Contam. Ambient. 3, 63-70, 1987

# SISTER CHROMATID EXCHANGES INDUCED BY THE ORGANOPHOSPHORUS INSECTICIDES METHYL PARATHION, DIMETHOATE, PHOXIM AND METHYL AZINFOS IN CULTURED HUMAN LYMPHOCYTES

SANDRA GOMEZ-ARROYO, NORMA NORIEGA-ALDANA, DOLORES JUAREZ-RODRIGUEZ and RAFAEL VILLALOBOS-PIETRINI

Laboratorio de Citogenética y Mutagénesis Ambientales, Centro de Ciencias de la Atmósfera, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D.F. México

(Recibido septiembre 1987, Aceptado diciembre 1987)

## ABSTRACT

The cytogenetic effect of the organophosphorus insecticides methyl parathion or folidol, dimethoate or rogor, phoxim or bay 77488 and methyl azinfos or gusathion was evaluated by means of the productions of sister chromatid exchanges (SCE) in cultured human lymphocytes. Positive results were obtained with methyl parathion, dimethoate and phoxim, while a negative response was achieved with methyl azinfos. The highest concentrations of all substances decreased the SCE, possibly due to their toxicity, producing the death of the damaged cells and therefore eliminating the SCE expression.

## RESUMEN

Se evaluó el afecto citogenético de los insecticidas organofosforados metil paration o folidol, dimetoato o rogor, foxim o bay 77488 y metil azinfos o gusation mediante el análisis de intercambio de cromátidas hermanas (ICH) en cultivo de linfocitos humanos. Se obtuvieron resultados positivos con metil paratión, dimetoato y foxim, en tanto que con metil azinfos la respuesta fue negativa. A las concentraciones más altas de todas las sustancias empleadas disminuyeron los ICH, debido posiblemente a su elevada toxicidad, provocando la muerte de las células más dañadas e impidiendo de esta manera la expresión del ICH.

## INTRODUCTION

The organophosphorus insecticides appear for the first time in 1945 in Germany. Most of them are esters of phosphoric acid. The common structure of the organophosphorus insecticides is P-O-C, the presence of phosphorus and carbon as electrophilic sites, gives the clue to understand the reaction with nucleophilics, suffering alkilation or phosphorilation.

These insecticides interfere with the neural transmission blocking the enzyme cholinesterase in vertebrates, that is esential to the neurotransmission function because it modulated the neurotransmitter acetil choline (O'Brien 1969, Karczmar *et al.* 1970, Aldrige 1971, Fukuto 1971). The mechanism of blockage is produced by phosphorilation of hydroxil-serine group in the active site of the enzyme (Fest and Schmidt 1973).

Also it has been mentioned that the organic phosphate insecticides are alkylant agents (Preussmann 1969, Bedford and Robinson 1972, Wooder and Wright 1981). These types of agents act as electrophilic sites capable of reacting with DNA specially with the nitrogen and oxygen atoms of adenine, guanine, and cytosine bases and with the phosphate groups, having a reference of $N_7$ of guanine (Verly and Brakier 1970).

To evaluate the damage at the genetical level induced by many physical and chemical mutagens of the environment and to know its risk to human health, various test systems using bacteria, cultured mammalian cells, fungi, plants and mammals as target cells or target organisms have been described (Environmental Mutagen Society 1975). Among them one of the most useful system has been the cultured human lymphocytes (Jasińska *et al.* 1970, Evans and O'Riordan 1975). The induction of sister chromatid exchanges (SCE) has been described as a rapid and sensitive end-point for mutagenicity test. In certain cases it can be induced by concentrations of a compound ten times lower to those producing chromosomal aberrations (Perry and Evans 1975, Stetka and Wolff 1976, Latt *et al.* 1981).

Due to the importance of organophosphorus insecticides as agricultural contaminants and to their alkylant capacities, it is important to determine the genetic risk to exposed individuals, for this reason in this paper the SCE induction of methyl parathion or folidol, dimethoate or rogor, phoxim or bay 77488 and methyl azinfos or gusathion is evaluated.

## MATERIAL AND METHODS

Cultures of peripheral blood of healthy individuals were made using the same donor for each experiment and its replicate.

All the insecticides purchased from Bayer were dissolved in distilled water and sterilized by means of Millipore membranes (0.45 $\mu$m). The concentrations used were established on basic of preliminary experiments: methyl parathion or folidol 0.5, 1, 2, 3, 4, 5, 6, 9, 10 and 13 ppm; dimethoate or rogor 5, 10, 20, 30, 40, 60, 80, 100 and 120 ppm; phoxim or bay 77488 4, 6, 7, 8 and 10 ppm; methyl azınfos or gusathion 2, 4, 8, 10, 20 and 30 ppm.

With a heparinized syringe, 5 ml of peripheral blood was extracted by vein puncture. Eight drops were put on a culture glass having 3 ml of McCoy's 5A (Microlab) plus 0.2 ml of phytohaemagglutinin (Gibco). This was incubated at 37°C for 72 hours. 24 hours later of the begining of culture 5-bromodeoxyuridine (Sigma) was added to the culture medium at final concentration of 5 $\mu$g/ml simultaneously with the corresponding concentrations of insecticides. After 70 hours 0.04 $\mu$g/ml of colchicine (Merck) was added. Then after 72 h the cells were harvested by centrifugation and the pellet was resuspended in hypotonic solution of 0.075 M KCl for 20 min. Cells were again centrifuged and finally fixed in methanol-acetic acid (3:1). The slides were made by dropping and air-drying and were immersed for 20 min in a solution of 0.05 $\mu$g/ml of Hoechst-33258 in deionized water in the dark, then rinsed and mounted with

a coverslip with phosphate buffer of 6.8 pH irradiated with UV light for one hour, after removal of the cover-glass, slides were stained in Giemsa distilled water (1:50) for 30 min.

For each concentration and its replicate, 50 second-division metaphases were analyzed. A terminal SCE was recorded as one exchange and an interstitial SCE as two exchanges. In order to avoid any bias, the slides were handled following in code to make their origin unknown.

The t student test was applied to the data obtained and a significant difference was found when $P < 0.001$

## RESULTS

Regarding the methyl parathion, the increase of concentration did not produce an increment of SCE frequencies. At 0.5, 1, 2, and 3 ppm no significant responses different from those of the controls were obtained, but with 4 ppm and higher than this concentration there were. At 13 ppm cell death was produced (Table I).

TABLE I. SCE FREQUENCIES INDUCED BY METHYL PARATHION IN HUMAN LYMPHOCYTES IN VITRO[a].

| Concentration (ppm) | SCEs/metaphase $\bar{X} \pm$ S.E. | "t" value |
|---|---|---|
| 0 | 6.04 ± 0.39 | |
| 0.5 | 6.37 ± 0.29 | 0.49 N.S. |
| 1.0 | 8.16 ± 0.36 | 2.83 N.S. |
| 2.0 | 8.68 ± 0.40 | 3.34 N.S. |
| 3.0 | 8.08 ± 0.40 | 2.58 N.S. |
| 4.0 | 10.30 ± 0.48 | 4.90 * |
| 6.0 | 9.40 ± 0.29 | 4.94 * |
| 9.0 | 9.08 ± 0.47 | 3.53 * |
| 10.0 | 9.42 ± 0.38 | 4.39 * |
| 13.0 | Cellular death | |

[a] n = 50 metaphases in 2 repetitions, the same donor was tested for each concentration.

* $P < 0.001$
N.S. not-significant.

Neither with dimethoate was a relationship concentration-response obtained, the peak value was reached at 30 ppm and except for 5 and 10 ppm, all other concentrations produced significant differences from that of the control. At 120 ppm cell death was produced (Table II).

With respect to phoxim in 4 and 6 ppm the response was not significant. The response increased at 7 and 8 ppm, and at 10 ppm cell death was produced (Table III).

Finally, with methyl azinfos no significant response was obtained and due to the fact the solubility limit was 30 ppm, higher concentrations were not applied (Table IV).

TABLE II. SCE FREQUENCIES INDUCED BY DIMETHOATE IN HUMAN LYMPHOCYTES IN VITRO[a]

| Concentration (ppm) | SCEs/metaphase $\bar{X} \pm$ S.E. | "t" value |
|---|---|---|
| 0 | 5.84 ± 0.28 | |
| 5 | 6.58 ± 0.37 | 1.14 N.S. |
| 10 | 7.54 ± 0.33 | 2.79 N.S. |
| 20 | 12.08 ± 0.33 | 10.23 * |
| 30 | 18.28 ± 0.48 | 16.37 * |
| 40 | 12.00 ± 0.40 | 9.06 * |
| 60 | 9.90 ± 0.39 | 6.06 * |
| 80 | 11.94 ± 0.47 | 8.13 * |
| 100 | 11.48 ± 0.56 | 6.71 * |
| 120 | Celullar death | |

[a] n = 50 metaphases in 2 repetitions, the same donor was tested for each concentration.

* $P < 0.001$

N.S. not-significant

## DISCUSSION

In spite of the fact that methyl parathion produced an increase of SCE frequencies (Table I), higher concentrations that might show more frequencies produced cell death. This insecticide has been widely studied, however there is a lot of controversy in respect to its genetic activity. In *Vicia faba* it induced chromosome clumping and stickness (de Kergommeaux *et al.* 1983), chromosomal aberrations (Gómez-Arroyo *et al.* 1985), and SCE (Gómez-Arroyo *et al.* in press). In barley, it caused chlorophilic mutations (Panda and Sharma 1979) and chromosomal aberrations in root meristems and in meiotic cells (Kaur and Grover 1985a,b).

TABLE III. SCE FREQUENCIES INDUCED BY PHOXIM IN HUMAN LYMPHOCYTES IN VITRO[a]

| Concentration (ppm) | SCEs/metaphase $\bar{X} \pm$ S.E. | "t" value |
|---|---|---|
| 0 | 5.60 ± 0.18 | |
| 4 | 5.66 ± 0.23 | 0.15 N.S. |
| 6 | 6.88 ± 0.26 | 2.91 N.S. |
| 7 | 9.44 ± 0.26 | 8.73 * |
| 8 | 8.66 ± 0.26 | 6.95 * |
| 10 | Celullar death | |

[a] n = 50 metaphases in 2 repetitions, the same donor was tested for each concentration.

* $P < 0.001$

N.S. not-significant.

In some mutagenic tests with microorganisms, methyl parathion was possitive (Fahrig 1974) and produced DNA breaks in El *Escherichia coli* plasmid (Griffin and Hill 1978). It also is mutagenic in *Salmonella typhimurium* and *Saccharomyces cerevisiae* (Waters *et al.* 1980). However, negative results were also obtained in *E. coli* (Mohn 1973), *S. typhimurium* (Simmon *et al.* 1976) and in mice dominant lethal test (Jorgenson *et al.* 1976). In three hematopoietic human cell lines, methyl parathion did not increase the frequency of chromosomal aberrations (Huang 1973), nor in the lymphocytes of workers occupationally exposed (de Cassia Stocco *et al.* 1982), but in the blood of people accutely toxified it increased the number of chromosomal aberrations (Yoder *et al.* 1973, van Bao *et al.* 1974). Chen *et al.* (1981) found significant increase of SCE in a Burkitt lymphoma B35M human cell line and in the V79 Chinese hamster cell line, and also a strong cell delay. Sobti *et al.* (1982) obtained an increase in SCE of human lymphoid cells in culture. In polichromatic erithrocytes of mice bone marrow it induced micronuclei (Grover and Malhi 1985).

With dimethoate, although a relationship concentration-effect was not produced, at 20. 30, and 40 ppm the values obtained were more than the double of the controls (Table II).

TABLE IV. 'SCE FREQUENCIES INDUCED BY METHYL AZINFOS IN HUMAN LYMPHOCYTES IN VITRO[a]

| Concentration (ppm) | SCEs/metaphase $\bar{X} \pm$ S.E. | "t" value |
|---|---|---|
| 0 | 5.38 ± 0.18 | |
| 2 | 6.28 ± 0.32 | 1.80 N.S. |
| 4 | 6.22 ± 0.27 | 1.87 N.S. |
| 8 | 7.00 ± 0.31 | 3.31 N.S. |
| 10 | 5.66 ± 0.21 | 0.72 N.S. |
| 20 | 6.24 ± 0.25 | 0.65 N.S. |
| 30 | 6.44 ± 0.24 | 2.52 N.S. |

[a] n = 50 metaphases in 2 repetitions, the same donor was tested for each concentration.

N.S. not-significant

Incongruent results have been obtained with the dimethoate or rogor, in barley it induced chromosomal aberrations in root tip meristems and in gametic cells (Kaur and Grover 1985a, b). In *Vicia faba*, it produced a decrement of the mitotic index and also chromosomal alterations (Amer and Farah 1974) as well as SCE (Gómez-Arroyo *et al.* in press). In *E. coli*, it caused resistance to 5-methyl tryptophane (Mohn 1973). It induced mitotic gene conversion in *S. cerevisiae* (Fahrig 1973, 1974). It is positive in the bacteria reversion test (Moriya *et al.* 1983). Chen *et al.* (1981) showed an increment in SCE frequency of V79 cell line of Chinese hamster. It induced micronuclei in mice and in the host mediated test, *S. typhimurium* gave positive results (Usna-Rani *et al.* 1980). However, Shirasu *et al* . (1976) found negative results in bacteria and in *Schizosaccharomyces pombe* did not increase the ade6 locus mutation frequency (Gilot-Delhalle *et al.* 1983).

Phoxim or bay 77488 showed similar effects to methyl parathion on the SCE induction, but it was more toxic because at 10 ppm produced cell death (Table III). Phoxim used meanly in grain storage, degradated rapidly and had low toxicity for mammals (Mason and Meloan 1976). However, in *Vicia faba* (Gómez-Arroyo *et al.* in press) and in human lymphocytes in culture it was very toxic as has been demonstrated in this work.

In the genetic point of view, the data are very few, phoxim has been shown as negative in bacteria reversion test (Moriya *et al.* 1983) but induced SCE in *Vicia faba* (Gómez-Arroyo *et al.* in press).

Methyl azinfos or gusathion did not produce SCE in human lymphocytes in cultu- (Table IV). This is in agreement with the results obtained in *Saccharomyces cerevisiae* D3 and D7, because it was not mutagenic (Riccio *et al.* 1981); it did not produce dominant lethals in mice (Jorgenson *et al.* 1976), did not increase SCE in Chinese hamster V79 cells (Chen *et al.* 1982) nor in *Umbra limi* (Vigfusson *et al.* 1983). However it produced mitotic genic recombination in *S. cerevisiae* D3 (Simmon *et al.* 1976) and chromosomal alterations in Chinese hamster CHO-KI line (Alam *et al.* 1974). It caused a c-mitotic effect (Grant 1973) and SCE in *Vicia faba* (Gómez Arroyo *et al.* in press).

Comparing the results obtained with these insecticides in human lymphocytes and in meristematic cells of *Vicia faba* (Gómez-Arroyo *et al.* in press), it was noted that *Vicia*, was more sensitive because SCE were produced by lower concentrations. In both cases no relationship concentration-effect was found.

Some organophosphorus insecticides had the capacity to affect cell membranes, thus modifying their permeability (Antunes-Madeira and Madeira 1979), and probably at low insecticide concentrations, the cell membrane permeability changed being less selective and the insecticides could penetrate more efficiently. Within the cell it accumulated and because it was not excreted easily, it produced high toxicity and cell death avoiding the expression of genetic damage.

## ACKNOWLEDGEMENTS

We thank Miguel Angel Meneses for his valuable technical assistance. This research was supported in part by a CONACyT grant PCCBBNA 021988.

## REFERENCES

Alam M. T., Corbiel M., Chagnon A. and Kasatiya S. S. (1974). Chromosomal anomalies induced by the organic phosphate pesticide guthion in Chinese hamster cells. Chromosoma (Berl.) *49*, 77-86.

Aldrige W. N. (1971). The nature of the reaction of organophosphorus compounds and carbamates with esterases. Bull. Wld. Hlth. Org. *44*, 25-30.

Amer S. M. and Farah O. R. (1974). Cytological effects of pesticides VI. Effect of the insecticide "Rogor" on the mitosis of *Vicia faba* and *Gossypium barbadense*. Cytologia *39*, 507-514.

Antunes-Madeira M. C. and Madeira V. M. (1979). Interaction of insecticides with lipid membranes. Biochem. Biophys. Acta *550*, 384-392.

Bedford C. T. and Robinson J. (1972). The alkylating properties of organophosphates. Xenobiotica *2*, 307-337.

Chen H. H., Hsueh J. L., Sirianni S. R. and Huang C. C. (1981). Induction of sister-chromatid exchanges and cell cycle delay in cultured mammalian cells treated with eight organophosphorus pesticides. Mutation Res. *88*, 307-316.

Chen H. H., Sirianni S. R. and Huang C. C. (1982). Sister-chromatid exchanges and cell-cycle delay in Chinese hamster V79 cells treated with 9 organophosphorus compounds (8 pesticides and 1 defoliant). Mutation Res. *103*, 307-313.

de Cassia Stocco R., Becçak W., Gaeta R. and Rabello-Gay N. (1982). Cytogenetic study of workers exposed to methyl parathion. Mutation Res. *103*, 71-76.

de Kergommeaux D. J., Grant W. F. and Sandhu S. S. (1983). Clastogenic and physiological response of chromosomes to nine pesticides in the *Vicia faba in vivo* root tip assay system. Mutation Res. *124*, 69-84.

Environmental Mutagen Society (1975). Environmental mutagenic hazards. Science *187*, 503-514.

Evans H. S. and O'Riordan M. L. (1975). Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutation Res. **31**, 135-148.

Fahrig R. (1973). Nachweis einer genetischen Wirkung von Organophosphor-In sektiziden. Naturwissenschaften *60*, 50-51.

Fahrig R. (1974). Comparative mutagenicity studies with pesticides. IARC Scientific Publications *10*, 161-181.

Fest C. and Schmidt K. J. (1973). The chemistry of organophosphorus pesticides, Springer-Verlag, Berlin.

Fukuto T. R. (1971). Relationships between the structure of organophosphorus compounds and their activity as acetylcholinesterase inhibitors. Bull. Wld. Hlth. Org. *44*, 31-42.

Gilot-Delhalle J., Colizzi A., Moutschen J. and Moutschen-Dahmen M. (1983). Mutagenicity of some organophosphorus compounds at the ade6 locus of *Schizosaccharomyces pombe*. Mutation Res. *117*, 139-148.

Gómez-Arroyo S., Baíza A. M., López G. and Villalobos-Pietrini R. (1985). A comparative study of the cytogenetic effects of the insecticides heptachlor, malathion, and methyl parathion in *Vicia faba*. Contam. Ambient. *1*, 7-16.

Gómez-Arroyo S., Castillo-Ruíz P., Cortés-Eslava J. and Villalobos-Pietrini R. *Vicia faba* - sister chromatid exchanges of the organophosphorus insecticides methyl parathion, dimethoate, oxydemeton methyl, azinphos methyl and phoxim. Cytologia (in press).

Grant W. F. (1973). Cytological effects of environmental mutagens pesticides. Mutation Res. *21*, 221-222.

Griffin D. E. III and Hill W. E. (1978). *In vitro* breakage of plasmid DNA by mutagens and pesticides. Mutation Res. *52*, 161-169.

Grover I. S. and Malhi P. K. (1985). Genotoxic effects of some organophosphorus pesticides. I. Induction of micronuclei in bone marrow cells in rat. Mutation Res. *155*, 131-134.

Huang C. C. (1973). Effect on growth but not on chromosomes of the mammalian cells after treatment with three organophosphorus insecticides Proc. Soc. Exp. Biol. Med. 142, 36-40. 36-40.

Jasińska J., Steffen J. A. and Michalowski A. (1970). Studies on *in vitro* lymphocyte proliferation in cultures synchronized by inhibition of DNA synthesis. II. Kinetics of the initiation of the proliferative response. Exp. Cell Res. *61*, 333-341.

Jorgenson T. A., Rushbrook C. J. and Newel G. W. (1976). *In vivo* mutagenesis investigation of ten commercial pesticides. Toxicol. Appl. Pharmacol. *37*, 109 (Abstr.).

Karczmar A. G., Nishi S. and Blaber L. C. (1970). Investigations, particularly by means of anticholinesterase agents, of the multiple peripheral and central cholinergic mechanisms and of their behavioral implications. Acta Vitaminol. Enzimol. *24*, 131.

Kaur P. and Grover I. S. (1985a). Cytological effects of some organophosphorus pesticides. I. Mitotic effects. Cytologia *50*, 187-197.

Kaur P. and Grover I. S. (1985b). Cytological effects of some organophosphorus perticides. II. Meiotic effects. Cytologia *50*, 199-211.

Latt S. A., Allen J., Bloom S. E., Carrano A., Falke E., Kram D., Schneider E., Schreck R., Tice R., Whitfield B. and Wo.ff S. (1981). Sister-chromatid exchanges: a report of the gene-tox program. Mutation Res. *87*, 17-62.

Mason W. A. and Meloan C. E. (1976). Degradation products of phoxim (Bay 77488) on stored wheat. J. Agric. Food Chem. *24*, 299-304.

Mohn G. (1973). 5-methyltryptophan resistance mutation in *Escherichia coli* K-12. Mutagenic activity of monofunctional alkylating agents including organophosphorus insecticides. Mutation Res. *20*, 7-15.

Moriya M., Ohta T., Watanabe K., Miyazawa T., Kato K. and Shirasu Y. (1983). Further mutagenicity studies on pesticides in bacterial reversion assay systems. Mutation Res. *116*, 185-216.

O'Brien R. B. (1969). Phosphorylation and carbamylation of cholinesterase. Ann. N. Y. Acad. Sci. *160*, 204-214.

Panda B. B. and Sharma C. B. (1979). Organophosphate induced chlorophyll mutations in *Hordeum vulgare*. Theor. Appl. Genet. *55*, 253-255.

Perry P. and Evans H. J. (1975). Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature (London) *258*, 121-125.

Preussmann R. (1969). Untersuchungen zum Nachweis alkylierender Agentien. Arzneim. Forsch *19*, 1059-1073.

Riccio E., Shepherd G., Pomeroy A., Mortelmans K. and Waters M. D. (1981). Comparative studies between the *S. cerevisiae* D3 and D7 assays of eleven pesticides. 12th Ann. Meeting Environ. Mutagen Soc. p. 82.

Shirasu Y., Moriya M., Kato K., Furuhashi A. and Kada T. (1976). Mutagenicity screening of pesticides in the microbial system. Mutation Res. *40*, 19-30.

Simmon V. F., Poole D. C. and Newell G. W. (1976). *In vitro* mutagenic studies of twenty pesticides. Toxicol. Appl. Pharmacol. *37*, 109.

Sobti R. C., Krishan A. and Pfaffenberger C. D. (1982). Cytokinetic and cytogenetic effects of some agricultural chemicals on human lymphoid cells *in vitro*: organophosphates. Mutation Res. *102*, 89-102.

Stetka D. G. and Wolff S. (1976). Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. II. *In vitro* test for compounds requering metabolic activation. Mutation Res. *41*, 343-350.

Usna Rani M. V., Reddy O. S. and Reddy P. P. (1980). Mutagenicity studies involving aldrin, endosulfan, dimethoate, phosphamidon, carbaryl and ceresan. Bull. Environ. Contam. Toxicol. *25*, 277-282.

Van Bao T., Szabó I., Ruzicska P. and Czeizel A. (1974). Chromosome aberrations in patients suffering acute organic phosphate insecticide intoxication. Humangenetik *24*, 33-57.

Verly W. G. and Brakier L. (1970). Mecanisme moleculaire de l'action toxique des agents alkylants. Rev. Europ. Etudes Clin. Et. Biol. *XV*, 483-488.

Vigfusson N. V., Vyse E. R., Pernsteiner C. A. and Dawson R. J. (1983). *In vivo* induction of sister-chromatid exchange in *Umbra limi* by the insecticides endrin, chlordane, diazinon and guthion. Mutation Res. *118*, 61-68.

Waters M. D., Simmon V. F.,Mitchell A. D., Jorgenson T. A. and Valencia R. (1980). An overview of short-term tests for the mutagenic and carcinogenic potential of pesticides. J. Environ. Sci. Health *15*, 867-906.

Wooder M. F. and Wright A. S. (1981). Alkylation of DNA by organophosphorus pesticides. Acta Pharmacol. Toxicol. *49*, (Suppl.) 51-55.

Yoder J., Watson M. and Benson W. W. (1973). Lymphocyte chromosome analysis of agricultural workers during extensive occupational exposure to pesticides. Mutation Res. *21*, 335-340.