

An Overview of Prechronic and Chronic Toxicity/Carcinogenicity Experimental Study Designs and Criteria Used by the National Toxicology Program

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Since the establishment of the National Toxicology Program (NTP), there have been gradual changes in strategies to evaluate the overall toxicity of chemicals as well as their carcinogenic potential. The spectrum of toxicologic information sought on selected chemicals has been broadened by the multidisciplinary approach to evaluating chemicals. This paper describes the scientific rationale and experimental processes used by NTP in designing studies. Also, an outline of current NTP protocols are given for prechronic and chronic toxicity/carcinogenicity studies.

Introduction

The National Toxicology Program (NTP) was established in November 1978 by the Secretary of the Department of Health and Human Services (DHHS). The primary rationale was to better integrate DHHS activities and resources concerned with determining the toxicologic potential of chemicals and to establish a more effective dialogue between the health research and regulatory agencies, enabling stronger links between the health research and regulatory needs. Four specific goals of NTP continue to broaden the spectrum of toxicologic information obtained on chemicals selected; increase the number of chemicals evaluated within resource limits; develop and validate a series of assays and protocols appropriate for regulatory needs; and communicate the plans and results to governmental agencies, the medical and scientific communities, and the public (1).

Since the establishment of the NTP, there have been gradual changes in strategies to evaluate the toxicity and carcinogenic potential of chemicals. The spectrum of toxicologic information sought on selected chemicals has been broadened by the multidisciplinary approach to

evaluate chemicals. The expertise in general toxicology, genetics, reproduction, pathology, chemistry, clinical pathology, animal care, immunology, statistics, and biochemical and molecular toxicology each play a major role in identifying toxic and carcinogenic properties of chemicals. A number of communications representing NTP efforts in meeting its goals have appeared in the literature (2-16).

The objectives of this paper are to present an overview of the scientific rationale involved in designing toxicology and carcinogenesis studies and to outline the experimental protocols used by the NTP in conducting these studies. The studies designed by the NTP are planned to provide maximal toxicology information on chemicals selected.

Toxicological Evaluation Process

Figure 1 diagrams the general sequence of events from chemical selection to toxicological characterization. Once a chemical is identified as an NTP priority chemical, the process of designing studies begins. The methods established and announced widely by the NTP for nominating, selecting, and designing studies give ample opportunity to industry, nominating/regulatory agencies, and the public to influence the final outcome of study designs (1). This process has resulted in developing studies and protocols that are used to advance the science and for regulatory purposes as well as developing a toxicity data base on chemicals. In general, these

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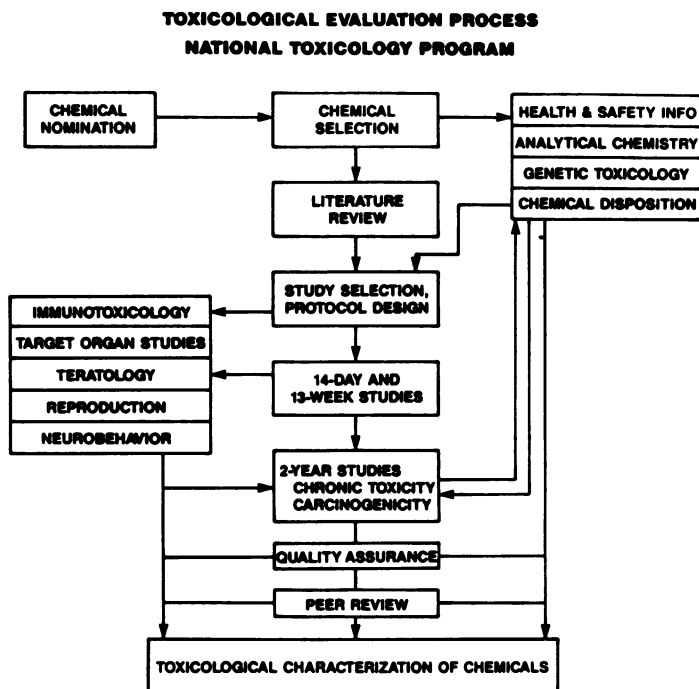


FIGURE 1. Toxicological evaluation process, National Toxicology Program.

studies are accomplished in two phases: prechronic or short-term studies and chronic or longer-term toxicity/carcinogenicity studies. Because of staff and laboratory space limitations these studies are ordinarily conducted at private laboratories and at national laboratories that have been approved by the NTP to perform such studies. The prechronic studies usually consist of those lasting 14 days and 90 days. Because few laboratories can perform studies in all disciplines, genetic toxicology, metabolism/disposition, immunotoxicology, and reproductive/developmental toxicology studies are conducted in different laboratories that are experienced and specialized in these areas. To the maximum extent possible data obtained from these labs, along with the prechronic toxicology data, are available in designing and interpreting the chronic toxicity/carcinogenicity studies.

After the 14-day and 90-day studies are completed, scientific decisions are made on whether or not a chronic study is required on a chemical. Some of the criteria used to justify a chronic toxicity/carcinogenicity study for a chemical are as follows: production for a chemical and human exposure levels, epidemiology studies indicative of the association of chemicals with increased incidence of cancer in human population, results of a prechronic toxicity study showing nonneoplastic lesions that could progress to neoplastic lesions during chronic exposure, chemicals that are structurally related to carcinogens, and a need for such studies by the nominating/regulatory agencies. Currently, nearly 50% of prechronic studies are followed by 2-year chronic toxicity/carcinogenicity studies.

General Considerations for Designing Studies

The NTP evaluates a large variety of chemicals for toxicity/carcinogenicity. The chemicals may be used in agriculture, manufacturing and pharmaceutical industries, or they may be food additives or occur as environmental contaminants. Designing studies for such diverse classes of chemicals requires a flexible approach and avoidance of rigid generic protocols. The NTP has adopted core study protocols in terms of species, strains, group size, and number of dose groups, and duration of exposure for prechronic and chronic studies. However, the toxicity end points studied for chemicals evaluated depend on a number of factors such as use patterns of the chemical, anticipated biological effects based on structural similarity to known classes of chemicals, and mechanism(s) of toxicity of chemicals in the same general class. Following are some of the major scientific and practical considerations in designing toxicology studies.

Background Information

A thorough knowledge is essential for background information on the chemicals selected for toxicity/carcinogenicity evaluation. To accomplish this the study toxicologist evaluates all information in the literature, interacts with the individual or agency nominator(s), and consults with manufacturer(s) of the chemical and representative regulatory agencies and other scientists. This background information generally includes physical and chemical properties of the chemical, production levels, human exposure data, results from previously conducted mutagenicity and short-term tests, metabolism/pharmacokinetics studies, teratogenicity, and reproductive toxicity studies. Background material also includes known information relative to carcinogenicity, epidemiology, mechanism of toxicity, neurobehavioral toxicity, immunotoxicity, and other target-organ toxicity. Based on the extent and adequacy of this information, strategies are developed for evaluating chemical toxicity/carcinogenicity, and important areas of toxicology needing more work are identified. Significant portions of the study design reflect an effort to fill these data gaps when appropriate. Table 1 gives the information on studies designed for 98 chemicals as an example of this effort.

Whenever possible, during the conduct of 90-day toxicity studies and where appropriate, genetic toxicity, metabolism/disposition, hematology, and reproductive/

Table 1. Types of studies designed on 98 nominated and selected chemicals.

Study	Number
14-day studies	69
90-day studies	75
Genetic toxicology	78
Metabolism/disposition	26
Immunotoxicology	13
Reproductive/developmental toxicity	24
Chronic toxicity/carcinogenicity	39

developmental toxicity studies are performed in parallel. Researchers should have results from these studies available before chronic studies are designed. The toxicity end points incorporated in 90-day studies are determined on a case-by-case basis for individual chemicals; Table 2 shows the frequency of toxicity end points incorporated into 75 recent 90-day studies. This table shows that selection of toxicity end points depends on the background information available on a particular chemical.

Table 3 gives key features of study design for 39 chemicals, further emphasizing the approach to evaluating chemical toxicity/carcinogenicity based on determining what additional information is needed for a chemical to best fill data gaps and, importantly, to best protect public health. The carcinogenicity studies generally consist of three exposure groups plus a control. These four groups are used to better define the dose response relationship that aids in the hazard assessment of chemicals having carcinogenic potential in humans (17,18). Each dose group consists of a minimum of 50 animals per species/sex, which is considered optimum by the International Agency for Research on Cancer (IARC) (19) and most others involved in these studies.

The adequacy of group size for carcinogenicity studies has also been dealt with in the report by the NTP Ad Hoc Panel (20). Each of the 39 NTP carcinogenicity studies

Table 2. Some features of 90-day studies.

Feature	Number
Sperm morphology and vaginal cytology	61
Hematology	58
Clinical pathology	61
Micronuclei in mouse erythrocytes	40
Urinalysis	29
Biochemical indices	21
Neurobehavioral evaluations	6
Routes of exposure	
Feed	30
Inhalation	21
Drinking water	6
Dermal	4
Gavage	3
Intraperitoneal	1
Comparison of routes (more than one route)	10

Table 3. Some features of 39 two-year toxicity/carcinogenicity studies.

Two-year studies at least have three dose levels plus controls
Three of these 39 studies were designed with more than three dose groups
All studies had additional groups from animals to be evaluated for toxicity and possible carcinogenicity at week 65
Fifteen studies had two or more interim evaluations
Seven studies had stop exposure studies
All designed for both sexes of two rodent species
All performed under extended good laboratory practices
All performed under strict health and safety standards
Routes of exposure
Feed, 12 studies
Gavage, 9 studies
Drinking water, 7 studies
Inhalation, 10 studies
Dermal, 1 study

designed had at least one interim evaluation group of 10 animals/sex at week 65. The purpose of interim evaluation is to identify chronic toxic effects of the chemical as well as any late appearing toxic effects that may help in changing the course of studies, if needed. In some cases, neoplasia is observed. Currently NTP is reviewing the use of the week 65 interim evaluation to determine if it is useful to have either a routine interim evaluation or to be selective in incorporating evaluation group(s) to carcinogenicity studies. Table 3 also shows that 15 studies had two or more interim evaluations at different time intervals. Again, the purpose was to establish relationships between the time of exposure and possibility of late-appearing lesions. Seven studies had chemical exposure of the animals stopped (stop exposure) at a specific time interval during the studies; regression/progression of specific lesions was followed up to the termination of experiment (2 years). Usually these lesions are first identified during 90-day studies.

Multidisciplinary Approach

Input from several scientific disciplines (e.g., toxicology, chemical disposition, immunotoxicity, pathology, genetic toxicology, laboratory animal management, chemistry, health and safety, statistics, etc.) is incorporated into the study design. The usefulness of toxicology procedures are critically reviewed by the various disciplines before they are incorporated into study designs. The toxicology procedures are continuously improved and validated by the NTP and contract laboratories. Recent publications on the development of a battery of assays for assessing chemical-induced immunotoxicity (15) and measurement of behavior indices of neurotoxicity are some of the examples for improvement of procedures (16,21).

Experimental Animals

The toxicology and carcinogenesis studies are conducted in rats and mice. Fischer 344 rats and B6C3F₁ (C57BL/6N × C3H/HEN^{MTV}-) mice are the selected experimental animals for carcinogenesis studies and will continue to be used for NTP prechronic and chronic toxicity/carcinogenicity studies until data show that other strains/species would be more relevant. The historical background on selection of these animals has been reviewed by Weisburger (22). Goodman et al. (23) have reviewed in depth the advantages and disadvantages of Fischer 344 rats and B6C3F₁ mice as compared to other strains of rats and mice as experimental animals for carcinogenesis studies. Their review suggests that, at present, Fischer 344 rats and B6C3F₁ mice should remain as the experimental animals of choice for long-term studies. In addition, NTP has the valuable and extensive historical control data base for these species.

An ad hoc panel on chemical carcinogenesis testing and evaluation reviewed NTP toxicity and carcinogenicity evaluation procedures (20). One of the recommendations of that panel was "if a determination is made to maintain a two species bioassay protocol, give serious

consideration to replacement of the B6C3F₁ mouse with a strain having an established lower and less variable spontaneous incidence of important tumors that are induced by chemicals. In addition, continued investigation of the use of other species as adjunct or replacement for the one now in use should be undertaken." To address the first part of the recommendation, the NTP formed an ad hoc committee and organized a workshop on Strains of Mice for Chemical Carcinogenicity Studies (24). The participants in that workshop reached a consensus to "continue to use the B6C3F₁ hybrid because of the extensive experience and historical data base with this strain." A better alternative species was not identified, and the consensus was reached that a third rodent species or replacement of the rat or mouse with another rodent species should not be a routine procedure. However, if the disposition of a chemical by hamsters or other rodents is more similar to humans than by the rat or mouse, then that other strain/species should be used either in addition or as a replacement.

NTP studies are designed to insure that the number of animals used is as near to optimum as is scientifically possible. Vigorous attempts are made to evaluate several toxicity end points in the same set of animals. For example, in 90-day studies clinical pathology parameters (enzymes, hematology), sperm motility and vaginal cytology evaluations (SMVCE), and histopathologic evaluations are carried out in the same group of animals. Furthermore, no single exposure (acute) studies have been undertaken for several years since data are often available. If sufficient information is available to select dose levels for 90-day studies, 14-day studies that usually precede 90-day studies may not need to be performed (Table 1).

Routes of Exposure

The awareness that human exposure to chemicals in the environment cannot be reproduced exactly in experimental animals is well recognized; however, attempts are made to expose animals under conditions as closely as possible to human exposures. Table 2 shows the different routes of exposure selected for seventy-five 90-day studies. Ten studies were designed to compare the toxicity of chemicals by two different routes. For the remaining 65 designs, 39 were performed by oral, 21 by whole body inhalation, 4 by dermal, and one by the IP route of exposure. Further breakdown of the 39 oral studies shows that dosed-feed was the most preferred route of exposure (30 studies), followed by drinking water (6 studies) and by gavage (3 studies). Oral administration by gavage is used only if other means of oral exposure are not feasible. This decision was made because the oral intubation route is more labor-intensive than other oral routes (feed, drinking water). Also it delivers chemical as a bolus (most reasonable for drugs and food additives), requires more direct handling of animals by technicians that can result in injury to the animals, and may result in tissue damage or death.

Table 4. Criteria used for selection of routes of exposure.

Mode of chemical administration	Major criteria used for selecting the mode of exposure
Feed	Major human exposure by oral route Chemical is absorbed by all routes of exposure and optimum systemic exposure Less labor-intensive
Drinking water	Water-soluble, major human exposure through drinking water
Gavage	Chemical reactive to oral mucous membrane by other oral modes Chemical unstable in feed or volatile Chemical not palatable in diet Difficult to make homogeneous in diet Mimicks human exposure (drugs or food additives) Limited amount of chemical available Containment of chemical needed to avoid exposure to laboratory personnel and to the environment
Dermal	Major human exposure by skin Skin as a target organ of toxicity Occupational exposure is primarily dermal Two-stage initiation-promotion studies
Inhalation (whole body)	Major occupational exposure is by inhalation Pulmonary system is primary target system of toxicity
Multiple routes	Selected to mimic human exposure when more than one route is common

Table 4 gives major criteria used for selection of the most frequently used routes of exposure. In addition to the criteria listed in the table, the input from a regulatory agency may play a major role in selecting a route that helps in hazard risk assessment. NTP has begun using microencapsulation of chemicals as an alternative means of incorporating those chemicals into animal feed when they can not be mixed easily or homogeneously in feed because of unpalatability, volatility, or reactivity (25,26).

Chemical

In general, the chemicals selected for evaluation of toxicity studies are representative of substances to which the human population is exposed. In most instances the materials used are commercial or technical grades (27,28). However, there are some instances when it is not practical to use commercial chemicals, which include the following:

- If the material being evaluated for toxicity is sparingly soluble or not soluble in water or unstable in the dosing mixture, the material can be used in its salt form to increase solubility and possibly stability.
- When a number of structurally related chemicals are evaluated to establish structure-activity relationships, the purest chemicals available are selected for toxicity/carcinogenicity studies.
- If the commercial grade of the material contains a

contaminant that could confound the interpretation of the results because of its own toxicity, the pure chemical is used to avoid possible interaction between the components; conversely, if the minor component(s) of a commercial product are suspected of being responsible for the overall toxicity of the product, then that contaminant in pure form may be selected for toxicity evaluation.

Structure-Activity Relationships

One of the NTP criteria for chemical selection is the understanding of structure-activity relationships (SAR). This understanding thereby assists in defining groups of chemicals that should be evaluated toxicologically. The Interagency Testing Committee (ITC) also uses structure-activity as one of the criteria for selecting chemicals. SAR may be used to evaluate the potential hazards of chemicals that have not undergone toxicity evaluation and also help in interpreting toxicity/carcinogenicity data. Helmes et al. (29) reviewed the SAR data in predicting potential carcinogenicity of chemicals that have not undergone carcinogenicity evaluation. They have suggested that chemical structure may be a predictor of carcinogenic activity if the information available on the ultimate forms of chemical carcinogens and the structural requirements for metabolic activation. NTP is developing a data base on a number of structurally related chemicals. Some of the examples are benzidine dye congeners, dioxin/dibenzofurans, short chain aliphatic hydrocarbons, aniline dyes, anthraquinones, benzene and methyl benzene, dinitrophenes, phenylenediamines, toluene and dinitro toluenes.

Health and Safety Considerations

The design and activities of the health and safety program are carried out to protect the workers handling chemicals under study and to safeguard the environment by complying with regulations and guidelines for disposing of chemicals and residues being studied (30). It is of the utmost importance that the health and safety aspects of chemicals being evaluated are considered early in the process, preferably at the time of the study designs. In general, adequate methods are available or can be developed to meet health and safety requirements. For inhalation studies where high flammability of materials may be a concern, the maximum concentration used could necessitate extensive safety precautions.

Selection of Dose Levels

The selection of dose levels especially for chronic toxicity/carcinogenicity studies is a topic that requires a separate and extensive review paper. Therefore, a brief description on dose selection is discussed here. For more details, the reader is referred to publications by Huff et al., the NTP, and Haseman (14,20,31).

The selection of dose levels for 90-day studies is generally based on the information from preceding 14-day

toxicity studies; however, there may be a number of instances when the dose levels are selected based on the information available in the literature, and no 14-day study is needed. Usually, the 90-day studies have five dose groups and a control group. The highest exposure group selected for studies is predicted to produce frank toxicity, and the lowest dose is expected to produce no adverse effects in the animals. The highest dose should ideally produce no mortality. The lower dose levels are generally spaced logarithmically using a factor of 2 or simply halving the concentrations. It is expected that a gradation of toxic responses will be observed at these dose levels.

The highest dose selected for carcinogenicity studies, also termed the maximum tolerated dose (MTD), is predicted to produce only minimal yet observable toxicity in the animals. The toxicity end points used in selecting that dose level are listed in Table 5. Depending on the toxicity end points used for an individual chemical during prechronic toxicity studies, one or several end points in combination may be used to select the highest dose. The selection of dose levels is essentially a professional judgment; it is based on input from various specialties and differs from chemical to chemical. However, the basic philosophy of dose selection for carcinogenicity studies by the NTP remains essentially the same as that described by Sontag et al. (32). The major change has been in broadening of the toxicology profile by the NTP in its 90-day studies, giving added confidence in estimating the dose levels selected, which according to NCI guidelines "causes no more than a 10% weight decrement as compared to the appropriate control group; and does not produce mortality, clinical signs of toxicity, or pathologic lesions (other than those that may be related to a neoplastic response) that would be predicted to shorten the animal's natural life span." The histopathologic and body weight changes have been major parameters in estimation of high dose. However, hematology data for nitroaromatics, biochemical parameters for organophosphate pesticides, hormonal levels for chemicals causing thyroid toxicity, clinical signs of toxicity for central nervous system depressants or stimulants, and behavioral and neurotoxicity parameters for chemicals primarily affecting the nervous system are some of the examples where these endpoints have been major determinants or aided in the selection of high-dose levels for carcinogenicity studies. Instances where criteria other than toxicity parameters are considered in selection of dose levels are listed in Table 6.

Table 5. Toxicity end points used in selecting dose levels for chronic studies.

Body weights	Hematology
Histopathologic changes	Clinical chemistry
Mortality	Metabolism/disposition
Clinical signs and toxicity	Biochemical parameters
Pharmacologic signs	Gross pathology
Food consumption and water consumption	Organ weights

Table 6. Maximum dose levels established based on criteria other than toxicity.

Route	Criteria
Feed	5% maximum in diet in absence of toxicity in prechronic studies
Gavage	5 mL/kg in rats and 10 mL/kg in mice is the maximum volume used for these studies; solubility and suspendibility of material are other limiting factors; usually stay below 1000 mg chemical/kg body weight
Dermal	0.1 mL for mice and 0.3 mL for rats is the maximum volume applied
Inhalation	Aerosol or particulate generation limits, explosive limits, and lung burden levels
Drinking water	Solubility, stability

The lower dose levels are usually one-half and one-fourth of the highest dose level and generally not below one-tenth of high dose level. Further information on the spread of dose levels for carcinogenicity studies is dealt with by Portier and Hoel (33) and Huff et al. (34).

Collaborative Efforts

Almost all of the prechronic and chronic studies are performed at private laboratories. The capabilities of these laboratories to perform specific toxicology experiments is one of the considerations used in selecting the laboratories. If a specific laboratory does not have expertise in certain toxicology procedures, specific segments may be accomplished in other laboratories, or such studies may be conducted in NTP laboratory facilities. For example, methyl isocyanate (MIC) studies required a multidisciplinary approach and could not be performed in a single laboratory. These studies were performed largely in NTP laboratories.

Peer Review of Study Designs

All designs of prechronic and chronic toxicity studies are reviewed and approved by the NTP Toxicology Design Review Committee. This committee is composed of individuals within NTP having expertise in general toxicology, reproduction, genetics, statistics, metabolism/disposition, and pathology. On an ad hoc basis committee members represent other disciplines such as clinical pathology, behavioral sciences, chemistry, animal care, health and safety, and immunotoxicology. The nominating, regulatory, and NTP participating agencies and representatives from industry are consulted on an ad hoc basis depending on their interest and expertise in an individual chemical.

Protocol Outlines of Prechronic and Chronic Studies

The conduct of prechronic and chronic toxicity/carcinogenicity studies are described in the official NTP Statement of Work document. This large document presents, in considerable detail, the NTP requirements and procedures for performing toxicology studies. These

detailed guidelines are essential to the success of doing our studies and, in particular, to be able to compare results across studies done at various laboratories (35). The following sections give an outline of core protocols for 14-day and 90-day toxicity studies and 24-month chronic toxicity/carcinogenicity studies, along with frequent toxicity end points incorporated into study protocols. All of these studies are performed under Good Laboratory Practices (36).

14-Day Toxicity Studies

The purpose of the 14-day studies is to characterize the toxicity associated with a substance administered to animals for 14 days. The objectives are to identify possible target organ(s), toxic effect similarities, and possible differences in sensitivity between sexes and species and dose-response relationships of toxicity to provide dose selection information for subsequent studies.

The usual protocol (Table 7) for 14-day studies consists of six groups (five dose and one control) of animals of each species and sex with five animals per dose group. The control animals receive the vehicle in which the substance is administered. The animals are observed two times daily, at least 6 hr apart (before 10:00 AM and 2:00 PM), including holidays and weekends, for clinical signs and pharmacologic and toxicologic effects, and moribundity or death. Body weights and organ weights (liver, thymus, right kidney, right testes, heart, brain, and lungs, plus other organs as appropriate) are determined for all animals surviving until the end of the study.

A complete necropsy is performed on all exposed and control animals and all tissues are preserved in formalin. If histopathological examination is required these tissues are trimmed, embedded, sectioned, and stained with hematoxylin and eosin, and evaluated.

90-Day Studies

The objective of 90-day studies is to characterize the toxicity associated with exposure over a period of usually 13 weeks including identification of target organ(s), lesions, similarities and differences in sensitivity between species and sexes, and the slope of dose-response curve. These studies may be extended up to 6 months if it is considered that the expression of toxic effects will take longer than 90 days or if the available

Table 7. 14-day studies.

Parameter	Animals	Species	Sex	Exposure levels	Totals
Exposure group	5	2	2	5	100
Controls	5	2	2	1	20
Total					120
Exposure duration	14 days				
Toxicity end points	Mortality, clinical signs of toxicity, body weights, food and water consumption, selected organ weights, gross pathology, histopathology on selected organs.				

information suggests a carcinogenic effect may be observed (e.g., benzidine dyes). Data from 90-day studies are the primary information source used for selecting dose levels for 2-year studies.

The core protocol (Table 8) for 90-day studies generally consists of five dosed groups plus one control group, ten animals/sex/species. The animals are exposed to the chemical for 13 consecutive weeks, after which they are killed without any recovery period. The treatment of animals, regimens for in-life observations, necropsy, and organ weight are the same as for 14-day studies. A complete histopathologic evaluation includes approximately 32 tissues/organs (Table 9) plus section of gross lesions, and it is conducted on all control animals, all animals in the highest dose group with at least 60% survival at the end of the experiment, plus all animals in the highest dose group where death occurred. After target organs of chemical toxicity are identified, these tissue/organs plus gross lesions are examined histopathologically in lower doses until chemically induced effects are no longer observable. A number of other

toxicity end points such as immunotoxicology, chemical disposition, behavioral toxicology, reproductive toxicity, etc., are incorporated into 90-day studies, depending on the information needed for an individual chemical. The additional toxicity end points are evaluated in core animals when feasible, and extra groups of animals may be incorporated into the study.

Two-Year Toxicity/Carcinogenicity Studies

The objectives of toxicity/carcinogenicity studies are to characterize long-term toxic effects and to identify the carcinogenic potential of chemicals in laboratory animals. These studies are usually performed at three dose levels, plus control groups of Fischer 344 rats and B6C3F₁ mice of both sexes. Additional characterization of chronic toxicity is achieved by studying 10 animals from each of the dose groups that are killed at 15 months (Table 10).

The chemical is administered for 2 years; 10 in feed and water studies, the chemical is available 24 hr per day, 7 days per week; for oral intubation and dermal studies the chemical is given or applied daily five times per week; inhalation exposures are given 6 hr per day 5 days per week. Individual animal body weights are recorded weekly for the first 13 weeks and at 4-week intervals thereafter. If significant morbidity or mortality occur during the study, the observation and weighing frequency may be increased. If considerable unanticipated deaths occur, the study may have to be modified or restarted. All animals that die or are killed during and at the end of the experiment receive a complete necropsy and microscopic examination of all tissues.

At 65 weeks into the study, up to 10 animals/dose/sex/species are killed and specific toxicologic parameters are determined. Generally, these include specific organ weight, hematology determinations, and complete necropsy and histopathologic evaluation of tissues from all animals in all dose groups plus control groups. Other toxicity end points are incorporated, depending on the chemical being evaluated. Information from these 15-month evaluations allows better planning and more astute pathology examinations; for example, more histopathologic sections can be taken for obvious target organs. Remaining animals that are exposed for 2 years are killed 1 week after cessation of treatment. A complete necropsy and a histopathologic evaluation (Table 9)

Table 8. 90-Day studies.

Parameter	Animals	Species	Sex	Exposure levels	Totals
Exposure group	10	2	2	5	200
Controls	10	2	2	1	40
Total					240
Exposure duration	90 days				
Toxicity end points	Mortality, gross pathology, clinical signs of toxicity, histopathology, selected organ weights, clinical pathology, hematology, sperm motility, body weights, vaginal cytology evaluation, food and water consumption, micronuclei in mice				

Table 9. Tissues for histopathologic evaluation.

Gross lesions and tissue masses (and regional lymph nodes)	Heart
Mandibular and mesenteric lymph nodes	Esophagus
Bronchial and mediastinal lymph nodes (inhalation studies)	Stomach (forestomach and glandular stomach)
Salivary gland	Uterus
Femur, including marrow	Brain (three sections, including frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons)
Thyroid gland	Thymus gland
Parathyroid glands	Larynx (inhalation studies)
Small intestine (duodenum, jejunum, ileum)	Trachea
Large intestine (cecum, colon, rectum)	Pancreas
Liver	Spleen
Gall bladder (mouse)	Kidneys
Prostate	Adrenal gland
Testes/epididymis/seminal vesicle	Urinary bladder
Ovaries	Pituitary gland
Lungs and mainstem bronchi	Spinal cord and sciatic nerve (if neurologic signs were present)
Nasal cavity and nasal turbinates (three sections)	Eyes (if grossly abnormal)
Preputial or clitoral glands	Mammary gland
Thigh muscle (13-week studies only)	Pharynx (if grossly abnormal)
	Skin

Table 10. Two-year toxicology and carcinogenesis studies.

Parameter	Animals	Species	Sex	Exposure levels	Totals
Exposure groups	60	2	2	3	720
Controls	60	2	2	1	240
Sentinel	15	2	2		60
Total					1020
Exposure duration	104 weeks				
Interim evaluations	Week 65				
Toxicity end points	Chronic toxicity, carcinogenicity				

are routinely conducted on all animals from all dose groups and controls including the 65-week groups.

Conclusions

This paper gives a brief overview of the toxicology and long-term carcinogenesis studies designed and conducted by the NTP. The core design in terms of number of dose groups and group sizes for 14-day and 90-day studies are not significantly different from the ones used in the past (32). The major difference in the prechronic studies performed in the past involve the primary objective of establishing doses to be used in subsequent carcinogenesis studies. The objectives of 90-day studies are generally 2-fold: to more completely characterize toxicity of the chemicals (stand-alone studies) and to develop data of a wide scope to better design toxicity/carcinogenicity studies. The objectives of chronic toxicity/carcinogenicity are to evaluate long-term toxicity as well as the carcinogenic potential of chemicals. In addition, chronic studies are conducted to evaluate dose-response relationships. An interim evaluation, usually at week 65, is included to evaluate long-term and/or late appearing toxic effects of the chemicals in the absence of old age lesions that are normally found at 104 weeks that can mask toxic lesions (37).

The NTP studies are designed to exploit the uniqueness of the chemical being evaluated; therefore, flexibility is important in the protocol development of each study. To accomplish this, NTP uses a multi-disciplinary team of experts within its organization who review and evaluate the appropriateness of toxicology procedures for each study design. As scientific advances in toxicology are discovered that are directly relevant to these studies, newer techniques will be adopted where appropriate.

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REFERENCES

1. NTP Annual Plan for Fiscal Year 1988. U.S. Dept. of Health and Human Services, National Toxicology Program, Research Triangle Park, NC, 88-200, 1988.
2. Huff, J. E., Moore, J. A., and Rall, D. P. The National Toxicology Program and preventive oncology. *The Cosmetic Industry* (N. F. Eskin, Ed.), Marcel Dekker, New York, 1984, pp. 647-673.
3. Haseman, J. K. Statistical issues in the design, analysis and interpretation of animal carcinogenicity studies. *Environ. Health Perspect.* 58: 385-392 (1984).
4. Haseman, J. K., Huff, J. E., and Boorman, G. A. Use of historical control data in carcinogenicity studies in rodents. *Toxicol. Pathol.* 12: 136-135 (1984).
5. Maronpot, R. R. Considerations in the evaluation and interpretation of long-term animal bioassays for carcinogenicity. *Handbook of Carcinogen Testing* (H. A. Milman and E. K. Weisburger, Eds.), Noyes Publication, Park Ridge, NJ, 1985, pp. 372-382.
6. Boorman, G. A., Montgomery, C. A., Eustis, S. L., Wolfe, M. J., McConnell, E. E., and Hardisty, J. F. Quality assurance in pathology for rodent carcinogenicity studies. In: *Handbook of Carcinogenesis Testing* (H. A. Milman and E. K. Weisburger, Eds.), Noyes Publications, Park Ridge, NJ, 1985, pp. 345-357.
7. Haseman, J. K., Huff, J. E., Rao, G. N., Arnold, J. E., Boorman, G. A., and McConnell, E. E. Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57B1/N6 X C3H/HeN) F1 mice. *J. Natl. Cancer Inst.* 75: 975-984 (1985).
8. Haseman, J. K., Tharrington, E. C., Huff, J. E., and McConnell, E. E. Comparison of site-specific and overall tumor incidence analyses for 81 recent National Toxicology Program carcinogenicity studies. *Regul. Toxicol. Pharmacol.* 6: 155-170 (1986).
9. Luster, M. I., and Thomas, P. T. Summary of an interlaboratory validation exercise for detecting chemically induced immune alterations: establishment and correlation of infecting models. *Advances in Modern Environmental Toxicology*, Vol. XIII (E. J. Burger, R. G. Tardeff, and J. A. Bellau, Eds.), Princeton Scientific Publishing Co., Princeton, NJ, 1986, pp. 181-194.
10. Rall, D. P., Hogan, M. D., Huff, J. E., Schwetz, B. A., and Tennant, R. W. Alternatives to using human experience in assessing health risks. *Annu. Rev. Public Health* 8: 355-385 (1987).
11. Zeiger, E. Carcinogenicity of mutagens: predictive capability of the Salmonella mutagenesis assay for rodent carcinogenicity. *Cancer Res.* 47: 1287-1286 (1987).
12. Tennant, R. W., Margolin, B. H., Shelby, M. D., Zeiger, E., Haseman, J. K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B., and Monor, R. Prediction of chemical carcinogenicity in rodents from *in vitro* toxicity assays. *Science* 236: 933-941 (1987).
13. Haseman, J. K., and Huff, J. Species correlation in long-term carcinogenicity studies. *Cancer Lett.* 37: 125-132 (1987).
14. Huff, J. E., McConnell, E. E., Haseman, J. K., Boorman, G. L., Eustis, S. L., Schwetz, B. A., Rao, G. N., Jameson, C. W., Hart, L. G., and Rall, D. P. Carcinogenesis studies: results of 398 experiments on 104 chemicals from the U.S. National Toxicology Program. *N.Y. Acad. Sci.* 534: 1-30 (1988).
15. Luster, M. I., Munson, A. E., Thomas, P. T., Holsapple, M. P., Fenters, J. D., White, K. L., Lancer, L. D., Germolec, D. R., Rosenthal, G. L., and Dean, J. H. Development of a testing battery to assess chemical induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. *Fundam. Appl. Toxicol.* 10: 2-19 (1988).
16. Tilson, H. A. Behavioral indices of neurotoxicity. What can be measured? *Neurotoxicol. Teratol.* 9: 427-443 (1987).
17. Organization for Economic Co-operations and Development (OECD). *OECD Guidelines for Testing Chemicals*. Paris, 1981.
18. U.S. Environmental Protection Agency. *Portions of EPA Health Effects Test Guidelines and FDA Toxicology Principles for Safety Assessment of Direct Food Additives and Color Additives Used in Food*. Office of Pesticides and Toxic Substances, EPA, Washington, DC, 1982.
19. IARC. Long-term and short-term screening assays for carcinogens: a critical appraisal. *In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Suppl. 2. International Agency for Research on Cancer, Lyon, 1980, pp. 21-83.
20. National Toxicology Program. Report of the NTP Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation. NTP Board of Scientific Counselors, August 17, 1984.
21. Pryor, G. T., Uyeno, E. T., Tilson, H. A., and Mitchell, C. L. Assessment of chemicals using a battery of neurobehavioral tests: a comparative study. *Neurobehav. Toxicol. Teratol.* 5: 91-117 (1983).
22. Weisburger, E. K. History of the bioassay program of the National Cancer Institute. *Prog. Exp. Tumor Res.* 26: 187-201 (1983).
23. Goodman, D. G., Boorman, G. A., and Standberg, J. D. Selection and use of the B6C3F₁ mouse and F344 rat in long-term bioassays for carcinogenicity. In: *Handbook of Carcinogenicity Testing* (H. A. Milman and E. K. Weisburger, Eds.), Noyes Publications, Park Ridge, NJ, 1985, pp. 282-325.
24. Rao, G. N., Birnbaum, L. S., Collins, J. J., Tennant, R. W., and Skow, L. C. Mouse strains for chemical carcinogenicity studies: overview of a workshop. *Fundam. Appl. Toxicol.* 10: 385-394 (1988).

25. Melnick, R. L., Jameson, C. W., Goehl, T. J., and Kuhn, G. O. Application of microencapsulation for toxicology studies. Principles and stabilization of trichloroethylene in gelatin-sorbitol microcapsules. *Fundam. and Appl. Toxicol.* 8: 425-431 (1987).
26. Melnick, R. L., Jameson, C. W., Goehl, T. J., Maronpot, R. R., Collins, B. J., Greenwall, A., Harrington, F. W., Wilson, R. E., Tomaszewski, K. E., and Agarwal, D. K. Application of microencapsulation for toxicology studies II. Toxicity of microencapsulated trichloroethylene in Fischer 344 rats. *Fundam. Appl. Toxicol.* 8: 432-442 (1987).
27. Woodhouse, E. J., Murrill, E. A., Stelting, K. M., Brown, R. D., and Jameson, C. W. Problems of testing commercial grade chemicals. In: *Chemistry of Toxicity Testing* (C. W. Jameson and D. W. Walters, Eds.), Butterworth Publishers, Stoneham, MA, 1984, pp. 31-49).
28. Jameson, C. W. Analytical chemistry requirements for toxicity testing of environmental chemicals. *Chemistry for Toxicity Testing* (C. W. Jameson and D. W. Walters, Eds.). Butterworth Publishers, Stoneham, MA, 1984, pp. 3-18.
29. Helmes, C. T., Sigman, C. C., and Papa, P. A. Structure-activity prediction of the carcinogenicity of chemicals. *Chemistry for Toxicity Testing* (C. W. Jameson and D. B. Walters, Eds.), Butterworth Publishers, 1984, pp. 15-29.
30. Walters, D. B. Chemical health and safety concerns for toxicity testing in the National Toxicology Program. *Chemistry for Toxicity Testing* (C. W. Jameson and D. W. Walters, Eds.), Butterworth Publishers, Stoneham, MA, 1984, pp. 3-20.
31. Haseman, J. K. Issues in carcinogenicity testing: dose selection. *Fundam. Appl. Toxicol.* 5: 66-78 (1985).
32. Sontag, J. M., Page, N. P., and Saffiotti, U. Guidelines for carcinogen bioassay in small rodents. U.S. Dept. of Health and Human Services Publication (NIH 76-801), National Cancer Institute, Bethesda, MD, 1976.
33. Portier, C., and Hoel, D. Optimal design of the chronic animal bioassay. *J. Toxicol. Environ. Health* 12: 1-19 (1983).
34. Huff, J. E., Haseman, J. K., McConnell, E. E., and Moore, J. A. The National Toxicology Program, toxicology data evaluation techniques, and long-term carcinogenesis studies. *Safety Evaluation of Drugs and Chemicals* (W. E. Lloyd, Ed.), Hemisphere Publishing Corporation, Washington, DC, 1986, pp. 411-446.
35. National Toxicology Program. General statement of work for the conduct of acute, fourteen-day repeated dose, 90-day subchronic, and 2-year chronic studies in laboratory animals. National Toxicology Program, Research Triangle Park, NC, 1987.
36. Bristol, D. W. National Toxicology Program Approach to Monitoring Study Performance in Managing Conduct and Data Quality of Toxicology Studies (B. K. Hoover, K. K. Baldwin, and A. F. Uelman, Eds.), Princeton Scientific Publishing, Princeton, NJ, 1986, pp. 79-97.
37. Solleveld, H. A., Haseman, J. K., and McConnell, E. E. National history of body weight gain, survival and nesplasia in the F344 rat. *J. Natl. Cancer Inst.* 72: 929-939 (1984).