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4-(Nitrobenzyl)Pyridine에 의한 알킬화합물들의 잠재적 변이원성에 대한 구조활성 및 광화학효과의 연구

김재현* · 엄애선 · 류재천
*동덕여자대학교 자연과학대학 보건관리학과
한양대학교 생활과학대학 식품영양학과
한국 과학기술연구원 독성연구실

Photosensitization Effect and Structure-Activity Relationship on Mutagenic Potential of Alkylating Agents by 4-(Nitrobenzyl)Pyridine (4-NBP) test

Jae-Hyoun Kim*, Ae-Son Om and Jae-Chun Ryu
**Department of Health Science, School of Natural Science, Dongduk Women's University*
Department of Food and Nutrition, College of Human Ecology, Hanyang University
Toxicology Laboratory, Korea Institute of Science and Technology P.O. Box 131, Seoul, Korea

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ABSTRACT **: The NBP assay was conducted to determine the photomutagenic or photocarcinogenic potential of alkylating agents. Using a 4-NBP *in vitro* technque, whereby photochemical treatment on CAS (Chemical Activation System) was performed to invetigate the enhancement effect, 20 compounds were shown to undergo alkylating mechanisms with 4-NBP. Chemically meaningful results were obtained with different sets of 20 compounds for the alkylating activities due to the UV irradiation, demonstrating that all of the testing compounds showed increasing photoalkylating effects either in the presence or absence of CAS in comparison with previously reported data, except furoic acid and fumaric acid that showed decreasing effect in the presence of a CAS. Caffeine did not show a meaningful result either. However, these findings demonstrate the effects of potential photoalkylating activity in chemical activation system (CAS) and suggest a potential risk-ranking system for the *in vivo* assays.**

Keywords : **NBP, CAS, photoalkylating, photomutagenic, risk-ranking**

Introduction

Intermediates in organic synthesis as well as in surfactants, fumigants, industrial sterilants, cosmetics, and pharmaceuticals posed potential hazards for biological through genetic interactions (Ames *et al.*, 1975; Hemminki and Suni, 1984; Dipple, 1995).

Data from animals and humans suggested that some photosensitizers enhanced UV-associated skin carcinogenesis (Mitchell and Nairn, 1988; Ashby *et al.*, 1993; Healy *et al.*, 1994).

Several epoxides have been demonstrated to be photoirritants and photochemical carcinogens in animal tests (De Flora *et al.*, 1989; Averbeck, 1997; Jacobs *et al.*, 1999). The potential human risk for photochemical carcinogenesis and mutagenesis of epoxides were correlated with the intensity of UV-B radiation because the association of carcinogenesis or mutagenesis with solar UV radiation is strong (De Flora *et al.*, 1989; Walles *et al.*, 1995).

Relatively few drug products have been tested to elucidate their potential for enhancing UV-mediated carcinogenic effects on the skin for the photosafety purposes. By itself, UV light is a carcinogen in humans (IARC, 1992). Photosafety testing (testing for adverse effects of drug products in the presence of light) is recommended when the results of testing would yield important safety information or be informative for the consumer and healthcare practitioner (Dean *et al.*, 1992; Guidance for Industry Pho-

*To whom correspondence should be addressed

tosafety Testing, 2000).

Many diverse classes of drugs including antimicrobials, NSAIDs, antidepressants, anticonvulsants, diuretics, and antihypertensives have been reported to be photosensitizers in the clinical setting (Johnson 1984; Holzle *et al.*, 1991). Muller and Kasper (1998) showed a good correlation between the photomutagenic and photocarcinogenic potential of pharmaceuticals, indicating *in vitro* photomutagenicity testing in mammalian cells might be an easy-to-measure predictor of the photocarcinogenic potential. The formation of 8-oxo-7,8-dihydro-2'-deoxyguanosisne (8-oxodG) by reactive oxygen radicals by HPLC was measured to evaluate the effect of ultraviolet light-induced DNA oxidation on the carcinogenicity (Rosen *et al.*, 1996). They have been engaged in evaluating ultraviolet light-induced DNA oxidation, especially, mediated by fluoroquinolone antibiotics (Rosen *et al.*, 1997). Photocarcinogenicity of several drugs were also tested (Sterns, 1998).

The phototoxicity testing models have been an important issue from the toxicologist's point of view now. *In vitro* phototoxicity testing methods for the phototoxicity were discussed (Spielmann *et al.*, 1999). Henderson *et al.* (1994) demonstrated the photomutagenic potential of *p*-aminobenzoic acid in the photomutagenicity assay using bacteria with various design factors. A number of studies have shown that *in vitro* experiments exhibit increased frequencies of photomutagenicity or photocarcinogenicity in the phototoxicity testing. Kornhauser *et al.* (1998) summarizes a few *in vitro* methods to assess photodamage in cells irradiated with UV of various wavelengths in the presence of a number of photosensitizers. Api (1997) developed a human skin model for testingan *in vitro* phototoxicity assay with seven water-insoluble materials. In addition, many other efforts have been made to develop assay models for detecting the photomutagenicity of chemicals with different mechanisms of phototoxicity and diverse cellular targets for injury. A number of phototoxicity models appears to be useful to predict these phototoxicity phenomena (Ellis, 1998; Kornhauser *et al.*, 1998; Forbes and Sambuco, 1998; Spielmann *et al.*, 1998; Okamoto *et al.*, 1999). Mechanistic relationship among mutagenicity, skin sensitization, and skin carcinogenicity was studied (Ashby *et al.*, 1993). Clearly, there was distinction between direct, enzyme-mediated and light-induced events in bacterial photomutagenicity (Utesch and Splittgerber, 1996).

The use of a chemical approach by the chemical activation system prove to be helpful in detecting and elucidating the structure-activity relationships of carcinogenic and mutagenic alkylating agents in environmental samples without employing complex and expensive biological systems. Many efforts have been made to test a number of compounds with a 4-NBP (4-(4-Nitrobenzyl)pyridine). Eugene *et al.* (1963) used a 4-NBP to test a mutagenic potential in the presence of alkylating agents. Later Agarwal *et al.* (1979) studied chemical reactivity of epoxides and other compounds, showing strong reactivity to 4-NBP. Furthermore, a *in vitro* model (CAS, Chemical Activation System) to mimic mammalian cell MFO (Mixed Function Oxidase) was developed and tested for comparing alkylating agents of bionucleophiles based on their reaction with 4-NBP to mutagenicity evaluation from DNA adducts of alkylating agents (Kim and Thomas, 1992; Thomas *et al.*, 1992).

However, little is known about the effect of UV on alkylating activities of 20 testing compounds, particularly in conjunction with mutagenicity or carcinogenicity. In this study, 20 compounds were tested in CAS to evaluate photosensitivity, photochemical carcinogenicity potential, or potential to enhance UV-associated skin carcinogenesis since there is a potential for false negatives or positives in metabolic incorporation of UV into normal metabolic activation system.

Materials and Methods

1. (A) To a reaction flask was added 3.0 ml of 0.2 M sodium acetate-acetic acid buffer (pH. 4.0), the test compound in 3.0 ml of water (or solvent), and 1.0 ml of 5% (w/w) 4-(4-nitrobenzyl)pyridine in acetone. Sufficient water was added to produce a total volume of 7.0 ml. The reaction flask was sealed. The samples were exposed to UV light in a merry-go-round Rayonet photoreactor with 12 10-watt photochemical lamps with major output at 350 nm. All test solutions received equal radiation from the light source with a distance from the test tubes to the lamp standardized at 12 cm. Triplicate samples were irradiated with *p*-nitro-acetophenone(PNAP)/pyridine actinometer for 5 min. Just after irradiation, the samples were placed in a boiling water bath (98°C). After 20 minutes, the solution was chilled in ice bath and 0.6 ml of ethyl acetate/acetone (5:2, v/v) was added, followed by 1.0 ml of 5 N sodium hydroxide. After mixing in a vortex mixer for about 30 seconds, the organic phase was separated in a separatory funnel, and the absorbance read at 540 nm. Following the addition of base, all the remaining procedures were carried out exactly 1.75 minutes after the addition of NaOH. All the reactions were performed at least in duplicate.

(B) To 3.0 ml of 0.1 M potassium phosphate buffer (monobasic, pH. 4.5) containing 10 mM EDTA was added 0.1 ml of 0.15 M ferrous sulfate and 0.1 ml of 1.0 M

ascorbic acid. The test compound in 2.0 ml of acetone (solvent) and 1.0 ml of 0.5 M hydrazine solution was thenadded. Finally, 30% (0.79 M) hydrogen peroxide was added to make 0.8 M. The reaction flask was then sealed with Teflon tape and its contents mixed by inverting twice. The samples were exposed to UV light in a merry-go-round Rayonet photoreactor with 10 20-watt photochemical lamps (black lamps) with major output at 350 nm. All test solutions received equal radiation from the light source with a distance from the test tubes to the lamp standardized at 7 cm. Triplicate samples were irradiated with *p*-nitro-acetophenone(PNAP)/pyridine actinometer for 5 min. Then the reaction mixture was incubated in a shaker bath at 37°C, and was removed. 1.0 ml of 5% 4-(4-nitrobenzyl) pyridine (NBP) in acetone was added and placed in a boiling water bath at 98°C. After 20 minutes, the reaction mixture was chilled on ice and 0.6 ml of ethyl acetate/acetone (5:2) added, followed by 1.0 ml of 5M sodium hydroxide. Following mixing in a vortex mixer for about 30 seconds, the organic phase was separated in a separatory funnel, and its absorbance read at 540 nm. From the addition of base on, additional handling was carried out rapidly in the dark. Absorbance was read exactly 1.75 minutes after the addition of NaOH. Blank solutions contained all components except the test compound.

Results and Discussion

Tables 1 and 2 allow absorption sensitivity comparisons of moles in the absence or presence of UV photolysis. All the compounds with the double bond produced a significant dose-related increase of alkylating activities, with no requirement for activation : as expected, the oxirane ring seems to a direct genotoxic activity (Table 1). 4-NBP reacts with epoxide intermediates to form a violent dye in an alkaline or acid medium. Other compounds are likely to undergo reaction mechanism *via* carbonium ion by resonance, as discussed (Thomas *et al.*, 1992). The result show the strong activity of ethylene dichloride, acrylamide acrylic acid and acrylonitrile without CAS, and of ethylene dichloride, trichloroethylene, 1,2-dibromoethylene, allyl alcohol, acrylic acid acrylonitrile, fumaric acid and 2-furoic acid especially.

Table 2 show more enhanced stronger electrophilic activity of compounds in the absence of a CAS than those in the presence of CAS. The electrophilic reactivity of the co mpounds were reduced following chemical activation.

The kinetic experiments with photosensitization were performed in order to determine whether UV can enhance mutagenicity or carcinogenicity under same experimental conditions used for CAS assays reported (Table 1). Table 2 indicated that UV photolysis may affect the alkylation of testing compounds. The rate constant with 20 compounds are largely enhanced without CAS after light exposure; about 6-fold to large extent for chloro- and bromoethylenes; large enhancement for DDT and their derivatives;

Table 1. Linear dose-response relationships of alkylating agents in the NBP-CAS assay with and without activation (Thomas *et al.*, 1992)

	Abs/mM without CAS	Abs/mM with CAS	R
Ethylene dichloride	0.41	5.8±0.7	0.47
Trichloroethylene	0.0	9.2±0.07	0.998
Tetrachloroethylene	0.0	0.0	–
1,2-Dibromoethylene	6.6	8.5±1.4	0.79
DDT	0.0	0.0	–
DDD	0.0	0.0	–
DDE	0.0	4.4±2.0	0.94
DDMU	0.0	0.0	–
DDNU	0.0	0.0	–
Allyl alcohol	0.0	10.6 5,4	0.50
Acrylamide	50	1.9±0.8	0.44
Acrylic acid	50	6.6±0.6	0.80
Acrylonitrile	19	8.6±0.9	0.93
Diethylnitrosamine	0.0	0.9±0.3	0.999
Fumaric acid	18	6.6±1.9	0.74
2-Furoic acid	0.0	8.5±1.9	0.58
2,4-Hexandienal	18	2.6±0.4	0.92
Caffeine	0.0	0.0	–
1-Methylhydrazine	0.0	0.03±0.02	–
1,1-Dimethylhydrazine	0.0	0.1±0.05	–

Table 2. Linear dose-response relationships of photosensitization effect of alkylating agents in the NBP-CAS assay with and without activation

	Abs/mM without CAS	Abs/mM with CAS	R
Ethylene dichloride	2.56	11.8±0.7	0.76
Trichloroethylene	1.8	8.8	–
Tetrachloroethylene	1.4	1.3	–
1,2-Dibromoethylene	15.1	9.3±1.4	0.88
DDT	0.4	0.43	–
DDD	1.3	0.8	–
DDE	1.6	3.6	–
DDMU	2.4	0.9	–
DDNU	1.8	2.5	–
Allyl alcohol	0.6	6.1±0.8	0.72
Acrylamide	84	6.7±0.7	0.56
Acrylic acid	79	7.3±0.6	0.87
Acrylonitrile	43	15.2±1.3	0.89
Diethylnitrosamine	1.6	2.8±0.8	0.93
Fumaric acid	11	3.6	–
2-Furoic acid	0.8	3.1	–
2,4-Hexadienal	32	4.2±0.9	0.97
Caffeine	0.1	0.4	–
1-Methylhydrazine	3.6	2.5±0.3	0.84
1,1-Dimethylhydrazine	4.1	3.1±0.1	0.82

about 2-fold for acrylamide, acrylic acid and acrylonitrile; large enhancement for 1-methylhydrazine and 1,1-dimethylhydrazine. Rest of compounds did not change or decreased more or less. Also, similar trends were foundin CAS with photolysis.

Halogenated compounds were found to be strong indirect alkylating agents to 4-NBP in this assay. Activation of the olefins can beexplained by epoxidation of the doulbe bond. From the result of PASS, we found that carcinogenicity and mutagenicity of trichloroethylene was the largest, and then other halogen compounds showed less activities. It does not seem to follow close relationship between results of Table 1 and those of Table 2. Interestingly the reactivity of acrylamide, acrylic acid and acrylonitrile were almost doubled with or without CAS incubation after photolysis. Whereas the reactivity of acids was decreased by half even with photolysis compared to those without photolysis. This finding indicates that acids do not properly undergo chain reactions than the other tested compounds.

Photosensitization effect

Photoalkylation of NBP with 20 compounds following chemical activation was observed for the concentration range 10-50 mM. The calculated light intensity of PNAP/pyridine actinometry was 9.5×10^{-5} einstein. $sec^{-1}L^{-1}$.

1. Halogenated hydrocarbons

Halogenated compounds were found to be strong indirect alkylating agents to 4-NBP in this assay. Alkylation of NBP following chemical activation was observed for the concentration range 0-10 mM range where compounds increased in a dose-dependent manner. A good correlation coefficient (R) indicated close conformity to Beer's law and it appears that the high precision indicated a stable oxidation product (Table 1). From the data shown in Table 2, the compounds increased the rate constants on photoactivation which was about 1-fold to 4-fold for DCE; 1-fold to 6-fold for TCE; large exertion for TTCE.

Ethylene dichloride (DCE) has been shown to be both direct acting and oxidatively activated in the CAS oxidation system. The mechanism ofdirect alkylation of NBP by DCE was most likely a general SN2 alkylation reaction whose chemical reaction was most likely epoxidation of double bond. Results demonstrated that oxidative activation followed by photolysis increased alkylation rate of DCE over direct acting mechanisms. Trichloroethylene (TCE) was not a direct alkylating agent but activated to NBP in the CAS (Table 1). Photolysis showed a significant elevation in photoalkylating activity with a 3-fold increase. Tetrachloroethylene (TTCE) was non-alkylating with or without chemical activation. However a positive result was obtained with and without the CAS on irradiation. Double increase of alkylation of NBP by 1,2-dibromoethylene was shown on the photosensitization.

2. DDT and its metabolites: DDD, DDE, DDMU, DDNU

DDD, DDE, DDMU, DDNU, which are non-mutagenic *per se* were slightly mutagenic with or without CAS in the presence of light. Only DDE decreased alkylating activity following activation in the presence of light (Table 1). Those compounds do not seem to have structure-activity correlationship with increasing number of chlorine atom.

3. Allyl alcohol, acrylamide, acrylic acid, acrylonitrile

These compounds rapidly increased absorbance of strong electrophilic activity without CAS about two-fold stronger in the presence of light than without photolysis. While other compounds did not increase the photoreactivity much, Acryl amide showed a two-fold enahancement of photoalkylation with CAS.

4. Diethylnitrosamine (DEN)

DEN had a strong photoalkylating activity with and without CAS although it was not alkylating agent without activation. A linear concentration-absorbance relationship was evident with a high degree of correlation (R=0.93). Activation of DEN was known to be initiated *in vitro* by a a *a*-hydroxylation mechanism.

5. Fumaric acid, 2-fluroic acid, 2,4-hexadienal, caffeine

Results indicated that fumaric acid was both a direct and an indirect photoalkylating compound whose activity became weaker on photolysis, which were probably due to the fact blue chromophore with NBP could not be extracted into ethyl acetate (Thomas *et al.*, 1992). A slight increase from 0 to 0.8 of photoalkylation of furoic acid occurs in the absence of CAS, but decreased to about half in the presence of chemical activation. A reduced activity seems to occur due to the fact that metabolic enzymes may play an active detoxifying role (Yamamoto and Miyachi, 2000). None of genotoxicity potential of furoic acid was reported *in vitro* unscheduled DNA synthesis assay (Aeron *et al.*, 1989). Strong photoalkylation of NBP by 2,4-hexadienal occurred with and without chemical activation, increasing the activity about two-fold. The alkylation of these compounds appears to be caused by epoxide formation or residual resonance forms.

6. 1,1-Dimethylhydrazine, 1-Methylhydrazine

The rates of the parent compounds are enhanced about to 3-fold to 10-fold on photolysis. The data indicate that the photolysis rates of two compounds are not much affected by the presence of CAS. None were direct alkylating compounds without UV. These hydrazine derivatives were

weakly active following activation (Table 1). Strong photoalkylation occurred with two compounds with and without CAS, to the extent that the compounds showed a rate of large enhancement over the system without photolysis.

PASS data and Experimental data (LC_{50} and LD_{50})

In the predicted biological activity epectrum obtained from PASS for testing compounds, Pa and Pi are the estimates of probably to be active and inactive respectively (Table 3). If Pa > 0.7, the compound is very likely to reveal this activity in experiments, but in this case the chance of being the analogue of the known pharmaceutical agents for this compound is also high. The ISIS/DRAW software (MDL Information Systems, Inc.) is used to draw structures and to export to molfiles with structures of compounds to get the activities.

Computer aided prediction of biological activities such as carcinogenicity, mutagenicity and teratogenicity. Three chlorinated aliphatic compounds (TCE=0.669, TTCE=0.860, DCE=0.774 respectively), DDT (Pa=0.967), DDE (Pa= 0.741) and Acrylic acid (Pa=0.721) have shown strong Pa value of about 0.7 or more than 0.7 for teratogenicity. Trichloroethylene (Pa=0.830), tetrachloroethylene (Pa= 0.800), ethylene dichloride (Pa=0.720), DDT (Pa=0.753) and 1-methylhydrazine (Pa=0.690) also showed high Pa values for carcinogenicity. Similarly, 1,1-dimethylhydrazine (Pa=0.736) demonstrated strong activities for mutagenicity respectively. Based on developed NBP-assay data in comparison with those obtained from PASS, we concluded that experimental data (Table 1) of CAS assay did not coincide with the prediction of 20 compounds tested with PASS program which was intended to use for optimization of chemical testing. In summary, the compounds need to be further tested according to the additional predicted activities.

The information of LC_{50} and LD_{50} of the testing compounds was obtained from the Tomes Plus database (Tomes Plus database, 1997, Micromedex Inc. U.S.A.) (Table 4). It was difficult to see the whole picture of experimental data, however, some of compounds clearly have a relevancy to the data set of oral LD_{50} in comparison with those in the presence of CAS in Table. 1.1-Methylhydrazine, acrylonitrile and allyl alcohol were shown to have the high toxicity with low oral LD_{50} values. DDT, DDD and dibromoethylene demonstrated a little higher values, indicating less toxic than three compounds (Table 4).

Table 3. Biological activity data obtained from PASS program

	Teratogen	Carcinogen	Mutagenic
Trichloroethylene	pi: 0.669	pi: 0.830	pi: 0.891
	pa: 0.019	pa: 0.005	pa: 0.003
Tetrachloroethylene	0.860	0.800	0.804
	0.007	0.006	0.005
Ethylene dichloride	0.774	0.720	0.705
	0.011	0.008	0.007
1,2-Dibromoethylene	*	0.666	0.197
		0.010	0.064
DDT	0.967	0.753	0.227
	0.004	0.007	0.050
DDE	*	0.551	0.405
		0.017	0.018
DDMU	0.444	0.673	0.634
	0.057	0.010	0.007
DDNU	0.533	0.396	0.179
	0.037	0.036	0.076
Allyl Alcohol	*	0.267	0.277
		0.067	0.035
Acrylamide	*	0.467	0.160
		0.025	0.090
Acrylic Acid	0.721	0.432	0.128
	0.015	0.030	0.121
Acrylonitrile	0.384	0.321	0.290
	0.078	0.051	0.031
Diethylnitrosamine	0.511	0.458	0.345
	0.042	0.026	0.023
Furamic Acid	*	*	*

Summary

The chemical activation system (CAS) proved to be use-

Table 4. Experimental values of LD50 and LC50 from the database

Chemical name	LD50 (mg/kg)		LC50 (ppm, 4h)
	Oral	Dermal	Inlalation
	rat/mouse	rabbit	rat/mouse
Trichloroethylene	5650/2402	>20000	/8450
Tetrachloroethylene	2629/8100	–	/5200
Ethylene dichloride	670/413	2800	1000 (7h)
1,2-dibromoethylene	117/	–	–
DDT	87/135	300	–
DDD	113/	1200	–
DDE	880/700	–	–
DDMU	2700/	–	–
DDNU	–	–	–
Allyl alcohol	64/96	45	76(8h)
Acrylamide	124/107	400(rat)	-
Acrylic acid	/2400	–	/5300mg/m3/2h
Acrylonitrile	78/27	250	425/
Diethylnitrosamine	220/200	–	–
Furamic acid	9300/	–	–
2-furoic acid	–	abdominal, 100(mus)	–
2,4-hexadienal	300/	270	–
Oleic acid	74000/	–	–
Caffeine	192/127	–	–
1-methylhydrazine	32/29	95	34/56
1,2-dimethylhydrazine	122/265	1060	252/172

ful in elucidating the reactivity of direct or indirect alkylating agents. Evidence of alkylation is presumed to be evidence of mutagenic risk, and thus the test may prove to be a simple non-biological indicator of carcinogenic risks. From the results presented herein, it is evident that certain olefins and other compounds have significant indirect photoalkylating potential indicating carcinogenic/mutagenic risks.

In the presence of UV, testing chemicals exerted in a dose-dependent manner, with a significant elevation of alkylating activities with maximum responses from 3-fold with halogenated aliphatics to large enhancement with other compounds, in comparison with those in the absence UV irradiation, ranging from 0 to 50 mM concentrations.

It was concluded that the toxicological risks from adducts need to be evaluated in conjunction with other data on genotoxicity and animal toxicity with following concepts:

(1) The olefinic bond may be a prerequisite for these types of activities since structurally-related molecules lacking the elefinic moiety are generally inactive in this respect in this chemical activation assay.

(2) The photochemical exposure really exerted the activities with and without chemical activation.

(3) The CAS system needs to be developed further to mimic a complexenzyme system of the body.

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