.19	17.42	19.78	0.9874	0.1473	5.4430

K, proteinase K; the abbreviations of drug names are the same as in Table 1

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2.3 Determination of cross-link ratio between DNA complementary pair bases

Based upon the previous experiments reported Dai indicated that cross-link between DNA non-complementary bases is very easy to be repaired depending upon the templets of their complementary bases, but cross-links between DNA complementary pair bases cannot be repaired even after a 24-hour culturing. Cross-link between DNA complementary pair bases induced by the tested potent carcinogen, aflatoxin G_1(+ + + +), remained completely without reparation undergoing a 24-hour culturing. Cross-link between DNA interstrands induced by tested carcinogenic N-nitrosodibutylamine (+ +), remained only up to 70%, but about 30% of that was repaired. Corresponding data and the values of γ, A and B in regression equations are listed in Table 4, and correlation figures are illustrated in Fig. 1.

Table 4. Cross-links induced by aflatoxin G_1 and N-nitrosodibutylamine and their 24-hour reparations

	Concentration (μmol/L)					Coefficient		
	0.1	0.2	0.3	0.4	0.5	γ	A	B
NDBA	4.51	13.30	14.96	18.76	19.00	0.9235	34.4400	3.7740
NDBA + K	3.69	4.92	6.35	7.58	10.04	0.9892	15.3600	1.9080
24, NDBA	3.73	13.46	15.32	17.60	20.29	0.9315	37.2600	2.9020
24, NDBA + K	2.62	3.23	5.04	6.25	7.46	0.9923	12.7000	1.1100
G1	4.21	8.82	9.42	11.02	12.00	0.9346	17.7800	3.7600
G1 + K	3.26	3.65	5.18	8.83	11.52	0.9610	21.7000	0.0220
24, G1	1.36	7.39	8.95	11.48	13.23	0.9621	27.8300	0.1330
24, G1 + K	0.96	5.37	7.49	10.36	11.71	0.9825	26.4900	-0.7690

K = proteinase K; 24 = Culture a further 24 hrs. Name abbreviations are the same as in Table 1

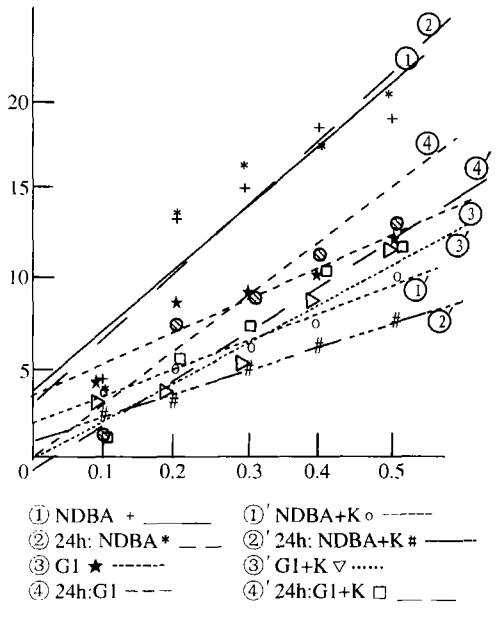

Fig. 1. Determination of cross-link between DNA complementary pair bases induced by aflatoxin G_1 and N-nitrosodibutylamine.

3 Discussion

The di-region theory summarized through the systematic quantum chemical research of the relationship between molecular structures and carcinogenic activities concluded[1-3] that various environmental carcinogens including the carcinogens in polycyclic aromatic hydro-carbons, N-nitroso compounds, aromatic amines, nitrogen containing heterocyclic compounds, azo dyestuffs, mycotoxins and metallic ions, initiate the covalent cross-linking between DNA complementary pair bases, which switches on the carcinogenesis event in a cell. Recently, it has been evidenced by DNA alkaline filter elution method in this laboratory that two carcinogenic aromatic hydrocarbons, four carcinogenic aromatic amines and two carcinogenic metallic salts, all induced cross-links between DNA interstrands with dosage correlation. However, the corresponding non-carcinogens in the above-mentioned three series as the controls, did not induce the cross-linking between DNA interstrands. As early as in 1982, Dai predicted based upon di-region theory that the endogenous carcinogen, estrogen, and the physical carcinogenic factor, asbestos, would induce the cross-linking between DNA interstrands which would switch on the endogenous and physical carcinogeneses. We have verified recently through DNA filter elution method too that two endogenous carcinogens[6], estrodiol and diethylstilbestrol, induced cross-links between DNA interstrands with dosage correlation after metabolic activation, but the non-carcinogen for control, cholesterol, cannot induce the detectable cross-links under the same condition. Furthermore, it was evidenced with DNA filter elution method that the asbestos[7] with carcinogenicity induced cross-links between DNA in-

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terstrands initiated by hydroxyl free radical HO·, but no cross-linking can be detected in the culture of glass fiber without carcinogenicity. Now, in this report, a series of typical carcinogens belonging to N-nitroso compound, mycotoxin, nitrogen containing heterocyclic compound and azo dyestuff series, all aroused cross-links between DNA interstrands or DNA-protein after metabolism, but the corresponding non-carcinogens in the above four series for control can not induce the cross-linking under the same condition. Therefore, it has been evidenced by systematic experiments with DNA filter elution method that the carcinogeneses switched on by various environmental carcinogens, endogenous carcinogens and physical carcinogenic factor, would be started up by the cross-linking between DNA complementary pair bases according to the rule discovered in di-region theory.

In terms of a large quantity of experimental materials of mutational spectra induced by various carcinogens, Dai indicated[3] that the mutational spectrum including point and frameshift mutations induced by a given carcinogen is brought out essentially by cross-linked DNA complementary pair bases which is very difficult to repair, but the mono-alkylation on DNA single strand which is very easy to repair depending upon its complementary templet cannot arouse any stable mutation. The mutations induced by DNA complementary base pair linking would switch on the cellular carcinogenesis undergoing a reverse transcription process, and the morphological deep mutations such as Philadelphia chromosome in cancer cells will appear at last. Carcinogenic 2-aminofluorene arouses almost only GC → TA point mutations, but carcinogenic 2-acetylaminofluorene gives surprisingly up to 95% frameshift mutations[12]. These different mutational spectra aroused by analogous carcinogens which cannot interpret in previous time, can be explained reasonably with the conception that the DNA complementary base cross-linked adducts bring out the mutations[3]. After metabolized *in vitro*, 2-aminofluorene(AF) through the sulfate of its —NHOH group reacts with the strongest nucleophile in DNA, i. e. guanine residual dG by a S_N2 like nucleophilic reaction. The N atom in AF will combine with dG-C8- carbon position to form an adduct dG-C8-N-AF which has been separated and its structure has been determined. We have predicted through AM1 theoretical calculation that the above adduct of AF would react further with the 2-position O atom in a tautomerized cytosine residual dC to form a cross-linked adduct, dG-C8yl-N-AF-C7yl-O^2-dC, through the 7-position carbenium ion in AF after its epoxidation. Thus the dG* residual would be rotated almost 180° in the DNA strand and the negative atoms would drift off from DNA small groove and cannot direct the reparation through the hydrogen bonds, but the negative atoms in dC* residual is a hydrogen-bonded equivalent of adenine A, which would direct the reparation with thymine T on its complementary position, then the AF cross-linked GC pair would transform to a TA pair by realizing a point mutation. However, the separated adduct of 2-acetylaminofluorene (AAF) after metabolism is dG-N^2-3-AAF, in which the 3-position carbon atom combines with 2-position N atom of guanine residual dG, in which the formed nitrenium ion with high stability from the sulfate of -AcNOH group in AAF will react with 2-position N atom in dG with the highest electron density through a S_N1 like nucleophilic reaction. The dG-N^2-3-AAF would react further with 2-position O atom in cytosine C through the AAF 7-position carbenium ion to form the cross-linked adduct, dG-N^2-3-AAF-C7yl-O^2-dC, in which the very long AAF residual would push away all the hydrogen-bonded atoms from the DNA small groove, and both the dG* and dC* residuals would rotate to be perpendicular to the plane of DNA double helix. The widely split dG* − dC* pair or with its neighbouring pair or pairs would be excised; then the broken DNA complementary double strands with cutting off base pair or pairs would be linked up through the reparation. Thus the − 1 or − 2 or even − 3 frameshift would occur. The results of NMR research[13] on the structures of AF and AAF DNA adducts are consistent with our above propounded models. The conformation of the modified DNA with the metabolized AF observed by NMR showed no detectable change, but the dG residual in DNA adduct of metabolized AAF rotated to constitute a *cis*-form configuration with the strand and the AAF residual inserted itself into the groove between the pair bases.

Obviously, the mutational spectrum induced by aflatoxin B_1 is decided also by its cross-linked adduct between DNA complementary pair bases. The metabolism of aflatoxin B_1 and its adduct with DNA have been investigated in details. It has been known already that an 8-position carbenium ion of aflatoxin B_1 is formed from the ring opening of its 8, 9-epoxide, the major metabolite of aflatoxin B_1, which reacts mainly with guanine G in DNA and combines with N^7-position on G to form a N^7-position ion. This

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dG-N^7-position ion of aflatoxin B_1 adduct may lose DNA strand to form DNA abasic site along two possible routes. Another more important route would be that the above aflatoxin B_1 DNA adduct reacts further to form cross-linking products between DNA interstrands, and indeed, the analogous aflatoxin G_1 forms a high ratio cross-linking between DNA complementary DNA pair bases as in the observation of present paper. After the configurations of aflatoxin B_1 and G_1 are optimized by AM1 theory, it may be noted that the OCH_3 group on the aromatic ring and two hydrogen atoms on the five-member ring of B_1 or on the six-member ring of G_1 will repulse vigorously with each other. The stereo-hindrances in aflatoxin B_1 or G_1 make their six-member ring lactones possess high acylation reactivities, and thus the cross-linking between complementary pair bases would be formed through the acylation on N^4 of cytosine C because the distances between these two reactive centers in aflatoxin B_1 or G_1 all approach 0.3 nm. It is possible that the cross-linking adducts induced by aflatoxin B_1 or G_1 occur only between complementary bases. Therefore, the cross-linking induced by aflatoxin G_1 cannot be repaired even after 24-hour cultivation in our experiments due to the lack of the complementary templet. The mutational spectrum induced by aflatoxin B_1[14] is constituted principally from the GC→TA point mutations, and GC→AT point mutations as well as −1 frameshift mutations are much less important contents in this spectrum. In the above-mentioned cross-linking model, the modified base G is twisted off from DNA chain, which cannot direct the repairing. However, the H atom on N^4 in the acylated N^4 of C will tautomerize to 3N of C, this tautomer of modified C has an analogous hydrogen-bond arrangement of base A, which would direct only the repairing with base T on the complementary strand, so that the point mutation, GC→TA, as the major trend is realized. If the H atom on N^4 tautomerizes to O^2, a hydrogen shift with energy higher than above tautomerization, the hydrogen- bond matching corresponding to T would guide the repairing on complementary strand with A. This explanation is consistent well with that GC→AT point mutation is a second important event in the mutational spectrum of aflatoxin B_1. Obviously, the observable −1 frameshift mutations of aflatoxin B_1 mutational spectrum originate from the simultaneous cutting off of the damaged complementary pair bases guided by bulky aflatoxin B_1 residual with a lesser possibility.

The interstrand cross-linking ratios of NDEA(++++), NMOR(++++) and NPRD(++) approach 95%, 80% and 75%, respectively; which shows that their carcinogenecities are correlated with the induced DNA interstrand cross-linking ratios in certain degree. Other than the formation of acetaldehyde from the α-position oxidation of ethyl group in NDEA, a diazonium hydroxide salt $C_2H_5^+N{\equiv}N\,OH^-$ is formed through a hydrogen shift of intermediate $C_2H_5NH{-}N{=}O$, from which a $C_2H_5^+$ carbenium ion will combine with DNA after releasing nitrogen immediately. The ethyl group of DNA adduct would form a β-position carbinol through the oxidation on its β-position which is a ω-position of ethyl group as well. This β-carbinol would form an alkylation species through the formation of sulfate under the catalysis of a sulfotransferase. The distance of both alkylating centers in this ethylene group approaches 0.28 nm which is just matching with the distance of the pairwise negative atoms; thus the cross-linking by this ethylene group would occur between DNA complementary pair bases. The β-position of butyl group in nitrosodibutylamine (NDBA) would be an alkylating center through the sulfate of its carbinol, and this substituted ethylene group would realize the cross-linking just between DNA complementary pair bases, which would occupy about 70% of the total cross-linked products and cannot be repaired after 24 hour cultivation. The ω-position, i.e. the δ-position, of the butyl group in NDBA is easy to be oxidated to form the sulfate of its δ-position carbinol, the bi-functional alkylating centers between α- and δ-positions would realize the cross-linkages between DNA non-complementary bases, which occupy about 30% and are repaired in our 24-hour reparation experiment. The cross-linkages induced by aflatoxin G_1 or NDEA have not been repaired completely in the first generation of the daughter L_{1210} cell. However, it has been found that the cross-linkages induced by both carcinogens tended to be repaired progressively in the latter successive L_{1210} cell generations. The researches on the fundamentals and extension of the 24-hour reparation are underway in this laboratory.

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