14 Structure–Activity Relationships: Experimental Approaches

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14.1 INTRODUCTION

Effects of genotoxic agents can vary by species, sex, and individual, as well as by cell type and stage in the same individual. Among the variables causing diversity in response are the types of adducts formed with DNA, their persistence, mutagen dose, and the time interval between the formation of premutagenic lesions and DNA replication. In spite of these variables, the question remains whether cause–effect relationships can be established for action spectra of genotoxicants that have significance beyond the experimental or environmental conditions under which the correlations are established. This problem is of foremost importance to assess relative risks due to exposure to genotoxic agents and to formulate conclusions from data obtained from different assays systems. Therefore, improvement of the understanding of underlying mechanisms by which genetic alterations are mediated is imperative. Therefore, all of the foregoing factors must be addressed before assessments of genetic hazards produced by synthetic and naturally occurring mutagenic chemicals can be made.

14.2 EARLY APPROACHES

Early attempts at classifying biological effects of mutagens occurred in the 1960s when fundamental chemistry principles were applied to catalogue the substitution reactions of alkylating agents (AAs) with nucleophiles in nucleic acids and proteins (Ross, 1962). The relative reactivity of various alkylating agents towards different nucleophilic centres in biomacromolecules was considered in terms of the rates of these substitution reactions, and according to the principle of Swain and Scott (1953). Swain and Scott developed a two-parameter equation to correlate the

Methods to Assess DNA Damage and Repair: Interspecies Comparisons
Edited by R. G. Tardiff, P. H. M. Lohman and G. N. Wogan

relative rates of reactions of various nucleophilic reagents (e.g., water, acetate, pyridine, thiosulphate) with a series of substrates (alkyl halides, esters, epoxides) in water solution. This linear relationship is described by

$$\log \left(\frac{k_y}{k_{H_2O}}\right) = s \times n \qquad (14.1)$$

where

k_{H2O} is a rate constant for reaction with water,

k_y is the corresponding rate constant for reaction with any other nucleophilic reagent,

s is a constant characteristic of the substrate, and

n is a constant characteristic of the nucleophilic reagent.

Usually, methyl bromide is chosen as a standard substrate, for which $s = 1.00$ to determine values of n; from n values, s values can be obtained for direct acting agents of unknown nucleophilic selectivity.

The Swain–Scott empirical linear relationship has proven to be highly valuable to understand the great diversity in genetic activity profiles (GAP) shown by the large group of alkylating agents. This diversity is primarily the consequence of varying alkylation patterns in DNA and proteins. These patterns have been reviewed in detail on several occasions (Hemminki, 1983; Singer and Grunberger, 1983; Coles, 1984–85; Saffhill *et al.*, 1985). For instance, AAs with high s values, typified by methyl bromide (MBr) or methyl methanesulphonate (MMS), show high selectivity in their reactions with nucleophiles, and are particularly efficient towards centers of high nucleophilicity, such as N^7 of G and N^3 of A in DNA, and of -RS^- and -RSH in proteins. The opposite type of AA has a strong affinity for exocyclic oxygen, and is represented by *N*-ethyl-*N*-nitrosourea (ENU) and *N*-ethyl-*N'*-nitro-N-nitrosoguanidine (ENNG). As a consequence of variation in intrinsic adduct distribution, GAPs of even closely related AAs vary considerably, as was first shown by Ehrenberg and coworkers (1966) in studies of plants. By systematically analyzing the biological effects of several monofunctional alkyl methanesulphonates in barley (Osterman-Golkar *et al.*, 1970), successful attempts have been made to obtain for AAs semi-quantitative correlations of mutagenic, clastogenic, and cytotoxic activities with chemical structure and physico-chemical characters. Similar studies on other higher plants (Gichner and Veleminsky, 1967), mammalian cells in culture (Couch and Hsie, 1978; Couch *et al.*, 1978), and *Drosophila* (Vogel and Natarajan, 1979a, b) further supported the feasibility of the concept.

To illustrate the value of physico-chemical properties in predicting general GAPs, the relative reactivities of AAs with different nucleophiles are compared in Table 14.1. The relative reaction rates with thiosulphate to acetate increase much faster for epichlorohydrin (ECH; factor = 2382) and methyl bromide (MBr; factor = 4380)

than for isopropyl methanesulphonate (iPMS; factor = 10), reflecting the higher nucleophilic selectivity of agents with a high *s* value. Consequently, most modifications by ECH and MBr are on proteins but not on DNA, that may cause cell lethality, and thus demonstrating mutagenic properties of high *s* value chemicals may often be difficult experimentally. Aside from reactivity, absorption and stability have a major impact on mutagenic or carcinogenic responses in a living organism (Figure 14.1).

Table 14.1 Relative reactivity of alkylating agents with nucleophiles compared with water

		$k_y \div k_{H2O}$			
		1	2	3	3/1
Substrate	*s*	Acetate (*n*=2.72)	Pyridine (*n*=4.43)	Thiosulfate (*n*=6.36)	
Isopropylmethane-sulfonate (iPMS)	0.28	6	12	60	10
Ethyl methane-sulfonate (EMS)	0.64	55	200	11300	205
β-Propiolactone (PL)	0.77	125	590	80000	640
Epichlorohydrin (ECH)	0.93	340	2250	810000	2382
Methyl bromide (MBr)	1.00	525	4000	2300000	4380

From Swain and Scott (1953) and Ross (1962)

This whole concept (i.e., analysis of multiple genetic endpoints in relation to specific chemical properties of the genotoxic agent) has not been applied. Progress in understanding how genotoxic chemicals exert their biological effects across species has been impeded by the lack of a concept considering both physico-chemical properties and GAPs of chemicals. The single end-point approach, relating induced genetic damage to exposure dose is of limited value for quantitative structure–activity considerations despite of its wide application in more than 150 tests for mutagenicity. For example, attempts to correlate the carcinogenic potency of chemicals with qualitative mutagenicity data obtained from a single

mutagenicity test turned out to be an oversimplification of a complex matter, as demonstrated for a set of AAs tested in the *Salmonella* assay (Bartsch *et al.*, 1983).

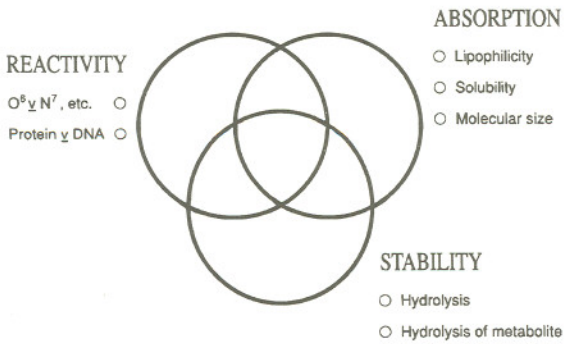

Figure 14.1 The three major physico-chemical factors influencing the extent to which a (DNA-reactive) parent chemical or metabolite may elicit a mutagenic or carcinogenic response in a living organism

14.3 CRITERIA AND METHODS FOR INTERSPECIES COMPARISONS

A most critical point in comparative mutagenesis studies with cellular systems or multicellular organisms is measurement of dose, i.e., the actual dose at the genetic material of the target cell under analysis. The external dose is not an adequate parameter to compare the genotoxic effects of chemicals, because of the large variation in their reactivity, stability, and absorption (Figure 14.1). Exact dose measurements at the genetically significant target are possible only when labelled mutagens or monoclonal antibodies are used (Drake, 1975); except for a few model compounds (mostly monofunctional AAs) such information is scanty. Another problem is that the types of DNA adducts and their relative distribution in DNA have been determined only for a minority of genotoxic chemicals.

To enable comparisons for a wide range of genotoxic agents, information is needed on the spectrum of genetic alterations produced in an assay system and the consistency of this spectrum over a range of organisms. Thus, if a genotoxic agent showed marked specificity for the production of a particular type of genetic damage, the consistency of this specificity must be defined over a wide range of organisms, and, if not, the variables causing heterogeneity in genotoxic effects must

be elucidated. The analytical tools available for such a comprehensive analysis comprise both traditional biological endpoints and recent molecular genetic techniques, that may be complemented by DNA dosimetry (Table 14.2). Some of the perspectives and limitations of the experimental possibilities are discussed below.

14.3.1 GENOTOXIC EFFECTIVENESS

Determinations of genotoxic effectiveness are based on the amount of chemical exposure (exposure concentration or dose) needed for the production of a specified frequency of mutations (Hussein and Ehrenberg, 1975). Factors that affect responses to a mutagen include uptake, penetration of and migration to the genetically significant target cells, rate of hydrolysis, and metabolic fate.
Consequently, even closely related genotoxicants may differ vastly in their mutagenic effectiveness. Typical examples include the small AAs: in *Salmonella typhimurium* strains TA98 and TA100 *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is more than 1000-fold more effective than dimethylnitrosamine (DMN) (Sugimura, 1982), despite their similar nucleophilic selectivity. Therefore, mutagenic effectiveness (i.e., expressing mutation induction per unit external dose) has little value to compare the response to mutagens among species.

14.3.2 GENOTOXIC EFFICIENCY

Ideally, the extent and the distribution of DNA adducts that result from interaction with genotoxic agents should be used for interspecies comparisons. Presently, such data exist mainly for the small group of methylating and ethylating carcinogens.

14.3.3 RELATIVE GENOTOXIC EFFICIENCY

14.3.3.1 Relative carcinogenic potency

Analysis of reactions of small AAs with DNA in animals showed that neither the absolute amount of reaction with DNA nor the reaction at the N^7 atom of guanine could be correlated with carcinogenic potency (Schoental, 1967: Swann and Magee, 1968; 1971). By contrast, the relative ability to react with the O^6 atom of guanine (O^6-G) correlated well with carcinogenic action (O'Connor *et al.*, 1979), confirming the prediction of Loveless (1969) that the induction of mutations and cancer is correlated with O^6-G alkylation.

In studying the complex relationships between types of DNA damage and biological endpoints such as tumor formation, Bartsch and coworkers (Bartsch *et al.*, 1983; Barbin and Bartsch, 1986, 1989) used median TD_{50} estimates for rodents,

and compared these to the nucleophilic selectivity of mono- and bifunctional direct-acting alkylating agents. Nucleophilic selectivity of AAs was expressed by Swain–Scott constants *s* and/or the ratios of O^7-/O^6-alkyl guanine. A positive linear relationship between the log of TD_{50} estimates and *s* values was established for 17, mostly monofunctional, AAs (Barbin & Bartsch, 1989; Vogel *et al.*, 1990). In contrast, seven bifunctional alkylating agents (all antineoplastic drugs); chloroethylene oxide (CEO) and two methyl halides were more potent carcinogens than the above 17 AAs. These agents showed a higher carcinogenic potency than would have been predicted on the basis of their nucleophilic selectivity. With the seven cross-linking agents, no simple correlation was apparent between the TD_{50} of each AA and its *s* value. Strikingly, their TD_{50} values remained within narrow limits for six of the seven bifunctional antitumor drugs (Table 14.3), despite a range of *s* values between 0.53 (BCNU) and 1.39 (melphalan; MEL). This observation suggests that their ability for cross-linking DNA, rather than a definite nucleophilic selectivity, is the predominant cause of their high carcinogenic potency (Vogel *et al.*, 1990).

Both in terms of risk assessment models and mechanisms of action, a crucial question raised by these studies is what causes the remarkable differences in tumorigenic potency existing even between closely related carcinogens such as the direct-acting methylating agents (Table 14.4)? Answers may be forthcoming from a comprehensive analysis of genetic action spectra of carcinogens including characterization of induced mutational spectra at the molecular level, the role of unstable and persistent DNA lesions in relation to DNA repair, and the dose-dependency of each process.

Multiple genetic endpoints in relation to nucleophilic selectivity

Mutational spectra produced in various repair backgrounds can provide information on the role of different premutagenic lesions and on the significance of repair for mutation fixation. Isolation and subsequent characterization of carcinogen-induced base-sequence changes is of particular relevance when attempting to relate specific DNA adducts to the product (i.e., induced genetic damage). However, analysis of mutational spectra should not be restricted to sequence determination of newly induced mutations, but should involve a broader elaboration of the genetic action spectrum of the genotoxic agent under study. This outcome may be achieved by a combined application of molecular and general genetic techniques. The eukaryotic systems available for such a combined approach are lower eukaryotes (*Neurospora*), mammalian cells in culture, *Drosophila*, and the mouse (Table 14.5).

Extensive work on small AAs revealed the feasibility to link, under repair-proficient conditions, certain types of DNA alkylation products with various genetic endpoints in mammalian cells (Natarajan *et al.*, 1984) and in *Drosophila* (Vogel and Natarajan, 1979a, b). In V79 cells, AAs of low *s* values were less clastogenic than those with high *s* values, as indicated by the ratio of *hprt* mutations/breaks per

Table 14.2 Strategy and experimental tools for comparative analysis

Analysis of	System(s)	Information
1. **Genotoxic effectiveness**		qualitative
Single genetic endpoint in relation to exposure dose	prokaryotic + eukaryotic	(less suited for analysis of SAR)
2. **Mutagenic/carcinogenic efficiency**		quantitative
Single or multiple biological endpoints in relation to DNA dose (DNA dosimetry)	eukaryotic + mammalian	
3. **Relative genotoxic efficiency**		semi-quantitative
3.1 TD_{50} in rodents versus nucleophilic selectivity	mouse, rat, + hamster	
3.2 Measurement of multiple genetic endpoints compared with nucleophilic selectivity of mutagen	*Neurospora*, *Drosophila*, + mammalian cells in culture	
3.3 Genetic damage in relation relation to altered DNA repair condition	*Neurospora*, *Drosophila*, + mammalian cells	
3.4 Biological dosimetry (= relating genetic endpoints to each other) mammalian cells,+ mouse (?)	*Neurospora*, *Drosophila*,	
3.5 Cross comparisons		
4. **Molecular spectra of procarcinogen-induced mutations**		
DNA base alterations induced by carcinogens requiring metabolic activation	*in vivo* systems (?)	types of ultimate electrophiles
5. **SAR considerations**		
Estimation and analysis of genotoxic properties from structural and physico-chemical properties		priority setting for in-depth analysis

100 cells (Table 14.5). A more recent analysis (Table 14.6), in which the ratios (CA/SLRL) of chromosome breakage events (CA, measured as ring-X loss) to recessive lethal mutation (SLRL) induction was compared with the nucleophilic

selectivity of monofunctional AAs, confirmed earlier conclusions (Zijlstra and Vogel, 1988). All these data suggest a correlation between the ability of AAs for base nitrogen alkylation in DNA and their chromosome-breaking efficiency. Conversely, AAs acting more extensively at the DNA base oxygen atoms, appeared to have a higher potential for mutation induction, as measured by traditional genetic techniques, such as the *Drosophila* sex-linked recessive lethal assay.

Table 14.3 Comparison of median TD_{50} (mg/kg bw) in rodents and s values for seven antitumour drugs with cross-linking properties

Compound	s	TD_{50}
Bis(chloroethyl)nitrosourea (BCNU)	0.53	34
Mitomycin C (MitC)	0.81	0.68
Busulfan (BUS)	0.90	<140
Thio-tepa (t-TEPA)	1.10	131
Mechlorethamine (MEC)	1.18	17
Chlorambucil (CA)	1.26	92
Melphalan (MEL)	1.39	67

The molecular techniques now available enable one to verify or modify earlier hypotheses and conclusions. In fact, direct miscoding mutagenesis due to oxygen modification seems the predominant mechanism leading to mutations with ENU-type agents for which O^6-EtG and O^4-EtT mediated transition mutations are the most common sequence changes in human cells (Eckert *et al.*, 1988), Drosophila (Pastink *et al.*, 1989), and in *Escherichia coli* (Richardson *et al.*, 1987b). Deletion mutations were either not found (Pastink *et al.*, 1989) or found very rarely (Kelley *et al.*, 1985). Compared with the effects of ENU, in *Drosophila*, the molecular mutational spectra changed for agents of higher nucleophilic selectivity, such as DES, EMS, MNU, and MMS (Nivard, 1991).

With MMS, next to intra-locus deletions (18%) and A:T→G:C transitions (20%), an increasing proportion of transversion mutations (56%) was found (Nivard *et al.*, 1992). By contrast, 95 to 100% of single base changes induced by DMS, EMS, MNU, and MNNG at the *lacI* gene of *E. coli* were transitions, mostly of the G:C→A:T type (Burns *et al.*, 1986, 1987, 1988; Richardson *et al.*, 1987a; Zielenska and Glickman, 1988). By analogy, all single base changes induced by EMS in mouse cells (Ashman and Davidson, 1987) and 53 of 54 mutations induced in human cells (Lebkowski *et al.*, 1986) were found to be G:C→A:T transitions. This discrepancy between *Drosophila* and cellular systems could be due to the shorter time for which the latter are at risk for mutagenesis. Alkylation at the N^7 and N^3 positions of purine bases, if not removed by repair enzymes, labilizes the glycosylic bond, leading to depurination (Loeb, 1985). This effect is time-dependent (Singer and Grunberger,1983), which could be one reason why in prokaryotic or other cellular systems, where the time interval between interaction with DNA and mutation fixation is short, mutation induction correlates best with O-alkylation.

Thus, altered spectra might be produced in more complex *in vivo* system, as recently shown for MMS for which 52% of mutations (mosaic mutations isolated from the F_2) fixed only after several rounds of replication represented A:T→T:A transversions. This value is significantly higher than the 25% A:T→T:A transversions found among *vermilion* mutations fixed immediately after fertilization (Nivard *et al.*, 1992).

Table 14.4 Tumorigenic potency of methylating AAs in relation to their nucleophilic selectivity

Chemical	*s*	O^6-/7-Alk-guanine[a]	TD_{50} (mg/kg bw)[b]
N-Methyl-*N*-nitrosourea (MNU)	0.42	0.11	28
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	0.42	0.11	103
Dimethylnitrosamine (DMN)	—	0.10/0.13	70.2[c]
Methyl methanesulfonate (MMS)	0.86	0.004	12200
Trimethyl phosphate (TMP)	0.91	—	167000

[a] Vogel and Natarajan (1982)
[b] Barbin and Bartsch (1989)
[c] procarcinogen

14.3.3.2 Altered DNA repair

Classification of genotoxic carcinogens with regard to the magnitude of altered mutation induction (hypermutability) in repair-defective condition revealed a positive linear relationship between enhanced mutagenic effectiveness in excision-repair defective (*exr*$^-$) cells in *Drosophila* and the nucleophilic selectivity for a series of 18 carcinogens, representing either monofunctional and cyclic alkylating agents:
where,

$r = 0.79$ ($p<0.01$)

f_{exr-}/f_{exr+} denotes the enhancement ratio for mutation induction, and

$$\frac{f_{exr^-}}{f_{exr+}} = 12.4s - 1.9 \qquad (14.2)$$

s is the Swain–Scott constant (Vogel, 1989).

Table 14.5 Enhancement ratios for mutation induction in three repair-deficient systems in comparison with TD_{50} values for mono-functional AAs

Chemical	s	TD_{50}[a]	*Drosophila* *mei-9*	*Neurospora* *uvs-2*	CHO[b] *27-1*
MMS	0.86	12200	8.2	7.7	2.2
β-Propiolactone (PL)	0.77	562	6.3	4.0	NT
EMS	0.67	[110][c]	2.7	2.0	3.0
MNU	0.42	28	5.8	5.0	NT
MNNG	0.42	103	2.6	2.0	NT
ENU	0.26	11	1.3	NT	1.0

[a] TD_{50} in mg/kg per bw
[b] HPRT locus
[c] TD_{50} for diethyl sulfate
$s = 0.64$
NT = no test

For some agents, similar studies were conducted with *Neurospora* and Chinese hamster ovary cell lines (Zdzienicka and Simons, 1986). Table 14.7 compares mutagenicity data from three different systems with TD_{50} estimates for rodents (Barbin and Bartsch, 1989). Median TD_{50} values decreased with decreasing s, and the relative ranking for hypermutability response in *Drosophila mei-9* paralleled that in *Neurospora uvs-2*. However, the similarity in response of the two genetic systems to monofunctional AAs was not found for cross-linking agents: both *mei-9* and *mus-201* mutants of *Drosophila* (both are *exr*$^-$) were insensitive to cross-linking agents (Vogel, 1989), while the nucleotide excision-repair defective *uvs-2* mutant is not (deSerres and Brockman, 1986).

The striking parallelism in response to AAs between the two repair mutants of *Drosophila* and *Neurospora* and the positive correlations of TD_{50} values and hypermutability indices with nucleophilic selectivity suggest efficient error-free repair of ring nitrogen alkylation products. Further support for this conclusion comes from analysis of base sequence changes of *Drosophila vermilion* mutations produced in *exr*$^-$ genotypes after treatment with MMS: 63% of all changes (vs. 25% in wild-type) resulted from a modified adenine (Nivard *et al.*, 1992).

Table 14.6 Endpoints for measuring relative genotoxic efficiency (RGE)

System	Measurement of RGE	Specific loci[a]
Neurospora	Point (or intracistronic mutations) versus multi-locus deletions	*ad-3A*, *ad-3B*
Mammalian cells in culture	Chromosomal aberrations, SCEs and point mutations in relation to cell killing	*hprt*[a]
Drosophila	Chromosome breakage effects (chromosome loss, translocations) relative to X-linked recessive lethals (700 loci)	*white*[a], *adh*[a], *vermilion*[a]
Mouse	Specific-locus mutations, dominant lethals, and cataracts, translocations	7 loci

[a]Used for analysis of DNA base sequence changes

Suggestive evidence also exists that other cellular factors also play a role in the hypermutability response (Vogel, 1989). These factors are (a) the faster rate of removal of methyl adducts compared with ethyl derivatives (also well-established from repair studies on mammalian cells; Brent *et al.*, 1988), and (b) the length of the time period between DNA modification and onset of replication. Despite the many variables, a positive correlation was found between relative efficiency ranking of carcinogens and enhancement ratios for hypermutability. This finding suggests a strong function of DNA repair in the initial process of tumorigenesis.

Active repair also seems to be the reason that a large series of genotoxic carcinogens, mostly of high nucleophilic selectivity, failed to produce significant numbers of specific-locus mutations in early germ cells in the mouse (Table 14.8).

14.3.3.3 Biological dosimetry

The most desirable dosimetry procedure would be that providing information on the extent and the distribution of DNA adducts resulting from interaction with genotoxic agents. Where such data are unavailable, as is often the case, two or more biological endpoints may be compared with one another. For instance, a perusal of Table 14.9 reveals that the results of studies with AAs in CHO cells or

Table 14.7 Nucleophilic selectivity (*s* value) and relative efficiency for chromosome aberrations versus mutation in *Drosophila* (CA/SLRL)[a] and V79 cells[b]

Chemical	*s*	CA/SLRL[a]	Mutation($\times 10^{-5}$) /breaks per 100 cells[b]
Ethylnitrosourea (ENU)	0.26	0.04	13.7/31.1[c]
N-Ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)	0.26	0.10	no test
Isopropyl methanesulphonate (iPMS)	0.29	0.17	no test
Methylnitrosourea	0.42	0.23	2.9
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	0.42	0.19	no test
Diethylsulfate (DES)	0.64	0.13	no test
Ethyl methanesulfonate (EMS)	0.67	0.32	0.7
Dimethylsulfate (DMS)	0.86	0.44	1.0/1.1
Methyl methanesulfonate (MMS)	0.86	0.55	0.4
Trimethylphosphate (TMP)	0.91	0.35	no test

[a]Ratio of ring-X chromosome loss (CA) to recessive lethals (SLRL) in *Drosophila*; data from Zijlstra and Vogel (1988).
[b]Ratio of *hprt*-locus mutations to the number of breaks in V79 cells; data from Natarajan *et al.* (1984).
[c]Two experiments.

Drosophila are in remarkable agreement, showing a relationship with *s* values in both systems, if mutation induction is related to cytotoxicity (efficiency measurement). For both *Drosophila* and the CHO system, however, no relationship exists between *s* values and genotoxic effect, when mutation induction is compared per unit of dose. This situation is not surprising, because analysis on an equal-molar basis (mutagenic effectiveness) takes no account of the differences among AAs in rate constants, rates of decomposition, and other characteristics (Osterman-Golkar *et al.*, 1970; Vogel and Natarajan, 1982). Biological dosimetry may also be used for genotoxicant classification, even when information on nucleophilic

selectivity is unavailable (Table 14.10). For example, in *Drosophila*, the ratio of ring-X loss (CA) to recessive lethals (SLRL) has prognostic value in terms of functionality of genotoxic agents (Zijlstra and Vogel, 1988). Thus, the 14 genotoxic agents listed in Table 14.10, based on their CA/SLRL ratios, clearly fall into two distinctly different groups: cross-linking versus monofunctional agents.

Table 14.8 Induction of specific locus mutations in (102/E1 x C3H/E1) F_1 male mice

Compound	Germ cell stage		Ref.
	Postspermatogonia[a]	Spermatogonia	
Chlormethine	+	-	[1]
Cyclophosphamide	+	-	[2]
Diethylsulfate	+	-	[3]
Ethyl methanesulfonate	+	-	[4]
Ethylnitrosourea	-/+[b]	+	[5,6]
Methyl methanesulfonate	+	-	[7,8]
Mitomycin C	-	+	[8]
Procarbazine, HCl	+	+	[9,10]
Vincristine	-	-	[11]

[a]For specification of different postspermatogonial germ cell stages.
[b]Among 15542 offspring, only one complete specific locus mutation, but nine mosaic mutations were obtained.

References:
[1] Ehling and Neuhäuser-Klaus (1989a)
[2] Ehling and Neuhäuser-Klaus (1988a)
[3] Ehling and Neuhäuser-Klaus (1988b)
[4] Ehling and Neuhäuser-Klaus (1989b)
[5] Favor *et al.* 1990)
[6] Ehling and Neuhäuser-Klaus (1984)
[7] Ehling and Neuhäuser-Klaus (1990)
[8] Ehling (1978)
[9] Kratochvilova *et al.* (1988)
[10] Ehling and Neuhäuser (1979)
[11] Ehling *et al.* (1988).

Comparisons of various endpoints have also been applied to the study of germ-line mutagenesis in the mouse, where tests for heritable translocations, dominant lethals, and specific-locus mutations can be combined with screening for dominant-cataract mutations, protein-charge mutations, and enzyme-activity mutations (Ehling *et al.*, 1985). This work is still in its inventory phase, since, apart from X-irradiation, only few model carcinogens (cyclophosphamide, ENU, MMS, mitomycin C, and procarbazine) have been analyzed by this multiple-endpoint approach (Ehling *et al.*, 1985; Ehling, 1988).

Table 14.9 Mutagenicity of alkylating agents in CHO cells and in *Drosophila*

Activity per unit exposure dose (mutagenic effectiveness):

CHO cells: EMS < ENU < DMS < MMS < MNU < ENNG < MNNG

Drosophila: EMS = iPMS < MNU < MMS+DMS < ENU

Activity relative to cytotoxicity (mutagenic efficiency):

CHO cells[a]: DMS < MMS < MNU < MNNG < iPMS < ENNG < ENU < DES < EMS

Drosophila[b]: DMS = MMS < MNU, iPMS < EMS < ENU

[a]HPRT mutants at 10% survival (Couch and Hsie, 1978; Couch *et al.* 1978; Natarajan *et al.*, 1982).
[b]Dose that produced 4% SLRL at 50% survival (Vogel and Natarajan, 1979a).

Cross-comparisons of TD_{50} and genotoxic properties

Two chemicals provide examples of the relationships between TD_{50} and genotoxic properties: Vinyl chloride (VC) and 7,12-dimethylbenz[*a*]anthracene (DMBA).

Apart from the well-studied class of small monofunctional AAs, few attempts have been made to relate carcinogenic potency estimates to formation and persistence of specific DNA adducts. An example is that of 2-chloroacetaldehyde (CAA) and of chloroethylene oxide (CEO) in VC carcinogenesis. Proteins and RNA, not DNA, are the major targets for CAA, and CEO is the metabolite responsible for VC carcinogenicity (Singer and Bartsch, 1986). Analysis of the genotoxic properties of both CAA and CEO provide strong support for such a conclusion (Table 14.11). CEO was shown to cause G:C→A:T transition mutations in *E. coli*, that could arise from the hypothetical N^4-(2-chlorovinyl)-deoxycytidine (Barbin and Bartsch, 1986). Interstrand cross-links have been demonstrated *in vitro* in salmon sperm DNA treated with CAA; its cross-linking property is also apparent from the CA/SLRL ratio of 19.0 in *Drosophila* (Table 14.11). In the *Drosophila* repair test with the excision-repair defective strain *mei-9*, CAA (a highly toxic agent) acts as a weak mutagen (a doubling of the spontaneous mutation rate was found) under exr^+ condition. In the absence of intact excision-repair, no effect was found. Its low genotoxic potential is in keeping with its *s* value of 1.3. Consequently, CAA could not be characterized by the *Drosophila* repair assay. The *s* value of CEO (0.71) is similar to that of glycidaldehyde (GA, 0.83), 1,3-propane sulphone (PS, 0.71), and ß-propiolactone (PL, 0.77); thus hypermutability effects comparable with these three agents are anticipated. By contrast, no effect at all was

found for CEO (Table 14.10). This observation is in remarkable agreement with the mechanism suggested for CEO-mutagenesis in *E. coli*. This agent mutated *recA*$^-$ strains more efficiently than a *recA*$^+$ strain. The absence of any elevated response in the *exr*$^-$ genotype in *Drosophila* (Vogel, 1989) suggests that direct miscoding may also be the mutagenic pathway of CEO in *Drosophila*.

7,12-Dimethylbenz[*a*]anthracene (DMBA) is another agent that gives no enhanced response in the *Drosophila* repair assay (Table 14.11). Thus, for three agents for which direct miscoding mutagenesis is likely to be a significant pathway (i.e., ENU, CEO, and DMBA), TD_{50} values indicated high tumorigenic potential (Table 14.11). Analysis is needed of DNA sequence changes of mutations induced in both repair-intact and repair-deficient cells, utilizing human and other mammalian cells, and *Drosophila*.

14.4 MOLECULAR GENETIC SPECTRA OF PROCARCINOGENS

Determination of molecular mutational spectra (i.e., "fingerprint" analysis) induced by both procarcinogens and their presumptive or established genotoxic metabolites is expected to provide information on the kind of DNA modifications involved. Yet unknown genotoxic action pathways may become apparent from such an analysis. For instance, for initial reaction products that they produced with DNA, methylating and ethylating AAs were among the best characterized genotoxic agents. Both *s* values and O^6-/O^7-alkylG ratios have been determined for most members of this group. However, in spite of close similarities of the O^6-/O^7-EtG ratio of DEN and ENU, the spectra of mutations found in the *Drosophila vermilion* gene are not the same (Pastink *et al.*, 1989; Sierra *et al.*, 1989). A considerable part of DEN-induced base sequence changes is transversions (46%), whereas 79% of those induced by ENU were transition mutations. By analogy, substantial numbers of transversions (C:G→A:T or A:T→T:A) were also found in activated *H-ras* genes from DEN-induced mouse liver tumor DNA (Stowers *et al.*, 1988). Whether a high constitutive activity of O^6-alkylG DNA-transferase, that is present in the liver (Pegg, 1983), or whether other factors are responsible for the unexpected mutational pattern remains unclear.

Another example of an unexpected finding is the significantly lower hypermutability indices of MNNG and DMN in *Drosophila* compared with MNU (Vogel, 1989). For DMN, bioactivation products other than the methylating species (e.g. formaldehyde and/or HO·-dependent activation) could be the reason that characterization of this group by their O^6-/O^7-metG ratios does not fully describe their genotoxic properties (Malaveille *et al.*, 1987). Thus substantial heterogeneity in DNA base change spectra can be expected even for "closely related" carcinogens.

Table 14.10 Cross-comparisons between genotoxic properties of carcinogens and their tumorigenic potency (TD_{50} estimates)

AAs	*s*	TD_{50}[a]	*exr*⁻/*exr*⁺ CA/SLRL *Drosophila*	27-1 CHO[c]	Suggested mechanism[b]
ENU	0.26	11	1.3	0.04	1.0	direct
DMBA	—	61	1.4	<0.04[c]	nt	direct?
CEO	0.71	72-0.43[d]	0.74	nt	nt	direct?
PS	0.71	1760	4.9	0.08	nt	indirect?
PL	0.77	562	6.3	0.39	nt	indirect?
GA	0.83	5960	12.3	2.56	nt	indirect?
MMS	0.86	12200	8.2	0.55	2.0	indirect/ mispairing
CAA	1.3	10000/ 15000[d]	inconcl	19	nt	crosslinks (weak)

[a]TD_{50} in mg/kg bw; from Barbin and Bartsch (1989).
[b]Predominant mechanism suggested to be either direct miscoding or indirect miscoding/misrepair mutagenesis.
[c]DMBA, a well established mutagen in *Drosophila*, did not measurably increase the frequency of ring-X loss (CA)
[d]The carcinogenic potency of CEO ranged from 0.43 to 72 mg/kg bw, and the estimate for CAA is 10 to 15 g/kg bw (Barbin and Bartsch, 1989).
[e]Zdzienicka and Simons (1986); *HPRT* locus.

14.5 SAR AMONG CARCINOGENS/MUTAGENS IN GENERAL

In the foregoing discussion, emphasis was placed on simple alkylating agents (AA); the fact that AAs are not a single uncomplicated series is perhaps the most important point to emerge from that discussion. Thus, the production of those mutations critical to carcinogenicity and the nature and extent of the carcinogenicity induced by an AA were shown to be dependent upon a matrix of variables, the major ones being either chemical (hydrolytic half-life, intrinsic reactivity, type of reactivity) or biological (extent and sites of reaction, response to adducts in terms of timing and fidelity of repair, or replication prior to repair). Within this context, no general and simple correlation should be expected—rather, subtle correlations between two parameters are to be expected once all other variables have been normalized. One could actually turn the matter around, and suggest that to expect simple axioms such as "gene mutagens will also always be clastogens" would be misleading. Once simple correlations are not sought or contrived, meaningful correlations are likely to emerge.

Moving away from relatively simple AAs into the broad middle ground of chemical carcinogenicity, the problems faced become immense, due mainly to the requirement for metabolic activation to produce a DNA-reactive (electrophilic)

Table 14.11 Ratios of ring-X loss (CA) to recessive lethals (SLRL) for a series of 14 genotoxic agents (Zijlstra and Vogel, 1988)

Chemical	CA/SLRL	Classification[a]
1,2-Dibromoethane (DBE)	0.37	monofunctional
Diethylnitrosamine (DEN)	< 0.04	monofunctional
Dimethylnitrosamine (DMN)	0.16	monofunctional
Dacarbazine (DTIC)	0.09	monofunctional
1-Phenyl-3,3-dimethyltriazene (PDMT)	0.22	monofunctional
Procarbazine (natulan, PC)	0.14	monofunctional
1-(2,4,6-Trichlorophenyl)-3,3-dimethyl-triazene (Cl_3PDMT)	0.29	monofunctional
Busulfan (BUS)	5.40	cross-linking
2-Chloroacetaldehyde (CAA)	19.0	cross-linking
2-Chloroethyl methanesulfonate (CEMS)	2.77	cross-linking
Cisplatin (DDP)	6.79	cross-linking
Cyclophosphamide (CPA)	8.54	cross-linking
Hexamethylphosphoramide (HMPA)	11.98	cross-linking
TEPA	14.60	cross-linking

[a]CA/SLRL values > 2 property for cross-linking

intermediate. Thus, once one includes the concept of metabolic transformation of a chemical, the possibility of forming several different electrophiles must be entertained, along with the likelihood of encountering metabolic pathways that will be directed towards elimination of the molecule from the target area, DNA. Adding to the complexity of attempting to simulate *in vitro* mammalian metabolism *in vivo* by an induced rodent liver subfraction (S9 mix), the situation approaches insolubility. This complexity must be accepted if progress is to be made. For example, the reference carcinogen benzo[*a*]pyrene is known to be mutagenic in the *Salmonella* assay (+S9 mix) via formation of the 4,5-epoxide, yet carcinogenic via formation of the bay region diol epoxide (Rosenkranz *et al.*, 1985). Likewise, the *N*-acetyl group on the 2-aminofluorene/DNA adduct drastically affects the response of DNA to the adduct—yet a complex of metabolic factors determines the presence or absence of this acetyl group, factors that vary among species and strains of rodents, and hence among the several S9 mixes used in routine *in vitro* testing. That reaction with isolated DNA of the model ultimate carcinogen for 2-acetylaminofluorene (2AAF), the *N*-acetoxy derivative 2-AAAF, failed to mimic the true interaction of 2AAF with cellular DNA emphasizes the complication of studying procarcinogens. Therefore, general SARs must be used as an adjunct to a scientific approach of estimating and understanding chemical carcinogenesis—not

as a means to bypass thought and experimentation. The following general considerations exist:

1. Chemical structure. The major chemical groupings associated with chemically induced cancer are shown in Figure 14.2 (Ashby *et al.*, 1989). These groups are usually associated with activity in short-term genotoxicity assays and with genotoxic carcinogenesis (Ashby and Tennant, 1988; Shelby, 1988; Ashby *et al.*, 1989; Bartsch and Malaveille, 1989; Tomatis *et al.*, 1989). An obvious hierarchy exists, and more agents of suspect structure are known than have been shown to be genotoxic *in vitro*, genotoxic *in vivo*, and/or carcinogenic.
2. Sites of carcinogenesis. Tomatis *et al.* (1989) recently warned against requiring coincidence between sites of carcinogenesis in humans and animals before animal data are regarded as an adequate model of these human effects. Nonetheless, certain patterns of carcinogenesis in rodents are emerging that can influence trans-species extrapolations, including those to humans. Thus, agents that are genotoxic (i.e., reactive to DNA) are more likely to be active across species and in several tissues, while non-genotoxic agents have a higher chance of being species- or sex- or site-specific in their rodent carcinogenicity. Furthermore, a sub-group of rodent tissues seems to be sensitive only to genotoxicants; this knowledge may contribute to trans-species extrapolations (tissues such as Zymbal's gland, skin, and circulatory system; Ashby *et al.*, 1989; Tomatis *et al.*, 1989). Thus, qualification of the term "carcinogen" is probably a necessary precursor to discern SARs of value useful in understanding trans-species extrapolation of cancer data.
3. Chemical studies. That small changes in chemical structure can produce large and predictable changes in biological activity, and that the synthesis and biological evaluation of key chemical entities are areas of this science that have been neglected, and which could be usefully reinstituted. The fact that replacement of the two protons on MNU with alkyl groups can extend the hydrolytic half-life of this agent 1000-fold (Figure 14.3), while retaining alkylating potential, is an illustration of this principle.
4. Metabolic Studies. Although not routinely practical, detailed metabolic and toxicokinetic studies are usually the surest way to rationalize the apparent species-specificity of a procarcinogen; such studies usually enable confident estimates of human carcinogenic potential for a rodent carcinogen.

14.6 CONCLUSIONS AND PERSPECTIVES

Much of the information in this review has been derived from work with alkylating carcinogens. This monograph illustrated how a combination of both classical and molecular genetic methods may be used to acquire information across species on cause–effect relationships in the action of genotoxicants. The analytical tools available for such a comprehensive analysis include determination of multiple

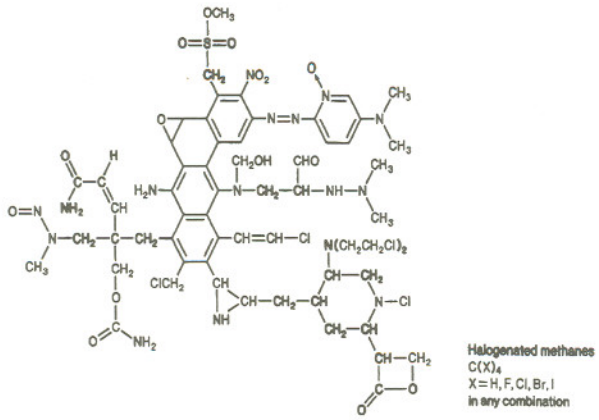

Figure 14.2 Main structural features of model chemical: DNA-reactivity, mutagenicity, and genotoxic carcinogenicity (structurally alerting fragments provided in Ashby *et al.*, 1989)

genetic endpoints related to fundamental physico-chemical properties of genotoxicants (such as Swain–Scott's *s* value), and modification of DNA repair condition and tumorigenic potency (TD_{50} compared with initial DNA interaction patterns). Although a systematic analysis of carcinogen-induced molecular mutational spectra has just begun, this approach promises to be particularly relevant to our understanding of the actions of genotoxic carcinogens in the following ways:

1. By comparing genetic action spectra of procarcinogens with those of their established or presumptive metabolites; such an analysis may help identify reactive intermediates relevant for the formation of premutagenic lesions.
2. By determining DNA base sequence changes of carcinogen-induced mutations produced in repair-intact versus repair-deficient condition; appropriate test systems (e.g., transgenic mice) have yet to be developed, before the likely link between DNA repair and carcinogenesis can be properly tested for mammals.

The correlations for relative potency ranking already established between tumorigenic potency in rodents, hypermutability response in *exr*$^-$ strains of *Drosophila* and *Neurospora*, and nucleophilic selectivity of AAs make it very likely that such a correlation is likely to be found for mammals.

To select proper model compounds, priority may be given to

1. Genotoxic carcinogens characterized as having low TD_{50} values (e.g., DEN,

DMBA, ENU, CEO, MitC), which is probably due to their ability for direct-miscoding (monofunctional agents) and/or cross-linking potential.

2. The mechanisms underlying the generally low tumorigenic potency of small AAs with a high *s* value; for human exposure, agents known to form cyclic adducts with nucleic acids are particularly relevant. However, one problem inherent with their generally high nucleophilic selectivity is their low mutagenic efficiency in relation to cytotoxicity, that makes the production of mutations at a single locus a cumbersome task. Some scientists hesitate to include this type of genotoxic agent into molecular mutational spectral analysis.

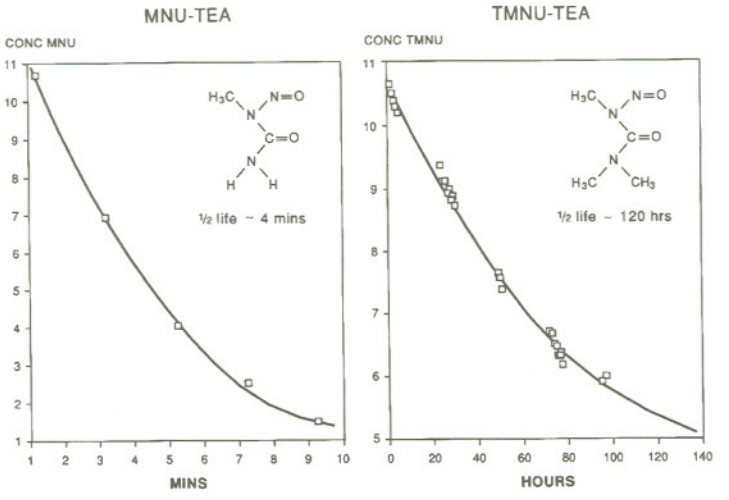

Figure 14.3 Extending the hydrolytic half-life 1000-fold results from replacing two protons on methylnitrosourea (MNU) to produce trimethylnitrosourea

14.7 REFERENCES

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