Medium-Term Bioassays for Carcinogenicity of Chemical Mixtures

Nobuyuki Ito,1 Katsumi Imaida,2 Masao Hirose,2 and Tomoyuki Shirai2

1Nagoya City University, Nagoya, Japan; 2Department of Pathology, Nagoya City University Medical School, Nagoya, Japan

Carcinogenic effects of chemical mixtures were examined with a medium-term liver bioassay for carcinogens or a multiorgan medium-term bioassay using male F344 rats. In the medium-term liver bioassay, rats were initially treated with diethylnitrosamine (DEN) at 200 mg/kg body weight, ip; after 2 weeks they received chemical mixtures such as 10 different heterocyclic amines at one-tenth or one-hundredth the dose levels used in carcinogenicity studies and the mixtures of 20 different pesticides, each at acceptable daily intake (ADI) levels or a mixture of 100 times ADI levels. All animals were subjected to two-thirds partial hepatectomy at week 3 and were sacrificed at week 8. The numbers and areas of glutathione S-transferase placental form (GST-P) positive foci (preneoplastic lesions in the liver) were compared between respective groups. When 10 heterocyclic amines were mixed in the diet at one-tenth dose level, clear synergism was observed, but no combined effects were evident with the one-hundredth dose levels. In the pesticide experiment, treatment of rats with the 20-pesticide mixture at the ADI dose level did not enhance GST-P-positive foci. In contrast, a mixture of 100 times the ADI significantly increased those values. In a multiorgan bioassay of 28 weeks, mixtures of 40 high-volume compounds and 20 pesticides (suspected carcinogens) added together at their respective ADI levels did not enhance carcinogenicity in any organs initiated by five different carcinogens (DEN, N-methylnitrosourea, dimethylnitrosamine, N-butyl-N-(4-hydroxybutyl)nitrosamine, and dihydroxy-di-n-propylnitrosamine) in combination. The combination effect of low dietary levels of five antioxidants, butylated hydroxyanisole, caffeic acid, sesamol, 4-methoxyphenol, and catechol, were also examined using the multiorgan bioassay. The incidence of forestomach papillomas was significantly increased only in the combination group and the results indicate that combination of the five antioxidants can exert additive/synergistic effects on tumorigenesis in the multiorgan bioassay. These results indicate that chemical mixtures at very low doses did not enhance preneoplastic lesions synergistically but the mixtures at certain doses show synergism in the target organ. The medium-term bioassays are particularly useful tools for this purpose.

Materials and Methods

Experiment I

Medium-Term Liver Bioassay for Carcinogens. Male F344 rats (group 1) were given a single ip injection of diethylnitrosamine (DEN) (200 mg/kg body weight) to initiate hepatocarcinogenesis; after a 2-week recovery period they received a test compound(s). The experimental protocol for the medium-term bioassay is shown in Figure 1. Groups 2 and 3 were given DEN alone and saline control test compound(s), respectively. All animals were subjected to two-thirds partial hepatectomy at week 3 and sacrificed at week 8. Liver splices were fixed in ice-cold acetone, embedded in paraffin, then immunohistochemically stained for glutathione S-transferase placental form (GST-P). Numbers and areas of GST-P-positive hepatic cell foci larger than 0.2 mm in diameter as well as the total areas of the liver sections examined were measured using a color video image processor. Quantitative values for GST-P positive foci were compared between the chemical-treated group and the control group. We consider this model eminently suitable for evaluation of combined effects of several carcinogenic agents may act in combination to induce cancers. Therefore, in addition to detecting carcinogens in our environment, examination of low-dose combination effects of such agents is an important area for research to evaluate human cancer risk.

Many combinations and scenarios are possible with exposure to multiple chemicals, depending on the mode of application and whether administration of carcinogenic and/or noncarcinogenic agents is simultaneous or sequential. The two-stage carcinogenesis hypothesis, now generally accepted as a basic theory of carcinogenesis, assumes sequential exposure to initiating agents and modifiers acting to promote the process of neoplasia.

To bridge the disadvantages of long-term carcinogenicity tests and in vitro mutagenicity assays, medium-term bioassays using preneoplastic lesions as end point markers have been proposed. A medium-term liver bioassay for carcinogens and a multiorgan bioassay can be used for detecting the effect of chemical mixtures at low dose levels, as well as detection of the carcinogenic potential of individual test chemicals.

Key words: medium-term liver bioassay for carcinogens, mixture, combination, heterocyclic amines, pesticide, antioxidant

---

Our environment contains a great variety of carcinogenic factors including naturally occurring and synthetic carcinogens, radiation, and viruses, all of which have been speculated to play major roles in the etiology of human cancers (1). Because humans are exposed concurrently or sequentially to a large variety of environmental carcinogens at very low individual doses over their lifetime, a strong possibility exists that
agents (2). Statistical analysis of differences between means was carried out using the Student’s t-test or the Welch’s t-test after application of the preliminary F-test for equal variance for each pair. For proportion data, the Fisher exact probability test was used. To determine whether the combined treatments acted additively or synergistically, one-tail analysis by the modified Colton’s z-test was carried out (3,4).

Experiment I-A
Low-Level Exposure to a Mixture of Heterocyclic Amines. Heterocyclic amines produced in foods under normal cooking conditions are highly mutagenic and induce carcinogenicity in rodents (5). Humans are chronically exposed to mixtures of these agents simultaneously at low dose levels in the diet (6). Therefore, potential synergism of 10 heterocyclic amines at various low doses was evaluated (2,7). The heterocyclic amines used and each carcinogenic dose (full dose) were 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole ([Trp-P-1] 150 ppm), 2-amino-9H-pyrido[2,3-b]indole ([Trp-P-2] 500 ppm), 2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline ([IQ] 300 ppm), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline ([MeIQ] 300 ppm), 2-amino-6-methylidipyrido[1,2a:3’,2’-d]imidazol ([Glu-P-1] 500 ppm), 2-amino-3-methyl-9H-pyrido[2,3-b]indol ([MeAoxC] 800 ppm), 2-amino-9H-pyrido[2,3-b]indol ([AtCoxC] 800 ppm), and 2-amino-1-methyl-5H-pyridine ([PhIP] 400 ppm). Purities of heterocyclic amines were > 99.5% and all 10 heterocyclic amines were added in the diet at dose levels of one-tenth or one-hundredth of the dose that was used in the carcinogenicity study of individual chemicals (3,7). The results were compared with those from groups given each chemical at the one-tenth dose level.

Experiment I-B
Low-Level Exposure to a Mixture of Twenty Pesticides. Male F344 rats 6 weeks of age were used. Concentrations of pesticides in the diet (milligrams per kilogram diet) were calculated based on reported food intake and body weight data (8). Food and water were available ad libitum.

Dose selection of each pesticide was decided on the base of the acceptable daily intake (ADI) value. The ADI values were proposed by the Ministry of Health and Welfare, Japan, with reference to the Joint Food and Agricultural Organization (FAO)/World Health Organization (WHO) Meeting on Pesticide Residues report (9) (Table 1). The pesticides investigated, along with concentrations in the diet, their purity, and ADIs (milligrams per kilogram body weight per day) are listed in Table 1. All pesticides examined were organophosphates, except for endosulfan.

The animals were initially given a single ip injection of DEN. After a 2-week recovery period, the rats received the pesticides either at the ADI (mixture one, group 1-A) or at 100 times higher doses (group 1-B) or were maintained on the basal diet throughout the experiment (group 2). Groups 3-A and 3-B were injected with saline and then the mixture one at the ADI and 100 times the ADI, respectively. All animals were subjected to two-thirds partial hepatectomy at week 3 and sacrificed at week 8. Liver slices were stained for GST-P. Numbers and areas of GST-P-positive hepatic cell foci larger than 0.2 mm in diameter and the total areas of liver sections examined were measured using a video image processor.

Table 1. Data for the 20 pesticides used in experiment 1-B.

| Pesticide     | CAS no. | Chemical class | Use | ADI, mg/kg bw/day | FAO report, year | Present experiment | Concentration in diet for the ADI level, ppm |
|---------------|---------|----------------|-----|-------------------|------------------|--------------------|--------------------------------------------|
| Acephate      | 30560-19-1 | OP             | IN  | 0.03              | 1990             | 99.3               | 0.3                                       |
| Butamifos     | 36335-67-8 | OP             | HB  | 0.0016            | 1971             | 97.9               | 0.016                                     |
| Chlorfenvinfos | 470-90-6  | OP             | IN, AC | 0.0015c           | 1989             | 93.3               | 0.015                                     |
| Chlorpyrifos  | 2921-88-2 | OP, HC         | IN  | 0.01              | 1993             | 99.3               | 0.1                                       |
| Dichlorvos    | 62-73-7   | OP             | IN, AN | 0.0033c           | 1987             | 98.9               | 0.033                                     |
| Dimethoate    | 60-51-5   | OP             | IN, AC | 0.01              | 1981             | 99.0               | 0.1                                       |
| Edifenphos    | 17109-49-8 | OP             | FU  | 0.0025f           | 1988             | 98.0               | 0.06                                     |
| Endosulfan    | 115-29-7  | OC, HC         | IN, AC | 0.006             | 1996             | 94.0               | 0.03                                     |
| Etrimfos      | 38260-54-7 | OP, HC         | IN, AC | 0.003             | 1998             | 96.7               | 0.05                                     |
| Fenitrothion  | 112-14-5  | OP             | IN  | 0.005             | 1997             | 99.0               | 0.03                                     |
| Iprobenfos    | 26087-47-8 | OP             | FU  | 0.003             | 1998             | 94.9               | 0.03                                     |
| Isoxathion    | 18854-01-8 | OP             | IN  | 0.003             | –                | 95.2               | 0.03                                     |
| Malathion     | 121-75-5  | OP             | IN, AC | 0.02              | 1996             | 96.6               | 0.2                                       |
| Methidathion  | 950-37-8  | OP, HC         | IN, AC | 0.001             | 1992             | 92.04              | 0.01                                     |
| Pirimiphos-methyl | 29232-33-7 | OP, HC       | IN, AC | 0.01f             | 1992             | 99.7               | 0.1                                       |
| Prothiophos   | 34643-46-4 | OP             | IN  | 0.0015           | –                | 94.7               | 0.015                                     |
| Pyraclofos    | 77458-01-6 | OP             | IN  | 0.001             | –                | 98.4               | 0.01                                     |
| Tocilcos-methyl | 57018-04-9 | OP             | FU  | 0.064             | –                | 99.5               | 0.64                                     |
| Trichlorfon   | 52-68-9   | OP, OC         | IN  | 0.01              | 1979             | 99.0               | 0.1                                       |
| Vanidochlor    | 2275-23-2  | OP             | IN, AC | 0.008             | 1998             | 99.0               | 0.08                                     |

Abbreviations: AC, acaricide; AN, anthelmintic drug; FU, fungicide; HC, heterocyclic; OC, organochlorine; OP, organophosphate/organothiophosphate; HB, herbicide; IN, insecticide. ADI levels were provided by the Ministry of Health and Welfare, Japan (9), except for dimethoate, endosulfan, and methidathion, for which data were cited from FAO/WHO (9). *Lower than the ADI levels from FAO/WHO (9).
Experiments

Experiment II

Multiorgan Bioassay. To detect carcinogenic or modifying potentials of test chemicals in multiple organs as well as the liver for the medium-term period, we also developed a medium-term multiorgan bioassay (DMBDD model) (4, 10). Figure 2 shows the experimental protocol of the multiorgan medium-term bioassay. As initiation, five known potent carcinogens were given in combination within the first 4 weeks: a single ip injection of DEN at a dose of 100 mg/kg body weight at the start of the experiment; four ip injections of N-methyl-N-nitrosourea (MNU) at a dose of 20 mg/kg body weight on days 2, 5, 8, and 11, and four sc injections of 1,2-dimethylhydrazine (DMH) at a dose of 40 mg/kg body weight on days 14, 17, 20, and 23, 500 mg/l N-buty1-N-(4-hydroxybutyl)nitrosamine (BBN) in the drinking water during weeks 1 and 2, and 1000 mg/l 2,2’-dihydroxy-di-n-propylbutynitrosamine (DHPN) in the drinking water during weeks 3 and 4. After this DMBDD treatment, rats received test chemicals for 24 weeks. Noninitiation controls were injected ip with saline and subcutaneously with corn oil and then given pesticide(s). At week 28 of the experiment, all surviving animals were sacrificed and completely autopsied. Livers were analyzed for GST-P-positive foci as described in Experiment I. The main organs and any macroscopic lesions were removed and fixed in formalin. The routinely prepared hematoxylin and eosin sections were examined for neoplastic and preneoplastic lesions.

Experiment II-A

Mixture of Twenty or Forty Pesticides. Pesticides selected for the mixtures were 40 chemicals of high-volume production (mixture one) and 20 chemicals for which carcinogenicity has been reported or suspected (mixture two). The pesticides and concentrations (milligrams per kilogram diet) in mixture one were acephate (0.3), bendicarb (0.04), benisulide (0.4), bentazone (0.9), chinomethionat (0.06), chlorbenzilate (0.2), chlorpropam (1), chlorpyrifos (0.1), clofentezine (0.086), cyfluthrin (0.2), cyhalothrin (0.085), cypermethrin (0.5), diflubenzuron (0.12), fenarimol (0.1), fenbutil oxide (0.3), fenvalerate (0.2), flucythrinate (0.125), flutolanil (0.8), glyphosate (1.5), imazalil (0.25), malathion (0.2), maneb (0.05), meipiquar chloride (0.75), metalaxyl (0.19), metolachlor (0.97), metribuzin (0.125), myclobutanil (0.12), oxamyl (0.2), pendimethalin (0.43), permethrin (0.48), pirimiphos-methyl (0.1), propiconazole (0.18), pyrifoxin (1), quinolacor (0.29), sethoxydim (1.4), thiobencarb (0.09), triadimefon (0.12), trichlorfon (0.1), vinclozolin (1.215), and zineb (0.05) Pesticides and concentrations in mixture two (milligrams per kilogram diet) were acephate (0.3), amitriz (0.012), captalof (0.5), clofentezine (0.086), cypermethrin (0.5), 2,4-dichlorophenoxyacetic acid (3), dichlorvos (0.033), dichlobenil (0.04), diochlor (0.25), fosetyl (8.8), glyphosate (1.5), mancozeb (0.5), maneb (0.05), mefolachlor (0.97), permethrin (0.48), phosmet (0.2), propiconazole (0.18), propoxur (0.63), triadimefon (0.12), and trifluralin (0.075).

Possible modifying effects of these pesticide mixtures on tumorigenesis were investigated using the DMBDD model. After the DMBDD treatment, groups of rats received one of the pesticide mixtures (group 1 received mixture one and group 2 received mixture two), captalof (1500 mg/kg in the diet) as a positive control (group 3) (11), or the basal diet (group 4) for 24 weeks. Rats were sacrificed after these treatments and multiple organs were analyzed as described in Experiment I.

Experiment II-B

Mixture of Five Antioxidants in a Multiorgan Bioassay. The carcinogenicity of low dietary levels of the antioxidants butylated hydroxyanisole (BHA), caffeine acid, sesamol, 4-methoxyphenol (4-MP), and catechol, known to target the forestomach or glandular stomach, were examined alone or in combination in a 2-year long-term experiment and their modifying effects assessed in a medium-term multiorgan bioassay model. In the carcinogenicity study, groups of 30 to 31 male F344 rats were treated with 0.4% BHA, 0.4% caffeic acid, 0.4% sesamol, 0.4% 4-MP, and 0.16% catechol either alone or in combination for up to 104 weeks and then sacrificed. In the medium-term bioassay, groups of 10 to 15 male F344 rats were given DEN, MNU, DMH, BBN, and DHPN for a total multiple initiation period of 4 weeks. BHA, caffeic acid, sesamol, and 4-MP, each at doses of 0.4 or 0.08%, and catechol at doses of 0.16 or 0.032%, were administered in the diet either alone or in combination after completion of the initiation regimen. All surviving animals were sacrificed at the end of week 28, and major organs were examined histopathologically.

Results

Experiment I-A

When 10 heterocyclic amines were mixed in the diet at the one-tenth dose level, clear synergism was observed, but no combined effects were evident with the one-hundredth dose levels (Table 2) (3). The fact that synergism clearly was observed at relatively low dose levels is important because mixtures of these heterocyclic amines may be generated in cooked food. Although individual compounds might be without obvious effects on carcinogenicity, they may present a hazard risk in combination at the levels present in food.

Experiment I-B

Data on the numbers and areas of GST-P-positive foci per unit area of liver section with and without DEN initiation are compared in Table 3. The number of GST-P-positive foci in group 1-A was 3.36 ± 1.29/cm² and the area of foci was...
Table 3. Quantitative data for GST-P-positive liver cell foci in treated rats (experiment I-B).

| Group | Treatment            | Effective no. of rats | GST-P-positive foci |
|-------|----------------------|-----------------------|---------------------|
|       |                      |                       | No./cm²             | Area, mm²/cm² |
| 1     | DEN–ADI mixture      | 19                    | 3.36 ± 1.29         | 0.29 ± 0.15  |
| 2     | DEN 100×ADI mixture  | 18                    | 4.51 ± 1.64*        | 0.44 ± 0.20**|
| 3     | DEN–basal diet       | 19                    | 3.50 ± 1.29         | 0.28 ± 0.13  |
| 4     | None–ADI mixture     | 10                    | 0                   | 0            |
| 5     | None 100×ADI mixture | 9                     | 0                   | 0            |

***Significantly different from the relevant group value at p < 0.05 and p < 0.01, respectively.

0.29 ± 0.15 mm²/cm². The levels were essentially the same as those in the control group (3.50 ± 1.29/cm² and 0.28 ± 0.13 mm²/cm²). However, the values obtained in the 100 times higher ADI mixture group (group 1-B) (4.51 ± 1.64/cm² and 0.44 ± 0.20 mm²/cm²) were both significantly higher than the control values. Without the DEN initiation, neither of the treatment schedules induced GST-P-positive liver cell foci larger than 0.2 mm in diameter (groups 3-A and 3-B).

**Experiment II-A**

In the liver, development of GST-P-positive foci was increased by captafol but not modulated by the mixtures (Figure 3). In the other organs, captafol showed promotion effects in the thyroid, whereas the pesticide mixtures did not influence the neoplastic development in any organ. No neoplastic and preneoplastic lesions were observed in noninitiated groups (groups 3-A to 3-C) (Table 4).

**Experiment II-B**

In the carcinogenicity study, slightly increased incidences of forestomach papillomas were found in the sesamol- (15.8%), caffeic acid- (14.8%), catechol- (3%), and 4-MP- (11.5%) treated groups as compared to the basal diet group (0%); a significant increase was observed with the five antioxidants in combination (42.9%, p < 0.001) (Table 5). In the medium-term multiorgan bioassay, incidences of forestomach papillomas and/or carcinomas were increased in

Table 4. Incidence of tumors (experiment II-A).^a

| Organ and type of tumors | 40 pesticides | 20 pesticides | Captafol | Basal diet |
|-------------------------|---------------|---------------|----------|------------|
| Thyroid                 |               |               |          |            |
| Follicular adenoma      | 2             | 6             | 9*       | 2          |
| C-cell adenoma          | 1             | 1             | 0        | 0          |
| Nasal cavity            |               |               |          |            |
| Papilloma               | 1             | 0             | 0        | 0          |
| Odontoma                | 0             | 1             | 0        | 0          |
| Lung                    |               |               |          |            |
| Adenoma                 | 4             | 5             | 3        | 5          |
| Carcinoma               | 1             | 1             | 0        | 2          |
| Oral cavity             |               |               |          |            |
| Odontoma                | 0             | 2             | 0        | 0          |
| Esophagus               |               |               |          |            |
| Squamous cell carcinoma | 0             | 0             | 1        | 0          |
| Forestomach             |               |               |          |            |
| Squamous cell papilloma | 3             | 8             | 2        | 4          |
| Squamous cell carcinoma | 0             | 1             | 1        | 0          |
| Small intestines        |               |               |          |            |
| Adenoma                 | 3             | 1             | 2        | 2          |
| Adenocarcinoma          | 0             | 2             | 2        | 1          |
| Large intestines        |               |               |          |            |
| Leiomyoma               | 0             | 1             | 0        | 0          |
| Adenocarcinoma          | 2             | 2             | 6        | 4          |
| Liver                   |               |               |          |            |
| Hyperplastic nodule     | 1             | 0             | 1        | 1          |
| Kidney                  |               |               |          |            |
| Nephroblastoma          | 2             | 4             | 7        | 2          |
| Transitional cell carcino| 0             | 1             | 0        | 0          |
| Urinary bladder         |               |               |          |            |
| Transitional cell papilloma | 0           | 1             | 0        | 1          |
| Prostate                |               |               |          |            |
| Leiomyosarcoma          | 0             | 1             | 0        | 0          |
| Sarcoma                 | 1             | 1             | 0        | 0          |
| Skin/subcutis           |               |               |          |            |
| Squamous cell papilloma | 0             | 1             | 0        | 0          |
| Lipoma                  | 0             | 1             | 0        | 0          |
| Abdominal cavity        |               |               |          |            |
| Mesothelioma            | 0             | 0             | 1        | 0          |
| Peripheral nerve        |               |               |          |            |
| Malignant schwannoma    | 0             | 1             | 0        | 0          |

^a A few tumors were observed only in the control group: thymic lymphoma (thymus), follicular carcinoma (thyroid), adenocarcinoma (nasal cavity), adenoma (seminal vesicle), keratoacanthoma (skin), schwannoma (peripheral nerve). No neoplastic lesions were found in the noninitiated groups. Number of animals in groups of 40 and 20 pesticides, captafol, and basal diet were 20, 20, 19, and 20, respectively. *Significantly different from control group at p < 0.05.
Table 5. Histopathologic findings in the forestomach of rats treated with DMNDD followed by antioxidants either alone or in combination (experiment II-B).

| Treatment              | Rats, no. | PN hyperplasia | Incidence, % | Multiplicity, no./slide | Papilloma | Incidence, % | Multiplicity, no./slide | Carcinoma incidence, % |
|------------------------|-----------|----------------|--------------|-------------------------|-----------|--------------|-------------------------|-------------------------|
| Combination            | 28        |                | 17 (61)**    | 1.04±0.105**           | 12 (43)** | 0.93±1.31**  | 1 (4)                   |                         |
| BHA                    | 26        |                | 0            | -                       | 0         | -            | 0                       |                         |
| 4-Methoxyphenol        | 26        |                | 8 (31)*      | 0.31±0.46               | 3 (12)    | 0.19±0.62    | 0                       |                         |
| Caffeic acid           | 27        |                | 17 (63)**    | 1.11±1.13**            | 4 (15)    | 0.19±0.47*   | 0                       |                         |
| Sesamol                | 19        |                | 5 (26)       | 0.37±0.67               | 3 (16)    | 0.21±0.52    | 0                       |                         |
| Catechol               | 29        |                | 6 (21)       | 0.69±2.73              | 1 (3)     | 0.03±0.18    | 0                       |                         |
| Basal diet             | 25        |                | 1 (4)        | 0.08±0.39               | 0 (4)     | -            | 0                       |                         |

PN, papillary or nodular. * **Significantly different from basal diet group at p<0.05 and p<0.01, respectively.

Discussion

In the liver model, the ADI mixture of organophosphorus pesticides exerted no effects on development of liver preneoplastic foci initiated by DEN, although the 100 times higher dose demonstrated lesion-promoting potential (8, 13, 14). In the multiorgan model, the ADI mixtures of 40 (mixture two) or 20 (mixture three) pesticides demonstrated no tumor-promoting potential in any organ or tissue (13, 14). Captafol, on the other hand, exerted apparent tumor-promoting effects in the liver, thyroid, and kidney, although the dose level was not comparable to the mixtures. The protocol has been developed in our laboratory over the last 15 years (10). Quantitative analysis of GST-P-positive foci has been established. The multiorgan method has been developed to supplement the liver model and is also a useful method for rapid detection of carcinogens at the whole-body level (4, 10).

With a safety factor approach, acceptable exposure levels such as ADIs are usually determined by dividing the no observed effect level from laboratory-based chronic toxicity tests by an appropriately chosen safety factor. The safety factor used for ADI by the Japanese Ministry of Health and Welfare and the FAO/WHO is usually 100, but WHO expert committees have used figures ranging from 10 to 2000 (15). Our experimental results indicate that this procedure is indeed appropriate and acceptable for risk evaluation at present. Furthermore, the chance of exposure to so many pesticides (20 or 40 chemicals) in concert might in practice be low (16).

Because most human cancers may be caused by trace environmental factors, it is of increasing importance that combined effects of chemicals at relatively low doses be examined. The medium-term bioassays used in these studies are particularly useful methods for this matter.

References and Notes

1. Doll R. Urban and rural factors in the aetiology of cancer. Int J Cancer 47:803–810 (1991).
2. Ito N, Shirai T, Hasegawa R. Medium-term bioassays for carcinogens. In: Mechanisms of Carcinogenesis in Risk Identification (Vainio H, Magee P, McGregor DB, McMichael AJ, eds). IARC Sci Publ No 116. Lyon:International Agency for Research on Cancer, 1992:353–388.
3. Hasegawa R, Tanaka H, Tamano S, Shirai T, Nagao M, Sugimura T, Ito N. Synergistic enhancement of small and large intestinal carcinogenesis by combined treatment of rats with five heterocyclic amines in a medium-term multi-organ bioassay. Carcinogenesis 15:2567–2573 (1994).
4. Hasegawa R, Miyata E, Futakuchi M, Hagihara A, Nagao M, Sugimura T, Ito N. Synergistic enhancement of hepatic foci development by combined treatment of rats with 10 heterocyclic amines at low doses. Carcinogenesis 15:1037–1041 (1994).
5. Wakanayashi K, Nagao M, Esumi H, Sugimura T. Food-derived mutagens and carcinogens. Cancer Res 52:2092S–2098S (1992).
6. Sugimura T. Food as source of complex mixture of mutagens and carcinogens. In: Complex Mixtures and Cancer Risk (Vainio H, Sorsa M, McMichael AJ, eds). IARC Sci Publ No 104. Lyon:International Agency for Research on Cancer, 1990:399–407.
7. Hasegawa R, Shirai T, Hako K, Wada S, Yamaguchi K, Takayama S. Synergistic enhancement of thyroid tumor induction by 2,4-diaminoazobenzene sulfate, NN'-diethythioleurea and 4,4'-thiodiannilnine in male F344 rats. Carcinogenesis 12:1515–1518 (1991).
8. Ito N, Hasegawa R, Imaida K, Kurata Y, Hagiwara A, Shirai T. Effects of ingestion of 20 pesticides in combination at acceptable daily intake levels on rat liver carcinogenesis. Food Chem Toxicol 33:159–163 (1995).
9. FAO/WHO. International Programme on Chemical Safety. Summary of Toxicological Evaluations Performed by the Joint FAO/WHO Meeting on Pesticide Residues [JMPR]. Geneva:World Health Organization, 1993.
10. Ito N, Tsuda H, Hasegawa R, Tatetatsu M, Imaida K, Asamoto M. Medium-term bioassay models for environmental carcinogens—two-step liver and multi-organ carcinogenesis protocols. In: Biologically-based Methods for Cancer Risk Assessment (Travis CC, ed.) New York:Plenum Publishing, 1989:209–230.
11. Tamano S, Kurata Y, Kawabe M, Yamamoto A, Hagiwara A, Cabral R, Ito N. Carcinogenicity of captafol in F344/DuCrj rats. Jpn J Cancer Res 81:1222–1231 (1990).
12. Hirose M, Takesada Y, Tanaka H, Tamano S, Kato T, Shirai T. Carcinogenicity of antioxidants BHA, caffeic acid, sesamol, 4-methoxyphenol and catechol at low doses, either alone or in combination, and modulation of their effects in a rat medium-term multi-organ carcinogenesis model. Carcinogenesis 19:207–212 (1997).
13. Ito N, Hagihara A, Tamano S, Hasegawa R, Imaida K, Hirose M, Shirai T. Lack of carcinogenicity of pesticide mixtures administered in the diet at acceptable daily intake (ADI) dose levels in rats. Toxicol Lett 82/83:513–520 (1995).
14. Ito N, Hagiwara A, Tamano S, Futakuchi M, Imaida K, Shirai T. Effects of pesticide mixtures at the acceptable daily intake levels on rat carcinogenesis. Food Chem Toxicol 34:1091–1096 (1996).

15. Lu FC, Sielken RL. Assessment of safety/risk of chemicals: inception and evolution of the ADI and dose-response modeling procedures. Toxicol Lett 59:5–40 (1991).

16. Yang RSH. Strategy for studying health effects of pesticides/fertilizer mixtures in groundwater. Rev Environ Commun Toxicol 127:1–22 (1992).