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STRUCTURE–MUTAGENICITY ANALYSIS WITH THE CHO/HGPRT SYSTEM*

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Summary—Using a mammalian cell gene mutational assay, the Chinese hamster ovary cell/hypoxanthine–guanine phosphoribosyl transferase (CHO/HGPRT) system, we have studied the structure–mutagenicity of ten alkylating agents and six platinum(II)chlorammines. In analysing the mutagenesis data, we describe the mutagenic activity as the number of mutants per 10^6 clonable cells induced by a 1 μM concentration of chemical tested. The mutagenicity of alkylating chemicals decreases with increasing size of the alkyl group; methylating agents are three to six times more mutagenic than the corresponding ethylating agents. *cis*-$Pt(NH_3)_2Cl_2$ is mutagenic, but its steric isomer, *trans*-$Pt(NH_3)_2Cl_2$, is very much less mutagenic. These results, together with determination of chemically induced DNA lesions permit analyses of certain aspects of mechanisms of chemical mutagenesis.

Introduction

Advances in the study of the genetics of mammalian somatic cells in culture have contributed much to the recent rapid progress in experimental biology and medicine. This article demonstrates the role that somatic-cell genetics has played in our gradual appreciation of the genetic aspects of chemical toxicology. The use of ten alkylating chemicals and six platinum(II) compounds for analysis of the effect of structure modification on chemical mutagenicity reflects a progression from studying pure model mutagens to examining antitumour agents used clinically.

Gene mutation in mammalian cells

Since the first demonstration that variants with altered nutritional requirements and drug sensitivity could be induced after treatment with a chemical mutagen in two near-diploid mammalian cell lines, CHO (Puck & Kao, 1967) and V79 (Chu & Malling, 1968), there has been interest in utilizing these cells for studying mechanisms of mammalian genetics. Many variants with a wide spectrum of phenotypes have since been isolated and characterized from these and other cells (Siminovitch, 1976; Thompson & Baker, 1973).

This article describes the use of a mutational system which affects the hypoxanthine–guanine phosphoribosyl transferase (HGPRT) activity in Chinese hamster ovary (CHO) cells, the CHO/HGPRT system (Hsie, Brimer, Mitchell & Gosslee, 1975a; Hsie, O'Neill, Couch, San Sebastian, Brimer *et al.* 1978; O'Neill, Brimer, Machanoff, Hirsch & Hsie, 1977), to illustrate aspects of gene mutation and its subsequent utilization in a quantitative assessment of structure–mutagenicity. To study the genetic, biochemical and molecular bases of gene mutation, one needs only to isolate one or a few mutants of the desired phenotypes; however, studies of quantitative mutagenesis require that a mutational protocol selects for mutants, a great majority (or all) of which are affected at a single gene. For mutagen screening a quantitative single-locus mutational assay must also have intra- and interlaboratory reproducibility (Hsie, 1980; Hsie *et al.* 1978).

The CHO/HGPRT system

We used CHO-K_1-BH_4 cells (Hsie *et al.* 1975a), a subclone of the near-diploid CHO cell line, for this study. CHO cells are genetically well characterized, grow well either on solid substrates or in suspension, with a population doubling time of 12–14 hr, and exhibit a high cloning efficiency and a relatively stable karyotype. They have been used extensively for studying mutagen-induced cytotoxicity and gene mutation and have been favoured for cytogenetic studies.

Using a previously standardized experimental procedure for culturing the cells, treatment with chemicals, and measurement of cytotoxicity and gene mutation (Hsie *et al.* 1975a; Hsie *et al.* 1978; O'Neill *et al.* 1977), we measured gene mutation by quantifying the frequency of mutants resistant to a purine analogue, 6-thioguanine (TG). A metabolic activation system derived from Aroclor 1254-induced male Sprague–Dawley rat livers was used to determine the mutagenicity of promutagens (Hsie, 1980; Machanoff, O'Neill & Hsie, 1981; O'Neill *et al.* 1977). For data presentation, we termed cytotoxicity as the effect of chemical treatment on the cloning efficiency of cells relative to the untreated control, which is routinely 80% or higher; mutagenicity was expressed as the number of TG-resistant mutants per 10^6 clonable cells; the spontaneous mutation frequency generally lies at $0–15 \times 10^{-6}$ mutants/cell.

*Joining the list of many, I am writing this article to honour the distinguished contribution of Dr Leon Golberg to toxicology, the journal *Food and Cosmetics Toxicology* and the British Industrial Biological Research Association.

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Questions concerning the genetic *versus* epigenetic origin of phenotypic variants have been raised earlier for studies of mutagenesis with mammalian cells. In the absence of direct evidence of gene mutation through analyses of the nucleotide sequence of the *hgprt* gene and the amino-acid sequence of the HGPRT protein for TG-resistant mutants, we have exhaustively accumulated genetic, biochemical, and physiological evidence that indicates that the CHO/HGPRT system fulfils the criteria for a specific-locus mutational assay (Hsie *et al.* 1978).

Quantitative mutagenesis with the CHO/HGPRT system

In our early studies we demonstrated that mutation induction by a direct-acting chemical mutagen, ethyl methanesulphonate (Hsie *et al.* 1975a), and a physical agent, ultraviolet light (Hsie, Brimer, Mitchell & Gosslee, 1974b), was quantifiable. Treatment of CHO cells with EMS (25–800 μg/ml) for 16 hr caused a concentration-dependent exponential killing after a distinct shoulder region where there was no appreciable loss of cell survival. However, mutation induction occurred over the entire concentration range tested (Fig. 1; Hsie *et al.* 1975a). Further studies using treatment times of 2–24 hr and various EMS concentrations demonstrated the existence of a limited reciprocity of EMS mutagenesis; that is, when different EMS concentrations (0·05–3·2 mg/ml) were multiplied by various treatment times (2–10 hr), the product [(mg/ml) × hr] yielded a constant mutation frequency and cytotoxicity (O'Neill & Hsie, 1977).

The quantitative nature of the CHO/HGPRT system has been used for studies of the structure–mutagenicity of ten alkylating chemicals: dimethyl sulphate (DMS), diethyl sulphate (DES), methyl methanesulphonate (MMS), ethyl methanesulphonate (EMS), isopropyl methanesulphonate (iPMS), *N*-nitroso-*N*-methylurea (MNU), *N*-nitroso-*N*-ethylurea (ENU), *N*-nitroso-*N*-butylurea (BNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG). Six platinum(II) compounds have also been investigated: *cis*-dichlorodiammine platinum(II) (*cis*-$Pt(NH_3)_2Cl_2$), *trans*-dichlorodiammine platinum(II) (*trans*-$Pt(NH_3)_2Cl_2$), potassium trichloramminepalatinate(II) ($K[Pt(NH_3)Cl_3]$), chlorotriammine platinum(II) chloride ($[Pt(NH_3)_3Cl]Cl$), potassium tetrachloroplatinate(II) ($K_2(PtCl_4)$], and tetraammine platinum(II) chloride ($[Pt(NH_3)_4]Cl_2$). All ten alkylating agents are carcinogenic in laboratory animals (IARC Working Group, 1974a,b & 1978; Schoental & Bensted, 1969; Ward & Weisburger, 1975), and of the platinum(II) compounds *cis*-$Pt(NH_3)_2Cl_2$, is an inorganic antitumour agent which is particularly effective in combination therapy against testicular and ovarian cancer (Einhorn & Donahue, 1977; Wittshaw & Kroner, 1976).

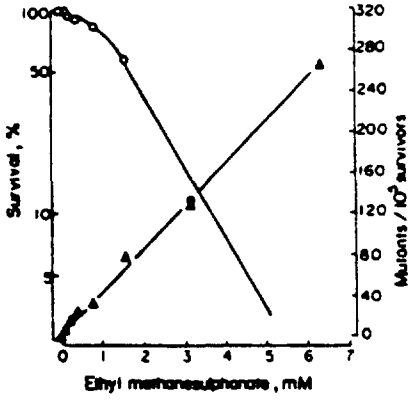


Fig. 1. Dose–response relationship of cytotoxicity (O) and mutagenicity (▲) of ethyl methanesulphonate. The average cloning efficiency of the untreated control culture is 80%, and the spontaneous mutation frequency is 12×10^{-6} mutants/clonable cell. [Redrawn from Hsie, Brimer, Mitchell and Gosslee (1975a).]

Alkylsulphates and alkanesulphonates

All alkylsulphates and alkanesulphonates have been found to be cytotoxic and mutagenic to CHO cells. Despite the vast difference in the size of the shoulder region and the slope of the survival curve, these alkylating agents have been shown to induce mutation with a linear increase over the entire survival curve (Couch, Forbes & Hsie, 1978; Couch & Hsie, 1978; Hsie *et al.* 1975a). The data in Table 1 show that the alkylsulphates vary considerably in cytotoxicity; for DMS and DES concentrations of 90 and 2800 μM, respectively, are required to reduce cell survival to 10%. At equitoxicity, DES is more mutagenic than DMS. Since DES is much less cytotoxic than DMS, DES is one-third as mutagenic as DMS when the comparison is made on a molar basis (Table 1).

Cytotoxicity has also been found to decrease with an increase in the size of the alkylating substituent among alkanesulphonates (Table 1); concentrations of 95, 4540 and 3700 μM MMS, EMS and iPMS, respectively, are required to reduce cell survival to 10%. MMS induces four- and 15-fold more mutations than EMS and iPMS, respectively, on a molar basis. However, EMS is ten- and four-fold more mutagenic than MMS and iPMS, respectively, on an equitoxic basis.

It appears that, when comparisons are made at equimolar rather than equitoxic concentrations, the cytotoxic and mutagenic effects of the alkylsulphates and alkanesulphonates decrease with increasing size of the alkylating group. These results also reinforce the notion that cytotoxic and mutagenic effects of EMS are dissociable (Hsie *et al.* 1975a; O'Neill & Hsie, 1977). In addition, mutagenicity does not appear to associate with S_N1 reactivity, since iPMS has the highest S_N1 reactivity (Lawley, 1974) but is the least mutagenic of these five chemicals (Table 1).

Nitrosamidines and nitrosamides

The five *N*-nitroso compounds that have been studied have been shown to produce a concentration-dependent exponential loss of clonal growth. While ENU and BNU exerted cellular lethality with a shoulder region of 100 and 400 μM, respectively, MNNG, ENNG and MNU caused an exponential lethality without any appreciable shoulder (Couch &

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Table 1. *Mutagenicity and cytotoxicity of alkylating chemicals**

Compound	Cytotoxicity†	Mutagenic activity‡ (mutants/10^6 cells)	
		At 10% survival	Per μM mutagen
Alkylsulphates:			
Dimethylsulphate	90	90	1·0
Diethylsulphate	2800	780	0·3
Alkylsulphonates:			
Methyl methanesulphonate	100	140	1·5
Ethyl methanesulphonate	3700	1600	0·4
Isopropyl methanesulphonate	4500	440	0·1
Nitrosamidines:			
MNNG	0·3	200	590
ENNG	6	520	90
Nitrosamides:			
N-Nitroso-*N*-methylurea	90	240	3
N-Nitroso-*N*-ethylurea	1200	550	0·5
N-Nitroso-*N*-butylurea	2500	200	0·1

MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine ENNG = *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine

*Data from Couch, Forbes & Hsie (1978) and Couch & Hsie (1978).

†Cytoxicity is expressed as the concentration of chemical (μM) required to reduce cellular cloning efficiency to 10%.

‡Mutagenicity is expressed as (1) the number of mutants per 10^6 cells at 10% survival with no reference to chemical concentration, and (2) the slope of the linear portion of the mutation induction curve as the number of mutants/10^6 cells/μM of chemical tested.

Hsie, 1978). All of the agents, however, produced a linear increase of mutation induction throughout the entire survival curve (Couch & Hsie, 1978).

Of the two *N*-nitrosamidines that have been studied (Table 1) MNNG is more toxic than ENNG: concentrations of 0·3 and 6 mM produce 10% cell survival for MNNG and ENNG, respectively. When mutagenicity is compared on a molar basis, MNNG is six times more active than ENNG; however, ENNG is about three times more mutagenic than MNNG when the comparison is made at 10% survival because MNNG is 20 times more toxic than ENNG (Table 1).

The three *N*-nitrosamides have been found to be much less toxic than the two nitrosamidines that have been studied: 90, 1200 and 2500 μM are required to reduce survival by 90% for MNU, ENU and BNU, respectively. Apparently the bulkier the alkylating group, the less toxic it becomes. This trend also applies to mutagenicity: MNU is six and 35 times more mutagenic than ENU and BNU, respectively, when compared on an equimolar basis; however both MNU and BNU are only half as mutagenic as ENU at 10% survival (Table 1).

These results suggest that, of the five *N*-nitroso compounds studied, both cytotoxic and mutagenic effects decrease with an increase in the size of the alkylating group. *N*-Nitrosoguanidines are more effective mutagens than nitrosourea, since on a molar basis MNNG and ENNG are 200 times more potent than their corresponding nitrosoureas on a molar basis.

N-Methyl-*N'*-nitro-*N*-nitroguanidine, a non-nitrosated analogue of MNNG, has been shown not to be mutagenic even at concentrations 100,000 times higher than tested for MNNG (J. R. San Sebastian & A. W. Hsie, unpublished data, 1978). Nitrosation appears to be essential for nitrosamidines to be mutagenic.

Relationship between DNA alkylation and mutation at the *hgprt* locus by MNU and ENU

Once we had established the effect of the modification of the alkylating moiety on the mutagenicity of ten alkylating chemicals, we examined the correlation between the degree of DNA alkylation and the mutagenicity of selected chemicals. For this purpose, we chose MNU and ENU because of the existence of well-documented comparisons of mutagenicity in microbial systems (Lawley, 1974; Neale, 1976) and in the CHO/HGPRT assay (Couch & Hsie, 1978), of animal carcinogenicity (Pegg, 1977), and of chemical reactivity (Veleminsky, Osterman-Golkar & Ehrenberg, 1970).

In cultures treated with MNU and ENU, we found that both alkylation and mutation induction by MNU and ENU increase linearly with increasing nitrosamide concentrations over the concentration range tested (Thielmann, Schröder, O'Neill, Brimer & Hsie, 1979). In this study, on an equimolar basis, MNU had 15 times the alkylating activity of ENU, but only three times the mutagenic activity. In terms of mutation induction per unit alkylation, ethylation of DNA by ENU appears to result in more than four times the number of mutagenic lesions than does methylation by MNU (Table 2). This may reflect either a higher miscoding frequency, such as O^6-guanine alkylation, or an effect of ethylation *per se* (Thielman *et al.* 1979).

Further studies will be directed toward determination of DNA adducts (alkylated nucleosides and phosphotriesters) and their relationship to mutation induction.

Platinum(II)chloramines

Data that have been obtained for the six platinum compounds are shown in Table 3. Three platinum(II)

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Table 2 *Mutation induction and DNA alkylation by MNU and ENU**

Compound	DNA alkylation†	Mutation induction‡	Ratio, mutation induction: DNA alkylation
MNU	57	2800	50
ENU	4	840	220
Ratio, MNU:ENU	15	3·3	

MNU = *N*-Nitroso-*N*-methylurea ENU = *N*-Nitroso-*N*-ethylurea

*Data from Thielmann, Schröder, O'Neill, Brimer & Hsie (1979).

†DNA alkylation is expressed as pmol of labelled radioactivity/μmol of cellular DNA/mM of unlabelled nitrosourea.

‡Based on the linear portion of the mutation induction curve; expressed as the number of mutants/10^6 cells selected/μM of nitrosourea.

compounds, *cis*-$Pt(NH_3)_2Cl_2$, $K[Pt(NH_3)Cl_3]$, and $[Pt(NH_3)_3Cl]Cl$, are mutagenic, the mutagenic potential of $K_2(PtCl_4)$ is marginal, and *trans*-$Pt(NH_3)_2Cl_2$ and $[(Pt(NH_3)_4]Cl_2$ are non-mutagenic. Based on the slope of the linear portion of the mutation induction curve, the relative mutagenicity of *cis*-$Pt(NH_3)_2Cl_2$: $K[Pt(NH_3)Cl_3]$: $K_2(PtCl_4)$: $[Pt(NH_3)_3Cl]Cl$: *trans*-$Pt(NH_3)_2Cl_2$: $[Pt(NH_3)_4]Cl_2$ is 100:9·0:4·0:3·0:0·04: 0·008; *trans*-$Pt(NH_3)_2Cl_2$ appears to lie on the lower limit of mutagenic response. Thus, *cis*-$Pt(NH_3)_2Cl_2$ is most mutagenic among these six platinum(II)chlorammines while its steric isomer, the *trans* form, is non-mutagenic; other structural congeners are less active or inactive in comparison with *cis*-$Pt(NH_3)_2Cl_2$. Analysis of the survival curve for treatment with *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ showed that the *cis* isomer is approximately 16 times more cytotoxic than the *trans* isomer, although the *cis* form is 2400 times more mutagenic than the *trans* form (Johnson, Hoeschele, Rahn, O'Neill & Hsie, 1980).

Binding of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ to cellular DNA

Both isomeric forms of $Pt(NH_3)_2Cl_2$ were taken up by CHO cells and found to be bound covalently to cellular DNA. Determination of the slope of the number of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ molecules bound per DNA nucleotide shows that the level of binding is three times higher per μM for the *cis* isomer than it is for the *trans* isomer (Table 3; Johnson *et al.* 1980).

A comparison of the mutagenicity and cytotoxicity per DNA lesion for *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ has shown that these two biological effects differ in their reactivity to DNA binding. The mutation induction by the *cis* isomer as a function of treatment concentration is 2400 times that of the *trans* isomer. However, the *cis* isomer binds only three times more to cellular DNA than does the *trans* isomer. The mutant yield per molecule bound to DNA is 800 times larger for the *cis* than for the *trans* isomer. Since 50% cellular lethality occurs at 1·3 and 35 μM for the *cis* and *trans* isomers, which correspond to 4×10^{-5} and 36×10^{-5} Pt molecules per DNA nucleotide, respectively, cytotoxicity per DNA lesion is a factor of nine greater for the *cis* than for the *trans* isomer. The *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ bind to cellular DNA to a similar extent (*cis*:*trans* = 3:1), yet the *cis* isomer is 2400 times more mutagenic and 16 times more cytotoxic than the *trans* isomer (Johnson *et al.* 1980). The subsequent expression and/or repair of these lesions

Table 3. *Mutagenicity, cytotoxicity and DNA binding of Pt(II)chlorammines**

Compounds	Mutagenic activity†		Cytotoxicity‡		
	mutants/10^6 cells/μM	max. no. of mutants/10^6 cells	log % survival/μM	LD_{10}	DNA binding§
cis-$Pt(NH_3)_2Cl_2$	31·5	140	−0·88	1·3	$2·8 \times 10^{-5}$
$K[Pt(NH_3)Cl_3]$	2·78	130	—	—	—
$[Pt(NH_3)_3Cl]Cl$	0·11	70	—	—	—
$K_2(PtCl_4)$	0·12	25	—	—	—
trans-$Pt(NH_3)_2Cl_2$	0·013	12	−0·005	35	$0·9 \times 10^{-5}$
$[Pt(NH_3)_4]Cl_2$	0·0025	17	—	—	—

*Data from Johnson, Hoeschele, Rahn, O'Neill & Hsie (1980).

†Expressed as (1) mutants/10^6 cells/μM based on the linear portion of the mutation induction curve, and (2) maximum number/10^6 cells derived from the highest observed number of mutants per 10^6 cells induced by a chemical among all concentrations tested. Spontaneous mutation frequency, 0–15 mutants/10^6 cells.

‡Expressed as (1) log % survival/μM based on the slope of the exponential portion of the survival (cloning efficiency) curve, and (2) LD_{10}, which is the concentration (μM) of a chemical required to reduce the cloning efficiency to 50%.

§The number of ^{195m}Pt-labelled molecules/DNA nucleotide/μM of platinum(II) compound.

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appear to account for the difference in potency of the two biological end points in these two steric isomers.

Concluding remarks

Conditions necessary for quantifying mutation induction to TG resistance, which selects for mutants deficient in the HGPRT activity in CHO cells, have been defined. The quantitative nature of the CHO/HGPRT system permits studies of the effect of chemical structural modification on the mutagenicity and cytotoxicity of alkylsulphates, alkanesulphonates, *N*-nitrosamidines, *N*-nitrosamides, and platinum(II)-chlorammines. Analyses of DNA alkylation by MNU and ENU as well as DNA binding by *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ demonstrate that both the quantity and quality of DNA adducts affect mutagenicity and cytotoxicity. The CHO/HGPRT system appears to be useful for studying genetic aspects of toxicology and to relate molecular parameters necessary for mutagenic and cytotoxic activities.

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