รายงานวิจัย

ทดสอบฤทธิ์ก่อมะเร็ง (carcinogenic effect) และฤทธิ์ส่งเสริม (promoting effect) การเกิดมะเร็งตับของสารเคมีกำจัดหญ้าบูตาคลอร์ (butachlor) โดยวิธีเหนี่ยวนำให้เกิดรอยโรคระยะก่อนมะเร็งตับ

โดย

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โครงการวิจัยนี้ได้รับการสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว) ในหมวดทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา พ.ศ. 2538-2540 ตามสัญญา BRG 04/2538 และได้รับการสนับสนุนบางส่วนในด้านเครื่องมือและคำแนะนำทางเทคนิคการทดลองจาก Professor Dr. Nobuyuki Ito และ Professor Dr. Tomoyuki Shirai ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ แห่งมหาวิทยาลัยนาโกย่า ซิตี้ เมืองนาโกย่า ประเทศญี่ปุ่น นอกจากนี้ยังได้รับความอนุเคราะห์อย่างดีจากหัวหน้าภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ (ร.ศ.นพ. วิญญู มิตรานันท์ และ ผ.ศ. พญ. สินีนาฏ กาลเนาวกุล) ในการใช้สถานที่และอุปกรณ์บางส่วนในการวิจัย ผู้วิจัยขอขอบพระคุณทุกท่านที่กล่าวถึงมา ณ โอกาสนี้

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Lack of Promoting Effect of Butachlor on Hepatocarcinogenesis of Wistar Rat by Medium-term Liver Bioassay

บทคัดย่อ:

ศึกษาฤทธิ์ก่อมะเร็ง (carcinogenic effect) และฤทธิ์ส่งเสริม (promoting effect) การเกิดมะเร็งตับ ของสารเคมีกำจัดหญ้าบูตาคลอร์ (butachlor) โดยเทคนิค medium-term liver biopssay

หลักการของเทคนิคนี้ยึดตามทฤษฎีการเกิดมะเร็งแบบ 2 ขั้นตอน โดยใช้ diethylnitrosamine (DEN) เป็นตัวเหนี่ยวนำ (initiator) ให้เกิดมะเร็งตับ และให้สารที่สงสัยว่าจะเป็นตัวส่งเสริม (promoter) สารก่อมะเร็งที่สมบูรณ์ (complete carcinogen) หรือตัวยับยั้ง (inhibitor) การเกิดมะเร็งตับร่วมด้วย การประเมินผลใช้ glutathione-S-transferase placental form (GST-P) positive foci ซึ่งถือว่าเป็น preneoplastic marker โดยสามารถมองเห็นได้ด้วยการย้อมทางวิธี immunohistochemistry ในการศึกษานี้ใช้หนูขาวเพศผู้สายพันธุ์ Wistar อายุ 6 สัปดาห์ แบ่งออกเป็น 3 กลุ่ม สองกลุ่มแรกมีกลุ่มละ 40 ตัว ส่วนกลุ่มที่ 3 มี 10 ตัว กลุ่มที่ 1 ได้รับ DEN (200 มก. / น้ำหนักตัว 1 กก.) ฉีดเข้าช่องท้อง 1 ครั้งในวันที่ 0 และบูตาคลอร์ (1/10 LD_{50}) ทางสายยางเข้ากระเพาะอาหารทุกวันจากวันที่ 14 เป็นต้นไป กลุ่มที่ 2 และ 3 ได้รับ DEN และบูตาคลอร์ อย่างเดียวตามลำดับ เมื่อครบ 21 วัน หนูทุกกลุ่มจะถูกตัดตับออก 2 ใน 3 ส่วน และปลายสัปดาห์ที่ 8 จะฆ่าหนูทุกกลุ่มเพื่อเก็บตับมาย้อม GST-P positive foci วิเคราะห์ผลฤทธิ์ก่อมะเร็ง ส่งเสริมการเกิดมะเร็ง และยับยั้งการเกิดมะเร็งด้วยการเปรียบเทียบความแตกต่างของจำนวน (number) และพื้นที่ (area)ของ GST-P positive foci โดย Student's t-test

ผลการวิจัยพบว่ากลุ่มที่ให้ DEN และบูตาคลอร์ มีทั้งจำนวนและพื้นที่ของ GST-P positive foci น้อยกว่ากลุ่มควบคุม แต่เฉพาะพื้นที่เท่านั้นที่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p < 0.01, 0.05$) และไม่พบ GST-P positive foci ในกลุ่มที่ให้บูตาคลอร์เพียงอย่างเดียว

จากผลการศึกษาครั้งนี้สรุปได้ว่า ภายใต้เงื่อนไขของการทดลองนี้ บูตาคลอร์ไม่มีฤทธิ์เป็นสารก่อมะเร็งหรือส่งเสริมการก่อมะเร็งตับ แต่กลับมีฤทธิ์ยับยั้งการเกิดมะเร็งตับได้ถึงแม้ว่าจะไม่แรงนัก และไม่สามารถสรุปได้ว่าบูตาคลอร์ จะไม่มีฤทธิ์ก่อมะเร็งหรือส่งเสริมการเกิดมะเร็งของอวัยวะอื่น ๆ จะต้องทำการศึกษาต่อไป เพราะวิธีการวิจัยนี้เป็นการทดสอบการก่อมะเร็งตับเป็นหลัก

Keywords : butachlor, medium-term liver bioassay, GST-P, hepatocarcinogenesis

Abstract:

The present study aimed to study the carcinogenic and carcinogenic modifier (promoter or initiator) effects of butachlor, a herbicide which is structurally similar to alachlor, a known carcinogen in animal by medium-term liver bioassay. Medium-term liver bioassay is primarily based two-step carcinogenesis hypothesis by using diethylnitrosamine (DEN) as initiator and testing whether a test compound is a complete carcinogen, tumor promoter or inhibitor. The end point marker is glutathione-S-transferase placental form (GST-P) positive foci in the preneoplastic nodule. Male Wistar rats of 6-week old were randomly allocated into 3 groups. Forty rats received intraperitoneal DEN (200 mg/kg body weight) in day 0 and butachlor (1/10 LD_{50}) by gastric lavage daily from day 14. Forty rats were given DEN and the other 10 were given test chemical alone at the same dose and route. All rats were 2/3 partially hepatectomised at day 21 and then killed at the end of week 8. The liver tissues were immunohistochemically stained with antibody to GST-P. Carcinogenic, promoting or inhibitory potential was assessed based on the difference in number and area of GST-P positive foci in the liver section of treated group and those of control groups by Student's t-test. Inhibitory effects were seen in the group receiving DEN followed butachlor. Rats receiving DEN and butachlor significantly reduced the area of GST-P positive foci at $p < 0.01$ and 0.05 of the focal size larger than 0.1 and 0.2 mm. respectivelty. However, the reduction in number of both focal sizes was not significant. No positive foci were developed in the group fed with butachlor alone. These findings suggest that butachlor in this condition does not possess the hepatocarcinogenic and promoting potential in rat. In contrast, it has a weak inhibitory effect of GST-P positive foci induction but the actual mechanism is not known. Detailed study of the mechanism is needed. In addition, further investigations of the carcinogenic, promoting and inhibitory potential of monocrtoophos on other organs are required.

Introduction:

Herbicides are used world-wide including in Thailand. Demands of herbicides are remarkedly high and increasing every year. In 1991, it was estimated that herbicide constituted 45% of global production of pesticide (2,100 thousand metric tons).[1] However, the actual use compared to calculation may be underestimated. There are direct hazards towards the farmers by direct exposure to the chemicals and indirect

hazards for the consumers by consumption of the contaminated products and by living in such a contaminated environment. Therefore, it is essential to assess the risk factors of the herbicides to the population in this country by screening the carcinogenic and tumor promoting potentials.

Criteria used to select the herbicide in this experiment were :1) evidence of toxicity described in the literature; 2) widespread use and 3) structure similar to the known carcinogen. According to the criteria mentioned, butachlor was chosen and its carcinogenic and promoting activities in rat liver carcinogenesis by medium-term liver bioassay were investigated.

Butachlor, N-(butoxymethyl)-2-chloro-N-(2-4diethylphenyl)-acetamide, is a systemic herbicide which is used widely for weed control in Thailand.[2] Its chemical structure is similar to alachlor (Fig.1), a known-carcinogenic herbicide found in several organs of rat and mouse.[3,4] Genotoxic data of butachlor obtained from short-term tests are available.[5,6] The carcinogenic evidence was observed in a two-year butachlor exposure. Butachlor induced poorly differrentiated gastric carcinoid in Sprague Dawley (SD) rats when high doses (3,000 ppm) were added to their diet and marginally effected females.[7] The mechanism responsible for this butachlor-induced tumor was through the hormonal effect involving stimulation of cell turnover driven by progressive fundic cell atrophy and hypergastrinemia. It thus indicated that the hormonal mediated butachlor-associated tumor was indirect pathway.[8]

The purpose of this study was to investigate whether butachlor is a carcinogen or a carcinogenic modifier (promoter or inhibitor) on liver carcinogenesis by medium-term liver bioassay.

Materials & Methods:

Chemicals: Diethylnitrosamine (DEN) was purchased from Sigma and butachlor (purity 98%) was gifted from the Monsanto Company, Thailand. Antibody to GST-P was from the Medical Biological Laboratory (Japan). Dose of butachlor used in this study was preliminarily tested regarding the highest dose of LD_{50} which the rats could tolerate. The LD_{50} of butachlor is 200 mg/kg/day and the selected dose was 1/10 LD_{50}. It was dissolved every two days in corn oil and kept in a dark brown glass bottle in a cool place or refrigerator.

Animals and treatments : The experimental protocol is shown in Figure 2. A total of 90 male Wistar rats, 5 weeks-old, were obtained from The National Animal Laboratory

Center at Salaya, Mahidol University. They were given basal diet and water ad libitum during one week of acclimatization and then were randomly divided into 3 groups. There were 40 rats in groups 1 and 2 and 10 rats in group 3. All rats were housed in group of 5 per cage in an air-conditioned room at 24±2°C. After one week of acclimatization, the rats in group 1 were given a single intraperitoneal injection of DEN (200 mg / kg body weight) at day 0 and butachlor by gastric lavage daily from day 14. All rats were subjected to 2/3 partial hepatectomy at day 21 and then killed at the end of week 8. Immediately upon killing, the liver were excised, weighed and then sliced into 4 pieces of 2-3 mm. thick. Three of them were immediately fixed in ice-chilled acetone for GST-P positive foci demonstration, another one was fixed in 10% neutral buffered formalin for hematoxylin and eosin (H&E) stain. Both kidneys were also removed, weighed and then fixed in 10% neutral buffered formalin for histologic examination.

Histological examination and immunohistochemistry: Histologic examination of the liver and both kidneys were viewed from H&E stain. GST-P positive foci staining was performed as previously described.[9] Briefly, the acetone-fixed liver sections were deparaffinized, rehydrated and treated sequentially with normal goat serum, rabbit anti GST-P (1:6,000), biotinylated goat anti rabbit Ig G (1:400) and avidin-biotin peroxidase. Sites of antigen antibody reaction were visualized by diaminobenzidine (DAB). Sections were then lightly stained with hematoxylin for histologic examination. The number and area of GST-P positive foci were measured by a color video image processor (model VIP-21C. Olympus Co., Japan). Measurement of GST-P positive foci is usually performed to measure the foci > 0.2 mm in diameters because they are clearly seen from a light microscope. In case when foci < 0.2 mm in diameter are more predominant than those > 0.2 mm in diameter both of them could be counted in order to test which focal size is significantly different from the control values.

Statistical Analyses: The carcinogenic and promoting effects were assessed based on the difference in numbers and areas of GST-P positive foci in the liver sections of the treated group versus the control group by Student's t-test. An enhancement or inhibition of hepatocarcinogenesis was considered a strong effect when the p-value was less than 0.05 of both numbers and areas of GST-P positive foci. Similarly, a borderline or a weak result determined the same p-value either in the numbers or areas. The negative result was justified when there was no or non-significant increase or decrease of both numbers and areas.

Results:

The survival, body, liver and kidney weights in this study are shown in Table 1. Four rats died after partial hepatectomy procedure. Macroscopic examinations during autopsy did not reveal any particular lesions. Mean final body weight of rats in the treated and control groups were 329.41+24.44 g. and 376.20+23.11 g. respectively. It was significantly lower in body weight of treated grooup compared to the control values at $p< 0.000$. In contrast, means of the liver and kidney weights were increased compared to the control group. However, the liver weight was not statistically significant ($p= 0.08$) but the kidney weight was ($p< 0.0025$).

Histological examination of the liver and kidney did not display any particular changes. In the liver, there was no cloudy swelling, fatty change, fibrosis or congestion except for, GST-P positive foci. However, these foci were not clearly seen in H&E stain compared to the immunohistochemical one. They were easily recognized only through the foci larger than 0.2 mm in diameter at 400 x magnification. Regarding the kidney, some of them showed red blood cells in glomeruli and mild dilatation of pelvis. No other pathologic evidences were noted.

Numbers and areas of GST-P positive foci per unit area of the liver section are summarized in Table 2. Rats receiving DEN and butachlor significantly reduced the area of GST-P positive foci at $p<0.01$ and 0.05 of the focal size that have the diameters larger than 0.1 and 0.2 mm. respectivelty. However, the reduction in number of both focal size was not significant. GST-P positive foci were not present in group fed with butachlor alone. Of interest was the prominence of the single cell GST-P positive and mini foci in the treated group. The mini focus was defined as any focus containing 2-5 cells positive for GST-P.[10] Usually, the measurement of GST-P positive foci is performed to measure the foci larger than 0.2 mm. In diameters because it is clearly seen from a light microscope. However we can count the foci less than 0.2 mm in diameter if they are prominent in order to test of which focal size is significantly different from the control values. Thus, the measurements of GST-P positive foci in this experiment were performed of both foci larger than 0.1 and 0.2 mm. In diameters as shown in Table 2

Discussion

The demands of herbicides are increasing in Thailand.[2] Both workers in the industrial or agricultural fields and the general population are at risk to expose to

herbicides in foods or a contaminated environment. Evaluation of carcinogenic and promoting potentials of herbicide, therefore, is clearly important for risk estimation in human beings. To achieve this purpose, the method used should be considered carefully in terms of reliability, rapidity, cost and practicality. The medium-term liver bioassay was selected to fulfill those criteria. This method is based on two-step model of hepatocarcinogenesis, by administrating DEN as an initiator to initiate the liver carcinogenesis and testing whether a test chemical is a promoter or an inhibitor.[11-12] The end point marker is GST-P positive foci induction in the liver. This marker was proved to be the most reliable marker for both genotoxic and non-genotoxic hepatocarcinogens.[13] Furthermore, it is comparable with the end point marker of the long-term test.[14-15]

Results from the present study demonstrated that rats fed with DEN and butachlor did not significantly enhance GST-P positive foci induction compared to the control values. In contrast, it reduced both number and area of foci but only the area was statistically significant from the control value at $p< 0.05$ and $p< 0.01$ of foci larger than 0.1 mm and 0.2 mm respectively. It thus, indicated that butachlor in this condition played the role as an inhibitor. Though it was weak in effect since it did not reduce both the number and area of GST-P positive foci significantly from the control groups. However, the mechanism of inhibitory effect was not determined and required further investigation.

This finding is an unexpected outcome since several data obtained from genotoxicity in short-term test and carcinogenicity in long-term test demonstratred that butachlor was mutagenic[5,6] and carcinogenic[7,8] Moreover, its chemical structure is similar to alachlor, a known carcinogen causing tumors of the thyroid, stomach and nasal cavity in rats and lung tumor in mice.[3,4] In adddition, The Environmental Protection Agency (EPA) stated that alachlor was the possible human carcinogen[4] and taking into account with the documentations of IARC[16] and the U.S. National Toxicology Program (NTP)[17] pointed out that 60% of any organ carcinogens are liver carcinogens. Therefore, butachlor was suspected to be the carcinogen or tumor promoter as well. However, in the present study butachlor exerted a different action of those previous data. Though butachlor is sometimes regarded as alachlor analogue since their chemical structures are similar (see Fig. 1) but the action of butachlor was different from alachlor in this condition. To our knowledge, so far, detailed results for experiment with intact animal regarding the inhibitory effect of butachlor on liver carcinogenesis was not

present. The present investigation thus provides a new information of the inhibitory action of butachlor in liver carcinogenesis model.

The mechanism responsible for the inhibitory effect of butachlor on GST-P positive foci induction in this study was not clear. Qu and Lin [18] found that there were several metabolites of butachlor in Long-Evans rat and the major one was butachlor glutathione conjugate (BGSC). BGSC was formed by the conjugation between butachlor and glutathione by the catalytic action of glutathione-s-transferase(GST). This occured mainly in the liver cells. As known, GST has an important function to catalyse the conjugation between reduced glutathione (GSH) with xenobiotic substances both generated from endogenous and exogenous sources. The reaction takes place in order to protect cells from those cytotoxic effects.[19] In mammals, including human, GST family are identified at least 4 distinct classes: alpha, mu, pi and theta. [20] GST-P is classified into pi class and it has very small proportion in the detoxification process in the physiologic condition.[21] Therefore, the expression of GST-P in the liver preneoplastic and neoplastic cells may be unrelated to detoxification activity but it is recognized as a preneoplastic and neoplastic markers.[21] In fact, its expression is not fully understood.[21] Since the result of Qu and Lin[18] did not specify exact class of GSTs so that it was not certain whether butachlor was converted to intermediate metabolite by GST-P. In according to the limited data of interaction between butachlor and GST-P. In this case, butachlor possibly acted as an inhibitor of GST-P focus formation by the conjugation process and GST-P may play the major role in the reaction resulting in reduction of number and area of GST-P positive foci in treated group study.

Recently, there were reports demonstrated that GST-P expression in putative initiated hepatocyte was controlled by some regulators during rat hepatocarcinogenesis. For instances, the positive regulator is a transcription factor AP-1, a complex of c-jun and c-fos oncoprotein [22] and the negative regulator is SF-B (silence factor-B).[23] In addition, Tamai's group found that GST-P was inactivated by cystine residues while other forms of GSTs were not [24] These evidences are conceivable that transcription and expression of GST-P are regulated by multiple factors. The inhibitory effect of butachlor might be due to its interaction with those factors or pathways involving in GST-P transcription and expression. Detailed study is needed in order to elucidate the exact mechanism.

The presence of prominent single cell GST-P positive foci and mini foci in the treated group compared to the control group was observed. However, the numbers of

GST-P single cell positive and minifoci were not quantitated according to the limitation of computer software. These lesions have been shown to be induced by an initiator but not a promoter and they are deserved as "initiated cell" or "precursor cell".[16] They can be detected at the earliest period at 48 hours[25] and for longer period, up to 37 weeks after single dose administration of hepatocarcinogen e.g., DEN, aflatoxin.[26] .These small foci finally progress to hepatocellular carcinoma.[15] Thus the higher number of GST-P positive single cell and mini foci observed in treated group more than the control group probably supported the evidence of the inhibitory effect of butachlor.

To clarify the carcinogenic, promoting or inhibitory effect of butachlor other than the inhibitory mechanism on GST-P positive foci induction are recommended. Firstly, to examine the dose-response relationship by using this approach is needed. Data from several studies exhibited the adverse action of pesticide.[27-28] For example, O-ethyl-O-4-nitrophenyl-phenylphosphonothioate (EPN), an insecticide at low dose reduced both number and area of GST-P positive foci while at high dose did not.[27] Secondly, to investigate the low dose mixture of butachlor with either pesticides or other compounds is essential. Since additive or synergistic effects of low dose combinations of chemicals have become increasing important because it mimics the real situation of human beings. Therefore, a risk estimation in human in this aspect is important. Thirdly, to make it relevant to the actual application of butachlor, its market formula is needed to evaluate the combination effects of active ingredient (pure butachlor) and the carrier substances. Usually the carrier substances are known as inert ingredients and most of them are considered non-genotoxic. The adverse effects of the inert ingredients, in some instances, may exceed those of the active ingredient.[1] Finally, the medium-term multi-organ model is suggested to investigate the carcinogenic, promoting or inhibitory effect of butachlor in the other organs rather than in the liver. This model is designed to examine the carcinogenic, promoting or inhibitory effect of chemical on various organs including the liver simultaneously in a rather short period of experiment ranging from 28-36 weeks.[29-30]

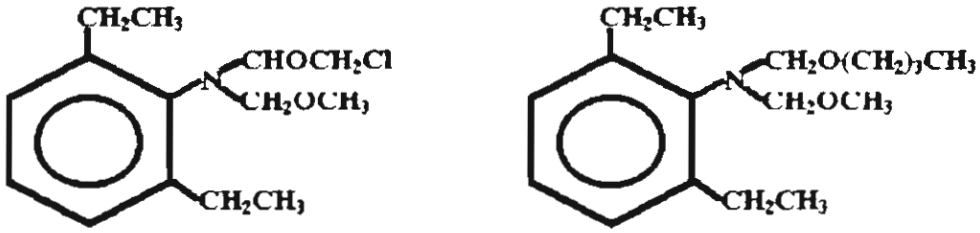
In summary, taking into consideration, butachlor may have the different action on differnt organs or different action at different dosage. Although it gave the inhibitory effect in this study it might have a carcinigenic or promoting effect on other organs. Further investigations are needed to reassure that this herbicide is safe for use.

References

1. Dich J, Zahn SH, Hanberg A, Adami HO. Pesticides and cancer. Cancer Causes and Control 1997;8:420-43.
2. Tayapatch N. Pesticides: Environmental and Health Hazards in Thailand. Technical Report.Division of Agricultural Toxicological Substances, Department of Agriculture. Bangkok Thailand 1990:1-71.
3. Environmental Protection Agency. Alachlor. Specific review position document 1. Office of Pesticides and Toxic Substances. Washington D.C 1984:89.
4. Environmental Protection Agency. Alachlor. Notice of intent to cancel registration: Conclusions of special review. Office of Pesticides and Toxic Substances. Washington D.C 1987:118.
5. Wang TC, Lee TC, Lin MF, Lin SY. Induction of sister chromatid exchange by pesticides in primary rat tracheal epithelium cell and Chiness hamster ovary cells. Mutat Res 1987;188:311-21.
6. Sinha S, Panneerselvam N, Shanmugam G. Genotoxicity of herbicide butachlor in cultured human lymphocytes. Mutat Res1995;344:63-7.
7. Hard GC, Iatropolous MJ, Thake DC et al. Identity and pathogenesis of stomach tumor in Sprague-Dawley rats associated with the dietary administration of butachlor. Exp Toxicol Pathol 1995;47:95-105.
8. Thake DC, Iatropolous MJ, Hard GC et al. A study of the mechanisms of butachlor associated gastric neoplasms in Sprague-Dawley rats. Exp Toxicol Pathol 1995; 47:107-16.
9. Hsu SM, Raine L, Faugen H. Use of avidin-biotin procedure compound (ABC) in immunoperoxidase technique: A comparison between ABC and unlabeled antibody (APA) procedures. J Hist Cytochem 1981;29:577-80.
10. Moore A, Nakamura T, Ito N. Immunohistochemically demonstrated glucose-6-phosphate dehydrogenase, g-glutamyl transpeptidase, ornithine decarboxylase and glutathione-S-Transferase enzymes: absence of direct correlation with cell proliferation in rat liver putative preneoplastic lesions. Carcinogenesis1986; 7: 1419-24.

11. Ito N, Hasegawa R. Medium-term in vivo bioassay for carcinogenicity of pesticides. Rev Pestic Toxicol 1993;2:115-32.
12. Ward LM, Ito N. Development of new medium-term bioassay for carcinogens. Cancer Res 1988;48:5051-4.
13. Ito N, Hasegawa R, Imaida K, Masui T, Takahashi S, Shirai T. Pathological markers for non-genotoxic agent-associated carcinogenesis Toxicol Letters 1992; 64/65:613-20.
14. Tatematsu M, Tsuda H, Shirai T, Masui T, Ito N. Placental glutathione-S-transferase (GST-P) as a new marker for hepatocarcinogenesis: In vivo short-term screening for hepatocarcinigens. Toxicol Pathol1987;15:60-8.
15. Ogiso T, Tatematsu M, Tamano S, Hasegawa R, Ito N. Correlaltion between medium term liver bioassay system data and results of long term technique in rat. Carcinogenesis 1990;11:561-6.
16. Anonymous. Overall evaulation of carcinogenicity: an updating of IARC monograph vols 1-42. In: IARC monographs on the evaulation of carcinogenic risks to humans. Suppl.7, IARC Scientific Publications, Lyon 1987.
17. Ashby J, Tennant RW. Definitive relationship among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the US NTP. Mutat Res 1991;257:229-306.
18. Qu YH, Lin JK. Biotransformation of butachlor through mercapturic acid pathway in rat tissue homogenate. J Toxicol Environ Health 1992;35:19-28.
19. Chasseaud LF. The role of glutathione and glutathione-s-transferase in metabolism of chemical carcinogens and other electrophilic agents. Adv Cancer Res 1979; 29:175-274.
20. Mannervik B, Alin P, Guthenberg C et al. Identification of three classes of glutathione transferase common to several mammalian species. Correlation between structure data and enzyme properties. Proc Natl Acad Sci USA1985; 82:7202-6.
21. Sato K. Glutathione-s-transferase and hepatocarcinogenesis. Jpn J Cancer Res 1988;79:556-77.
22. Okuda A, Imagawa M, Maeda Y, Sakai M, Muramatsu M. Structural and functional analyses of an enhancer GPEI having a phobol 12-O-tetradecanoate 13-acetate responsive element-like sequence found in the rat glutathione transferase P gene. J Biol Chem 1989;264:16919-26.

23. Imagawa M, Osada S, Koyama Y et al. SF-B that binds to a negative element in glutathione transferase P gene is similar or identical to transactivator LAP/IL 6-DBP. Biophys Res Commun 1991; 179: 293-00.
24. Tamai K, Shen H, Tsuchida S et al. Role of cysteine residues in the activity of rat glutathione transferasa p (7-7). Biochem Biophys Res Commun 1991; 179:790-7.
25. Moore MA, Nakagawa K, Satoh K, Ichigawa T, Sato K. Single GST-P positive liver cell-putative initiated hepatocytes. Carcinogenesis 1987;8:483-6.
26. Takahashi S, Tsutsumi M, Nakae D, Denda A, Kinugasa T, Konishi Y. Persistent effect of pre-administrated diethylnitrosamine on the induction of enzyme-altered foci in rat liver. Carcinogenesis 1987;8:509-13.
27. Kato T, Ogiso T, Kato K et al. Lack of promoting activity of four pesticides on induction of preneoplastic liver cell foci in rats. Teratogenesis Carcinogenesis & Mutagen 1995;15:251-7.
28. Tanaka H, Hasegawa R, Takesada Y, Ogiso T, Hirose M, Shirai T. Effects of carbamate insecticides in a rat medium term bioassay for hepatocarcinigens. J Toxicol Environ Health 1996;47:493-8.
29. Ito N, Imaida K, Tsuda H et al. Wide-spectrum initiation models: possible applications of medium-term multiorgan bioassay for carcinogenic modifiers. Jpn J Cancer Res 1988;78:413-7.
30. Fukushima S, Hagiwara A, Hirose M, Yamaguchi S, Tiwawech D, Ito N. Modifying effects of various chemicals on preneoplastic and neoplastic lesion development in a wide-spectrum organ carcinogenesis model using F344 rats. Jpn J Cancer Res 1991;82:642-9.



Alachlor **Butachlor**

Figure 1. Chemical structures of Alachlor and Butachlor

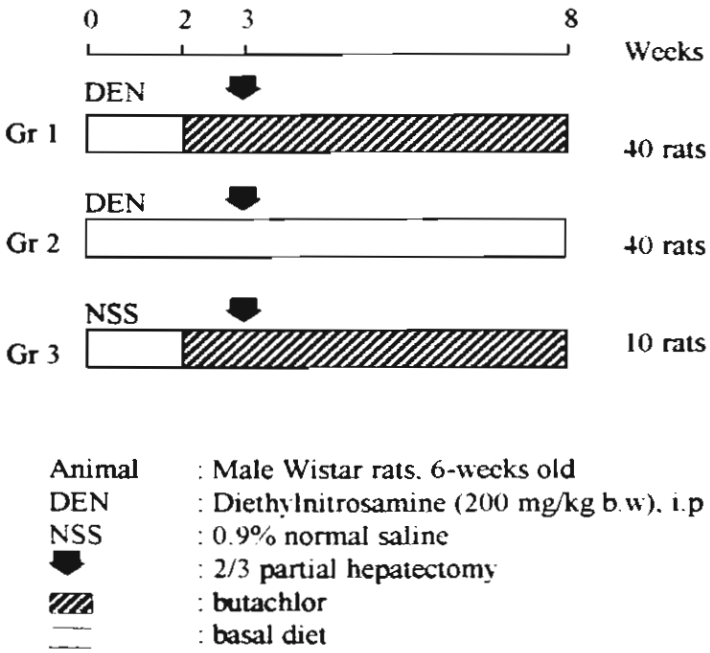


Figure 2 Experimental protocol

Table 1. Survival, body, liver and kidney weights of rats exposed to DEN and butachlor

Treatment	Body weight (mean±S.D.,g)				Liver weight (g)	Kidney weight (g)
	No. of rats	At start	No. of rats	At sacrifice		
1. DEN-butachlor	40	181.15±15.50	39	329.41±24.44 [a]	12.16±2.16	2.41±0.30 [b]
2. DEN-basal diet	40	184.76±15.00	37	364.54±29.75	11.42±1.40	2.21±0.25
3. NSS-butachlor	10	181.70±09.41	10	376.20±23.11	13.87±1.56	2.58±0.36

a, b, Significantly different from group 2 at $p < 0.000$, 0.0025 respectively

Table 2. Numbers and areas of glutathione-s-transferase placental form (GST-P) positive foci in rats treated with DEN and butachlor

Treatment	No. of rats	No. of GST-P (No./cm^2)		Area GST-P (mm^2/cm^2)	
		≥0.1 mm	≥0.2 mm	≥0.1 mm	≥0.2 mm
1. DEN- butachlor	39	13.99±10.90	4.31±3.78	0.36±0.31[a]	0.29±0.27 [b]
2. DEN-basal diet	37	18.25±10.51	6.18±4.59	o.67±0.52	0.48±0.41

a, b, Significantly different from group 2 at $p < 0.01$, 0.05 respectively