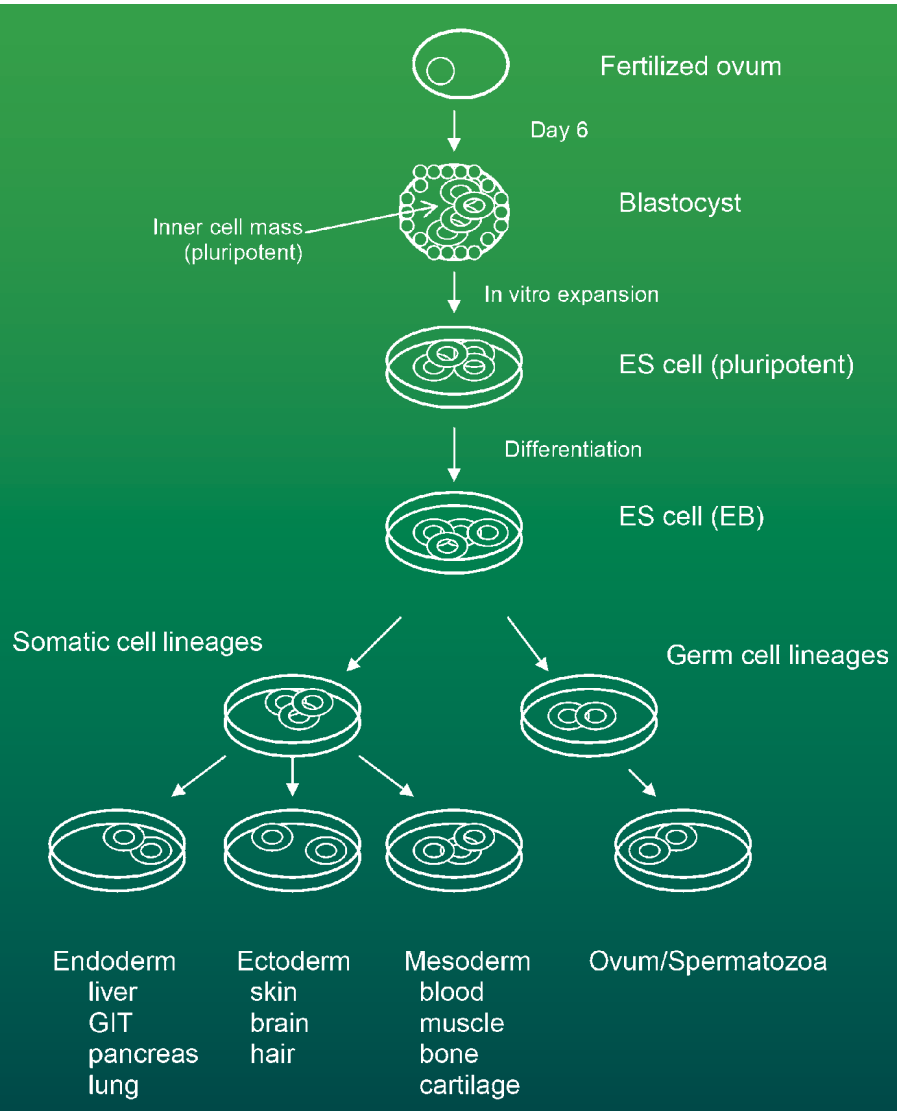
SECOND EDITION

# PRINCIPLES OF Toxicology Testing




FRANK A BARILE

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# Principles of Toxicology Testing

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## Second Edition

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# *Dedication*

*To Pauline*

# Contents

# Preface

In the first edition, I commented in the Preface that the science of toxicology testing has evolved in the last few decades from an applied and supportive science to its own refined and technical discipline. I also mentioned that the development and maturity of the discipline has been sporadic. In the last 5 years since the first edition, however, the latter description has not held true. In fact, the field has soared in its applications of the techniques and principles that define the field. Its progress has been prompted not by public health initiatives and needs but by the discovery of new disciplines, such as epigenetics, toxicogenetics, and toxicodynamics (for which new chapters have been added in the second edition). Thus, new roles have emerged for the application of both *in vivo* and *in vitro* techniques. These new roles have allowed for unique investigations in toxicology testing, followed by introduction of exciting principles to the field. No sooner have the burgeoning advances of biotechnology allowed for the development of corresponding *in vitro* systems that complement traditional animal toxicology testing methods, but the new disciplines have been fitted as well to important applications within the toxicology arena.

As in the first edition, the book begins with an introduction into the fundamentals of toxicology (Section I) to prepare students for the subsequent topics and continues through with a discussion of toxicokinetics and human risk assessment. This introductory material is useful in understanding the applications of toxicology testing.

Section II describes the fundamental principles of toxicology testing in animals in greater detail. This section describes acute toxicity studies as well as subchronic and chronic studies performed in animals. Special emphasis is placed on study design and determination of classical indicators for acute and chronic testing, such as the LD50. Other short- and long-term animal toxicity testing methodologies including dermal, ocular, and reproductive toxicity testing are discussed. Mutagenicity and carcinogenicity studies are also discussed in separate chapters.

Section III introduces and discusses *in vitro* alternatives to animal toxicology tests. This section emphasizes cell culture methodology and cellular methods for acute systemic toxicity, target organ toxicity, and local toxicity. The advantages and disadvantages of alternative methods are presented. Special features of this section describe the use of high-throughput screening and its applications, the concepts of standardization and validation of *in vitro* techniques, especially large, organized validation efforts currently supported by US and EU regulatory agencies, and the theories supporting the development of *in vitro* methodologies. Undergraduate and graduate toxicology students and industrial and academic research laboratories will find the text useful for the entry level students in the discipline or for establishing a toxicology testing laboratory, respectively.

The juxtaposition of the principles of animal toxicology testing in the same text as *in vitro* alternative methods highlights the importance of both fields for interpretation of the significance and relevance of the other. Thus, the discussions continuously refer to the corresponding methods available and the potential results from complementary designs of studies. In fact, both animal and *in vitro* toxicology testing methods are currently employed,

often together, in toxicological analysis, derivation of mechanisms of toxicity, mutagenicity testing, and preclinical drug development.

Several excellent texts are available in the field concerning the details of individual protocols. Consequently, although some procedures are outlined in detail, the emphasis is on the principles of the disciplines rather than on the particular steps of the techniques. In fact, the title, *Principles of Toxicology* (rather than *Toxicity*) *Testing*, emphasizes the universal application of the field as a scientific discipline as opposed to amplification of laboratory techniques. In addition, the book highlights contemporary issues in toxicology testing including the various means of possible exposure to chemicals, high-throughput screening of chemicals for preclinical drug development, and an overview of applications. Overall, the reader is challenged to interpret the significance of toxicology testing results and to construct a logical approach toward the ultimate purpose of testing. Thus, the information contained herein is presented with great enthusiasm, particularly for the students prepared to dedicate their careers to this intriguing and fascinating scientific discipline.

# Acknowledgments

I would like to extend my appreciation to the editorial staff at Informa Healthcare, Inc., for their interest, professionalism, and commitment to the project, in particular to Ms. Amber Thomas, Ms. Claire Bonnett, and Mr. Oscar Heini, as well as the staff at Exeter Premedia Services. My colleagues and staff at the College of Pharmacy, Department of Pharmaceutical Sciences, afforded invaluable comments, suggestions, and support throughout the project. Finally, I have had the pleasure to know and been fortunate to guide undergraduate and graduate toxicology and pharmacy students during the writing of this text, especially Angela Aliberti, Sanket Gadhia, Tak Lee, and Sanjay Dholakiya. This work would not have progressed fluently or competently without their continuous feedback.

# Biography

**Frank A. Barile, Ph.D.,** is a full professor in the Toxicology Division of the Department of Pharmaceutical Sciences at St. John's University College of Pharmacy and Health Sciences, New York.

Dr. Barile received his B.S. in Pharmacy (1977), M.S. in Pharmacology (1980), and Ph.D. in Toxicology (1982) at St. John's University. After a postdoctoral fellowship in Pulmonary Pediatrics at the Albert Einstein College of Medicine, Bronx, New York, he moved to the Department of Pathology, Columbia University, St. Luke's Roosevelt Hospital, New York, as a research associate. In these positions, he investigated the role of pulmonary toxicants on collagen metabolism in cultured lung cells. In 1984, he was appointed as an assistant professor in the Department of Health Sciences at City University of New York. Sixteen years later, he rejoined St. John's University in the Department of Pharmaceutical Sciences and became an instrumental part of the toxicology program in the College of Pharmacy.

Dr. Barile holds memberships in several professional associations, including the US Society of Toxicology, American Association of University Professors, American Association for the Advancement of Science, American Society of Hospital Pharmacists, New York City Pharmacists Society, New York Academy of Sciences, and New York State Council of Health System Pharmacists. He has been appointed as a consultant scientist with several professional groups, including the Department of Pediatrics, Schneider's Children's Hospital, Long Island Jewish/Cornell Medical Centers, New York, as a committee member of SACATM, ICCVAM/NICEATM, and NIEHS, and as President and Past-President of the *In Vitro* and Alternative Methods Specialty Section, US Society of Toxicology. He is also the recipient of the *Public Health Service Medallion* from the Director of the NIEHS, Dr. Linda Birnbaum (2009). He was recently appointed as Editor-In-Chief (Rest of the World) for *Toxicology In Vitro*.

Dr. Barile has been the recipient of Public Health Service research grants from the National Institutes of Health (NIGMS), including awards from the Minority Biomedical Research Support program, the Minority High School Student Research Apprentice program, and AREA program.

Dr. Barile has authored and coauthored approximately 75 papers and abstracts in peer-reviewed biomedical and toxicology journals as well as three books and one contributed chapter. He contributed original *in vitro* toxicology data to the international Multicenter Evaluation for Cytotoxicity program, along with distinguished international investigators. He lectures regularly to toxicology and pharmacy undergraduate and graduate students in the toxicological and pharmaceutical sciences (he was awarded Professor of the Year for the College of Pharmacy by the University Student Government Association in 2003). Dr. Barile continues to perform fundamental research on the cytotoxic effects of environmental chemicals and therapeutic drugs on cultured human and mammalian stem cells.

# 1 Introduction to principles of toxicology

## INTRODUCTION

From its inception in the medieval era to its maturity as a distinct and separate discipline in the twenty first century, toxicology was taught primarily as an applied science. The field incorporated the various approaches from a variety of disciplines, not limited to the broad sciences of chemistry and biology. In particular, toxicology evolved from applications of analytical and clinical chemists whose job definitions included chemical identification and analysis of body fluids.

The first modern toxicologists were chemists who had specialized training in inorganic separation methods including chromatographic techniques. Analytical chemists later employed thin layer and gas chromatography. The development of methods for forensic analysis greatly promoted the field. These advances were particularly important for acceptance within the legal community and in jurisprudence arenas. Furthermore, as the instrumentation evolved and the technology became more exacting, high-performance liquid chromatography was incorporated into the analytical arsenal in order to isolate minute quantities of compounds from complex mixtures of toxicological importance. Eventually, biological applications exerted their influence, incorporating such specialties as microbiology, genetics, and cell culture methodology. Today, the field has burgeoned into areas of specialization, some of which are hardly identifiable with their ancestral origins.

## TYPES OF TOXICOLOGY

### General Toxicology

General toxicology involves studies of exposure to chemical, biological, or physical agents and the untoward consequences that affect biological systems. The term, however, has been replaced by descriptions of areas that more closely reflect the specialized fields of study within the discipline. The development of advanced methodologies in biotechnology, the requirement for increased training, and the involvement of toxicology in legal applications have made it necessary to more accurately label the discipline according to the expanding body of specialties. As a result, a variety of descriptions further define the field of toxicology.

### Mechanistic Toxicology

Mechanistic toxicology involves the identification of the cause, pathway, reaction, and cellular modification associated with the toxicity of a chemical at the level of the cell, tissue, or organ. The classification of toxicity of a chemical, therefore, may be expressed in terms of its mechanism of toxicity, its site of action, target area, or the organ most affected by the toxic insult. A similar expression, *mechanism of action*, is universally applied in the study of

pharmacology. Thus, mechanistic toxicology seeks to determine the biochemical, physiological, or organic basis of a toxic agent's effects on biological systems.

### Regulatory Toxicology

Regulatory toxicology relates to the administrative dogma associated with the potential exposure to toxic agents encountered in the environment, in occupational settings, and in the home. Regulatory toxicology defines, directs, and dictates the rate at which an individual may encounter a synthetic or naturally occurring toxin and establishes guidelines for its maintenance in the environment, for risks due to possible exposure within the community and within the remedial market. The guidelines are generally promulgated by agencies whose jurisdiction and regulations are established by federal, state, and local authorities.

### Descriptive Toxicology

Descriptive toxicology is a subjective attempt to explain toxic agents and their applications. The list of descriptive areas developed principally as a method for bridging the vacuum between science and the public's understanding of the field, especially when it became necessary for nonscientific sectors to comprehend and interpret the importance of toxicology. This is especially true for public sector interpretation so that they could translate the information for the development of regulations and guidelines. For instance, the study of metals in the environment (metal toxicology) has become a popular discipline for toxicologists interested in examining the roles of heavy or trace metals in the environment.

### Forensic Toxicology

From its inception in the medieval era to its maturity as a distinct and separate discipline in the 1950s, toxicology was taught primarily as an applied science. More recently, toxicology has evolved from applications of analytical and clinical chemists whose job definition was the chemical identification and analysis of body fluids. Thus the first modern toxicologists were chemists with specialized training in inorganic separation methods, including chromatographic techniques. Eventual evolution toward liquid chromatographic methods allowed analysis and isolation of minute quantities of compounds from complex mixtures of toxicological importance. Forensic toxicology thus integrates these techniques to identify compounds of sometimes unrelated poisons from biological specimens as a result of incidental or deliberate exposure. Initially, forensic sciences profited from the application of the principles of chemical separation methods for the identification of controlled substances in body fluids. Later, forensic toxicology applied biological principles of antigen–antibody interaction for paternity testing. By using the principles of blood grouping and "exclusion" of the potential outcomes of paternal contribution to offspring phenotype, it was feasible to eliminate the possibility of a male as the father of a child. Antigen–antibody interactions also became the basis for enzyme-linked immunosorbent assays (ELISA) currently used for specific and sensitive identification of drugs in biological fluids. Radioimmunoassays (RIAs) utilize similar antigen–antibody reactions while incorporating radiolabeled ligands as indicators. DNA separation and sequencing techniques have now almost totally replaced traditional paternity exclusion testing. These methods are also the basis for inclusion or exclusions of evidence in criminal and civil cases.

### Clinical Toxicology

Clinical toxicology is also considered a descriptive category. However, toxic agents with clinical applications are also characterized in other toxicological fields. Clinical toxicology has evolved and branched from its counterpart, forensic toxicology, to include identification,

**TABLE 1.1**
**Other Descriptive Fields of Toxicology**

| Descriptive Field | Definition |
|---|---|
| Genetic toxicology | Incorporates molecular biology principles in applications of toxicological sciences, as applied to toxic agents that interfere with normal physiological function |
| Occupational toxicology | Examines hazards associated with toxic exposure in the workplace, including industrial, agricultural, and communal sectors |
| *In vitro* toxicology | Development of cell culture and biochemical techniques as alternatives to animal toxicity testing; a current definition of this field includes *alternative methods to animal toxicology*, which more accurately describes the applications of *in vitro* methods |
| Analytical toxicology | Chemical and biochemical procedures and methods associated with identification, analysis, reactions, and detection of toxic substances in specimens |
| Developmental toxicology | Study of toxic substances and their potential effects on biological reproduction, mating, fetal development, and embryogenesis |
| Immunotoxicology | Study of toxic substances and their potential effects on immunity and resistance |
| Neurotoxicology | Study of toxic substances and their potential effects on the function and activity of the nervous systems |

diagnosis, and treatment of a condition, pathology, or disease resulting from environmental, therapeutic, or illicit exposure to chemicals or drugs. Treatment may involve amelioration of the signs and symptoms or controlling the underlying pathology. In clinical toxicology, exposure is commonly understood to include individual risk of contact with a toxin, either deliberate, accidental, or intentional. Exposure may further be defined to include population risk.[1] Table 1.1 defines other descriptive fields of toxicology. More recently, the field has blossomed into more broadly defined areas including the study of apoptosis, receptor-mediated signal transduction, gene expression, proteomics, oxidative stress, and toxicogenomics, among others.

## COMMON TERMS AND NOMENCLATURE

The classic definition of toxicology has traditionally been understood as the study of xenobiotics, the science of poisons, and refers particularly to the interactions of exogenous agents with biological systems. For purposes of organizing the nomenclature, chemicals, compounds, and drugs are often referred to as *agents* or *substances*. Because such agents induce undesirable effects, they are usually alluded to as *toxins*. Consequently, toxicology involves internal and external physiological exposures to toxins and their interactions with organisms. Gradually, the term has evolved to include many chemically or physically unrelated classes of agents. What transforms a chemical into a toxin, therefore, depends more on the duration of exposure, dose (or concentration), or route of exposure and less on the chemical structure, product formulation, or intended use of the material. As a result, almost any agent has the potential for toxicity and thus falls within the broad definition of toxicology.

[1]Risk to groups of persons from exposure to radiation, pollutants, and chemical or biological threats, which necessitates identification, diagnosis, and treatment.

## APPLICATIONS OF TOXICOLOGY

### Research

#### Academic Applications

In the academic arena, research toxicologists examine the broad issues of toxicology in the laboratory setting. Academic concerns include all the public health areas in which progress in understanding toxicological sciences is necessary and include the elucidation of mechanistic, clinical, and descriptive toxicological theories. Research methods are modified to answer specific questions that arise from toxicological concerns that affect public health.

#### Industrial Applications

Research toxicologists employed in toxicity testing in the biotechnology and pharmaceutical industries perform toxicity testing—the screening of chemicals and drugs that have toxic potential—before they are marketed. Preclinical testing in the pharmaceutical industry involves the conduct of phase I trials to test the toxicities of candidate agents that have been chemically and biochemically screened as potentially useful therapeutic drugs. The toxicity testing procedures include both in vitro and animal protocols.

### Regulatory Toxicology

Regulatory toxicologists are employed primarily in government administrative agencies, as consultants to government and industry or as representatives of industrial concerns. In this role, they sanction, approve, and monitor the uses of chemicals by establishing rules and guidelines. The guiding principles are promulgated through laws enacted by appropriate federal, state, and local jurisdictions that grant regulatory agencies their authority. Thus, through these regulations, an agency determines who is accountable and responsible for manufacturing, procurement, distribution, marketing, and, ultimately, release and dispensing of chemical substances to the public.

### Forensic Toxicology

Forensic toxicologists integrate appropriate techniques to identify compounds arising from mixtures of sometimes unrelated poisons as a result of incidental or deliberate exposure. Initially, forensic sciences profited from the application of the principles of chemical separation methods for the identification of controlled substances in body fluids. Later, forensic toxicologists applied biological principles of antigen–antibody interaction for paternity testing. By using the principles of blood grouping and the exclusion of the possible outcomes of paternal contributions to offspring phenotypes, it became possible to eliminate a male as a possible father of a child.

Antigen–antibody interactions also became the basis for ELISA and enzyme multiplied immunoassay technique, which are currently used for specific and sensitive identification of drugs in biological fluids. RIAs utilize similar antigen–antibody reactions while incorporating radiolabeled ligands as indicators. DNA separation and sequencing techniques have now almost totally replaced traditional paternity exclusion testing. These methods are also the basis for inclusion or exclusion of evidence in criminal and civil cases.

### Clinical Toxicology

Clinical toxicologists have evolved and branched away from their corresponding forensic applications. The clinical toxicologist is interested in the identification, diagnosis, and treatment of a condition, pathology, or disease resulting from an environmental, therapeutic, or illicit exposure to chemicals or drugs. Exposure is commonly understood to include the individual risk of contact with a toxin.

## CLASSIFICATION OF TOXIC AGENTS

Classification of toxic agents is a daunting task, considering the vast numbers and complexities of chemical compounds in the public domain. The availability of the variety of chemicals, drugs, and physical agents, along with their varied toxicological and pharmacological effects, means that a single agent may be listed in several different categories. Even compounds with similar structures or toxicological actions may be alternatively grouped according to their activities or physical states. The following outline of the classification system is generally accepted for demonstrating the complex nature of toxins.

### Classification According to Use

#### Pesticides

The U.S. Environmental Protection Agency defines a pesticide as a substance or a mixture of substances intended to prevent, destroy, repel, or mitigate a pest. In general, pesticides are classified according to their biological targets. The four major classes of pesticides are insecticides, herbicides, rodenticides, and fungicides. Table 1.2 lists these categories and their general subclasses. Because of the physiological and biochemical similarities of target species and mammalian organisms, an inherent toxicity is associated with pesticides in mammalian organisms. In addition, within each classification, compounds are identified according to mechanism of action, chemical structure, or semisynthetic source. For instance, although many fungicide categories exist, fungicidal toxicity in humans is mostly low order, with the exception of therapeutic antifungal agents, principally because of their specific mechanisms of action. Similarly, fumigants range from carbon tetrachloride to ethylene oxide and are used to kill insects, roundworms, and fungi in soil, stored grain, fruits, and vegetables. Their toxicity, however, is limited to occasional occupational exposure.

#### Food and Industrial Additives

Direct food and color additives are intentionally incorporated in foods and food-processing operations for purposes of changing, enhancing, or masking color. They are also used for a variety of functionalities ranging from anticaking agents to stabilizers, thickeners, and texturizers. Food and industrial additives fall into the field of food toxicology and readers are referred to review articles listed at the end of this chapter for information concerning food ingredients and contaminants.

#### Therapeutic Drugs

Toxicological classification of therapeutic agents follows their pharmacological mechanisms of action or their principal target organs of toxicity. Several important references address clinical toxicologies of therapeutic drugs as extensions of their adverse reactions and direct effects resulting from their excessive use.

**TABLE 1.2**
**Classification of Pesticides**

| Pesticide Class | Classification According to Target |
|---|---|
| Insecticide | Organophosphorus esters |
| | Organochlorine compounds |
| | Carbamate esters |
| | Pyrethroid esters |
| | Botanical derivatives |
| Herbicide | Chlorophenoxy compounds |
| | Bipyridyl derivatives |
| | Chloroacetanilides |
| | Phosphonomethyl amino acids |
| Rodenticide | Anticoagulants |
| | α-Napthyl thiourea |
| | Miscellaneous metals, inorganic, natural products |
| Fungicide | General areas of agricultural, domestic, and therapeutic antifungal agents |

## SOURCES OF TOXINS

### Botanical

Contact dermatitis caused by poison ivy is a well-characterized syndrome of acute inflammation. Today, many compounds of botanical origin are classified as herbal supplements, implying that their origins are botanical. Their importance in maintaining health is also related to their natural derivations. The toxicities of these agents, however, are poorly understood.

### Environmental

As a result of industrialization, many chemicals are associated with and classified according to their continuous presence in the environment, that is, water, land, and soil. The phenomenon is not limited to developed Western nations and is becoming a problem among developing South American, Asian, and African countries.

*Environmental toxicology* is a distinct discipline encompassing the areas of air pollution and ecotoxicology. A discussion of air pollution necessarily includes outdoor and indoor air pollution, presence of atmospheric sulfuric acid, airborne particulate matter, interaction of photochemicals with the environment, and chemicals found in smog. *Ecotoxicology* is the branch of environmental toxicology that investigates the effects of environmental chemicals on the ecosystems in question. Readers are referred to the review articles at the end of this chapter for further discussion.

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