MUTAGENICITY AND ANTIMUTAGENICITY OF TRICYCLIC COMPOUNDS: ACRIDINES AND PHENOTHIAZINES

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Publication List:

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7. **Tanaka M**, Molnár J, Kidd S: Antimutagenicity of phenothiazine-metal co-ordination complexes in chemically induced mutagenesis. Anticancer Research, 17 (1), 1997 (in press).
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9. Motohashi N, Kurihara T, Kawase M, Hevér A, Nacsa J, **Tanaka M**, Yamanaka W, Kerim A, Molnár J: The effect of phenothiazines on the antibiotic resistant bacteria and multidrug resistant tumor cells. (submitted to Anticancer Research).
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Abstract

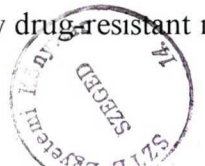
Acridines and phenothiazines are tricyclic aromatic hydrocarbons which have been shown to have a broad spectrum of biological activities, some of which are widely employed in the therapeutic regimen. Generally speaking, acridines are mutagenic; phenothiazines are not mutagenic, but antimutagenic. However, mutagenicity and antimutagenicity of the tricyclics depend on the substitution of (a) functional group(s) and (a) side chain(s) at the tricyclic skeleton, and the relationships between their mutagenicity/antimutagenicity and the chemical structure of the tricyclic compounds have not yet been established.

The mutagenicity of structurally related groups of newly synthesized acridines and phenothiazines were examined in the Ames' strains of *Salmonella typhimurium*. Eleven methylbenz[*c*]acridines showed diverse mutagenic activity in the Ames' strains; however, their mutation spectra do not correlate well with the epithelioma index. Discussing two contradictory hypotheses of the ultimate carcinogenic metabolite of methylbenz[*c*]acridines, the study suggests that an innovative mutagenicity assay be necessary to predict the culprit chemicals in mutagen-induced carcinogenesis in humans.

The study on the mutagenicity of antitrypanosomal acridines showed that the mutagenic activity depends on the tricyclic skeleton and the functional groups substituted, and a series of studies will establish the structure-activity relationships which enable the development of nonmutagenic antimicrobic agents.

Although some of the antipsychotic phenothiazines in clinical use were reported to be clastogenic and mutagenic in the presence of ultraviolet (UV) light, all of the phenothiazines examined in the model were found not to be mutagenic. A comparison of the mutagenicity of structurally similar benzo[*a*]phenothiazines and benz[*c*]acridines concluded that regardless of (a) side chain(s), the incorporation of sulfur atom at the *ortho*-position to nitrogen atom in heterocyclic hydrocarbons plays a crucial role in nullifying mutagenic activity not only of acridines, but also of benz[*c*]acridines. Noticing that some mutagenic acridines have quite specific mutational spectra and action sites at the bases of DNA, the author proposes the mutagenic tricyclics as possible gene therapeutic agents of *in vivo* site-specific mutagenesis.

The study of tricyclic antimutagenesis have at least two implications: development of cancer chemopreventive agents which arrest a successive mutation in the multistage carcinogenesis and the prevention of the emergence of new drug-resistant mutant microbics



and cancer cells such as antibiotic-resistant mutants, drug-resistant quasi-species of AIDS virus and multi-drug resistant (MDR) cancer cells. The models employed in a series of experiments did not supply more information on the antimutagenic activity of the phenothiazines in spontaneous mutagenesis, but in chemically induced mutagenesis the author presented, for the first time in the literature, the study of the antimutagenicity of groups of structurally related phenothiazines against a direct-acting mutagen to DNA. The DNA-blocking action of the phenothiazines was further evidenced by ultraviolet (UV) spectroscopy.

Furthermore, the correlation of the antimutagenicity of benzo[*a*]phenothiazines and the ground/first excited singlet-state dipole moments was presented. The correlation of antimutagenicity of phenothiazines and some more of their molecular and physicochemical properties are expected to be established for the structure-activity relationships (SAR), which make it feasible to design more powerful antimutagenic agents out of numerous possibly synthesizable tricyclics by computer-assisted drug design (CADD).

The data of the mutagenicity and antimutagenicity of the structurally related tricyclic compounds are expected to be stored in the database accessible to the public on the Internet. Thus novel tricyclic and related compounds will rationally be designed, synthesized and assayed for the development of nonmutagenic drugs, *in vivo* site-specific mutagenesis agents, spontaneous and chemical-induced mutation suppressing agents and cancer chemopreventive agents.

Contents

7.1.5. Tanaka M, Mucsi I, Wayda K, Molnár J, Motohashi N: Antiherpetic activity, antimutagenicity and mutagenicity of benzo[*a*]phenothiazines. Abstract. Acta Microbiologica et Immunologica Hungarica, 1996 (in press).

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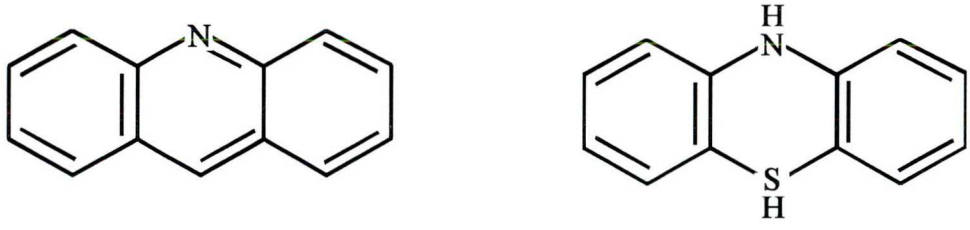
Figures and Tables

1. Introduction

1.1. Structures, biological activities, mutagenicity, antimutagenicity and their potentials

Acridines and phenothiazines are a group of tricyclic aromatic hydrocarbons in which a nitrogen atom is incorporated in the ring system in acridines and in addition a sulfur atom is incorporated in phenothiazines (Figure 1). Both heterocyclic compounds have been known to have a broad spectrum of biological activities such as antimicrobial, antitumor and antipsychotic effects (1-9). While tumoricidic acridines and antipsychotic phenothiazines have been already applied as therapeutic regimens (1), the synthesis of new tricyclic compounds is continuously attempted in search of new biological properties of novel derivatives and at the improvement of therapeutic effects of the lead compounds with substitution of various functional groups to different positions of the tricycle skeleton.

Both acridines and phenothiazines share structural similarity in their tricyclic ring system where a carbon atom is replaced with a sulfur atom in phenothiazines (Figure 1). However, they have contrastive properties in mutagenicity and antimutagenicity. Acridines are generally known as weak to strong mutagens, while phenothiazines are not mutagenic, but they were observed as antimutagens. Furthermore, the mutagenicity of acridines and the antimutagenicity of phenothiazines strikingly change with a position of various side chains at the tricyclic skeleton.



acridine phenothiazine

Figure 1. Structures of acridines and phenothiazines

Mutagens are defined as chemical and physical agents which alter and/or lead to the alteration of a base of deoxyribonucleic acids and mutagenicity is defined as property of chemical and physical agents to cause mutation (10).

The identification of environmental mutagens has been emphasized in search of carcinogenic compounds which transform normal cells to carcinogenic cells by mutation, that is, (a) change(s) of base components of deoxyribonucleic acids, resulting in the

alteration of biochemical properties of translated protein products. A large number of chemicals have been identified as mutagens and classified according to chemical structures in various experimental models (10).

Meanwhile, antimutagens are defined as chemical and physical agents which reduce the occurrence of mutations either by directly or indirectly inhibiting action of mutagens. Antipsychotic phenothiazines were observed to suppress both spontaneous and chemically induced mutation (11, 12). Antimutagenic activity of the phenothiazines is of particular interest not only to suppress the emergence of new drug-resistant mutant microbes (11) and multi-drug resistant cancer cells but also to develop antimutagenic cancer chemopreventive agents which arrest mutation sequence at a single point in multistage carcinogenesis (13).

1.2. Purposes and background

A number of novel acridines and phenothiazines have been synthesized from their parent compounds in search of the drugs of higher potency, the drugs of less side effects and new therapeutic values. A great majority of the compounds which are not found to be effective in the *in vitro* target-specific bioassays are not to be studied for their toxicological properties including mutagenicity assays. The data on a number of candidate drugs which reach the stage of toxicological assays are stored only in the database of private pharmaceutical companies and rarely appear in the scientific literature.

The study of the mutagenicity of structurally related tricyclic compounds is of great importance to identify environmental mutagens (10) which are to be avoided and to rationally design drugs for the development of nonmutagenic drugs which do not induce secondary diseases in the treated patients (14).

The mutagenicity of the groups of the acridines was studied to:

1) identify the mutational spectrum of methylbenz[*c*]acridines,
2) compare their mutagenic activity with carcinogenicity of methylbenz[*c*]acridines,
3) identify the mutagenicity of the antitrypanosomal acridines,
4) establish the structure-activity relationships (SAR) of the antitrypanosomal acridines and
5) store the data on the mutagenicity of the structurally related acridines in the database.

Generally speaking, phenothiazines are known not to be mutagenic. However, some antipsychotic phenothiazines in clinical use were reported to be clastogenic to the human chromosomes sampled from the psychiatric patients on thioridazine (15). Phenothiazines were found to be mutagenic in the presence of ultraviolet (UV) light (16). The observations lead to the suggestion of a more careful clinical use of the antipsychotic phenothiazines (15).

The mutagenicity of the groups of the phenothiazines was studied to:

1) screen the mutagenicity of the newly synthesized phenothiazines and
2) compare the mutagenicity of structurally related benz[*c*]acridines and benzo[*a*]phenothiazines.

Schizophrenic patients treated with phenothiazines were observed to have less incidence of cancer than those who were not treated (17), which suggests that phenothiazines have an inhibitory effect on the development of cancer. Antipsychotic phenothiazines were found to be antimutagenic both to strong mutagens, benzo[*a*]pyrene in *Salmonella typhimurium* (12, 18, 19) and to aflatoxin B_1 in the *in vitro* DNA-binding assay (20).

The antimutagenicity of the groups of the phenothiazines in spontaneous and chemically induced mutagenesis was studied to:

1) screen the antimutagenicity of the phenothiazines,
2) discover the antimutagenic activity of the phenothiazines against the direct-acting mutagens to DNA,
3) identify the antimutagenic action of the phenothiazines against the mutagens,
4) establish the structure-activity relationships (SAR) of the phenothiazines,
5) correlate the antimutagenicity and the physicochemical properties of the phenothiazines and
6) store the data on the antimutagenicity of the structurally related phenothiazines in the database.

2. Materials and Methods

2.1. Materials

2.1.1. Methylbenz[*c*]acridines

Eleven methylbenz[*c*]acridines: 7-methylbenz[*c*]acridine, 8-methylbenz[*c*]acridine, 9-methylbenz[*c*]acridine, 10-methylbenz[*c*]acridine, 11-methylbenz[*c*]acridine, 5,7-dimethylbenz[*c*]acridine, 7,9-dimethylbenz[*c*]acridine, 7,10-dimethylbenz[*c*]acridine, 7,11-dimethylbenz[*c*]acridine, 7,9,10-trimethylbenz[*c*]acridine and 7,9,11-trimethylbenz[*c*]acridine were synthesized and purified according to the literature (21), and they were dissolved in DMSO.

2.1.2. Antitrypanosomal acridines: acridinones, thio-substituted acridines and thioacridinones

Antitrypanosomal acridines including acridinones, thio-substituted acridines and thioacridinones: 9-(hydrazinothiazolo[5,4-a])acridinone, 9-(1,4-dioxano[5,6-a])acridinone, 2,3-dimethoxy-9-acridinone, 2,7-dimethoxy-9-thioacridinone, 2,7-dimethoxy-9-(3'-aminopropyl)thioacridine, 2,7-dihydroxy-9-acetamidothioacridine, 2,7-dihydroxy-9-(3'-aminopropyl)thioacridine and 1,4,7,10,13,16,19-heptaoxanonadecyl-2,7-[(10-methyl)-9-thioacridinone] were synthesized according to the procedures previously described (22, 23). The compounds were dissolved in water or DMSO according to their solubility.

2.1.3. Phenothiazine, promethazine, promazine and chlorpromazine

Phenothiazine, promethazine, promazine and chlorpromazine were purchased from Sigma-Aldrich Co., and they were dissolved in DMSO.

2.1.4. Phenothiazine-metal co-ordination complexes

Twelve phenothiazine-metal co-ordination complexes: phenothiazine-copper co-ordination complex, phenothiazine-palladium co-ordination complex, phenothiazine-gold co-ordination complex, promethazine-copper co-ordination complex, promethazine-palladium co-ordination complex, promethazine-gold co-ordination complex, promazine-copper co-ordination complex, promazine-palladium co-ordination complex, promazine-

gold co-ordination complex, chlorpromazine-copper co-ordination complex, chlorpromazine-palladium co-ordination complex and chlorpromazine-gold co-ordination complex were synthesized and purified by Sue Kidd. Phenothiazine, promethazine, promazine, chlorpromazine, copper, palladium and gold salts were obtained from Sue Kid. They were all dissolved in DMSO.

2.1.5. Benzo[*a*]phenothiazines

Seven benzo[*a*]phenothiazines: 12*H*-benzo[*a*]phenothiazine, 9-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 5-oxo-5*H*-benzo[*a*]phenothiazine and 6-hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine were synthesized as described before (24) and were dissolved in DMSO.

2.1.6. 10-[n-(Phthalimido)alkyl]-2-substituted-10*H*-phenothiazines

10-[3-(Phthalimido)propyl]-10*H*-phenothiazine, 10-[4-(phthalimido)butyl]-10*H*-phenothiazine, 10-[3-(phthalimido)propyl]-2-chloro-10*H*-phenothiazine. 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine, 10-[3-(phthalimido)propyl]-2-trifluoromethyl-10*H*-phenothiazine and 10-[4-(phthalimido)butyl]-2-trifluoromethyl-10*H*-phenothiazine were prepared as described before (25) and the compounds were dissolved in DMSO.

2.1.7. 1-(2-Chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkyl-1-ureas

1-(2-Chloroethyl)-3-(10*H*-phenothiazin-10-yl)propyl-1-urea, 1-(2-chloroethyl)-3-(10*H*-phenothiazin-10-yl)butyl-1-urea, 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)propyl-1-urea, 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea, 1-(2-chloroethyl)-3-(2-trifluoromethyl -10*H*-phenothiazin-10-yl)propyl-1-urea and 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)butyl-1-urea were prepared as described before (25) and the compounds were dissolved in DMSO.

2.2. Experimental models for mutagenicity assay

More than a hundred models have been proposed in detection of mutagens. Prokaryotic organisms such as *Escherichia coli* and *Salmonella typhimurium* are widely used for their convenience, while yeast is applied in mutagen detection assay to simulate the eukaryotic system. Furthermore, transgenic mice have been developed as mammalian *in vivo* mutagenicity assay (Table 1).

Table 1. Representative models for mutagenicity assay (26).

Salmonella typhimurium
Escherichia coli
Yeast
Fungi
Host mediated assay
Body fluid analysis
Plants
Drosophila sex linked recessive lethal
Chinese hamster lung cells (V79)
Chinese hamster ovary cells (CHO)
Mouse lymphoma L5178Y cells
Mouse spot test
Mouse visible specific locus test

2.2.1. Ames' *Salmonella* mutagenicity assay

Ames' *Salmonella* mutagenicity assay has been widely accepted as a method for detecting carcinogens and mutagens. The test has also been used to determine the mutagenicity of complex environmental and biological mixtures. A considerable number of mutagens first detected by the *Salmonella* test have been shown subsequently to be carcinogenic in animal tests, including the hair dye amines: 10-nitropyrene from diesel exhaust, the flame retardant: tris-2,3-dibromopropyl-phosphate and several of the protein pyrolysis products produced by cooking foods. The test data on more than 5000 chemicals were published in 1982 (27).

2.2.1.1. Bacterial strains

A set of histidine-requiring strains is used for mutagenicity assay. Each tester strain contains a different type of mutation in the histidine operon. In addition to the histidine

mutation, the standard tester strains contain other mutations that greatly increase their ability to detect mutagens. One mutation (*rfa*) causes a partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability of large molecules such as benzo[*a*]pyrene that do not penetrate the normal cell wall (29). The other mutation (*uvrB*) is a deletion of a gene coding for the DNA excision repair system (Δ*uvrB*), resulting in greatly increased sensitivity in detecting many mutagens (29, 30).

Table 2. *Salmonella typhimurium His*⁻ Strains for Ames' *Salmonella* mutagenicity assay (27, 28).

Strains	Genotypes	Histidine Mutation	Permeability	Repair Mechanism	R-factor (pKM101[a])
TA97a	*hisO1242,hisD6610*/Δ*uvr* /*rfa*/pKM101	*his O1242* *hisD6610*	*rfa*[b]	Δ*uvr*[c]	+
TA98	*hisD3052*/Δ*uvr*/*rfa*/ pKM101	*his D3052*	*rfa*	Δ*uvr*	+
TA100	*hisG46*/Δ*uvr*/*rfa*/ pKM101	*hisG46*	*rfa*	Δ*uvr*	+
TA102	*hisG428*(pAQ1)/+[d]/ *rfa*/pKM101	*hisG428* on a multicopy plasmid (pAQ1)	*rfa*	+	+
TA104	*hisG428*(pAQ1)/Δ*uvr*/*rfa*/ pKM101	*hisG428* on the chromosome	*rfa*	Δ*uvr*	+

Notes: All strains were originally derived from *S. Typhimurium* LT2. [a]pKM101: conforms error-prone repair to the cell. [b]*rfa*: deep rough (defective lipopolysaccharide). [c]Δ*uvr*: deletion of the ultraviolet-repair B gene, this deletion also removes the *gal* operon which includes nitrate reductase (*chl*) and biotin (*bio*) genes. [d] +: wild-type genes.

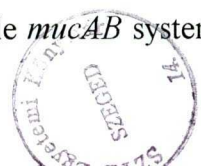
For technical reasons, the deletion excising the *uvrB* gene extends through the *bio* gene and as a consequence, these bacteria also require biotin for growth. TA102 does not contain the *uvrB* mutation because it was constructed primarily for detecting mutagens that require an intact excision repair system. The standard tester strains, TA102 and TA104 contain the R-factor plasmid, pAQ1, which carries the *hisG428* mutation and tetracycline resistance gene. These R-factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains (31, 32). In both *Escherichia coli* and *Salmonella typhimurium*, pKM101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms (31, 33, 34) (Table 2).

2.2.1.2. Target sequences

The *G46* mutation in TA100 and TA1535 exists in the *hisG* gene coding for the first enzyme of histidine biosynthesis (30). This mutation determined by DNA sequence analysis substitutes -GGG-, -CCC- proline for -GAG-, -CTC- leucine in the wild-type organism (35). TA 1535 and its R-factor derivative, TA100 detect mutagens that cause base-pair substitutions, primarily at one of these G-C pairs. The *his D3052* mutation in TA1538 and TA98 is in the *hisD3052* gene coding for histidinol dehydrogenase. TA1538 and its R-factor derivative, TA98 detect various frameshift mutagens. Frameshift mutagens can stabilize the shifted pairing that often occurs in repetitive sequences or hot spots of the DNA, resulting in a frameshift mutation which restores the correct reading frame for histidine synthesis. The *hisD3052* mutation has the sequence -CGCGCGCG-, -GCGCGCGC- in 8 repetitive -GC- residues near the site of a -1 frameshift mutation in the *hisD* gene (36). This mutation is reverted by mutagens such as 2-nitrosofluorene and daunomycin. The new frameshift strain, TA97, replaces the less sensitive TA1537 that was previously included in the standard set of strains. This new strain has an added cytosine resulting in a run of six cytosines at the site of the *hisD6610* mutation (28). The mutagenic specificity of TA97 is similar to that of the *hisC3076* mutation in TA1537 but because TA97 also has the second hot spot of alternating -GC- base pairs near the run of cytosines, it is sensitive to some of the mutagens that revert TA1538 and TA98. TA102 contains the ochre mutation -TAA-, -ATT- in the *hisG428* gene. This strain efficiently detects a variety of mutagens such as formaldehyde, glyoxal, various hydroperoxides, bleomycin, phenylhydrazine, X rays, ultraviolet (UV) light, streptonigrin, and cross-linking agents such as psoralens and mitomycin *C*. These mutagens are not detected or are poorly detected by the standard set of tester strains.

2.2.1.3. New tester strains: detection of all six substitution mutations

A detection and classification system for mutagens that identifies their six possible base-pair substitution mutations has been developed. A set of six *Salmonella typhimurium* strains has been constructed, each of which carries a unique missense mutation in the histidine biosynthetic operon. In addition to the *his*$^-$ mutation, these strains carry different auxiliary features that enhance the mutability of the target *his*$^-$ mutation. These include the R-factor pKM101 (31, 33, 34), which has the SOS-inducible *mucAB* system, a deletion of



the *uvrB* component of excision repair (29, 30) and *rfa* mutations to increase the accessibility of bulky chemicals in the bacteria (29). Another set of strains contains a wild-type *rfa* gene. Reversion via the base substitution unique to each strain was verified by sequence analyses of 800 revertants obtained from different types of mutagens. The strains have considerably lower spontaneous reversion frequencies and detect a variety of mutagens at a sensitivity comparable to the *Salmonella* tester strains TA100, TA102 and TA104. The low spontaneous frequency of the reversion of the mixture of the six tester strains enables a single mutation assay that is followed by classification of the type of mutation with the individual strains (38).

Table 3. Mutation of *Salmonella typhimurium His*⁻ Strains (37).

Strains	Mutated gene	Mutation	Gene sequence	
			Wild types	Mutant tester strains
TA97a	*hisD6610* *hisO6610*	+1 frameshift	-GUC ACC CCC UGA- [a]	-GUC ACC CCC CUG-
TA98	*hisD3052*	-1 frameshift	-CGC CTG TGG CGG GCC-	-CGC CTG TGG CGG CCG- [b]
TA100	*hisG46*	base-pair substitution	-GAG- (leucine) -CTC-	-GGG- (proline) -CCC-
TA102	*hisG428* (pAQ1)	ochre codon	-AG AGC AAG CAA GAG C-	-AG AGC AAG TAA GAG C-

Notes: a: pseudo-wild type which has 60% of the wild-type enzyme activity. b: No back-mutation to wild-type was recognized. All revertants were double frameshift (+1, −1) mutants.

2.2.1.4. Design of standard protocols

An attempt was made to construct a scientifically based, internationally harmonized protocol at the International Workshop on the Standardization of Genotoxicity Test Procedures in Melbourne, 1993. The consensus opinion was reached that the bacterial test battery should consist of *Salmonella typhimurium* TA1537, TA1535, TA98 and TA100 and 3 strains: TA97a, TA97 and TA1937 could be used interchangeably. The majority of the researchers agreed that strains for the detection of mutagens acting specifically on AT base pairs should be routinely included within the test battery. These strains may be *Salmonella typhimurium* TA102 or *Escherichia coli* WP2 strains. With regard to study design, 5 doses of test compound with 3 plates per dose should be used in each experiment. The use of 2 plates per dose is acceptable only if the experiment is repeated. The negative controls may consist of solvent control alone provided that historical data are available to

demonstrate lack of effect of the solvent in question. Positive control compounds should be included in all experiments, although the nature of these control compounds need not be specified in the guidelines. For non-toxic freely soluble test agents, an upper limit of 5mg/plate should be tested (5μl per plate for liquids). A consensus agreement was reached on the need to carry out further tests if equivocal results are obtained in the initial test, although the design of repeat study should be left flexible. Negative results in the test should be further investigated by either conducting a modified repeat (e.g. S9 titration) or by conducting the alternative methodology. If a preincubation assay is carried out, an incubation time within the range of 20-60 minutes is recommended, usually at a temperature of 37 degrees in Celsius. No consensus agreement on the acceptable criteria for a positive or negative result could be reached. The majority agreed that a reproducible dose-response was necessary for a chemical to be classified as positive. A number of limitations of the proposed test methodology were recognized and the adequate testing of specific chemical classes or groups such as azo-dyes and diazo compounds, gases, volatile chemical and glycosides were included. Such special cases should be evaluated using alternative published procedures (39).

2.2.1.5. Procedures

TA97a, TA98, TA100 and TA102 strains of *Salmonella typhimurium* were obtained from Prof. Bruce Ames. The plate incorporation test or spot test was carried out as described (27). The bacterial strains were cultured in Oxoid nutrient broth No.2 for 10-12 hours for turbidity to reach the optical density of 0.3-0.5 at 600nm. 0.1 ml Of bacterial culture, 0.1 ml of 100μg/ml test chemical and 0.5 ml of 0.1 mM sodium phosphate buffer (pH 7.4) were mixed, where the phosphate buffer replaced S9 microsomal fraction for the direct mutagenicity assay. Addition of top agar was followed by twenty-minute preincubation period at 37 degrees in Celsius in the turbulating incubator. After 48 hours the presence of the background lawn on all plates was confirmed and the number of back-mutant colonies was counted. The assay was performed in triplicate on two separate occasions (37).

2.3. Experimental models for antimutagenicity assays

A number of experimental models for antimutagenicity assays have been proposed using bacteria, yeast and culture cell-lines, most of which are modification of models of mutagenicity assay in which antimutagenic test chemicals are administered either prior to, simultaneously with or successively to mutagenic compounds. (40-43)

2.3.1. Antimutagenicity assay in Ames' *Salmonella* strains

Traditionally, TA98 and TA100 strains of *Salmonella typhimurium* have been used for antimutagenicity assay (12) and the TA98 strain was selected for antimutagenicity assay of antipsychotic phenothiazines in benzo[*a*]pyrene-induced mutagenesis (18).

2.3.1.1 Antimutagenicity assay of spontaneous mutation

2.3.1.2. Procedures

TA98 and TA100 strains of *Salmonella typhimurium* were obtained from Prof. Bruce Ames. The plate incorporation test was carried out as described (27) for antimutagenicity assay of spontaneous mutation. The bacterial strains were cultured in Oxoid nutrient broth No.2 for 14-16 hours for turbidity to reach the optical density of 0.3 at 600nm. 0.1 ml of bacterial culture, 0.1 ml of test chemical in various concentration and 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) were mixed and incubated for twenty minutes at 37 degrees in Celsius in the turbulating incubator. Five doses of the test chemicals were tested below their minimum inhibitory concentration (MIC) value. The phosphate buffer replaced S9 microsomal fraction for the direct antimutagenicity assay. 0.1 M Sodium phosphate buffer (pH 7.4) were used as control. After 48 hours the presence of the background lawn on all plates was confirmed and the number of back-mutant colonies was counted. The assay was performed in triplicate on two separate occasions.

2.3.1.3. Antimutagenicity assay of chemically induced mutation

2.3.1.4. Procedures

The plate incorporation test was carried out as described (27) with some modification for antimutagenicity assay of chemically induced mutation. The bacterial strains were cultured in Oxoid nutrient broth No.2 for 14-16 hours for turbidity to reach the optical density of 0.3 at 600nm. 0.1 ml of bacterial culture, 0.1 ml of test chemical in various concentration and 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) were mixed and incubated for twenty minutes at 37 degrees in Celsius in the turbulating incubator. 10 μg/ml 4-Nitro-*o*-phenylenediamine was then added to the mix and incubated again as described above. Five doses of the test chemicals were tested below their minimum inhibitory concentration (MIC) value. The phosphate buffer replaced S9 microsomal fraction for the direct antimutagenicity assay. 4-Nitro-*o*-phenylenediamine and chlorpromazine were used as positive control for mutagenicity and antimutagenicity assay, respectively. After 48 hours the presence of the background lawn on all plates was confirmed and the number of back-mutant colonies was counted. The assay was performed in triplicate on two separate occasions (44).

2.4. Ligand-DNA binding assay by ultraviolet (UV) spectroscopy

2.4.1. Procedures

DNA binding study of promazine-palladium co-ordination complex $[Pd(pzH)Cl_3]$ was performed by ultraviolet (UV) spectrophotometer at room temperature, 25 degrees in Celsius in 10 mM Tris-HCl, pH 7.0, 1mM EDTA. Ultraviolet (UV) absorption was measured in the ligand absorption region (190-400 nm). The presence of isosbestic points during titration with DNA allowed an evaluation of free and DNA-bound complex. Calf thymus DNA, sodium salt, Tris-HCl and EDTA were purchased from Sigma-Aldrich Co. (Budapest, Hungary) and spectrophotometric measurements were performed with a UNICAM SP. 800 B Ultraviolet Spectrophotometer (45).

3. Results

3.1. Mutagenicity

3.1.1. Methylbenz[*c*]acridines

8-Methyl-benz[*c*]acridine (II), 5, 7-dimethylbenz[*c*]acridine(VI), 7, 10-dimethyl-benz[*c*]acridine (VIII), and 7, 9, 10-trimethylbenz[*c*]acridine (X) showed an increased number of back-mutant colonies on TA97 strain by −1 frameshift back-mutation. 7, 10-dimethylbenz[*c*]acridine (VIII) was a strong mutagen, while 7, 5-dimethylbenz[*c*]acridine (VI) and 7, 9, 10-trimethylbenz[*c*]acridine (X) were moderately strong mutagens and 8-methylbenz[*c*]acridine (II) was weakly mutagenic. 7, 10-Dimethylbenz[*c*]acridine (VIII), 7, 11-dimethylbenz[*c*]acridine (IX). 7, 9, 11-trimethylbenz[*c*]acridine (XI) caused double frameshift (+1, −1) mutation on TA98 strain, each of which is weak to moderately strong mutagen. Transition base-pair substitution was induced on TA100 strain strongly by 7, 9, 11-trimethylbenz[*c*]acridine (XI), moderately strongly by 7, 10-dimethylbenz[*c*]acridine (VIII) and only weakly by 8-methylbenz[*c*]acridine (II), 9-methylbenz[*c*]acridine (III), 10-methylbenz[*c*]acridine (IV) and 5, 7-dimethylbenz[*c*]acridine (VI). T→C transition single base substitution was observed on TA102 strain treated by all methylbenz[*c*]acridines whose mutagenicity was weak to moderate (37) (Table 4).

3.1.2. Antitrypanosomal acridines

Back-mutation was significantly increased on 9-aminoacridine-sensitive TA97a strain with 9-(hydrazinothiazolo[5,4-a])acridinone 1, 2,7-dimethoxy-9-thioacridinone 4, 2,7-dimethoxy-9-(3'-aminopropyl)thioacridine 5 and 2,7-dihydroxy-9-acetamidothioacridine 6 and only slightly increased with 9-(1,4-dioxano[5,6-a])acridinone 2. Sodium azide-sensitive TA100 strain showed markedly increased back-mutation with 9-(hydrazinothiazolo[5,4-a])acridinone 1, 9-(1,4-dioxano[5,6-a])acridinone 2 and 2,3-dimethoxy-9-acridinone 3, and slightly increased with 2,7-dimethoxy-9-thioacridinone 4, 2,7-dimethoxy-9-(3'-aminopropyl)thioacridine 5, 2,7-dihydroxy-9-acetamidothioacridine 6 and 2,7-dihydroxy-9-(3'-aminopropyl)thioacridine 7 (46) (Table 5).

1,4,7,10,13,16,19-heptaoxanonadecyl-2,7-[(10-methyl)-9-thioacridinone] 8 can be recommended for further *in vitro* and *in vivo* studies for antiparasitic drug design because of the absence of its mutagenic activity on both strains. The mutagenic activity of 9-(1,4-

dioxano[5,6-a])acridinone 2 and 2,7-dihydroxy-9-(3'-aminopropyl)thioacridine 7 can probably be reduced by well-planned substitution of the tricyclic skeleton.

3.1.3. Phenothiazine, promethazine, promazine and chloropromazine

None of the four phenothiazines: phenothiazine, promethazine, promazine and chloropromazine showed any mutagenic activity on TA98 and TA100 strains of *Salmonella typhimurium*.

3.1.4. Phenothiazine-metal co-ordination complexes

All the twelve phenothiazine-metal co-ordination complexes: phenothiazine-copper co-ordination complex, phenothiazine-palladium co-ordination complex, phenothiazine-gold co-ordination complex, promethazine-copper co-ordination complex, promethazine-palladium co-ordination complex, promethazine-gold co-ordination complex, promazine-copper co-ordination complex, promazine-palladium co-ordination complex, promazine-gold co-ordination complex, chlorpromazine-copper co-ordination complex, chlorpromazine-palladium co-ordination complex and chlorpromazine-gold co-ordination complex were not mutagenic on TA98 strain of *Salmonella typhimurium* and copper, palladium and gold salts did not show mutagenic activity on the strains.

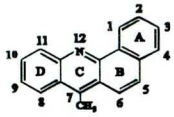
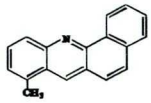
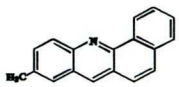
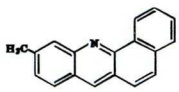
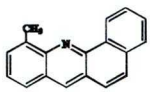
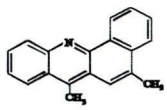
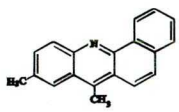
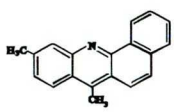
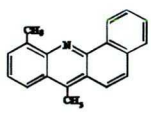
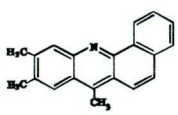
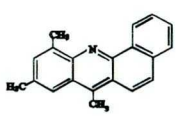
3.1.5. Benzo[*a*]phenothiazines

None of the seven benzo[*a*]phenothiazines: 12*H*-benzo[*a*]phenothiazine, 9-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 5-oxo-5*H*-benzo[*a*]phenothiazine and 6-hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine showed mutagenic activity on TA98 and TA100 strains of *Salmonella typhimurium* (47).

3.1.6. 10-[n-(phthalimido)alkyl]-2-substituted-10*H*-phenothiazines

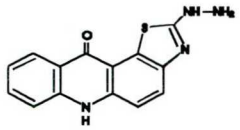
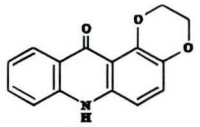
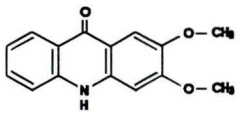
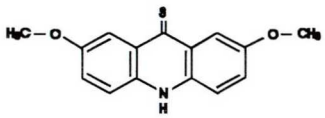
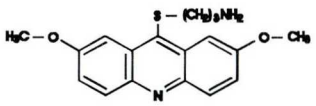
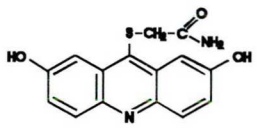
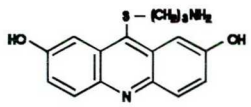
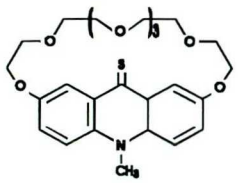
10-[3-(Phthalimido)propyl]-10*H*-phenothiazine, 10-[4-(phthalimido)butyl]-10*H*-phenothiazine, 10-[3-(phthalimido)propyl]-2-chloro-10*H*-phenothiazine. 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine, 10-[3-(phthalimido)propyl]-2-

Table 4. Mutagenic activity of methylbenz[*c*]acridines on *Salmonella typhimurium His*⁻ strains (37).

No.	test chemicals (10μg/sample)	colony numbers				mutagenicity index[a]				epithelioma index[b]
		TA97a	TA98	TA100	TA102	TA97a	TA98	TA100	TA102	
I.		0	inh	inh	234	0	-	-	23	63
II.		280	0	29	390	15	0	14	39	0
III.		0	0	270	270	0	0	13	27	0
IV.		0	inh	380	36	0	-	19	4	0
V.		0	0	inh	36	0	0	-	4	0
VI.		560	0	420	360	30	0	21	36	0
VII.		0	inh	0	246	0	-	0	24	81
VIII.		1280	336	990	372	70	25	49	37	56
IX.		0	126	inh	258	0	9	-	26	14
X.		760	inh	0	390	41	-	0	39	48
XI.		160	498	2920	129	9	37	144	13	29
	positive control	1840	1359	2030	1008	100	100	100	100	100

Note: 7-methylbenz[*c*]acridine **(I),** 8-methylbenz[*c*]acridine **(II),** 9-methylbenz[*c*]acridine **(III),** 10-methylbenz[*c*]acridine **(IV),** 11-methylbenz[*c*]acridine **(V),** 5, 7-dimethylbenz[*c*]acridine **(VI),** 7, 9-dimethylbenz[*c*]acridine **(VII),** 7, 10-dimethylbenz[*c*]acridine **(VIII),** 7, 11-dimethylbenz[*c*]acridine **(IX),** 7, 9, 10-trimethylbenz[*c*]acridine **(X),** 7,9,11-trimethylbenz[*c*]acridine **(XI)**. a: mutagenicity index = colony numbers of test chemical / colony numbers of positive control × 100. b: (37). Negative control values were substracted. inh represents growth inhibition.

Table 5. Mutagenicity of acridinones, thioacridines and thioacridinones (46).

	test chemicals	dose (μg/sample)	colony numbers TA97a	colony numbers TA100	mutagenicity index[a] TA97a	mutagenicity index[a] TA100
1		200	132	-	1.4	-
		20	296	88	3.1	2.8
2		200	23	41	0.2	1.3
		20	30	23	0.3	0.7
3		200	5	32	0.1	1.0
		20	0	26	0.0	0.8
4		200	-	-	-	-
		20	118	12	1.3	0.4
5		200	-	-	-	-
		20	122	19	1.3	0.6
6		200	85	7	0.9	0.2
		20	0	18	0.0	0.6
7		200	0	4	0.0	0.1
		20	0	16	0.0	0.5
8		200	3	5	0.0	0.2
		20	14	0	0.1	0.0
	positive control	-	94	31	1.0	1.0

Note: **1.** 9-(hydrazinothiazolo[5,4-a])acridinone **2.** 9-(1,4-dioxano[5,6-a])acridinone **3.** 2,3-dimethoxy-9-acridinone **4.** 2,7-dimethoxy-9-thioacridinone **5.** 2,7-dimethoxy-9-(3'-aminopropyl)thioacridine **6.** 2,7-dihydroxy-9-acetamidothioacridine **7.** 2,7-dihydroxy-9-(3'-aminopropyl)thioacridine **8.** 1,4,7,10,13,16,19-heptaoxanonadecyl-2,7-[(10-methyl)-9-thioacridinone] **a**: mutagenicity index = colony numbers of test chemical / colony numbers of positive control × 100. Negative control values were subtracted. 9-Aminoacridine (15μg/sample) was used as a positive control for TA97a strain; sodium azide (0.4μg/sample) for TA100 strain. -: inhibition due to toxicity.

trifluoromethyl-10*H*-phenothiazine and 10-[4-(phthalimido)butyl]-2-trifluoromethyl-10*H*-phenothiazine were not mutagenic on TA98 strain of *Salmonella typhimurium* (25).

3.1.7. 1-(2-Chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkyl-1-ureas

1-(2-Chloroethyl)-3-(10*H*-phenothiazin-10-yl)propyl-1-urea, 1-(2-chloroethyl)-3-(10*H*-phenothiazin-10-yl)butyl-1-urea, 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)propyl-1-urea, 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea, 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea and 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)butyl-1-urea did not show mutagenic activity on TA98 strain of *Salmonella typhimurium* (25).

3.2. Antimutagenicity

3.2.1. Antimutagenicity to spontaneous mutation

3.2.1.1. Phenothiazine, promethazine, promazine and chlorpromazine

None of the four phenothiazines: phenothiazine, promethazine, promazine and chlorpromazine showed antimutagenic activity in spontaneous mutation of TA98 strain of *Salmonella typhimurium*.

3.2.1.2. Phenothiazine-metal co-ordination complexes

None of the twelve phenothiazine-metal co-ordination complexes: phenothiazine, promethazine, promazine and chlorpromazine-copper, -palladium and -gold co-ordination complexes showed antimutagenicity in spontaneous mutation of TA98 strain of *Salmonella typhimurium*.

3.2.1.3. Benzo[*a*]phenothiazines

None of the seven benzo[*a*]phenothiazines: 12*H*-benzo[*a*]phenothiazine, 9-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 5-oxo-5*H*-benzo[*a*]phenothiazine and 6-hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine showed

antimutagenic activity in spontaneous mutation of TA98 and TA100 strain of *Salmonella typhimurium*.

3.2.2. Antimutagenicity to chemically induced mutation

3.2.2.1. Phenothiazine, promethazine, promazine and chlorpromazine

All four phenothiazines: phenothiazine, promethazine, promazine and chlorpromazine showed antimutagenic activity in 4-nitro-*o*-phenylenediamine-induced mutagenesis. Of the four phenothiazines, promethazine showed the highest percent inhibition at 33 percent, while promazine showed the lowest percent inhibition of 8 percent. Phenothiazine and chlorpromazine showed the percent inhibition at 12 percent and 22 percent, respectively (45).

3.2.2.2. Phenothiazine-metal co-ordination complexes

All the twelve phenothiazine-metal co-ordination complexes: phenothiazine, promethazine, promazine and chlorpromazine-copper, -palladium and -gold co-ordination complexes showed antimutagenicity against 4-nitro-*o*-phenylenediamine. All the three metals increased percent inhibition of their parent compounds by being complexed with phenothiazine and promazine; however, it was not always true for promethazine and chlorpromazine. All palladium complexes increased percent inhibition of its parent compounds; palladium co-ordination complexes showed the highest percent inhibition among all co-ordination complexes (45) (Figure 2).

3.2.2.3. Benzo[*a*]phenothiazines

All the seven benzo[*a*]phenothiazines: 12*H*-benzo[*a*]phenothiazine, 9-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 10-methyl-12*H*-benzo[*a*]phenothiazine, 5-oxo-5*H*-benzo[*a*]phenothiazine and 6-hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine showed antimutagenic activity against 4-nitro-*o*-phenylenediamine. 9-Methyl-12*H*-benzo[*a*]phenothiazine [3] was the most potent antimutagen of 30 percent inhibition, while 5-oxo-5*H*-benzo[*a*]phenothiazine [5] was the least potent antimutagen of 15 percent inhibition. 12*H*-Benzo[*a*]phenothiazine [2], 9-methyl-12*H*-benzo[*a*]phenothiazine [3], 6-

hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine [6] and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine [7] showed higher percent inhibition than chlorpromazine [1] (44) (Figure 3).

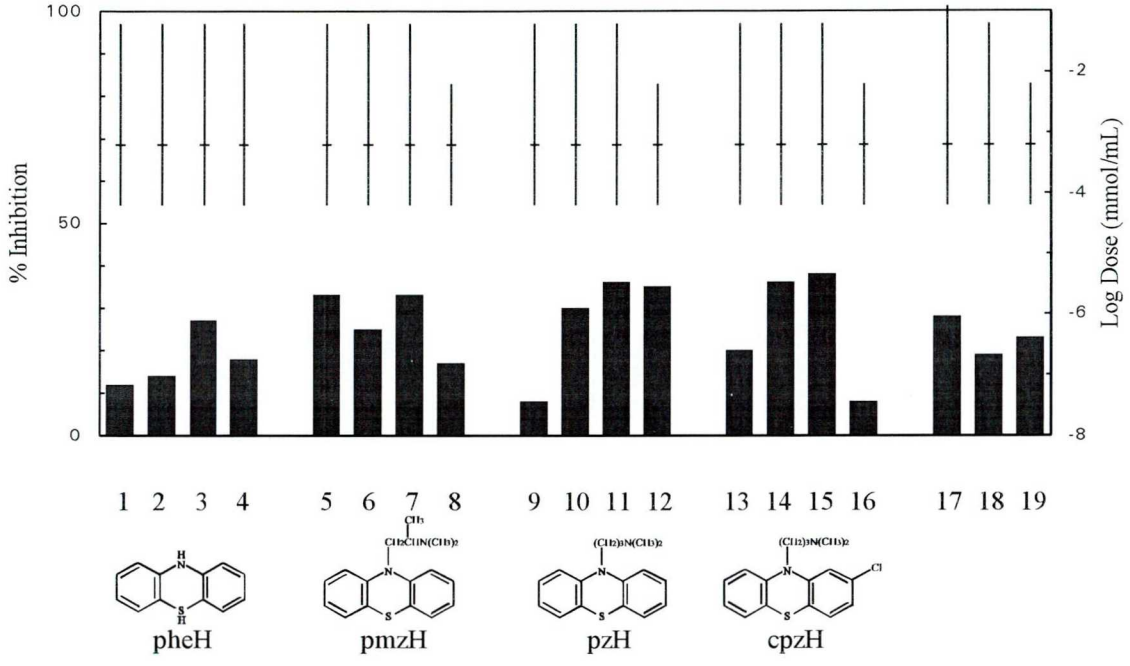


Figure 2. Antimutagenicity of phenothiazine-metal co-ordination complexes (45).
▪: antimutagencity. |: test chemical dose. –: mutagen dose. 1: $Cu(pheH)Cl_3$. 2: $Pd(pheH)Cl_3$. 3: $Pd(pheH)Cl_3$. 4: $Au_2(pheH)Cl_5$. 5: pmzH. 6: $Cu(pmzH)Cl_3$. 7: $Pd(pmzH)Cl_3$. 8: $Au_2(pmzH)Cl_5$. . 9: pzH. 10: $Cu(pzH)Cl_3$. 11: $Pd(pzH)Cl_3$. 12: $Au_2(pzH)Cl_5$. 13: cpzH. 14: $Cu(cpzH)Cl_3$. 15: $Pd(cpzH)Cl_3$. 16: $Au_2(cpzH)Cl_5$. 17: $CuCl_3$. 18: K_2PdCl_4. 19: $AuCl_3$. pheH: phenothiazine. pmzH: promethazine. pzH: promazine. cpzH: chlorpromazine.

3.3. Ligand-DNA binding assay by ultraviolet (UV) spectroscopy

In the ligand absorption region (190-400nm) the bathochromic and hypochromic shifts were observed in the spectrophotometric titration of promazine-palladium co-ordination complex $[Pd(pzH)Cl_3]$ with calf thymus DNA, suggesting the ligand bound to DNA. In titration experiments an isosbestic point was observed at 285 nm (Figure 4). The data supports that the antimutagenic action of the promazine-palladium co-ordination complex $[Pd(pzH)Cl_3]$ is attributed to its binding to DNA, blocking an electrophilic mutagen from nucleophilic sites of DNA (45) (Figure 4).

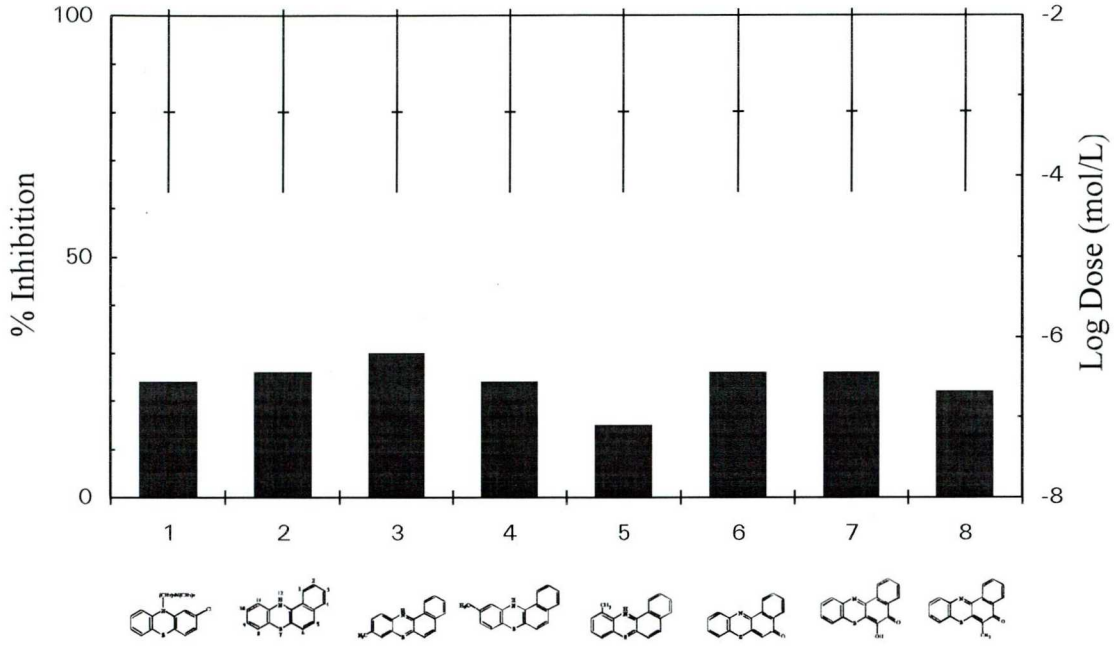


Figure 3. Antimutagenicity of benzo[*a*]phenothiazines (44).
▮: antimutagenicity. |: test chemical dose. –: mutagen dose. chlorpromazine [1], 12*H*-benzo[*a*]phenothiazine [2], 9-methyl-12*H*-benzo[*a*]phenothiazine [3], 10-methyl-12*H*-benzo[*a*]phenothiazine [4], 11-methyl-12*H*-benzo[*a*]phenothiazine [5], 5-oxo-5*H*-benzo[*a*]phenothiazine [6], 6-hydroxy-5-oxo-5*H*-benzo[*a*]phenothiazine [7] and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine [8].

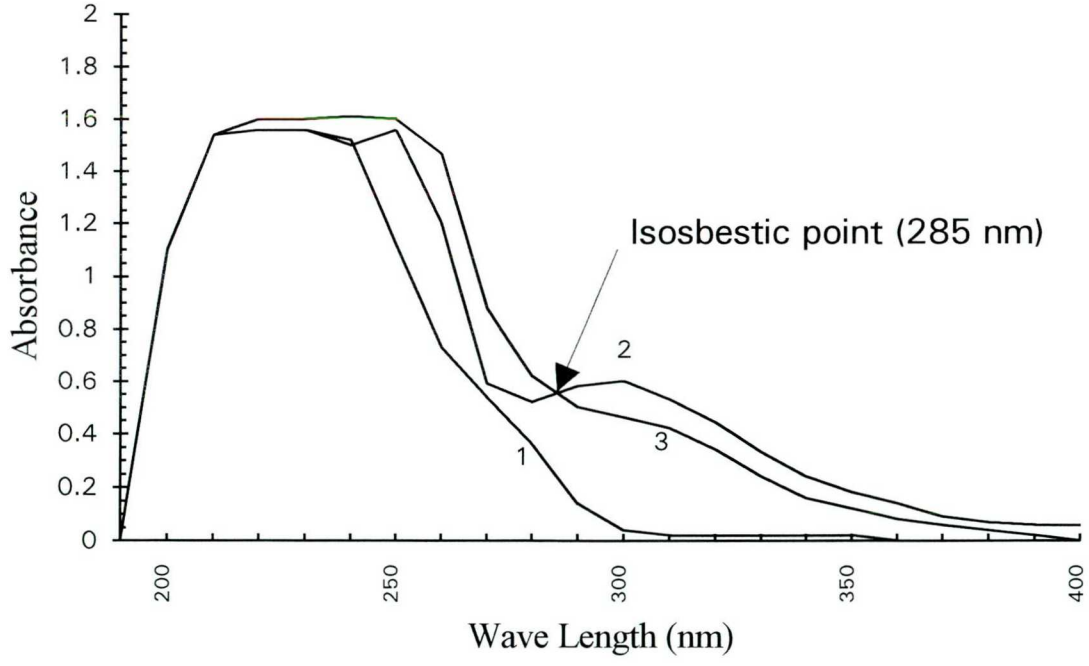


Figure 4. Ultraviolet (UV) spectra of calf thymus DNA (1), promazine-palladium co-ordination complex [Pd(pzH)Cl_3] (2) and [Pd(pzH)Cl_3]-DNA complex (3) (45).

4. Discussion

4.1. Mutagenicity of acridines

4.1.1. Methylbenz[*c*]acridines

4.1.1.1. An environmental mutagen and its methyl substitution

Aza-arenes (polycyclic aromatic nitrogen heterocyclics) are known to be environmental carcinogens formed by the incomplete combustion of organic material (48). Benz[*c*]acridines (B[*c*]ACRs) are polycyclic aza-aromatic compounds in which nitrogen is incorporated in the aromatic ring system, being detected in the various environmental samples such as tobacco smoke (49), airborne particles (50) and others (51). Benz[*c*]acridines have been known to be carcinogenic and their carcinogenicity is, furthermore, enhanced by the induction of methyl substituents on the benz[*c*]acridine (52). It is also known that only a few of the theoretically possible methylbenz[*c*]acridine isomers show carcinogenicity, which depends on the position of the methyl-substitution of benz[*c*]acridine (53).

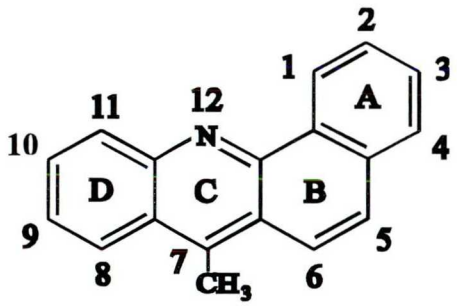


Figure 5. Structure and numbering system of 7-methylbenz[*c*]acridine.

4.1.1.2. Effects of methyl substitution on mutagenicity

Benz[*c*]acridines which induced strong mutation in TA97a, TA98, TA100 and TA102 strains of *Salmonella typhimurium* mostly possess a methyl substituent at C-7 position of benz[*c*]acridine. Interestingly, monomethyl, 7-methylbenz[*c*]acridine and 10-methylbenz[*c*]acridine did not show mutagenic activity on three tester strains except for TA102 and TA100, respectively. In the presence of methyl substituent(s) at C-9 and/or C-10 of benz[*c*]acridine, a C-7 methyl substituent suggests to play a key role in the direct

mutagenic effect of methylbenz[*c*]acridines to the *Salmonella typhimurium* tester strains. The carcinogenicity of benz[*c*]acidine and methylbenz[*c*]acridines shown by the epithelioma index depends on the location of methyl-substituent(s) on benz[*c*]acridine (52). The mutagenicity of 7, 10-methylbenz[*c*]acridine and 7, 9, 11-methylbenz[*c*]acridine on the *Salmonella typhimurium His*$^-$ strains correlates well with the carcinogenic activity on the epithelium. However, noncarcinogenic methylbenz[*c*]acridines such as 8-methylbenz[*c*]acridine, 9-methylbenz[*c*]acridine, 10-methylbenz[*c*]acridine, 11-methylbenz[*c*]acridine and 7, 9-methylbenz[*c*]acridine exhibited mutagenic activity in the assay (Table 4, Figure 6).

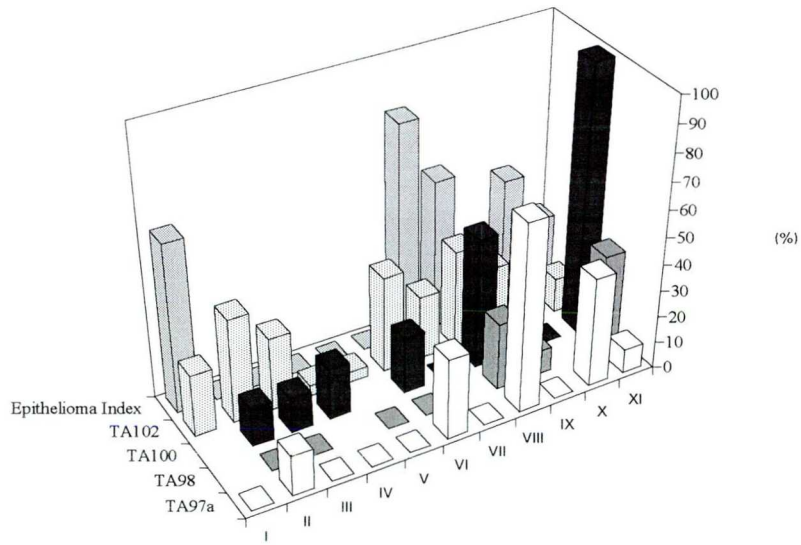


Figure 6. Mutagenicity index and epithelioma index of benz[*c*]acridines.
7-methylbenz[*c*]acridine **(I),** 8-methylbenz[*c*]acridine **(II),** 9-methylbenz[*c*]acridine **(III),** 10-methylbenz[*c*]acridine **(IV),** 11-methylbenz[*c*]acridine **(V),** 5, 7-dimethylbenz[*c*]acridine **(VI),** 7, 9-dimethylbenz[*c*]acridine **(VII),** 7, 10-dimethylbenz[*c*]acridine **(VIII),** 7, 11-dimethylbenz[*c*]acridine **(IX),** 7, 9, 10-trimethylbenz[*c*]acridine **(X),** 7,9,11-trimethylbenz[*c*]acridine **(XI)**.

4.1.1.3. Carcinogenicity of methylbenz[*c*]acridines: physicochemical approaches

The mechanism of chemical carcinogenesis of benz[*c*]acridines has been attempted to elucidate by various approaches and the mechanism of the enhanced carcinogenicity of benz[*c*]acridines by C-7 methyl substitution has been speculated (54). The interaction of benz[*c*]acridines with DNA was studied by various spectroscopic methods. Ultraviolet (UV) spectroscopy showed that the double helical conformation of DNA appeared to be important to the interaction (55). The fluorescence spectra and Raman spectra of DNA-benz[*c*]acridine and DNA-methylbenz[*c*]acridine complex showed that there are two

different binding sites: one at the base pair and the other at the sugar-phosphate of DNA (56, 57). Meanwhile, free radical formation of carcinogenic and non-carcinogenic benz[*c*]acridines was compared in the presence of proteins, showing that electron spin resonance (ESR) signals of carcinogenic benz[*c*]acridines were higher in intensity than those of noncarcinogenic ones (58). This study, furthermore, suggested that the carcinogenicity of benz[*c*]acridines correlates with the charge of the K-region.

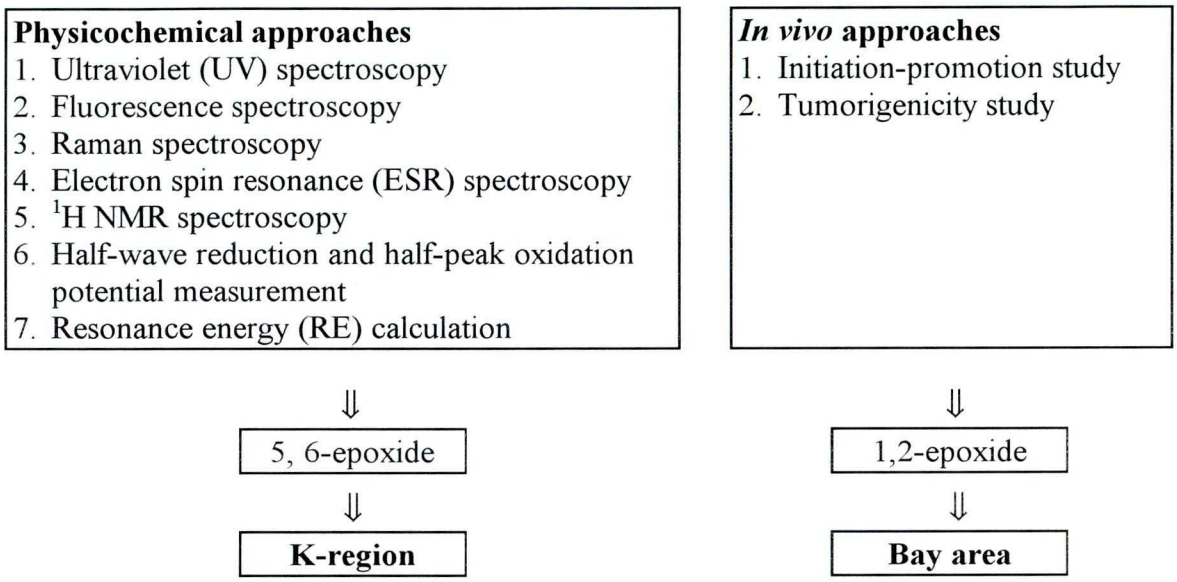


Figure 7. Carcinogenicity of benz[*c*]acridines

Physicochemical approaches to the mechanism of chemical carcinogenesis of benz[*c*]acridines are in favor of the hypothesis that the K-region plays a crucial role in carcinogenesis of benz[*c*]acridines. The ^{1}H NMR spectra showed that the K-region protons correspond to the epithelioma index of benz[*c*]acridines (59). Half-wave reduction and half-peak oxidation potential measurement indicated that the electrophilic reactivity of the K-region and the ring nitrogen atom of methylbenz[*c*]acridines were important to the carcinogenic activity (60). The charge in the K-region was correlated with the rate constant of the second-order reaction with osmium tetroxide, and the relationship among the K-region reactivity, mutagenicity and carcinogenicity, supported 5, 6-epoxide as an ultimate carcinogenic metabolite in which the nucleophilic property of the K-region react with electrophilic regions of DNA (61). Furthermore, the resonance energy (RE) of carcinogenic methylbenz[*c*]acridines was calculated to be significantly lower per π-electron than that of noncarcinogenic counterparts (62). The carcinogenic activity of benz[*c*]acridines was also related to the charge of K-region (QK), nitogen atom (QN) and

L-region (QL) and high electron density on π-electron highest occupied molecular orbital (π-HOMO) by resonance energies (RE), circuit resonance energies (CRE) and bond currents (BC) (63). Mutagenic and carcinogenic benz[*c*]acridines showed the out-of-phase in the L-region and their energy was accumulated in the K-region of the molecular orbitals (64) (Figure 7, 8).

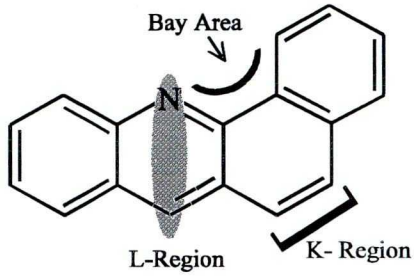


Figure 8. Proposed area and regions responsible for carcinogenicity of benz[*c*]acridine

4.1.1.4. Carcinogenesis of methylbenz[*c*]acridines: *in vivo* experiments

On the other hand, the initiation-promotion experiments on mouse skin and tumorigenicity study in newborn mouse of benz[*c*]acridine metabolites stand in favor of the hypothesis that the bay-region diol-epoxide plays an ultimate role in chemical carcinogenesis of benz[*c*]acridines. *Trans*-3,4-dihydroxy-3,4-dihydrobenz[*c*]acridine was shown to have significant tumor-initiating activity (65) and tumorigenic activity (66); of two diastereomeric isomers of bay-region diol-epoxides to the benzylic hydroxyl group, trans isomer of 3,4-diol-1,2-epoxide-benz[*c*]acridine was as active as *Trans*-3,4-dihydroxy-3,4-dihydro-benz[*c*]acridine (65, 66). However non-bay-region diol-epoxides including K-region were inactive (65). Furthermore, the tumor-initiating activity and tumorigenicity of 7-methylbenz[*c*]acridine and its metabolites were studied. 3,4-Dihydroxy-3,4-dihydro-7-methylbenz[*c*]acridine, the metabolic precursor of a bay-region-diol-epoxide was more active than 7-methylbenz[*c*]acridine and addition of a methyl substituent at C-7 of benz[*c*]acridine resulted in significant increase in the tumor-initiating activity (67) (Figure 7, 8).

4.1.1.5. Mutagenicity/carcinogenicity discrepancy

Ames' *Salmonella* mutagenicity assay has been widely applied to detect chemical mutagens, having once claimed some 90 percent identification rate of chemical

carcinogens. The mutagenicity of 7, 10-methylbenz[*c*]acridine and 7, 9, 11-methylbenz[*c*]acridine on the *Salmonella typhimurium His*⁻ strains correlates well with the tumorigenic activity on the epithelium. However, some of the non-carcinogenic methylbenz[*c*]acridines exhibited mutagenic activity in the assay such as noncarcinogenic methylbenz[*c*]acridines including 8-methylbenz[*c*]acridine, 9-methylbenz[*c*]acridine, 10-methylbenz[*c*]acridine, 11-methylbenz[*c*]acridine and 7, 9-methylbenz[*c*]acridine exhibited mutagenic activity in the assay. Regarding the chemical carcinogenesis of benz[*c*]acridines, furthermore, physicochemical and biological experimental approaches have presented contradictory hypotheses: the K-region versus the bay-region. Further study is expected to elucidate the mechanism of carcinogen-DNA interaction, chemical mutagenesis and carcinogenesis.

4.1.2. Antitrypanosomal acridines

4.1.2.1. Mutagenicity of antitrypanosomal acridines

Newly synthesized antitrypanosomal and antiplasmid acridinones, thio-substituted acridines and thioacridinones were tested for a direct genotoxic effect on TA97a and TA100 tester strains by Ames' *Salmonella* mutagenicity assay. Of acridinones, only 9-(hydrazinothiazolo[5,4-a])acridinone showed strong mutagenic activity on TA97a strain. However, mutagenic activity was manifested invariably by all three acridinones on TA100 strain. Of thioacridines, 2,7-dimethoxy-9-thioacridinone, 2,7-dimethoxy-9-(3'-aminopropyl) and 2,7-dihydroxy-9-acetamidothioacridine were explicitly mutagenic on TA97a, but on TA100 they were just weakly mutagenic (Table 5). The data improves the drug design against protozoas by establishing relationships between the types and sites of substitution and the mutagenic properties of acridines. Thereby, harmful mutagenic acridine derivatives should be excluded from further *in vivo* experiments and the data of the substitution-mutagenicity relationships further facilitate the rational drug design (46) (Figure 9).

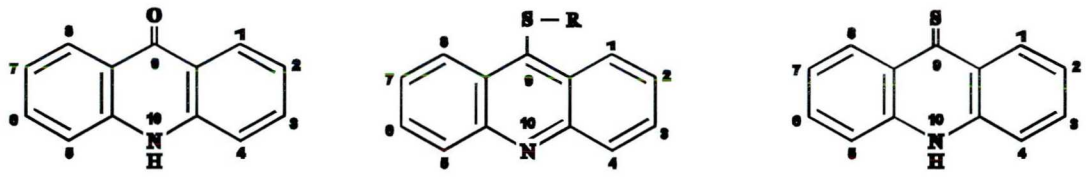
4.1.3. Biological activities and mutagenicity of acridines

Acridine compounds are known to have a broad spectrum of biologic activities such as antibacterial (68, 69), antiparasitic (70-73), mutagenic (75) and antitumor ones (76-79). The effects of acridines on bacteria were studied for their antibacterial and antiplasmid

actions. Their activities on bacteria seem to depend on the substitution on the tricyclic skeleton (68). Acridinones have been proved to be trypanocidal by being capable of intercalation into the DNA of the *Trypanosoma cruzi* (71, 73). The trypanocidal activity was also shown against the *Trypanosoma brucei* strains with very low concentration of substituted acridines with simultaneous brief irradiation (74) and without irradiation (80).

The antitumor activity of acridine derivatives attributes to their ability of intercalation to DNA, stabilizing the DNA-topoisomerase II intermediate complex. This cleavable complex appears to be toxic to cancerous cells (76). The DNA-topoisomerase II inhibiting acridines are primarily chromosomal mutagens in mammalian cells, although they are frameshift mutagens in bacterial and bacteriophage systems (75).

The mutagenicity of various acridine compounds have been studied by Ames' *Salmonella* mutagenicity assay. Acridines are planar polycyclic aromatic molecules which bind noncovalently and reversibly to DNA by intercalation. Simple acridines are frameshift mutagens. However, benzacridines are mainly base-pair substitution mutagens by interacting covalently with DNA following microsomal activation (61). Acridine mustards such as the ICR compounds, acting as carriers to target alkylating agents to DNA are frameshift as well as base-pair substitution mutants. Nitroacridines may act as either simple acridines or alkylating agents, depending on the position of the nitro group (75).



(1) (2) (3)

Figure 9. Molecular formulas and numbering system of acridinone (1), thioacridine (2) and thioacridinone (3).

4.1.4. Acridines as potential therapeutic agents

The biological activities of some newly synthesized acridine compounds have been shown by *in vitro* studies. Thioacridine derivatives are cytotoxic against K562 and Raji cell lines, and also inhibitory to nucleic acid synthesis in K562 (76). Anti-amebic activity of thioacridanones was also found to be against *Naegleria* and *Acanthamoeba* species (73).

Furthermore, acridines bearing aromatic rings inhibited the mitogen-induced blast transformation of human-peripheral lymphocytes, showing their immunomodulatory activity, possibly by acridine-mitogen complex formation (81). A correlation between the biological activity of tricyclic compounds such as benz[*c*]acridines and some molecular orbital parameters was suggested by quantum mechanical analysis (64). But the fourth ring attached to acridines does not favor the antiplasmid action. Antiplasmid and mutagenic compounds appear to have basically different molecular orbitals responsible for their actions to DNA analysis (64).

The effective dose against *Trypanosoma* was shown to be very low in the range of 0.1 μM (74) and the minimal inhibitory concentration (MIC) was determined on Escherichia coli to be some 200-280 mg/ml (82), indicating a possibly large therapeutic window. At near MIC values, however, some acridinones and thioacridines tested in this experiment were capable to induce both frameshift and base-pair substitution mutation; the others caused either of them or even none. The nature of mutagenicity of the related acridinones and thioacridines appears to depend mostly on the substitution on the tricyclic rings. The characterization of biological activities of acridine derivatives are important because of their considerable potential for antibacterial, antiprotozoal, immunoregulatory and even antitumor drugs.

4.2. Mutagenicity of phenothiazines

Phenothiazine and its derivatives generally do not covalently bind to DNA and considered not to be mutagenic in spite of their structural similarity to acridines which are known as moderate to weak mutagens. However, genotoxic effects of pharmaceutical phenothiazines have been recognized and a more careful use of the drugs was suggested (15). Chlorinated phenothiazines were found to be frameshift mutagens in the presence of ultraviolet (UV) light in *Salmonella typhimurium* (16). Thioridazine was observed as a clastogen to human chromosomes in lymphocyte cultures sampled from the peripheral blood of psychiatric patients on therapeutic doses of thioridazine (15). Furthermore, electron spin resonance (ESR) has shown that chlorpromazine cation radical interacts with DNA, but the observation was considered to support its antimutagenic effect by blocking the covalent intercalation of mutagenic benzo[*a*]pyrene metabolites with DNA-base pairs (83).

Mutagenicity of a parent compound, phenothiazine and three therapeutic derivatives, promethazine, promazine and chlorpromazine was studied in *Salmonella typhimurium* strains, TA98 and TA100. All four compounds failed to revert the *His*$^-$ strains, suggesting nonmutagenic ones (84). Mutagenicity of dioxochlorpromazines, 6, 9-dioxochlorpromazine and 7, 8-dioxochlorpromazine and 3, 7, 8-trihydroxychlorpromazine was also examined, all of which were found not to be mutagenic in the non-toxic range (85).

Mutagenicity of benzo[*a*]phenothiazine and its derivatives with methyl-, hydroxy- and/or oxo-substituent(s) was screened on TA98 and TA100 strains of *Salmonella typhimurium*. No mutagenic activity was recognized (86) in spite of their structural similarity to benz[*c*]acridines, which are strong to weak mutagens and whose mutagenicity depends on the methyl substitution(s) (37). The result further suggests that regardless of a side chain(s), the incorporation of sulfur atom at the *ortho*-position to nitrogen atom in heterocyclic hydrocarbons plays a crucial role in nullifying mutagenic activity not only of acridines and but also benz[*c*]acridines.

Six 10-[n-(Phthalimido)alkyl]-2-substituted-10*H*-phenothiazines and six 1-(2-chloroethyl)-3-(2-substituted-10*H*-phenothiazin-10-yl)alkyl-1-ureas were synthesized in attempt to increase the penetrating property of two strong alkylating agents into the blood brain barrier (BBB) (87). Those compounds showed no mutagenic activity on TA98 strain of *Salmonella typhimurium* (25), suggesting that the tricyclic skeleton of phenothiazine alters the alkylating property of the side chain.

Metals are known to have mutagenic activity by directly acting to DNA in change of the redox state and by acting to DNA repair proteins (88). Phenothiazine-metal co-ordination complexes, phenothiazine, promethazine, promazine and chlorpromazine co-ordinately complexed either with copper, palladium or gold, did not show mutagenic activity in TA98 strain of Salmonella typhimurium. Interestingly, metal co-ordination complexes increased antimutagenic activity of phenothiazines and ultraviolet (UV) spectroscopy showed enhanced interaction to DNA (45).

Mutagenicity of phenothiazines is expected to be further investigated, especially in compound situation with physical agents such as ultraviolet (UV) light and other ubiquitous environmental chemicals. It is of particular interest in rational drug design to significantly decrease mutagenicity of heterocyclic hydrocarbons by the incorporation of sulfur atom to the tricyclic skeleton and to the substitution of phenothiazine to mutagenic compounds in chemical synthesis.

4.3. Targets of mutagens

Most chemical mutagens or their metabolites are electrophilic and covalently bind to cellular macromolecules such as proteins, RNA and DNA. The four classes of molecules have been recognized for high-fidelity DNA synthesis: the DNA template, dNTPs and their precursors, DNA replication proteins and DNA repair proteins (88) (Table 6).

4.3.1. DNA as a target: DNA-adduct formation

The DNA template is obviously a critical target for mutagens (89). DNA damaged by ultraviolet (UV) irradiation yields predominantly transition mutations at thymine residues (90), while acetyl aminofluorene (AAF)-damaged DNA yields small frameshift mutations (91). In order for a damaged base to induce a mutation it must affect the fidelity of DNA synthesis. Mutations occurring at the site of damage presumably arise as a result of either ambiguous base-pairing (89) or error-prone repair of the damaged base (92). Probably the best characterized promutagenic DNA adducts are those formed by simple alkylating mutagens such as the dialkyl sulfates, sulfonates and nitrosamines. It has been shown that O^6-alkylguanine and O^4-alkylthymine adducts exhibit ambiguous base-pairing properties and characteristically induce G:C $\rightarrow$ A:T and A:T $\rightarrow$ G:C transition mutations, respectively (93, 94).

4.3.2. Nucleotide pools

Besides direct damage to the DNA template, mutagens attack to cellular deoxyribonucleoside triphosphates (dNTPs) which upon the DNA polymerization are incorporated into the nascent DNA. Being more abundant in nucleophilic sites than double stranded DNA, unincorporated nucleotides, nucleosides and bases are better targets for mutagens (89). An alkylating agent, *N*-methyl-*N*-nitrosourea (MNU) was observed to produce more deoxynucleotide adducts in the uncorporated nucleotide pool than in DNA (95). One mechanism in which modified dNTPs might induce mutation, is via ambigous base-pairing during or after incorporation into DNA (96). The efficiency of incorporation varies among the different adducts studied and appears to be dependent on both the size of the adducted side group as well as the position of modification on the dNTP molecule (97).

Table 6. Targets of mutagens (88)

Targets	Mutagens
1. Electrophilic sites of DNA base-pairs	ultraviolet light (UV), aminofluorene, O^6-alkylguanine, O^4-alkylthmine
2. Nucleotide pools	
a. dNTPs or precursors	*N*-methyl-*N*-nitrosourea (MNU), ultraviolet light (UV), 2-aminopurine, 6-amino-2-hydroxypurine, 5-bromodeoxyuridine, mitomycin *C*, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), ethyl nitrosourea (ENU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)
b. dNTP balance	ultraviolet light (UV), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), mitomycin *C*, cytosine arabinoside, bromouridine
i) Nucleotide metabolizing enzyme inhibitors	fluorodeoxyuridine, antifolates aminopterin, methotrexate, hydroxyurea, 8-aminoguanosine, 8-amino-9-benzylguanine, azaserine, 6-diazo-5-oxonorleucine
3. DNA replication proteins	γ-rays, *N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), metals (Mg, Co, Ag, Be, Cd, Cr, Ni, Pb, Cu)
4. DNA repair proteins	methanesulfonate (MMS), dimethyl-methanesulfonate (DMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), reactive oxygen species

It is well established that perturbations of dNTP pools elicit a broad range of genetic changes, including mutations, recombination, chromosome loss, chromosome aberrations and breakage, sister chromatid exchange and DNA strand breaks (98). Mutations by dNTP pool imbalances are thought to occur via a decrease in the fidelity of DNA synthesis (99). DNA sequence analyses of mutations induced by nucleotide pool imbalances in cells in culture strongly support this kinetic mechanism of mutagenesis (100). Disruptions of nucleotide pool ratios also produce a next nucleotide effect on mutagenesis that appears to be due to a decrease in the efficiency of mispair excision by proofreading 3' → 5' exonucleases that are associated with some DNA polymerases (101). Thus dNTP imbalances can affect polymerase fidelity in two ways: by increasing the rate of misincorporation and by decreasing proofreading efficiency.

4.3.3. Mutagen-protein interaction: DNA polymerase and DNA repair proteins

The accuracy of DNA synthesis is ultimately determined by the proteins at the replication fork. Central to this process is the DNA polymerase itself, which plays a critical role in reading the template DNA and in selecting correct dNTPs for incorporation (99). Thermodynamic measurements indicate that the hydrogen bond energies of G:C and A:T base-pairs are only sufficient to direct DNA synthesis with an accuracy of about 1 error per 100 nucleotides polymerized (102). Yet DNA synthesis *in vitro* with purified DNA polymerase is highly accurate showing error rates as low as 1 in 10^4 to 1 in 10^5 nucleotides copied in the absence of exonucleolytic proofreading (100). Thus DNA polymerases contribute at least 100-fold to the fidelity of DNA synthesis. In the presence of proofreading 3' $\rightarrow$ 5' exonucleases, this accuracy is increased by another 10- to 100-fold to achieve an error rate of 10^{-6} to 10^{-7} for certain polymerases (103). Single-strand DNA binding proteins also increase the fidelity of DNA synthesis (104) and processivity factors, such as proliferating cell nuclear antigen (PCNA) are likely to affect the frequency of certain types of base substitution mutations (105). Other proteins essential to DNA replication include primase, Rnase H, ligase, helicase, topoisomerase, gyrase, and initiation factors (106). Since these enzymes are closely associated with the processes of DNA replication and repair, damage to any of these could cause significant aberrations in DNA structure and result in mutations.

The major pathways of DNA repair fall into two general categories. The first involves the repair of DNA damage and can act either before or after DNA synthesis. The second setoff pathways act postreplicationally to repair nucleotide mismatch errors made by DNA polymerases. The importance of these repair systems is clearly demonstrated by genetic studies which show that removal of the functional repair proteins results in a mutator phenotype (107). It is reasonable, therefore, that a mutagen could similarly affect mutagenesis by damaging one or more of these repair proteins.

4.4. Mutagens leave fingerprints

Mutagens are recognized to leave fingerprints in DNA, whether the damage is a result of endogenous or exogenous insult (108). Distinct mutational spectra for different mutagens in the *lacI* gene of *Escherichia coli* were demonstrated (109). The S_N1 (substitution, nucleophilic, unimolecular) alkylating agent *N*-methyl-*N'*-nitrosoguanidine

(MNNG) produced almost exclusively G:C → A:T transitions with a preference for target sites preceded by a guanine. In contrast, the S_N2 (substitution, nucleophilic, bimolecular) alkylating agent ethylmethanesulphonate (EMS) produced a slightly broader spectrum of base substitutions although still predominately G:C → A:T transitions (110). G:C → A:T transitions induced by both alkylating agents are thought to be medicated by the miscoding properties of the O^6-alkylguanine adduct (111). G:C → A:T transitions also predominate in the spectrum of ultraviolet (UV)-induced mutation in the *lacI* gene (112), but these occur almost exclusively at cytosine-containing dipyrimidine sites as would be expected if cyclobutane pyrimidine dimers (CPD) or pyrimidine-pyrimidone lesions were reponsible for the mutational spectra of ultraviolet (UV) and ethylmethanesulphonate (EMS). Furthermore, mutational spectra obtained in bacteria are quite similar to those recovered in mammalian cells.

4.5. New models for mutagenicity assay

4.5.1. *In vivo LacI/LacZ* transgenic rodent mutagenicity assay

The Big Blue transgenic rodent mutagenesis assay is a standardized tissue-specific, *in vivo*, whole-animal mutagenesis assay system. Mutations are identified in any tissue containing DNA, including germ cells. Transgenic rodents carry a lambda phage shuttle vector suitable for *in vivo* mutagenesis test and genetic toxicology studies (113, 114). Genetically identical C57BL/6 transgenic mice, hybrid $B6C3F_1$ transgenic mice or new Fischer 344 transgenic rats are chosen. The extensively studied mutable target gene, *LacI*, is highly sensitive to a variety of mutation endopoints, and is easily excised and sequenced. Transpack packaging extract for *in vitro* recovery of transgenic *LacI* target is used. Mutation detection and the characterization of mutations by sequence analysis are easily made.

4.6. Mutagenicity assays in the future: gene-specific, gene-functional mutagenicity assays

Development of gene-specific, gene-functional mutagenicity assays of human tissue cell lines for *in vitro* and transgenic mouse for *in vivo* study are expected. The assays are to be specific for various oncogenes and tumor suppressor genes whose pathological mutations are implicated in carcinogenesis and also applicable to diseases of mutation in relevance of environmental and nutritional factors.

Popular mutagenicity assays such as the Ames' test and the Big Blue mutagenesis assay have been widely accepted for standard chemical mutagenicity tests. Taking a high proud of its cost-effectiveness, little sacrifice of animals and the application of S9 microsomal fraction to the system, the Ames' test nevertheless has a flaw as a prokaryotic model which is too different to simulate the physiology of humans (30). Big Blue mutagenesis assay, on the other hand, is an innovative rodent model, simulating *in vivo* physiology of the living mammals (113, 114). Big Blue mutagenesis assay still identifies only 50 percent of known carcinogens. Furthermore, both assays employ irrelevant gene targets to human DNA sequence which are known to assume particular secondary structures depending on the base composition of DNA and intracellular environment (115).

Mutagens are known to have preferential target sites in DNA sequence, leaving fingerprints in mutated DNA (116). Pathological mutation takes place in the relatively limited regions of a gene (109). Silent mutations in genes allow the normal biochemical functions of their translated protein products, but the traditional assays detect all mutations of their target sites, overexpressing the mutagenicity of test chemicals. This may also lead to misconcept that even beneficial mutation is regarded as harmful. All the observations now necessitate the development of a new experimental model for a specific and functional gene which simulates more realistic human physiology. A gene-specific, gene-functional mutagenicity assay not only analyzes chemical mutagenesis of human genome more precisely, but also leads to the identification of ultimate mutagens, to the understanding of mechanisms of genotoxic activity and to the comparative analysis of mutagenesis and carcinogenesis.

4.7. Antimutagens and antimutagenicity

Antimutagens are defined as chemical and physical agents which reduce the occurrence of mutations either by directly or indirectly inhibiting action of mutagens. Antimutagens are further devided into bioantimutagens and desmutagens. Bioantimutagens are also called true mutagens, defined as inhibitors of mutation by DNA replication/repair intracellularly. Bioantimutagens reduce mutation by increasing in DNA replication fidelity, enhancing the DNA repair mechanism and inhibiting the error-prone repair pathway. Desmutagens are also called apparent mutagens, defined as inhibitors of mutation by reacting to promutagens/mutagens and/or to enzymes intracellularly or extracellularly. Intracellular desmutagens reduce mutation by modulating metabolic enzymes and blocking

Table 7. Classification of antimutagens (117, 118, 119)

Class	Mechanism	Antimutagen
I. Bioantimutagens	Modulators of DNA replication/repair	
	a) increase in DNA replication fidelity	cobaltous chloride, sodium arsenite
	b) enhance DNA repair	cinnamalde-hyde, coumarin, umbellifero-ne, vanillin, thiols
	c) inhibit error-prone repair pathways	protease inhibitors
II. Desmutagens		
i) Intracellular	A) Metabolism modulators	retinoids, thiols, cruciferous plants
	B) Reactive molecule blockers	
	a) electrophile reaction	sulfur compounds
	b) reactive oxygen scavenging	antioxidants
	c) blocking the nucleophilic sites of DNA	Ellagic acids, retinoids
ii) Extracellular	A) Mutagen promutagen uptake inhibitors	fatty acids, putrescin, aromatic acids, iodide, fibers
	B) Endogenous mutagen formation inhibitors	ascorbic acid, tocopherols, phenols, fermented dairy products
	C) Mutagen inactivators	physical pH, antioxidants, peroxidase

reactive molecules. This blocking mechanism takes place by electrophilic reaction, reactive oxygen scavenging and protecting the nucleophilic sites of DNA. Extracellularly, desmutagens act as mutagen/promutagen uptake inhibitors, endogenous mutagen formation inhibitors and mutagen inactivators (117-119) (Table 7).

4.7.1. Phenothiazines: their biological activities and antimutagenicity

Phenothiazines are the heterocyclic aromatic hydrocarbons in which nitrogen and sulfur atoms are incorporated in the tricyclic system and are the drugs of choice widely employed in the therapeutic regimen of psychotic disorders including mania, paranoia, schizophrenia and alcoholic hallucinosis, drug-induced nausea, allergy and even intractable hiccup (1). In addition, newly synthesized phenothiazines with side chains of various functional groups have shown many other biological activities such as antiviral, antibacterial, antiparasitic and antitumor ones (2-9), expected to be a new therapeutic choice of other diseases. Furthermore phenothiazines have been shown to have antimutagenic activities presumably by inactivating cytochrome P_{450} and shielding the nucleophilic sites of DNA.

4.7.1.1. Antimutagenesis of phenothiazines

Phenothiazines have been known to reduce spontaneous mutation (11) and chemically induced mutation (12). However, no exact antimutagenic mechanism of phenothiazines against chemically induced mutation is known. Two hypotheses were proposed. Phenothiazines were observed to be metabolized by aromatic hydrocarbon hydroxylase system, cytochrome P_{450} enzymes, competitively inhibiting the activation of mutagen precursors (120, 121). The hypothesis was supported by the observation of a quantitative decrease in benzo[*a*]pyrene metabolites after *in vivo* metabolism of benzo[*a*]pyrene with and without phenothiazine by high performance liquid chromatography (HPLC) analysis (122). The second hypothesis holds that phenothiazines block the covalent intercalation of mutagenic benzo[*a*]pyrene metabolites with DNA-base pairs, supported by electron spin resonance (ESR) analysis which showed the interaction of the chlorpromazine cation radical with DNA (83). However, phenothiazines have not yet shown to bind covalently to DNA with the exception of chlorinated phenothiazines in the presence of ultraviolet (UV) light, which function as frameshift mutagens (16).

Antimutagenicity of phenothiazines was examined against model mutagens such as aflatoxin B_1 and benzo[*a*]pyrene. In aflatoxin B_1-induced mutagenesis, phenothiazines reduced their mutagenic activity by 92 percent in covalent DNA binding assay (123). Against benzo[*a*]pyrene phenothiazine inhibited its mutagenic activity by 75 percent in TA98 strain of Ames' *Salmonella* mutagenicity assay (18, 19). Both aflatoxin B_1 and benzo[*a*]pyrene require metabolic enzymes, the cytochrome P_{450} to be the ultimate electrophilic mutagens, and primary antimutagenic action of phenothiazines is considered to be an antioxidant which inhibit metabolite mutants either by competitive inhibition or by selective changes in the activation mechanism: change in the ratio of primary to secondary metabolism, for example. Thus phenothiazines were studied as desmutagens. On the other hand, an antimutagen is most likely to have multiple target sites and multiple inhibitory mechanisms to mutagens, being influenced by many parameters in the living cells.

4.7.1.2. Antimutagenicity of phenothiazines against a direct-acting mutagen

Antimutagenic and mutagenic agents are known to generally have multiple inhibition and activation sites in the mutational cascades, rendering the exact mechanism of action ambiguous. Antimutagenicity of phenothiazines was studied against benzo[*a*]pyrene and aflatoxin B_1 (27, 18, 19), both of which require microsomal liver enzymes for their activation (123). Furthermore, their antimutagenicity against a direct-acting mutagen to DNA, 4-nitro-*o*-phenylenediamine, which does not require the enzymatic activation of the cytochrome P_{450}, was examined in TA98 strain of *Salmonella typhimurium*. All four phenothiazines: phenothiazine, promethazine, promazine and chlorpromazine showed antimutagenic activity in 4-nitro-*o*-phenylenediamine-induced mutagenesis; promethazine being the most potent (33 percent inhibition), while promazine being the least potent (8 percent) (45). The experimental design further informed on the action site of phenothiazines as bioantimutagens and/or direct DNA blocking agents in living cells.

4.7.1.3. Antimutagenesis of metals

Metal salts have been applied in the past and present time to the treatment of diseases: mercury against syphilis, gold against rheumatoid arthritis and magnesium and calcium against peptic ulcer, for example (1). Cisplatinum, an inorganic co-ordination complex, is one of the most potent anticancer drugs against testicular, ovarian and breast

cancer, whose cytotoxic effects attribute to covalent DNA adduct formation which is not repairable by DNA polymerase (124-126). Recently, due to cisplatinum-resistance tumors, the cytotoxic effects of not only inorganic, but also organic platinum co-ordination complexes are intensively studied, some of which are already under phase II clinical trial (127). On the other hand, cobaltous chloride was shown to have antimutagenic activity against benzo[*a*]pyrene-induced mutation, presumably by increasing the fidelity of DNA repair (117).

Many other inorganic metals such as arsenic, cobalt, copper, iron, molybdenum, selenium and zinc, have also been shown to have antimutagenic activity in various experimental models (128). Cobalt chloride (129), sodium arsenite (130), sodium selenite (131, 132) showed antimutagenicity against *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) in *Escherichia coli* WP2, *Escherichia coli* WP2 uvrA and chromosomal aberrations of Chinese hamster cells *in vitro*. Cobalt chloride reduced its mutagenicity by 99 percent. In aflatoxin B_1-induced mutagenicity, ammonium molybdate, cobalt sulfate, cupric sulfate, ferric chloride, ferrous sulfate, manganese chloride, sodium selenite and zinc sulfate were studied in *Salmonella* mutagenicity assay and covalent DNA binding assay. Cupric sulfate (100 percent inhibition) was the most active (100 percent inhibition) followed by manganous chloride (83 percent) and ferrous sulfate (72 percent inhibition) (133, 134). Interestingly, the inhibition of aflatoxin B_1-induced hepatocarcinogenesis by the metallic salts has not been reported (133).

Against benzo[*a*]pyrene mutagenicity, sodium selenite was studied in *Salmonella* mutagenicity assay, reducing its mutagenicity by 61 percent (12, 132, 135). In addition sodium selenite was shown to inhibit the mutagenicity of both direct-acting mutagen, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and *N*-acetoxy-acetylaminofluorene (*N*-acetoxy-AAF) and metabolically activated carcinogens (132, 136, 137).

Against 4-nitro-*o*-phenylenediamine, a direct-acting mutagen, copper, palladium and gold showed antimutagenic activity of 26 percent, 19 percent and 22 percent, respectively (45).

Furthermore, the mechanism of antimutagenesis of metallic salts has been speculated. Selenium appears to have multiple inhibition mechanism in addition to the prevention of oxygen radical-induced peroxidation as a constituent of glutathion (GSH) peroxidase (138). Cobaltous chloride appears to inhibit radiation- and chemical-induced mutations in bacteria by increasing DNA replication fidelity and by enhancing

recombination repair (117). Arsenite inhibits the *umu*C gene expression, enhancing error-free repair in bacteria (118).

4.7.1.4. Antimutagenicity of phenothiazine-metal co-ordination complexes

Metals are known to have specific affinity to the secondary and the double helical structures of DNA. Organometalics are of great interest in their interaction to DNA and its conseuence as biochemical modifiers. Antimutagenicity of phenothiazine-metal co-ordination complexes was studied to investigate the effect of the metals on DNA-binding properties of phenothiazines against a direct-acting mutagen, 4-nitro-*o*-phenylenediamine. Chlorpromazine-palladium co-ordination complex showed the highest antimutagenicity of 33 percent, which is much higher that that of chlorpromazine of 23 percent. All palladium complexes increased percent inhibition of its parent compounds; palladium co-ordination complexes showed the highest percent inhibition among all co-ordination complexes (45). More organometalic compounds are expected to be synthesized and examined for their site-specific affinity to DNA and their antimutagenicity.

4.7.1.5. Antimutagenicity of benzo[*a*]phenothiazines

Phenothiazines are major tranquilizers employed in the treatment of psychotic disorders such as mania, paranoid states, psychosis and schizophrenia (139). Schizophrenic patients treated with phenothiazines were observed to have less incidence of cancer than those who were not treated (17), which suggests that phenothiazines have an inhibitory effect on the development of cancer. Meanwhile cancer has been shown to be caused by a series of mutations of oncogenes (140) and tumor suppresser genes (141) in which cancer progression takes place in multistage carcinogenesis (142).

Antimutagenic activity of seven benzo[*a*]phenothiazines including methyl-, oxo- and hydroxy-derivatives was examined against a direct-acting mutagen to DNA, 4-nitro-*o*-phenylenediamine in TA98 strain of *Salmonella typhimurium* mutagenicity assay and was compared with that of chlorpromazine. All seven benzo[*a*]phenothiazines showed antimutagenic activity against 4-nitro-*o*-phenylenediamine; 9-methyl-12*H*-benzo[*a*]phenothiazine being the most potent antimutagen of 30 percent inhibition; 12*H*-benzo[*a*]phenothiazine, 9-methyl-12*H*-benzo[*a*]phenothiazine, 6-hydroxy-5-oxo-5*H*-

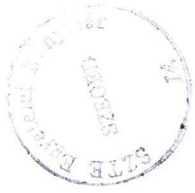
benzo[*a*]phenothiazine and 6-methyl-5-oxo-5*H*-benzo[*a*]phenothiazine showed higher percent inhibition than chlorpromazine (44. 47) (Figure 3).

4.7.1.6. Correlation of antimutagenicity and computer-assisted molecular calculation

Antimutagenicity of seven benzo[*a*]phenothiazines was compared with the experimental and calculated ground/first excited singlet-state dipole moments. The experimental ground-state dipole moments of the moderately antimutagenic benzo[*a*]phenothiazines were higher than those of the weakly antimutagenic benzo[*a*]phenothiazines. The first excited singlet-state dipole moments depend on the change of the π-component of the dipole moment [HOMO → LUMO ($\pi \rightarrow \pi^*$)] transition while the σ-contribution remains unchanged. The first excited singlet-state dipole moments of the moderately antimutagenic benzo[*a*]phenothiazines were higher than those of the weakly antimutagenic benzo[*a*]phenothiazines. Therefore, the ground and first excited singlet-state dipole moments governed by the substitution on benzo[*a*]phenothiazines correlated well with antimutagenic activity (47). The study will further make it possible to design more potent antimutagenic phenothiazines by computer-assisted molecular modeling.

4.7.1.7. Antitumor activity

Benzo[*a*]phenothiazes have been reported to inhibit tumor growth of implanted Walker carcinoma 256 in albino rats (143), which indicates that the induction of a methyl substituent at the 12th or the 9th position of the aromatic skeleton increases the activity. Furthermore, the cumulative effect of methyl substituents on the benzo[*a*]phenothiazines was recognized (144, 145). Against Ehrlich carcinoma 9-methyl-12*H*-benzo[*a*]phenothiazine was highly active (21). Interestingly, the result correlates well with antimutagenic activity studied in this experiment. Nitrogen mustard derivatives of benzo[*a*]phenothiazines were tested in several tumor cell lines, none of which showed significant antitumor activity (145).



4.7.2. Antimutagenic phenothiazines, suppression of mutants and cancer chemoprevention

Phenothiazines have been reported to possess antimutagenic activity in spontaneous mutation (11) and chemically induced mutation (12). As regards to spontaneous mutation, Ames' strains of *Salmonella typhimurium* employed in the experiments did not provide promising data on the antimutagenicity of phenothiazines to spontaneous mutation. Nevertheless some other experimental models are expected to be used to investigate their antimutagenic activity in spontaneous mutation. For the research on the suppression of spontaneous mutation hopefully leads to the prevention of the emergence of new mutant microbial strains such as antibiotic-resistant mutants, quisi-species of AIDS virus and multi-drug resistant (MDR) cancer cells.

On the other hand, phenothiazines showed to have powerful antimutagenic action not only to benz[*a*]pyrene (18, 19) and aflatoxin B_1 (123), but also to a direct-acting mutagen to DNA, 4-nitro-*o*-phenylenediamine (44, 45, 47). A series of experiments on structurally related antimutagenic phenothiazines is expected to be stored in the database accessible on the Internet. Furthermore, the correlation between the antimutagenicity of phenothiazines and their molecular and physicochemical properties will establish the structure-activity relationships (SAR) which make it possible to design more poweful antimutagenic phenothiazines by computer-assisted drug design (CADD).

The development of powerful antimutagenic agents is of great interest to cancer chemoprevention. Cancer chemoprevention is one of the most extensively studied disciplines in cancer chemotherapy both for general and targeted populations. Cancer chemoprevention is defined as inhibition of cancer development by administration of natural or synthetic compounds that are non-toxic during carcinogenesis (13, 146), proposed to be one of the most cost-effective prophylactic treatments of cancer in targeted high-risk populations in which a precancerous state or an inherited mutation is positively diagnosed. Of many natural (147), synthetic (148) and nutritional (149) compounds which have shown inhibitory effects on cancer development at various stages, antimutagenic agents are considered to be a group of the ideal cancer chemopreventive agents which are expected to halt successive mutation sequence of neoplastic cells in multistage carcinogenesis. However, the mechanism of chemical antimutagenesis against induced mutations is complex and is not understood well yet due to the presence of multiple active metabolites and multiple inhibitory target sites in the living cells. Nevertheless well-planned experimental models elucidate the antimutagenic mechanism step by step. Based on such

experimental data and computer-assisted molecular modeling, novel antimutagenic compounds are expected to be designed and synthesized to further enhance mutation inhibition and ultimately arrest multistep carcinogenesis.

6. Conclusion and further consideration

The data on the mutagenicity and antimutagenicity of the tricylc aromatic hydrocarbons, namely, acridines and phenothiazines obtained in a series of the experiments covers those of only a small part of the numerous, theoretically possibly synthesizable tricyclic compounds. However, the relationships of the mutagenicity and antimutagenicity of the closely related compounds in structure and biological function, and their correlations to the molecular and physicochemical properties make it possible to extrapolate the desirable information of a number of groups of chemicals, such as the design of nonmutagenic drugs of maximum potency, the most poweful antimutagenic compounds, DNA-sequence specific mutators, *etc*.

Meanwhile, various combinatorial libraries are expected to be constructed based on the similarity and diversity in molecular structure, molecular and physicochemical properties. The similarity and diversity mesurement should be made by the molecular weight, hydrophobicity, polarity and electronic status, hydrogen-bonding donor/acceptor status, flexibility, shape indices, pharmacophore profiles, quantum mechanical descriptors, and so on (150). The data calculated by computers should be cross-referenced with the data on a number of *in vitro* and *in vivo* bioassays such as ligand-based and binding-site directed assays, animal experiments and three phases of clinical trials. All the data are desirably stored in the database and accessible to the public on the Internet.

Limited data on the bioassay of mutagenicity and antimutagenicity of the tricyclic compounds will be maximized together with the development of computer-assisted molecular profiling, molecular modeling, well-planned bioassays and the database accessibility.

(MNNG) produced almost exclusively G:C → A:T transitions with a preference for target sites preceded by a guanine. In contrast, the S_N2 (substitution, nucleophilic, biimolecular) alkylating agent ethylmethanesulphonate (EMS) produced a slightly broader spectrum of base substitutions although still predominately G:C → A:T transitions (110). G:C → A:T transitions induced by both alkylating agents are thought to be medicated by the miscoding properties of the O^6-alkylguanine adduct (111). G:C → A:T transitions also predominate in the spectrum of ultraviolet (UV)-induced mutation in the *lacI* gene (112), but these occur almost exclusively at cytosine-containing dipyrimidine sites as would be expected if cyclobutane pyrimidine dimers (CPD) or pyrimidine-pyrimidone lesions were reponsible for the mutational spectra of ultraviolet (UV) and ethylmethanesulphonate (EMS). Furthermore, mutational spectra obtained in bacteria are quite similar to those recovered in mammalian cells.

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