Available online at www.sciencedirect.com
SCIENCE DIRECT®
Environmental Toxicology and Pharmacology 18 (2004) 65–81
ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY ETAP
www.elsevier.com/locate/etap

Chemical mixture toxicology: from descriptive to mechanistic, and going on to in silico toxicology

Raymond S.H. Yang[a,b,*], Hisham A. El-Masri[a,d,1], Russell S. Thomas[a,1], Ivan D. Dobrev[a,b,1], James E. Dennison Jr.[a,b], Dong-Soon Bae[a,1], Julie A. Campain[a,b], Kai H. Liao[a,c], Brad Reisfeld[a,b,c], Melvin E. Andersen[a,b,1], Moiz Mumtaz[d]

[a] *Quantitative and Computational Toxicology Group, Center for Environmental Toxicology and Technology, Colorado State University, Foothills Campus, Ft. Collins, CO 80523-1690, USA*
[b] *Departments of Environmental and Radiological Health Sciences, Atlanta, GA, USA*
[c] *Chemical Engineering, Colorado State University, Fort Collins, CO, USA*
[d] *ATSDR, Atlanta, GA, USA*

Accepted 16 January 2004

Abstract

Because of the pioneering vision of certain leaders in the biomedical field, the last two decades witnessed rapid advances in the area of chemical mixture toxicology. Earlier studies utilized conventional toxicology protocol and methods, and they were mainly descriptive in nature. Two good examples might be the parallel series of studies conducted by the U.S. National Toxicology Program and TNO in The Netherlands, respectively. As a natural course of progression, more and more sophistication was incorporated into the toxicology studies of chemical mixtures. Thus, at least the following seven areas of scientific achievements in chemical mixture toxicology are evident in the literature: (a) the application of better and more robust statistical methods; (b) the exploration and incorporation of mechanistic bases for toxicological interactions; (c) the application of physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling; (d) the studies on more complex chemical mixtures; (e) the use of science-based risk assessment approaches; (f) the utilization of functional genomics; and (g) the application of technology. Examples are given for the discussion of each of these areas. Two important concepts emerged from these studies and they are: (1) dose-dependent toxicologic interactions; and (2) "interaction thresholds". Looking into the future, one of the most challenging areas in chemical mixture research is finding the answer to the question "when one tries to characterize the health effects of chemical mixtures, how does one deal with the infinite number of combination of chemicals, and other possible stressors?" Undoubtedly, there will be many answers from different groups of researchers. Our answer, however, is first to focus on the finite (biological processes) rather than the infinite (combinations of chemical mixtures and multiple stressors). The idea is that once we know a normal biological process(es), all stimuli and insults from external stressors are merely perturbations of the normal biological process(es). The next step is to "capture" the biological process(es) by integrating the recent advances in computational technology and modern biology. Here, the computer-assisted Reaction Network Modeling, linked with PBPK modeling, offers a ray of hope to dealing with the complex biological systems.

Keywords: Chemical mixture toxicology; Historical; Present status; Future development; In silico toxicology

1. Introduction

This, being the opening scientific talk of the International Conference on Chemical Mixtures (ICCM), carries with it certain obligatory responsibilities for the areas of toxicology of chemical mixtures and risk assessment. These re-

* Corresponding author. Tel.: +1 970 491 5652; fax: +1 970 491 8304.
E-mail address: raymond.yang@colostate.edu (R.S.H. Yang).
[1] Present addresses: HAEl, ATSDR, Atlanta, GA; RST, CIIT Centers for Health Research, RTP, NC; IDD, Environ Int'l, Ruston, LA; DSB, NCI, Bethesda, MD; MEA, CIIT Centers for Health Research, RTP, NC.

1382-6689/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.etap.2004.01.015

sponsibilities are personal perceptions by the speaker, the lead author of this paper. As an introduction, it is fitting to first outline the purposes and the scope of this scientific deliberation.

Whenever an area of research has been developed to the point of warranting an international conference, it is inevitably the effort of many investigators over a very long period of time. More often than not, hundreds of publications are already in the scientific literature by these investigators. Thus, it is impossible to summarize all the scientific contributions and to pay tribute to all the investigators in the field. The speaker's bias is a certainty and, in this case, a special emphasis has been given to those contributions which have the most profound influence to the professional development of the speaker.

This paper follows the keynote speech closely in presenting the following recent development in the area of toxicology of chemical mixtures: (1) "Earlier Days": Descriptive Toxicology; (2) Mechanistic Toxicology of Chemical Mixtures and Applications of Physiologically Based Pharmacokinetic/Pharmacodynamic (PBPK/PD) Modeling; (3) PBPK Modeling of More Complex Chemical Mixtures; (4) The Concept of "Interaction Threshold"; (5) The Concept of "Dose-Dependent Toxicological Interactions"; (6) Applications of Genomics to Chemical Mixture Toxicology; and (7) In Silico Toxicology: Is it for real? Certain concluding remarks summarize the future perspectives of the authors.

2. "Earlier Days": Descriptive Toxicology

This is a discussion of recent development of the area of toxicology of chemical mixtures; thus, by "Earlier Days" we are referring to the period of from approximately the late 1970s to the early 1990s. During this period of time, with some exceptions, the toxicology studies of chemical mixtures were mainly descriptive in nature. By that, we are referring to experimental work which followed existing and commonly accepted toxicology protocols for that time with endpoints related to morphological, cellular, or biochemical changes. In those days, scientists working on chemical mixture toxicology research were like cosmologists; they had to work sort of "in the dark" with very little useful information and data. Almost any approach one took would end up with numerous criticisms by others. To para-phrase Lev Landau, a Russian physicist, who was poking fun at cosmologists: ". . . Mixture toxicologists were often wrong, but never in doubt . . ." Under those circumstances, it is particularly noteworthy to recognize Drs. David P. Rall and Barry L. Johnson, then Director of the National Institute of Environmental Health Sciences/National Toxicology Program (NIEHS/NTP) and Assistant Administrator of the Agency for Toxic Substances and Disease Registry (ATSDR), respectively, who had the vision and courage to support very controversial research activities in chemical mixtures.

Looking back, it is also most gratifying to witness the recent progress made in this area by many groups. Some notable examples are reflected by the following special panels/workshops/conferences/symposia:

- The effort by the National Research Council (NRC) Safe Drinking Water Committee, Subcommittee on Mixtures, led by Dr. Ronald Wyzga (NRC/NAS, 1989).
- The Scientific Advisory Panel to Electric Power Research Institute (EPRI)'s complex mixture toxicology program related to the "town gas site" at manufactured gas plant sites organized and led by Drs. Ronald Wyzga and Lawrence S. Goldstein between the late 1980s and the early 1990s (Wyzga and Goldstein, 1994).
- The Society of Toxicology (SOT) symposium on "Risk Assessment of Chemical Mixtures—Biologic and Toxicologic Issues, 1992, Seattle, WA.
- The IV European ISSX Meeting on Toxicological Evaluation of Chemical Interactions, organized by Professors Giorgio Cantelli-Forti, Luciano Vittozzi, and Marvin S. Legator, at Bologna, Italy in July 1992 (Cantelli-Forti et al., 1994).
- The USEPA Symposium on "Chemical Mixtures and Quantitative Risk Assessment" organized and held by Dr. Jane Ellen Simmons et al. at Raleigh, NC in November 1994 (Simmons, 1995).
- The International Congress of Toxicology (ICT) VII symposium on "Chemical Mixtures: Toxicological Impacts and Interactive Effects," organized by Professors H. Greim, GSF-Institüte für Toxikologie, Germany, and G. Sipes, University of Arizona, USA in 1995.
- The European Conference on Combination Toxicology, at Veldhoven, The Netherlands, organized by Professor Victor J. Feron, TNO Toxicology, The Netherlands, and Professor Hermann M. Bolt, Institut für Arbeitsphysiologie an der Universität Dortmund, Germany (Feron and Bolt, 1996).
- The two Conferences on Current Issues on Chemical Mixtures and Application of Technology to Chemical Mixture Research sponsored by NIEHS and Colorado State University, at Colorado State University in 1997 and 2001, respectively (Yang and Suk, 1998, 2002).
- The International Working Conference on Exposure to Combinations of Substances: A System for Assessing Health Risks, organized by Dr. P.W. van Vliet, Health Council of The Netherlands, at Den Dolder, The Netherlands, October 2000.
- The formation of the National Occupational Research Agenda (NORA) Mixed Exposures Team organized and led by Frank Hearl, P.E., National Institute of Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention (CDC).
- The SOT effort, under the leadership of Drs. Jay I. Goodman, Linda K. Teuschler, and James E. Klaunig on Risk Assessment of Mixtures: Development of Testable Hypotheses as Science Input into Policy

Decisions (Teuschler et al., 2002) in the past few years.
- Of course, the most recent notable event is this International Conference on Chemical Mixtures (ICCM), spearheaded and led by Drs. Christopher De Rosa, Moiz Mumtaz, Hana Pohl, and Hisham El-Masri at ATSDR.

In addition to the national and international activities on chemical mixture toxicology and risk assessment, several books also appeared since the late 1980s, specifically on chemical mixtures; these include "Methods for Assessing the Effects of Mixtures of Chemicals," edited by Drs. Velimir B. Vouk, Gordon C. Butler, Arthur C. Upton, Dennis V. Parke, and Susan C. Asher, and published in 1987 by John Wiley & Sons on behalf of World Health Organization (WHO), the United Nations Environmental Programme (UNEP), and the International Labour Organization (ILO); the NRC book on "Complex Mixtures. Methods for In Vivo Toxicity Testing," developed by the Committee on Methods for the In Vivo Toxicity Testing of Complex Mixtures and published by the National Academy Press in 1988; "Multiple Chemical Interactions," authored by Dr. Edward J. Calabrese and published in 1991 by Lewis Publishers, Inc.; "The Toxicology of Chemical Mixtures. An Introduction to Recent Development in Toxicology," authored by Dr. John K. Pollak and published in 1993 by The Center for Human Aspects of Science and Technology (CHAST), University of Sidney, Australia; and "Toxicology of Chemical Mixtures. Case Studies, Mechanisms, and Novel Approaches," edited by Dr. Raymond S. H. Yang and published in 1994 by Academic Press.

In 1987, Yang and Rauckman reviewed the available toxicology studies in chemical mixtures in the scientific literature and indicated that, although drug/chemical interaction studies were not new, most past studies involved two chemicals, and their emphasis was generally on acute biochemical, physiological, or toxicological effects. Yang and Rauckman (1987) went on and provided several examples where chemically defined mixtures of 10 or more components were studied; these included amino acid toxicity (Gullino et al., 1956), subchronic toxicities of persistent contaminants in the Great Lakes (Chu et al., 1981; Cote et al., 1985), and the chronic toxicity/carcinogenicity studies of 11 volatile halogenated hydrocarbon drinking water contaminants (Webster et al., 1985). Further, toxicological studies on complex mixtures such as hazardous dump site samples (Plotkin and Ram, 1984; Silkworth et al., 1984), combustion products from tobacco (Heckman and Dalbey, 1982; Bassi et al., 1984; Rylander, 1984), diesel fuel and gasoline (Dalbey and Lock, 1982; MacFarland, 1984), contaminated water samples (Bull, 1984; Kool et al., 1985), contaminated fish (Villeneuve et al., 1981; Chu et al., 1984), and by-products from synfuel operations and coal combustions (Kirchner et al., 1983; Mahlum, 1983; Benson et al., 1984; Gray, 1984; Hackett et al., 1984; Reiley et al., 1985; Springer et al., 1986) were also reported during the 1980s.

This is not meant to be an exhaustive review of the toxicology of chemical mixtures; however, several research groups or their respective derivative groups have contributed significantly to the development of this area. These groups deserve special recognition.

Dr. Jerry Stara and his many fine colleagues at the then Environmental Criteria Assessment Office [ECAO; now known as National Center for Environmental Assessment (NCEA)], the U.S. Environmental Protection Agency (USEPA) were probably the pioneers, in those "earlier days," in the toxicology and risk assessment of chemical mixtures. As evident from the 1984 Monograph entitled *Selected Approaches to Risk Assessment for Multiple Chemical Exposures* (Stara and Erdreich, 1984), scientists at ECAO organized and sponsored three major workshops that brought together expert scientists to review and discuss guidelines for health risk assessment of chemical mixtures. This stemmed from USEPA's recognition, as all Agency program officers realized, that environmental exposures are with mixtures of chemicals, not single pollutants. The ECAO scientists, led more recently by Drs. Richard C. Hertzberg and Linda K. Teuschler, continued to contribute in a major way in the risk assessment of chemical mixtures by developing and publishing a number of landmark documents including *Guidelines for the Health Risk Assessment of Chemical Mixtures* (USEPA, 1986), *Technical Support Document on Risk Assessment of Chemical Mixtures* (USEPA, 1988), *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (USEPA, 2000).

In the mid 1980s, research efforts at the U.S. NIEHS/NTP under the auspices of Superfund legislation via interagency support of USEPA and the ATSDR contributed to the advancement of experimental toxicology of chemical mixtures. Under this program, a series of studies were conducted on mixtures of groundwater water contaminants from hazardous waste disposal sites and pesticide applications; the areas covered included analytical chemistry (Yang et al., 1989), immunotoxicity (Germolec et al., 1989), myelotoxicity (Hong et al., 1991, 1992, 1993), genetic toxicology (Shelby et al., 1990), and reproductive and developmental toxicities (Chapin et al., 1989; Heindel et al., 1994, 1995). Because of interagency collaboration, scientists at the USEPA conducted hepato and renal toxicity studies (Simmons et al., 1994) and in vivo cytogenetic studies (Kligerman et al., 1993) on these chemical mixtures. Collectively, these studies stimulated the thinking and provided some impetus for the further development of the area of toxicology of chemical mixtures. Through this effort, the most significant findings were: (1) potential interactive immuno- and cytogenetic toxicities were seen in mixtures at low component concentrations which were not likely to cause toxicities individually (Germolec et al., 1989; Kligerman et al., 1993); and (2) residual chemical mixture effects persisted in animals which showed no outward clinical signs; such residual effects might interact with radiation to cause enhanced myelotoxicity (Hong et al., 1991).

In the early 1990s, one of the key scientists of USEPA/ECAO, Dr. Christopher De Rosa, joined ATSDR as Direc-

tor of the Division of Toxicology; thus, among other initiatives, started a "derivative research group" on chemical mixture toxicology. Dr. De Rosa was soon joined by another ECAO key scientist on chemical mixtures, Dr. Moiz Mumtaz. Thus, a Mixtures Workgroup was established that includes, apart from the aforementioned, Drs. Hana Pohl and Hugh Hansen. The ATSDR chemical mixtures research program is a university-based experimental research and a risk assessment program that has flourished; it developed key issues and published both experimental findings and mixtures risk assessments in a series of documents called "Interaction Profiles" (ATSDR, 2002a, 2002b). More recently, a computational component has been added to this program following the addition of Dr. Hisham El-Masri to the group. Their collective activities eventually led to the organization of this International Conference on Chemical Mixtures (ICCM). ICCM was hosted by ATSDR in Atlanta 10–12 September 2002 and co-sponsored by several national and international organizations including the SOT, USEPA, NIEHS, NIOSH, Food and Drug Administration (FDA), International Joint Commission and the Health Council of The Netherlands.

Meanwhile, independently in the early 1990s across the Atlantic Ocean, the TNO in The Netherlands was very active in pursuing the toxicology of chemical mixtures. Professor Victor J. Feron led the effort as early as 1986 heading up an international panel of scientists considering testing complex chemical mixtures for carcinogenicity. Subsequently, in a series of papers, acute and repeated-dose toxicity studies were conducted on a number of chemical mixtures either arbitrarily chosen (Jonker et al., 1990), or with the same target organ (Jonker et al., 1993a, 1993b; Feron et al., 1995), or with different target organs (Feron et al., 1995). These studies were carried out at either less or equal to no-observed-adverse-effect-level (NOAEL) or minimal-observed-adverse-effect-level (MOAEL) of each individual component chemicals. The most significant conclusion from their studies appeared to be that, when mixtures were made up by at each individual component's NOAEL or lower, no acute or subacute (repeated dose up to 4 weeks) toxicities were observed. Among others, their scientific contribution also included a number of recent reviews (Casse et al., 1998; Groten, 2000; Groten et al., 2001; Feron and Groten, 2002). Currently, this group is conducting collaborative research with the ATSDR scientists to test chemical mixtures and improve methods for the evaluation of joint toxicity of chemical mixtures and to study the role of chemical interactions through experimental laboratory studies (Mumtaz et al., 1998, 2000, 2003).

Another research group which has a long distinction and very active in toxicological interactions and chemical mixture toxicology was originally led by Professors Gabriel L. Plaa and Jules Brodeur, and more recently by Professor Kannan Krishnan, Université de Montréal, Canada. Professor Krishnan's contribution will be recognized in a later section on PBPK modeling of more complex chemical mixtures. Other international research groups active in chemical mixture related research include Dr. I. Eide, Statoil Research Centre, Norway; Professors N. Ito and R. Hasegawa, Nagoya City University, Japan; Professors H.G. Neumann and W.K. Lutz, University of Wurzburg, Germany; Professor H.M. Mehendale, University of Louisiana at Monroe, USA; Professor G. Poch, University of Graz, Austria; Professor J. Suhnel, Institut fur Molekulare Biotechnologie, Germany; Dr. Y.T. Woo, USEPA, and their respective colleagues; their contributions have been reviewed elsewhere (Yang, 1994a; Arcos et al., 1995; Feron et al., 1998; Yang and Suk, 1998, 2002; Feron and Groten, 2002).

3. Mechanistic Toxicology of Chemical Mixtures and Applications of PBPK/PD Modeling

In the above section, we provided a glimpse of the development of chemical mixture toxicology in the "early days". As the field grew more and more mature, the natural progression proceeded from descriptive work to mechanistic-based research. In this and the next few sections, we will provide a few examples which reflect the development of chemical mixture toxicology in our own laboratory and Professor Kannan Krishnan's laboratory at Université de Montréal, Canada.

Mechanistic studies on toxicological interactions between two chemicals have been around for a long time; it is particularly true for drug–drug interactions. However, what we would like to emphasize in the sections below are specifically related to: (1) the integration of PBPK or PBPK/PD modeling with chemical mixture toxicology; and (2) the toxicology of more complex chemical mixtures.

One of our earlier examples was the PBPK/PD modeling of a toxicological interaction between Kepone (also known as chlordecone) and carbon tetrachloride (CCl_4) based on mechanisms of interactive toxicity and the application of computer technology in acute toxicity studies. This was a collaboration among three research groups: Dr. Melvin E. Andersen, CIIT (presently as CIIT Centers for Health Research), Dr. Harihara M. Mehendale, Northeast Louisiana University (presently as University of Louisiana at Monroe), and our Quantitative and Computational Toxicology group at Colorado State University. The details of this study may be found elsewhere (El-Masri et al., 1996a). Briefly, CCl_4 is a well-known hepatotoxin. Following free radical formation through the P450 enzyme system, the toxicity of CCl_4 can be an accumulation of lipids (steatosis, fatty liver) and degenerative processes leading to cell death (necrosis). Kepone is found in the environment as a result of photolytic oxidation of Mirex, a pesticide used for the control of fire ants, or as a pollutant from careless and irresponsible discharge. At relatively low levels (e.g., 10 ppm in the diet), even repeated dosing of Kepone in the diet up to 15 days caused no apparent toxicity to the liver. The toxicological interaction between Kepone and CCl_4 was reported by Curtis et al. (1979). They illustrated that a 15-day dietary exposure of male rats to Kepone at 10 ppm, an environmentally realistic level, markedly enhanced liver toxicity

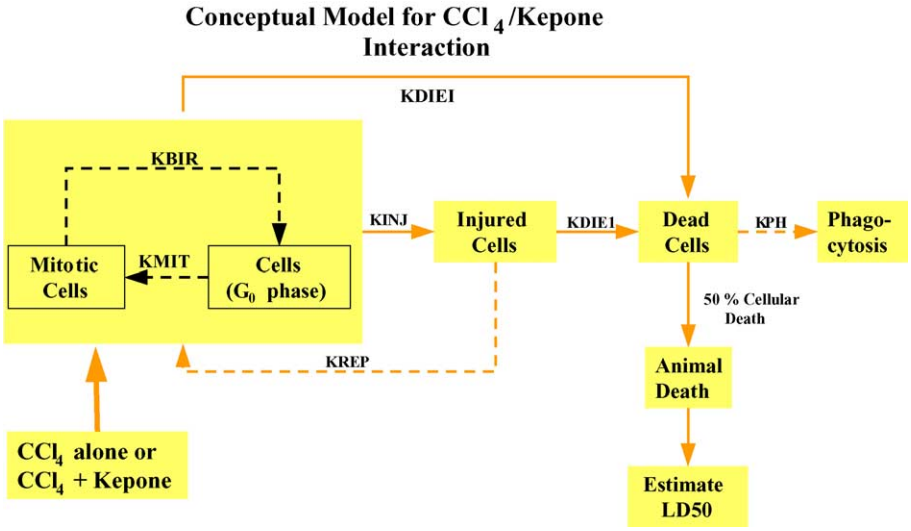

Fig. 1. A conceptual physiologically based pharmacodynamic (PBPD) model for CCl_4 and Kepone interaction. KMIT is the rate constant for mitosis; KBIR the rate constant for cell birth; KINJ the rate constant for cell injury; KDIEI the rate constant for general cell death; KDIE1 the rate constant for cell death due to injury; and KPH is the rate constant for phagocytosis. From: El-Masri et al., 1996. Arch. Toxicol. 70, 704–713.

produced by an i.p. injection of a marginally toxic dose of CCl_4 (100 μl/kg). This toxicological interaction is unique in that: (1) unlike many other toxicological interaction studies which were usually dealing with acute toxicity at very high doses, Kepone in this instance was administered at a very low environmental level; (2) CCl_4 was also dosed at a low, marginally toxic level; and (3) the magnitude of toxicological interaction, 67-fold, is very large. The mechanism of this toxicological interaction was elucidated by Dr. Mehendale's group through a series of studies to be the impairment, by Kepone, of the liver's regeneration process. These mechanistic studies were summarized in a number of publications (Mehendale, 1984, 1991, 1994).

As shown in Fig. 1, a PBPD model was constructed by El-Masri et al. (1996a) based on the mechanism of toxicological interaction between Kepone and CCl_4. This PBPD model was verified by literature information and it was capable of providing time-course computer simulations of mitotic, injured, and pyknotic (dead) cells after treatment with CCl_4 alone or with Kepone pretreatment. This PBPD model was further linked with Monte-Carlo simulation to provide predictability of the acute lethality of CCl_4 alone and in combination with Kepone. As shown in Table 1, the a priori predictions of lethality were in very good agreement with experimentally-derived values except at very high CCl_4 levels. In this latter case, the under-prediction of lethality was due to toxicity other than the liver, i.e., the neurotoxic effects of CCl_4 on the central nervous system. When this study was first presented at the ICT symposium in 1995, a reporter for Food and Chemical News wrote a section in the News titled "Colorado Researchers Use Electronic Rats." Although it was somewhat amusing at the time, the term "Electronic Rats" nevertheless reflects our ultimate goal of in silico toxicology.

Table 1
PBPK/PD modeling and Monte-Carlo simulation of Kepone/CCl_4 toxicological interaction

Dose given		Model prediction[a]		Observed[b]	
Kepone (ppm)	CCl_4 (μl kg^{-1})	Dead rats	Dead (%)	Dead rats	Dead (%)
0	100	0	0.0	0	0.0
0	1000	1–2	13.2	1	11.1
0	3000	3	32.8	4	44.4
0	6000	4–5	47.8	8	88.8
10	10	0	0.0	0	0.0
10	50	4–5	47.5	4	44.4
10	100	8–9	84.0	8	88.8

Modified after Yang et al., 1995. Toxicol. Lett. 82/82, 497–504.
[a] Mortalities in 48 h, given a hypothetical condition of $n = 9$; Monte-Carlo simulation, $n = 1000$.
[b] Actual lethality studies ($n = 9$).

4. PBPK Modeling of More Complex Chemical Mixtures

Pioneering efforts in the PBPK modeling of more complex chemical mixtures were from a research group led by Professor Kannan Krishnan, Université de Montréal, Canada. Earlier work from this group concentrated on interactions and PBPK modeling between two chemicals (Tardif et al., 1993, 1995; Pelekis and Krishnan, 1997). As progress was made, these investigators began to build up the mixtures and devoted their effort to PBPK modeling of more and more complex chemical mixtures (Tardif et al., 1997; Haddad et al., 1999, 2000a, 2000b). So far, these investigators have successfully carried out PBPK modeling on the pharmacokinetic interactions on chemical mixtures involving up to five chemicals (Haddad et al., 2000b; Krishnan et al., 2002); however, they advanced the hypothesis that pharmacokinetic interactions

of complex chemical mixtures, regardless of the number of components, may be predicted based on the PBPK modeling of binary mixtures of the component chemicals (Haddad and Krishnan, 1998; Krishnan et al., 2002). According to their concept, thus, PBPK models for mixtures of any complexity can be created, as long as the quantitative information on the mechanism of interaction for each interacting pair (e.g., competivitive inhibition rate constant) is available (Krishnan et al., 2002).

Applying the same approach created by Krishnan et al., investigators in our laboratory have studied PBPK modeling of a ternary mixture of trichloroethylene (TCE), tetrachloroethylene (PERC), and 1,1,1-trichloroethane (methyl chloroform, MC) in rats and humans (Dobrev et al., 2001, 2002). These studies are summarized in the following section in relation to the concept of "Interaction Threshold." Furthermore, Dennison et al. (2003) in our laboratory characterized the pharmacokinetics of gasoline, a very complex mixture, in rats using an integrated PBPK modeling and lumping approach. The PBPK model tracks selected target components (benzene, toluene, ethylbenzene, *o*-xylene, and *n*-hexane) and a lumped chemical group representing all non-target components. Competitive inhibition was the principal mechanism of pharmacokinetic interactions among these five selected target single chemicals and a pseudo-chemical from the lumped components. Computer simulation results from the six-chemical interaction model matched well with gas uptake pharmacokinetic experimental data from single chemicals, five-chemical mixture, and the two blends of gasoline. The PBPK model analyses indicated that metabolism of individual components was inhibited up to 27% during the 6 h gas uptake experiments of gasoline exposures.

5. The Concept of "Interaction Threshold"

In 1996, we introduced the idea of "Interaction Thresholds" as the minimal level of change in tissue dosimetry associated with a significant health effect (El-Masri et al., 1996b). When two or more interactive chemicals are studied together, theoretically there could be infinite interaction thresholds. However, if we specify certain occupational or environmental exposure concentrations for all the other chemicals in the mixture except one, we may obtain an interaction threshold for that set of exposure conditions. This is important because human risk from exposure to multiple chemicals may not always obey the rule of additivity. In a 2001 publication from our laboratory, Dobrev et al. estimated the interaction thresholds of three common volatile organic solvents, TCE, PERC, and MC under different dosing conditions (Dobrev et al., 2001). Briefly, an interactive PBPK model was built where PERC and MC are competitive inhibitors for TCE. The model was developed and validated by gas uptake pharmacokinetic studies in F344 rats at relatively high doses of single chemicals, binary mixtures, and the ternary mixture. Using computer simulation to extrapolate from high to low concentrations, we investigated the toxicological interactions at occupational exposure levels, specifically at around threshold limit value/time weighted average (TLV/TWA). Since long term toxicity/carcinogenicity of these three solvents is clearly associated with their metabolism, and TCE being the most extensively metabolized among them, we focused our study on changes in internal TCE dose measures related to the mixture co-exposure. Using a 10% elevation in parent compound blood level as a criterion for significant interaction, we estimated interaction thresholds with two of the three chemicals held at constant concentrations. Under the above exposure conditions (i.e., TCE and PERC at their TLVs but varying MC concentrations), Dobrev et al. (2001) reported that, the interaction threshold for the ternary mixture was 50, 130, and 25 ppm for TCE, MC, and PERC, respectively. This work has recently been extended, using computer simulation (i.e., in silico toxicology), to human exposure to this three-chemical mixture and the estimation of interaction thresholds for humans (Dobrev et al., 2002). We found that increases in the TCE blood levels led to higher availability of the parent compound for glutathione conjugation, a metabolic pathway associated with kidney toxicity/carcinogenicity. The simulated change in production rates of toxic conjugative metabolites exceeded 17% for a corresponding 10% increase in TCE blood concentration, indicating a nonlinear risk increase due to combined exposures to TCE. This study (Dobrev et al., 2002) and the related discussion above reveal that evaluation of metabolic interactions and their thresholds illustrates a unique application of PBPK modeling in risk assessment of occupational exposures to chemical mixtures.

6. The Concept of "Dose-Dependent Toxicological Interactions"

This concept development was the result of collaborative research between statisticians (Drs. Chris Gennings and Walter H. Carter, Virginia Commonwealth University) and experimental toxicologists (Drs. Dong-Soon Bae, Julie A. Campain, and Raymond S.H. Yang, Colorado State University). Such interdisciplinary collaboration is vital for synergistic creativity and further development for chemical mixture research (Yang, 1998).

Dose response assessment of toxicologic interactions of metal mixtures, using the human keratinocyte systems, revealed that toxicological responses are highly dose-dependent. From very low dose levels (about 0.3–10 ppb) to the highest dose levels (about 200 ppb to 8 ppm), the responses varied from a growth stimulatory effect (hormesis), to additive, synergistic, and finally antagonistic cytotoxicity (Bae et al., 2001; Gennings et al., 2002). Table 2 is an example of such dose-dependent toxicological interactions in normal human epidermal keratinocytes (NHEK) following treatment of a metal mixture containing arsenic, chromium, cadmium, and lead. More examples on RHEK-1, HaCaT, and NM1 cell lines of human keratinocytes were given in Bae

Table 2
Observed mean % cell viability and predicted responses in NHEK cells exposed to a metal mixture

Mixture dilution	Total Concentration of metals (μM)[a]	Observed % viability	Variance	Predicted response	95% prediction intervals under additivity
0.0014	0.163	116.6[b]	35.0	100.9	[88.5, 113.3][c]
0.004	0.475	95.7	12.1	100.7	[92.8, 108.7]
0.0123	1.465	96.0	24.0	100.2	[89.8, 110.5]
0.037	4.39	76.5	10.2	98.4	[91.3, 105.4][d]
0.111	13.18	64.3	1.56	91.8	[87.0, 96.7][d]
0.333	39.56	50.1	29.1	60.8	[47.9, 73.6]
1	118.7	31.1	23.0	8.76	[0,19.5][e]

Modified after Ginnings et al., 2002. J. Agr. Biol. Environ. Stat. 7, 58–73.

[a] The predetermined LD_{50}'s were 7.7, 4.9, 6.1, and 100 μM for arsenic, chromium, cadmium, and lead, respectively. These levels were used for the 1X mixture (i.e., the highest dose level).

[b] More than 100% viability here means more cells were alive in the MTT assay in this group than the control group; in separate clonogenic assays, more clones were present in this group than the control group.

[c] Indicates a possible hormesis effect.

[d] Indicates a greater than additivity relationship (i.e., synergistic cytotoxicity).

[e] Indicates a less than additive relationship (i.e., antagonistic cytotoxicity).

et al. (2001). The growth stimulatory effects, or hormesis, at very low ppb range of these metals have been reported in many publications with other chemicals. The leading scientists in this area are Dr. Edward Calabrese and his colleagues of the University of Massachusetts. Hormesis may be the cumulative consequence of transient and sustained "overcompensation" by homeostatic mechanisms in response to low levels of inhibitory challenge (Stebbing, 1997; Calabrese and Baldwin, 1997). At higher levels of the metal mixture, the response is additive (Table 2). Still higher concentrations bring about synergistic cytotoxicity and at the highest concentration tested antagonistic cytotoxicity is apparent (Table 2). Particularly interesting is the somewhat abrupt switch from synergistic to antagonistic interactions in cells exposed to the metal mixture (Table 1; Bae et al., 2001). Mechanistic studies involving cellular levels of glutathione and metallothioneins I and II suggested that synergistic cytotoxicity turned to antagonistic cytotoxicity at highest mixture exposure concentrations because cellular defense mechanisms were enhanced (Bae et al., 2001).

7. Applications of Genomics to Chemical Mixture Toxicology

The NTP and its predecessor, the National Cancer Institute's Carcinogenesis Bioassay Program, collectively, form the world's largest toxicology program (NTP, 2002a). In its nearly 40 years of operation, fewer than 600 chemicals have been studied for carcinogenicity and other chronic toxicities (NTP, 2002a, 2002b). These "gold standard" chronic toxicity/carcinogenicity studies are extremely expensive (i.e., up to several million dollars/chemical), require large number of animals (i.e., about 2000 animals per chemical), and are lengthy (i.e., 5–12 years/chemical). Thus, considering the approximately 80,000 chemicals in commerce (NTP, 2002a), the number of compounds for which we have adequate toxicology information for risk assessment so far is minuscule. With the mode and rate of studying these chemicals as indicated above, it is unlikely that our society will ever have thorough toxicology information on the majority of the chemicals that we use now or may use in the future. Considering further the "real-world" issue of the health effects of chemical mixtures, it would be impossible to obtain adequate information on most of the chemicals or chemical mixtures that humans might be exposed to using the conventional approach (Yang, 1994b, 1997).

It is apparent that alternative, scientifically sound, less animal-intensive, shorter-term, and less expensive toxicology methods must be developed if we are to have a reasonable chance to deal with the hundreds of thousands of chemicals, as well as the near infinite number of chemical mixtures, in the environment. With the recent advances in molecular biology and computational technology, the applications of genomics and computer modeling to toxicology, particularly chemical mixture toxicology, appear to be inevitable. In this section we emphasize some of the recent research activities related to the application of genomics to toxicological studies, including work from our laboratory on chemical mixtures. In subsequent sections, we will discuss the application of computer technology to chemical mixture research at present and into the future.

Chemicals that exert either acute or chronic toxic effects at the cellular level may do so through direct or indirect alterations in gene expression. Signal transduction pathways that culminate in a transcriptional response mediate the toxicity of most chemical agents (Afshari et al., 1999; Nuwaysir et al., 1999; Thomas et al., 2001). Toxicity is commonly manifested as inflammation, proliferation, apoptosis, necrosis, and/or alterations in normal cellular differentiation (Thomas et al., 2001). Previous studies have demonstrated that chemicals with similar toxicological effects induce gene expression alterations in a common battery of genes following either in vitro (Waring et al., 2001a) or in vivo (Hamadeh et al., 2002; Thomas et al., 2001; Waring et al., 2001b) exposure. Analysis of 1200 transcripts by cDNA microarray for changes in

liver gene expression after exposure of mice to 24 chemical treatments that fall into five well-studied toxicological categories (peroxisome proliferators, aryl hydrocarbon receptor agonists, noncoplanar PCBs, inflammatory agonists, and hypoxia-inducing agents) resulted in a predictive gene set (Thomas et al., 2001). This diagnostic set of 12 transcripts provided an estimated 100% predictive accuracy. In a more recent study, patterns of gene expression in liver tissues derived from four chemically exposed rats revealed distinct profiles between a class of peroxisome proliferators and a class of enzyme inducers (Hamadeh et al., 2002). Gene expression analysis on RNA from the livers of rats treated with 15 different known hepatotoxins showed strong correlation between the histopathology, clinical chemistry (necrosis, DNA damage, cirrhosis, hypertrophy, and hepatic carcinoma), and gene expression profiles induced by the agents (Waring et al., 2001b). In addition, a similar conclusion that gene expression profiles for compounds with similar toxic mechanisms indeed formed clusters was demonstrated in studies by Waring et al. (2001a) utilizing the rat hepatocyte system with the same chemical treatments.

In our own laboratory, we have also demonstrated the potential utility of gene expression profiling by identifying a common suite of genes altered during malignant transformation of human keratinocytes by diverse chemical carcinogens (Bae, 2002; Bae et al., 2002, 2003). For instance, we have demonstrated that a single treatment with MNNG enhanced malignant transformation of the human keratinocyte cell line, RHEK-1. In contrast, chronic low-level exposure of cells to arsenic alone or in a mixture containing arsenic, cadmium, chromium, and lead inhibited malignant conversion (Bae et al., 2002). To identify changes in gene expression that influence these different outcomes, cDNA microarray technology was utilized. Analysis of multiple human arrays, including the Atlas Human Cancer 1.2, the NEN Human 2400, and the NEN Oncogene/Tumor Suppressor arrays, in MNNG-transformed RHEK-1 cells, designated OM3, and those treated with arsenic or the arsenic-containing metal mixture showed unique patterns of gene expression (Bae et al., 2002). Genes that are overexpressed in OM3 include oncogenes, cell cycle regulators, and those involved in signal transduction, while genes for DNA repair enzymes and inhibitors of transformation and metastasis were suppressed. In arsenic-treated cells, multiple DNA repair proteins were overexpressed. Arsenic containing metal mixture-treated cells showed increased expression of a variety of genes including metallothioneins and integrin β4. These cells showed decreased expression of oncogenes, DNA repair proteins, and genes involved in the MAP kinase pathway. Many of these changes have been validated using quantitative Real-Time RT-PCR (RT RT-PCR) (Bae et al., 2003).

We have recently obtained several RHEK-1 lines that were malignantly transformed in the laboratory of Dr. J. Rhim (Center for Prostate Disease Research, Rockville, MD) with MNNG, NQO, or TCDD (Rhim, 1989; Rhim et al., 1986). In collaborative studies with these investigators, we are comparing gene expression patterns or "molecular biosignatures" in OM3 (MNNG-transformed RHEK-1 cells in our laboratory) and these different transformed cell lines using the Clontech Human 3.8 II microarray. These studies have been specifically designed to minimize intrinsic variation due to the microarray technology itself and to allow statistical analysis of the resulting data, with triplicate arrays run for each cell sample and numerous internal control spots. Rigorous analysis of this data set using appropriate software program(s), such as ScanAlyze and Cluster (Michael Eisen, Stanford University), and validation studies with highly quantitative RT RT-PCR are currently being carried out. These studies will allow us to identify a battery of genes that is consistently up- or down-regulated in response to chemicals that confer upon RHEK-1 anchorage-independent growth (AIG+) and the tumorigenic phenotype in vitro. The genes identified in studies to date, along with their known or putative function are listed in Table 3.

Among the genes commonly up-regulated in all four transformed RHEK-1 sublines, are a member of the ras gene family, p21/cdc42, RNB6, a low fidelity DNA polymerase (κ), and several genes involved in growth and differentiation. Commonly down-regulated genes include five membrane transporters, two Ser/Thr protein kinases involved in apoptosis, cadherin 12, and several novel proteins.

Genes that were uniquely up- or down-regulated in response to the three different chemical agents are shown in Table 4. Additionally, there are several families of genes whose members (albeit different) are consistently altered in their expression in the four malignant RHEK-1 lines; these families include the cyclins, the histones, and the matrix metalloproteinases.

8. In Silico Toxicology: Is it for Real?

The short answer is "Yes." Our laboratory, the Quantitative and Computational Toxicology Group at Colorado State University, has been practicing "*In Silico Toxicology*" for over 10 years. In essence, it means integrating computer modeling with focused, mechanistic, animal experimentation such that experiments which are impractical (e.g., too large, too expensive) or impossible (e.g., human experiments with carcinogens) to perform are conducted on computer. Earlier examples, using PBPK/PD modeling and other computer modeling techniques such as Monte Carlo simulation, include: (1) acute toxicity studies, at 1000 rats per group, of toxicologic interactions between Kepone and carbon tetrachloride (El-Masri et al., 1996a); (2) human biological exposure indices (BEIs) studies on six industrial solvents (Thomas et al., 1996a); (3) "Interaction Thresholds" studies on binary and ternary chemical mixtures (El-Masri et al., 1996b, 1996c; Dobrev et al., 2001, 2002); (4) studies on age- and dosing-related pharmacokinetic changes in mice in a 2-year chronic toxicity/carcinogenicity bioassay (Thomas et al., 1996b); and (5) clonal growth modeling of early stages of carcinogenesis

Table 3
Genes commonly affected by MNNG, NQO, and TCDD in RHEK-1 cells

Regulation	Name	Fold[a]	Function(s)
Up	Ca activated chloride channel	2.3/2.5/2.3/2.2	Membrane channel
	CGI-120 protein	2.6/2.1/2.6/2.8	A novel gene found with Caenorhabditis elegans template
	COP9 subunit 6	6.3/5.1/11/2.7	RNA binding and macromolecule assembly; control of cell growth
	Differentiation related protein dif13	3.4/8.9/14/2.3	Differentially expressed gene in leukemia cell line k562 differentiation; homology with oncodevelopmental soluble placental tissue protein 17
	DNA-directed polymerase κ	9.1/6.1/2.9/3.3	A newly identified low fidelity polymerase
	Flavin containing monooxygenase 1	2.6/9.6/2.6/3.1	Chemical metabolism
	p21/cdc42/Rac1-activated kinase 1	4.4/4.4/11/2.6	Regulate actin organization/cell motility
	Purine-rich element binding protein B	3.9/3.7/6.1/2.3	Histone family
	Ras homolog gene family	3.1/8.2/3.4/2.0	Oncogene
	Recombining binding protein suppressor of hairless (Drosophila)	3.8/5.1/11/3.5	Recombination signal binding protein gene
	RNB6	6.1/25/4.6/3.3	Homology with a novel rat cDNA; control of cell motility through actin filament assembly
Down	Apoptosis-inducing ser/thr kinase 17b	4.4/8.1/4.2/3.4	Apoptosis
	Aquaporin 4	3.8/6.2/6.2/3.6	Membrane transporter
	Cadherin 12 type 2	4.1/5.7/5.0/3.6	Cell–cell interaction
	CD164 antigen, sialomucin	5.3/8.1/5.3/8.6	An adhesive glycoprotein; a novel core protein in gastric carcinoma
	CGI-68 protein	9/10/12/10	Leucine carboxyl methyltransferase for protein phosphatase 2A
	Cold shock domain protein A	4.4/4.7/4.5/3.4	DNA binding protein
	Cytokine receptor-like factor 1	4.1/4.5/3.8/3.2	A novel soluble protein: a possible regulatory role in the immune system
	Ephrin A1 (human B61 mRNA)	4.6/18/11/5.7	A novel secreted protein
	Fibroblast growth factor (acidic) intracellular binding protein	15/6.4/5.3/4.6	An intracellular protein
	G protein-coupled receptor 64	6.0/3.6/3.8/5.3	A novel member of the 7 transmembrane domain receptor family
	Homeo box A1 (HOX A1)	3.4/3.4/3.7/3.2	Transcription factor
	Low density lipoprotein-related protein 1	3.8/5.9/6.5/4.4	Ca binding cell surface protein
	Nebulin	3.0/3.0/3.9/5.5	A giant filamentous protein specific for vertebrate skeletal muscles
	Phosphodiesterase 6A, cGMP-specific, rod, α	3.3/3.1/2.4/2.7	Photoreceptor signal transduction
	Proprotein convertase subtilisin/kexin type 7	3.7/10/4.7/11	Ca-dependent serine protease prohormone convertase PC8
	Rhodopsin kinase	5.1/14/7.0/3.4	β-adrenergic receptor kinase
	Serine threonine protein kinase	7.3/6.8/8.4/4.5	Fas-mediated apoptosis
	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	5.0/4.1/3.4/2.9	Membrane transporter
	Solute carrier family 12 (potassium/chloride transporters), member 7	3.9/3.2/5.3/4.0	Membrane transporter
	Solute carrier family 21 (organic anion transporter), member 6	3.1/2.7/3.6/3.0	Membrane transporter
	Ubiquilin 2	15/3.9/33/20	Possible involvement in apoptosis
	Ubiquitin specific protease 21	4.4/3.5/6.9/4.3	A novel USP23
	U2 small nuclear ribonucleoprotein auxiliary factor (65 kD)	9.6/8.1/3.4/5.7	An essential mammalian splicing factor

To identify commonly or uniquely regulated genes in tumorigenic RHEK-1 cell lines obtained by exposure to MNNG, NQO, or TCDD, the expression analysis of 3,800 genes on Human 3.8II array was performed as described in previous studies (Bae et al., 2002; 2003). The genes commonly and significantly changed (>2-fold) in three chemical-treated cell populations compared to vehicle-treated ones are available in Bae et al., 2002. Two selection criteria for choosing these genes were applied: (i) genes consistently altered in all triplicate arrays, and (ii) genes that have a known or putative function.

[a] Gene expression fold in OM3, and MNNG, NQO, TCDD-transformed cell line compared to vehicle-treated cells (left to right).

Table 4
Genes uniquely affected by MNNG, NQO, and TCDD in RHEK-1 cells

Chemical	Name	Fold[a]	Function(s)
MNNG	CD24 antigen	⇑ 3.4	Small cell lung and hepatocellular carcinoma antigen
	Jumping translocation breakpoint	⇑ 4.9	A novel membrane protein; expressed in malignant cells
	Nucleolar protein 3	⇑ 17	Apoptosis inhibitor
	REV1 (yeast homolog)-like	⇑ 10	Required for mutagenesis induced by UV light
	HSPC037 protein	⇓ 2.8	Undefined gene from CD34+ hematopoietic stem/progenitor cells
	Neutral sphingomyelinase	⇓ 2.5	Signal transduction
	Pancreas enriched phospholipase Cε	⇓ 2.2	Signal transduction
NQO	c-Type lectin-like receptor 2	⇑ 4.7	Novel lectin-like receptor
	cystatin F (leukocystatin)	⇑ 30	Liver metastasis; proteinase inhibitor
	E1B-55 kD associated protein 5	⇑ 5.0	A cellular protein with RNA binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs
	Friend leukemia virus integration 1	⇑ 3.7	Ets oncogene family
	MAD2 (mitotic arrest deficient)-like 2	⇑ 4.9	Mitotic arrest deficient, yeast, homolog
	SEC10 (*S. cerevisiae*)-like 1	⇑ 32	Post-Golgi traffic
	Transient receptor potential channel 6	⇑ 14	Ca-like channel
TCDD	ANKHZN protein (a novel one)	⇑ 17	Ankyrin repeats hooked to a zinc finger motif
	Follistatin-like 1	⇑ 94	Activin binding protein; Kazal-type S protease inhibitor; novel autoantigen in systemic rheumatic diseases
	Homeobox D13	⇑ 20	Transcription factor HOX; development regulation
	Retinoblastoma binding protein 7 (RbAp46)	⇑ 16	Shares significant homology with a known yeast protein, MSI1, a negative regulator of the ras signal transduction pathway; regulation of cell proliferation/differentiation
	transmembrane 4 superfamily member (tetraspan NET-2)	⇑ 4.3	Cell migration
	Ubiquitin specific protease 8 (USP8)	⇑ 30	Oncogenic fusion product of the PI-3-kinase p85 subunit and HUMORF8, a putative deubiquitinating enzyme
	Hepatocyte nuclear factor 4 α	⇓ 4.2	Transcription factor

For each chemical, seven representative up- or down-regulated genes were chosen according to the same criteria used in Bae et al. (2002, 2003).
[a] Gene expression fold in chemical-transformed cell line compared to vehicle-treated cells. For MNNG, average fold is shown from MNNG-transformed cell lines at two different concentrations.

(Ou et al., 2001, 2003; Thomas et al., 2000). We believe that utilization of computer modeling is essential in the studies of chemical mixture toxicology and risk assessment. The area of toxicology, in general, will be well served by the application of computer technology as an alternative research method to minimize the killing of laboratory animals. Looking into the future, we believe that the linkage of PBPK modeling and "Reaction Network Modeling," described in details in the following two subsections, has the potential of providing a computer simulation platform for complex biological systems involving chemical mixtures and/or multiple stressors (Liao et al., 2002, 2004; Yang et al., in press).

8.1. Building a "Second Generation" PBPK/PD model

In classical pharmacokinetics and physiologically based pharmacokinetics (PBPK), human or animal bodies were often described by a few compartments. By "Second Generation" PBPK/PD modeling, we are referring to integrating PBPK with reaction network modeling, thus including many more compartments (i.e., a delumping process). Our thoughts may best be explained in this way: if one draws a parallel between an oil refinery, where the application of reaction network modeling approach has been very successful, and a human body, the individual processing units in the oil refinery may be considered as equivalent to the vital organs of the human body. Even though the cell or organ may be much more complicated, the complex biochemical reaction networks in each organ may be similarly modeled and linked much the same way as the modeling of the entire oil refinery through linkage of the individual processing units.

In the following paragraphs, we outline the traditional processes involved in building a PBPK model. This traditional approach will still be followed. However, because we are interested in complex biological processes in which detailed knowledge of the underlying interdependent biochemical reaction pathways is vital, the integration of PBPK modeling and "Reaction Network Modeling" will be necessary.

The fundamentals of PBPK modeling are to identify the principal organs or tissues involved in the disposition of the chemical of interest and to correlate the chemical absorption, distribution, metabolism, and excretion within and among these organs and tissues in an integrated and biologically plausible manner. A scheme is usually formed where the normal physiology is followed in a graphical manner. Within the boundary of the identified compartment (e.g., an organ or tissue or a group of organs or tissues), whatever 'comes' in must be accounted for via whatever 'goes out' or whatever is transformed into something else. This "mass balance" is expressed as a mathematical equation with appropriate parameters carrying biological significance. A series of such equations representing all of the interlinked compartments are formulated

to express a mathematical representation, or model, of the biological system. This model can then be used for computer simulation to predict the time course behavior of any given parameter. Three sets of parameters are needed for PBPK model building: physiological parameters (e.g., ventilation rates, cardiac output, organs as % body weight), thermodynamic parameters (e.g., tissue partition coefficients, flow rates), and biochemical parameters (e.g., K_m and V_{max}). Most, if not all, of the parameters for laboratory animals are available in relevant literature such as the Biological Data Book, and the special report by the International Life Sciences Institute (ILSI) on the compilation of physiological parameters for PBPK models (Brown et al., 1997). When information gaps exist, the solution is either an empirical one via experimentation or through allometric extrapolation, usually based on a power function of the body weight (Lindstedt, 1987).

Our approach goes beyond the traditional PBPK modeling in that we plan to embody the concept of "Reaction Network Modeling" (see next subsection for details) through the inclusion of the essential biological pathways into the PBPK model. If we think of a reaction network (e.g., metabolic pathway for a specific xenobiotic) as a spider web, because of shared enzymatic pathways many spider webs may overlap one another. Thus, for instance, reaction networks with overlapping metabolic reactions of many xenobiotics may be linked into the liver compartment of a PBPK model, or reaction networks of overlapping signaling pathways of certain neurotransmitters under the influence of environmental pollutants may be linked to a brain compartment of a PBPK model. In this way, pharmacodynamic interaction(s) will be incorporated into the PBPK modeling to transform the model to a PBPK/PD model.

We believe that such a development of the "Second Generation PBPK/PD Model" is a natural course of progression for this area by drawing a parallel between the evolution of pharmacokinetics and the evolution of reaction network modeling or structure-oriented lumping (SOL) in the field of chemical/petroleum engineering. As shown in Fig. 2, the classical pharmacokinetic compartment models such as one-, two-, or three-compartment models may be considered equivalent to the one-lump model (Blanding, 1953) for the entire gasoil feedstock in chemical/petroleum engineering. The kinetic modeling, under these circumstances, was understandably crude. As the respective areas progressed, particularly correlated with advancement of computer technology and analytical methodologies, a delumping process ensued. Thus, in pharmacokinetics, scientists began to develop PBPK modeling which, once upon a time, had considered a "Minimammal" model containing up to 54 compartments (Bischoff and Brown, 1966). Similarly, in chemical/petroleum engineering, the 10-lump Mobil Fluid Catalytic Cracking (FCC) model was successfully developed (Jacob et al., 1976). The resulting kinetic modeling in both fields was considerably more sophisticated which improved the accuracy of computer simulations. In the case of PBPK modeling, it became a powerful tool for extrapolation among species, routes, and doses.

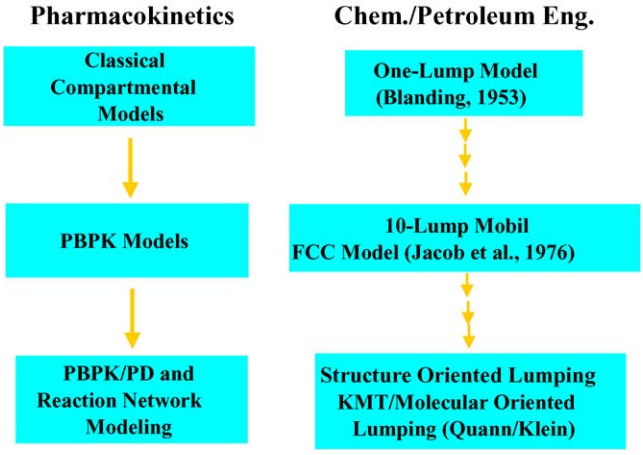

Fig. 2. Parallel comparison of the development of pharmacokinetics and reaction network modeling of petroleum refinery engineering processes. FCC is the fluid catalytic cracking; and KMT is the Kinetic Modeler's Toolkit.

Furthermore, incorporation of mechanistic information in recent years in the modeling process transformed PBPK modeling to PBPK/PD modeling. Such advancement in pharmacodynamic modeling might be the inescapable result of NIH's emphasis on mechanistic-based research. Meanwhile, further delumping in kinetic modeling continued in chemical/petroleum engineering as computational power and the knowledge of process chemistry in petroleum refining increased exponentially. Thus, as shown in Fig. 2, Dr. Richard Quann and colleagues at Mobil Technology Company created SOL and Dr. Michael Klein and colleagues at University of Delaware formulated the basis for reaction network modeling by creating the Kinetic Modeler's Toolkit (Quann and Jaffe, 1992, 1996; Broadbelt et al., 1994a, 1994b, 1996; Klein, 2000). Parallel to this type of delumping, we believe that the recent advances in computational technology and molecular biology will push the PBPK/PD modeling into more sophistication and finer-grain compartments. As illustrated in Fig. 3, the classical compartmental pharmacokinetic models may be considered an embryonic form of "Virtual Human." Indeed, despite the crude nature of these models as compared with the human body, classical pharmacokinetics has contributed very significantly to the field of medicine. The advancement of PBPK modeling, as a result of "delumping" and the incorporation of physiology into the modeling process, result in a better "Virtual Human," as depicted in Fig. 3 with a shadow of a human. We believe that the integration of reaction network modeling into the appropriate compartment of PBPK models, as shown in Fig. 3 with the incorporation of many "spider webs" (i.e., reaction networks) into the PBPK models, will create the "Second Generation PBPK/PD models" which will bring the concept of "Virtual Human" closer to reality.

8.2. Reaction Network Modeling

A "Reaction Network Model," from the perspective of its original application in petroleum engineering, is a tool for

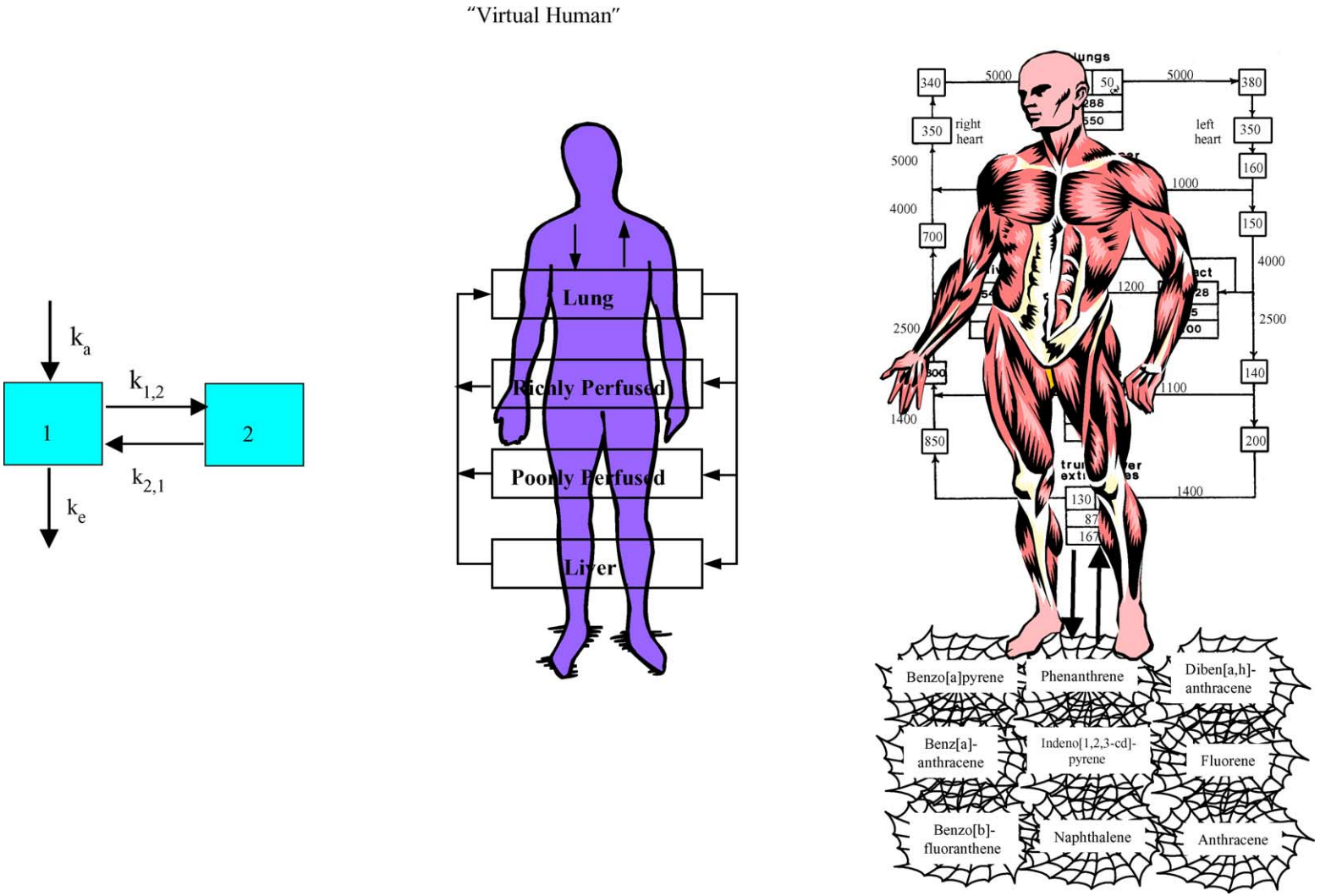

Fig. 3. Various forms of "Virtual Human" from the simple classical two-compartment pharmacokinetic model, to a four-compartment physiologically based pharmacokinetic (PBPK) model, to a complex PBPK model linked with a reaction network model.

predicting the amounts of reactants, intermediates, and products as a function of time for a series of coupled chemical reactions (potentially numbering in the tens of thousands of reactions for some systems). It is usually a mathematical or symbolic formulation, suitable for solution on the computer. A "Reaction Network Model" builder is a tool for the computer generation of a "Reaction Network Model." The model builder can thus be used to not only solve the kinetic equations of interest, but also generate the reaction mechanisms, rate constants, and reaction equations themselves.

Essentially, the model builder works as follows:

1. The concentrations of the species to be reacted or metabolized are input to the model builder.
2. For each species in turn, the model builder performs a test against each of a set of 'reaction rules' to determine whether or not the species is susceptible to a particular chemical reaction.
3. If it is not susceptible to any reactions, no further action is taken on this species.
4. If it is susceptible, a transformation of the species into one or more product species is performed, based on the particular chemical reaction.
5. Each of these product species then undergoes the same susceptibility tests and a similar transformation sequence. This leads to a linking of all reactants with intermediates, and ultimately, with final products. This linking forms the structure of the chemical 'reaction network'.
6. After the reaction network is established, the rate constants for the reactions are retrieved or are computed.
7. The coupled differential equations governing the reaction kinetics for the network are then formulated by the model builder.
8. Finally, the kinetic equations, i.e., the model equations, are solved numerically, leading to the concentrations of all species as a function of time.

More details on reaction network modeling, particularly the initial application to biomedical research, are available in a number of recent publications (Klein et al., 2002; Liao et al., 2002; Yang et al., in press; Reisfeld and Yang, 2004); one (Reisfeld and Yang, 2004), in particular, is a companion paper in this volume.

As an illustration, the biological application of reaction network modeling may be pursued with a "bottom up" approach such as what we have been doing with benzo[a]pyrene (BaP) and some of its metabolites in the overall BaP reaction network. We have been mining the literature or generate kinetic data on some of the metabolic processes experimentally for this modeling effort. Some preliminary information from this research may be seen in Liao et al. (2002); more complete information is reported in Liao (2004). Expanding on the "bottom up" approach, once BaP reaction network modeling is complete, we will link it with reaction networks of other carcinogenic or non-carcinogenic polycyclic aromatic hydrocarbons (PAHs). An ultimate goal of such a program can be the successful and accurate computer simulation of reaction networks of all the PAHs from air pollution in our body by integrating reaction network modeling and PBPK modeling.

9. Conclusion

In pursuing an understanding of toxicological risk, it is impractical to investigate all of the vast number of combinations of chemical mixtures and multiple stressors because the numbers approach infinity. A more realistic strategy is to focus on finite number of entities; thus, we should investigate biological processes which, though complex, represent finite entities. If we consider the collective effects of any given combination of chemical mixtures and multiple stressors are merely perturbations of normal biological processes, then a seemingly impossible task becomes a reachable goal through the utilization of biologically-based computer modeling. Therefore, we propose to utilize "Reaction Network Modeling" to analyze the reaction networks for metabolism of toxicologically important compounds and other stressors, the physiological and toxicological consequences of such chemicals and stressors, and the biological processes involved. Coupling such "Reaction Network Modeling" with PBPK modeling, we should be able to handle very complex chemical mixtures and other stressors as well as the related complex biological systems. Such an approach has great potential and represents pioneering work in adapting an engineering modeling approach that has been used successfully for many years in petroleum industry to solving problems in biomedical research.

In an AAAS Plenary Lecture on February 13, 1998, Dr. Harold Varmus, then Director of NIH, emphasized, among others, two specific themes: ". . .. Discoveries in biology and medicine depend on progress in many fields of science . . ." and ". . .. Methods that dramatically expand biological data also demand new modes of analysis and new ways to ask scientific questions . . ." He said: ". . .. In short, biology is not only for biologists . . ." Here we present an engineering approach and its possible application in biomedical research. We believe that this technology has the potential for the eventual development of a "virtual human."

Acknowledgements

The concepts and work discussed in this presentation were partially contributed by many colleagues associated or collaborating with the Quantitative and Computational Toxicology group at Colorado State University; we are grateful to their contribution and intellectual stimulation. Any advances in science require funding support from many agencies. We thank ATSDR (Cooperative Agreement U61/ATU881475), NIEHS (Superfund Basic Research Program Project P42 ES05949; research grants R01 ES09655 and RO3 ES10116 ZES1; training grant T32 ES 07321; and Career Development Award K25 ES11146), NCI (two Career Development Award 1 K08 CA72396 and 1 K01 CA75955-01A1), US

Air Force (research grants F33615-91-C-0538; F49620-94-1-0304), and NIOSH/CDC (1 RO1 OH07556-01). Without the generous support of these agencies, the development of research described herein could have never been possible.

References

Afshari, C.A., Nuwaysir, E.F., Barrett, J.C., 1999. Application of complementary DNA microarray technology to carcinogen identification, toxicology, and drug safety evaluation. Cancer Res. 59, 4759–4760.

Arcos, J.C., Argus, M.F., Woo, Y.T., 1995. Chemical Induction of Cancer. Modulation and Combination Effects. Birkhäuser, Boston, 711 pp.

ATSDR, 2002a. Interaction profiles for persistent chemicals found in breast milk. Chlorinated dibenzo-p-dioxins (CDDs), hexachlorobenzene, dichlorodiphenyl dichloroethane (p,p′-DDE), methyl mercury, and polychlorinated biphenyls (PCBs). Draft for public comments. Available at http://www.atsdr.cdc.gov.

ATSDR, 2002b. Interaction profile for 1,1,1-trichloroethane, 1,1-dichloroethane, trichloroethylene, and tetrachloroethylene. Draft for public comments. Available at http://www.atsdr.cdc.gov.

Bae, D.S., Gennings, C., Carter Jr., W.H., Yang, R.S.H., Campain, J.A., 2001. Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes. Toxicol. Sci. 63, 132–142.

Bae, D.S., 2002. Use of human keratinocytes for assessing the cytotoxicity and carcinogenic potential of chemical mixtures. Ph.D. Dissertation.

Bae, D.S., Hanneman, W.H., Yang, R.S.H., Campain, J.A., 2002. Characterization of gene expression changes associated with MNNG, arsenic, or metal mixture treatment in human keratinocytes: application of cDNA microarray technology. Environ. Health Perspect. 110 (Suppl. 6), 931–941.

Bae, D.S., Handa, R.J., Rhim, J.S., Yang, R.S.H., Campain, J.A., 2003. Gene expression patterns as potential molecular biomarkers for malignant cellular transformation in human keratinocytes treated with MNNG, arsenic, or metal mixture. Toxicol. Sci. 74, 32–42.

Bassi, J.A., Rosso, P., Moessinger, A.C., Blanc, W.A., James, L.S., 1984. Fetal growth retardation due to maternal tobacco smoke exposure in the rat. Pediatrics Res. 18, 127.

Benson, J.M., Hanson, R.L., Royer, R.E., Clark, C.R., Henderson, R.F., 1984. Toxicological and chemical characterization of the process stream materials and gas combustion products of an experimental low Btu coal gasifier. Environ. Res. 33, 96.

Bischoff, K.B., Brown, R.G., 1966. Drug distribution in mammals. Chem. Eng. Prog. Symp. Series. 62, 33–45.

Blanding, F.H., 1953. Reaction rates in catalytic cracking of petroleum. I.E.C. 45, 1186.

Broadbelt, L.J., Stark, S.M., Klein, M.T., 1994a. Computer generated pyrolysis modeling: on-the-fly generation of species, reactions, and rates. Ind. Eng. Chem. Res. 33, 790–799.

Broadbelt, L.J., Stark, S.M., Klein, M.T., 1994b. Computer generated reaction networks: on-the-fly calculation of species properties using computational quantum chemistry. Chem. Eng. Sci. 49, 4991–5010.

Broadbelt, L.J., Stark, S.M., Klein, M.T., 1996. Computer generated reaction modeling: decomposition and encoding algorithms for determining species uniqueness. Comp. Chem. Eng. 20, 113–129.

Brown, R.P., Delp, M.D., Lindstedt, S.L., Rhomberg, L.R., Beliles, R.P., 1997. Physiological parameter values for physiologically based pharmacokinetic models. Toxicol. Ind. Health 13, 407–484.

Bull, R.J. 1984. Toxicology of natural and man-made toxicants in drinking water. In: Proceedings of the 14th Annual Conference on Environmental Toxicology. Air Force Aerospace Medical Research Laboratory Technical Report No. 83-099, pp. 259–266.

Calabrese, E.J., Baldwin, L.A., 1997. The dose determines the stimulation (and poison): development of a chemical hormesis database. Int. J. Toxicol. 16, 545–559.

Cantelli-Forti, G., Vittozzi, L., Legator, M.S., 1994. Toxicological evaluation of chemical interactions. Environ. Health Perspect. 102 (Suppl. 9), 5–167.

Casse, F.R., Groten, J.P., van Bladeren, P.J., Feron, V.J., 1998. Toxicological evaluation and risk assessment of chemical mixtures. Crit. Rev. Toxicol. 28, 73–101.

Chapin, R.E., Phelps, J.L., Schwetz, B.A., Yang, R.S.H., 1989. Toxicology studies of a chemical mixture of 25 groundwater contaminants: (III) male reproduction study in B6C3F1 mice. Fundam. Appl. Toxicol. 13, 388–398.

Chu, I., Villeneuve, D.C., Becking, G.C., Lough, R., 1981. Subchronic effects of a mixture of inorganic substances present in the Great Lakes ecosystem in male and female rats. Bull. Environ. Contam. Toxicol. 26, 42–45.

Chu, I., Villeneuve, D.C., Valli, V.E., Ritter, L., Norstrom, R.J., Ryan, J.J., Becking, G.C., 1984. Toxicological response and its reversibility in rats fed Lake Ontario or Pacific salmon for 13 weeks. J. Environ. Sci. Health B19, 713–731.

Cote, M.G., Plaa, G.L., Valli, V.E., Villeneuve, D.C., 1985. Subchronic effects of a mixture of “persistent” chemicals found in the Great Lakes. Bull. Environ. Contam. Toxicol. 34, 285–290.

Curtis, L.R., Williams, W.L., Mehendale, H.M., 1979. Potentiation of hepatotoxicity of carbon tetrachloride following preexposure to chlordecone (Kepone) in the male rat. Toxicol. Appl. Pharmacol. 51, 283–293.

Dalbey, W., Lock, S. 1982. Airborne mixtures. Inhalation toxicology of diesel fuel obscurant aerosol in Sprague–Dawley rats, Phase I, acute exposures. Oak Ridge National Laboratory Report No. ORNL/TM-8867, p. 48.

Dennison, J.E., Andersen, M.E., Yang, R.S.H., 2003. Characterization of the pharmacokinetics of gasoline using PBPK modeling with a complex mixture chemical lumping approach. Inhal. Toxicol. 15, 961–968.

Dobrev, I., Andersen, M.E., Yang, R.S.H., 2001. Assessing interaction thresholds for trichloroethylene, tetrachloroethylene, and 1,1,1-trichloroethane using gas uptake studies and PBPK modeling. Arch. Toxicol. 75, 134–144.

Dobrev, I., Andersen, M.E., Yang, R.S.H., 2002. In silico toxicology: simulating interaction thresholds for human exposure to mixtures of trichloroethylene, tetrachloroethylene, and 1,1,1-trichloroethane. Environ. Health Perspect. 110, 1031–1039.

El-Masri, H.A., Thomas, R.S., Sabados, G.R., Phillips, J.K., Constan, A.A., Benjamin, S.A., Andersen, M.E., Mehendale, H.M., Yang, R.S.H., 1996a. Physiologically based pharmacokinetic/pharmacodynamic modeling of the toxicologic interaction between carbon tetrachloride and Kepone. Arch. Toxicol. 70, 704–713.

El-Masri, H.A., Tessari, J.D., Yang, R.S.H., 1996b. Exploration of an interaction threshold for the joint toxicity of trichloroethylene and 1,1-dichloroethylene: utilization of a PBPK model. Arch. Toxicol. 70, 527–539.

El-Masri, H.A., Constan, A.A., Ramsdell, H.S., Yang, R.S.H., 1996c. Physiologically based pharmacodynamic modeling of an interaction threshold between trichloroethylene and 1,1-dichloroethylene in Fischer 344 rats. Toxicol. Appl. Pharmacol. 141, 124–132.

Feron, V.J., Groten, J.P., van Zorge, J.A., Cassee, F.R., Jonker, D., van Bladeren, P.J., 1995. Toxicity studies in rats of simple mixtures of chemicals with the same or different target organs. Toxicol. Lett. 82/83, 505–512.

Feron, V.J., Bolt, H.M., 1996. Combination Toxicology. Proceedings of a European Conference. Food Chem. Toxicol. 34, 1025–1185.

Feron, V.J., Cassee, F.R., Groten, J.P., 1998. Toxicology of chemical mixtures: international perspective. Environ. Health Perspect. 106 (Suppl. 6), 1281–1289.

Feron, V.J., Groten, J.P., 2002. Toxicological evaluation of chemical mixtures. Food Chem. Toxicol. 40, 825–839.

Gennings, C., Carter Jr., W.H., Campain, J.A., Bae, D.S., Yang, R.S.H., 2002. Statistical analysis of interactive cytotoxicity in human epidermal keratinocytes following exposure to a mixture of four metals. J. Agric. Biol. Environ. Stat. 7, 58–73.

Germolec, D.R., Yang, R.S.H., Ackermann, M.P., Rosenthal, J.G., Boorman, G.A., Thompson, M., Blair, P., Luster, M.I., 1989. Toxicology studies of a chemical mixture of 25 groundwater contaminants: (II) immunosuppression in B6C3F1 mice. Fundam. Appl. Toxicol. 13, 377–387.

Gray, R.H., 1984. Chemical and toxicological aspects of coal liquefaction and other complex mixtures. Reg. Toxicol. Pharmacol. 4, 380.

Groten, J.P., 2000. Mixtures and interactions. Food Chem. Toxicol. 38 (Suppl. 1), S65–S71.

Groten, J.P., Feron, V.J., Suhnel, J., 2001. Toxicology of simple and complex mixtures. Trends Pharmacol. Sci. 22, 316–322.

Gullino, P., Winitz, M., Birnbaum, S.M., Cornfield, J., Otey, M.C., Greenstein, J.P., 1956. Studies on the metabolism of amino acids and related compounds in vivo. I. Toxicity of essential amino acids, individually and in mixtures, and the protective effect of L-arginine. Arch. Biochem. Biophys. 64, 319–332.

Hackett, P.L., Rommereim, D.N., Sikov, M.R., 1984. Developmental toxicity following oral administration of a high-boiling coal liquid to pregnant rats. J. Appl. Toxicol. 4, 57–62.

Haddad, S., Krishnan, K., 1998. Physiological modeling of toxicokinetic interactions: implications for mixture risk assessment. Environ. Health Perspect. 106 (Suppl. 6), 1377–1384.

Haddad, S., Tardif, R., Charest-Tardif, G., Krishnan, K., 1999. Physiological modeling of the toxicokinetic interactions in a quaternary mixture of aromatic hydrocarbons. Toxicol. Appl. Pharmacol. 161, 249–257.

Haddad, S., Charest-Tardif, G., Tardif, R., Krishnan, K., 2000a. Validation of a physiological modeling framework for simulating the toxicokinetics of chemicals in mixtures. Toxicol. Appl. Pharmacol. 167, 199–209.

Haddad, S., Charest-Tardif, G., Krishnan, K., 2000b. Physiologically based modeling of the maximal effect of metabolic interactions on the kinetics of components of complete chemical mixtures. J. Toxicol. Environ. Health 61, 209–223.

Hamadeh, H.K., Bushel, P.R., Jayadev, S., DiSorbo, O., Bennett, L., Li, L., Tennant, R., Stoll, R., Barrett, J.C., Paules, R.S., Blanchard, K., Afshari, C.A., 2002. Prediction of compound signature using high density gene expression profiling. Toxicol. Sci. 67, 232–240.

Heckman, C.A., Dalbey, W.E., 1982. Pathogenesis of lesions induced in rat lung by chronic tobacco smoke inhalation. J. Natl. Cancer Inst. 69, 117–129.

Heindel, J.J., Chapin, R.E., Gulati, D.K., George, J.D., Price, C.J., Marr, M.C., Myers, C.B., Barnes, L.H., Fail, P.A., Grizzle, T.B., Schwetz, B.A., Yang, R.S.H., 1994. Assessment of the reproductive and developmental toxicity of pesticide/fertilizer mixtures based on confirmed pesticide contamination in California and Iowa groundwater. Fundam. Appl. Toxicol. 22, 605–621.

Heindel, J.J., Chapin, R.E., George, J., Gulati, D.K., Fail, P.A., Barnes, L.H., Yang, R.S.H., 1995. Assessment of the reproductive toxicity of a complex mixture of 25 groundwater contaminants in mice and rats. Fundam. Appl. Toxicol. 25, 9–19.

Hong, H.L., Yang, R.S.H., Boorman, G.A., 1991. Residual damage to hematopoietic system in mice exposed to a mixture of groundwater contaminants. Toxicol. Lett. 57, 101–111.

Hong, H.L., Yang, R.S.H., Boorman, G.A., 1992. Alterations in hematopoietic responses in mice caused by drinking a mixture of 25 groundwater contaminants. J. Environ. Pathol. Toxicol. Oncol. 11, 1–10.

Hong, H.L., Yang, R.S.H., Boorman, G.A., 1993. Enhancement of myelotoxicity induced by repeated irradiation in mice exposed to a mixture of groundwater contaminants. Arch. Toxicol. 7, 358–364.

Jacob, S.M., Gross, B., Voltz, S.E., Weekman, V.W., 1976. A lumping and reaction scheme for catalytic cracking. AIChE J. 22, 701.

Jonker, D., Woutersen, R.A., van Bladeren, P.J., Til, H.P., Feron, V.J., 1990. 4-Week oral toxicity study of a combination of eight chemicals in rats: comparison with the toxicity of the individual compounds. Food Chem. Toxicol. 28, 623–631.

Jonker, D., Jones, M.A., van Bladeren, P.J., Woutersen, R.A., Til, H.P., Feron, V.J., 1993a. Acute (24 h) toxicity study of a combination of four nephrotoxicants in rats compared with the toxicity of the individual compounds. Food Chem. Toxicol. 31, 45–52.

Jonker, D., Woutersen, R.A., van Bladeren, P.J., Til, H.P., Feron, V.J., 1993b. Subacute (4-week) oral toxicity of a combination of four nephrotoxicants in rats: comparison with the toxicity of the individual compounds. Food Chem. Toxicol. 31, 125–136.

Kirchner, F.R., Reilly Jr., C.A., Cuddihy, R.G., Jones, R.K., Mauderly, J.L., Wolff, R.K., 1983. Inhalation toxicology of diesel exhaust particles. In: Lewtas, J. (Ed.), Toxicological Effects of Emissions From Diesel Engines. Elsevier Biomedical, New York, pp. 99–120.

Klein, M.T. 2000. Faculty activities publication. Department of Chemical and Biochemical Engineering, Rutgers University.

Klein, M.T., Hou, G., Quann, R., Wei, W., Liao, K.H., Yang, R.S.H., Campain, J.A., Mazurek, M., Broadbelt, L.J., 2002. BioMOL: a computer-assisted biological modeling tool for complex chemical mixtures and biological processes at the molecular level. Environ. Health Perspect. 110 (Suppl. 6), 125–129.

Kligerman, A.D., Chapin, R.E., Erexson, G.L., Germolec, D.R., Kwanyuen, P., Yang, R.S.H., 1993. Analyses of cytogenetic damage in rodents following exposure to simulated groundwater contaminated with pesticides and a fertilizer. Mutat. Res. 300, 125–134.

Kool, H.J., Kuper, F., van Haeringen, H., Koeman, J.H., 1985. A carcinogenicity study with mutagenic organic concentrates of drinking-water in The Netherlands. Food Chem. Toxicol. 23, 79–85.

Krishnan, K., Haddad, S., Béliveau, M., Tardif, R., 2002. Physiological modeling and extrapolation of pharmacokinetic interactions from binary to more complex chemical mixtures. Environ. Health Perspect. 110 (Suppl. 6), 989–994.

Liao, K.H. 2004. Reaction Network Model for the Prediction of Mammalian Metabolism of Benzo[a]pyrene. Ph.D. dissertation, Colorado State University, 214 pp.

Liao, K.H., Dobrev, I., Dennison Jr., J.E., Andersen, M.E., Reisfeld, B., Reardon, K.F., Campain, J.A., Wei, W., Klein, M.T., Quann, R.J., Yang, R.S.H., 2002. Application of biologically based computer modeling to simple or complex mixtures. Environ. Health Perspect. 110 (Suppl. 6), 957–963.

Lindstedt, S.L., 1987. Allometry: body size constraints in animal design. Pharmacokinetics in Risk, Assessment, Drinking Water, Health, vol. 8. National Academy Press, Washington, DC, pp. 65–79.

MacFarland, H.N. 1984. The toxicity of complex mixtures. In: Proceedings of the 14th Annual Conference on Environmental Toxicology. Air Force Aerospace Medical Research Laboratory Technical Report No. 83-099, pp. 239–245.

Mahlum, D.D., 1983. Initiation/promotion studies with coal-derived liquids. J. Appl. Toxicol. 3, 31–34.

Mehendale, H.M., 1984. Potentiation of halomethane hepatotoxicity: chlordecone and carbon tetrachloride. Fundam. Appl. Toxicol. 4, 295–308.

Mehendale, H.M., 1991. Role of hepatocellular regeneration and hepatolubular healing in the final outcome of liver injury. Biochem. Pharmacol. 42, 1155–1162.

Mehendale, H.M., 1994. Mechanism of the interactive amplification of halomethane hepatotoxicity and lethality by other chemicals. In: Yang, R.S.H. (Ed.), Toxicology of Chemical Mixtures: Case Studies, Mechanisms, and Novel Approaches. Academic Press, San Diego, CA, pp. 299–334.

Mumtaz, M.M., De Rosa, C.T., Groten, J., Feron, V.J., Hansen, H., Durkin, P.R., 1998. Estimation of toxicity of chemical mixtures through modeling of chemical interactions. Environ. Health. Perspect. 106 (Suppl. 6), 1353–1360.

Mumtaz, M.M., De Rosa, C.T., Groten, J., Feron, V.J., Hansen, H., Durkin, P.R., 2000. In vitro evaluation of the weight of evidence method for the toxicologic interactions of chemical mixtures. Toxicologists 54, 1059.

Mumtaz, M.M., El-Masri, H., De Rosa, C.T., Moffett, D., Durkin, P., Schoen, E., Jonker, D., Groten, J.P., van Zorge, J., 2003. Assessment of the weight-of-evidence method using mixtures of benzene, lead, methyl mercury, and TCE. Toxicologists 72, 670.

NRC/NAS 1989. Drinking Water and Health. Selected Issues in Risk Assessment, vol. 9, Part II. Mixtures, National Academy Press, 268 pp.

NTP 2002a. National Toxicology Program: Annual Plan for Fiscal Year 2001. National Toxicology Program, U.S. Department of Health and Human Services, Washington, DC.

NTP 2002b. National Toxicology Program, Management Status Report. U.S. Department of Health and Human Services, 14 January 2002.

Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C., Afshari, C.A., 1999. Microarrays and toxicology: the advent of toxicogenomics. Mol. Carcinog. 24, 153–159.

Ou, Y.C., Connolly, R.B., Thomas, R.S., Xu, Y., Andersen, M.E., Chubb, L., Pitot, H.C., Yang, R.S.H., 2001. A clonal growth model: time-course simulations of liver foci growth following penta- or hexachlorobenzene treatment in a medium-term bioassay. Cancer Res. 61, 1879–1889.

Ou, Y.C., Connolly, R.B., Lapidot, S.A., Thomas, R.S., Gustafson, D.L., Long, M.E., Dobrev, I., Chubb, L., Xu, Y., Andersen, M.E., Pitot, H.C., Yang, R.S.H., 2003. Stochastic simulations of liver foci development in a medium-term bioassay for four chlorobenzene congeners. Toxicol. Sci. 73, 301–314.

Pelekis, M., Krishnan, K., 1997. Assessing the relevance of rodent data on chemical interaction for health risk assessment purposes: A case study with dichloromethane-toluene mixture. Regul. Toxicol. Pharmacol. 25, 79–86.

Plotkin, S., Ram, N.M., 1984. Multiple bioassays to assess the toxicity of a sanitary landfill leachate. Arch. Environ. Contam. Toxicol. 13, 197–206.

Quann, R.J., Jaffe, S.B., 1992. Structure-oriented lumping: describing the chemistry of complex hydrocarbon mixtures. I&EC Research 31, 2483–2497.

Quann, R.J., Jaffe, S.B., 1996. Building useful models of complex reaction systems in petroleum refining. Chem. Eng. Sci. 51, 1615–1635.

Reiley Jr., C.A., Peraino, C., Haugen, D.A., Mahlum, D.D., Springer, D.L., 1985. Promotion of preneoplastic changes in liver by coal-derived organic mixtures applied to skin. Cancer Lett. 28, 121–125.

Reisfeld, B.,Yang, R.S.H., 2004. A reaction network model for CYP2E1-mediated metabolism of toxicant mixtures. Environ. Toxicol. Pharmacol. 28, 173–179.

Rhim, J.S., Fujita, J., Arnstein, P., Aaronson, S.A., 1986. Neoplastic conversion of human keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science 232, 385–388.

Rhim, J.S., 1989. Neoplastic transformation of human epithelial cells in vitro. Anticancer Res. 9, 1345–1365.

Rylander, R., 1984. Environmental tobacco smoke and lung cancer. Eur. J. Resp. Dis. Suppl. 65, 127–133.

Shelby, M.D., Tice, R.R., DeMarini, D.M., Yang, R.S.H., 1990. Toxicity and mutagenicity of a mixture of 25 chemicals found in contaminated ground water. In: Vainio, H., Sorsa, M., McMichael, A.J. (Eds.), Complex Mixtures and Cancer Risk. International Agency for Research on Cancer, Lyon, pp. 314–332.

Silkworth, J.B., McMartin, D.N., Rej, R., Narang, R.S., Stein, V.B., Briggs, R.G., Kaminsky, L.S., 1984. Subchronic exposure of mice to Love Canal soil comtamination. Fundam. Appl. Toxicol. 4, 231.

Simmons, J.E., 1995. Chemical mixtures and quantitative risk assessment. Toxicology 105, 109–441.

Simmons, J.E., Yang, R.S.H., Svendsgaard, D.J., Thompson, M.B., Seely, J.C., McDonald, A., 1994. Toxicology studies of a chemical mixture of 25 groundwater contaminants: hepatic and renal assessment, response to carbon tetrachloride challenge, and influence of treatment-induced water restriction. J. Toxicol. Environ. Health 43, 305–325.

Springer, D.L., Miller, R.A., Weimer, W.C., Ragan, H.A., Buschbom, R.L., Mahlum, D.D., 1986. Effects of inhalation exposure to a high-boiling (288–454 °C) coal liquid. Toxicol. Appl. Phamacol. 82, 112–131.

Stara, J.F., Erdreich, L.S., 1984. Selected approaches to risk assessment for multiple chemical exposure. Progress Report on Guideline Development at ECAO-Cincinnati, EPA-600/9-84-014a. June 1984, 103 pp.

Stebbing, A.R.D., 1997. A theory for growth hormesis. BELLE Newsletter 6, 1–11.

Tardif, R., Lapare, S., Krishnan, K., Brodeur, J., 1993. Physiologically based modeling of the toxicokinetic interaction between toluene and *m*-xylene in the rat. Toxicol. Appl. Pharmacol. 120, 266–273.

Tardif, R., Lapare, S., Charest-Tardif, G., Brodeur, J., Krishnan, K., 1995. Physiologically- based pharmacokinetic modeling of a mixture toluene and xylene in humans. Risk Analysis 15, 335–342.

Tardif, R., Charest-Tardif, G., Brodeur, J., Krishnan, K., 1997. Physiologically based pharmacokinetic modeling of a ternary mixture of alkyl benzenes in rats and humans. Toxicol. Appl. Pharmacol. 144, 120–134.

Teuschler, L., Klaunig, J., Carney, E., Chambers, J., Connolly, R., Gennings, C., Giesy, J., Hertzberg, R., Klaassen, C., Kodell, R., Paustenbach, D., Yang, R., 2002. Support of science-based decisions concerning the evaluation of the toxicology of mixtures: a new beginning. Regulat. Toxicol. 36, 34–39.

Thomas, R.S., Bigelow, P.L., Keefe, T.J., Yang, R.S.H., 1996a. Variability in biological exposure indices using physiologically based pharmacokinetic modeling and monte carlo simulation. Am. Ind. Hyg. Assoc. J. 57, 23–32.

Thomas, R.S., Yang, R.S.H., Morgan, D.G., Moorman, M.P., Kermani, H.R.S., Sloane, R.A., O'Connor, R.W., Adkins Jr., B., Gargas, M.L., Andersen, M.E., 1996b. PBPK modeling/Monte-Carlo simulation of methylene chloride kinetic changes in mice in relation to age and acute, subchronic, and chronic inhalation exposure. Environ. Health Perspect. 104, 858–865.

Thomas, R.S., Conolly, R.B., Gustafson, D.L., Long, M.E., Benjamin, S.A., Yang, R.S.H., 2000. A physiologically-based pharmacodynamic analysis of hepatic foci within a medium-term liver foci bioassay using pentachlorobenzene as a promoter and diethylnitrosamine as an initiator. Toxicol. Appl. Pharmacol. 166, 128–137.

Thomas, R.S., Rank, D.R., Penn, S.G., Zastrow, G.M., Hayes, K.R., Pande, K., Glover, E., Silander, T., Craven, M.W., Reddy, J.K., Jovanovich, S.B., Bradfield, C.A., 2001. Identification of toxicologically predictive gene sets using cDNA microarrays. Mol. Pharmacol. 60, 1189–1194.

USEPA 1986. Guidelines for the Health Risk Assessment of chemical mixtures. Federal Register vol. 51, pp. 34014–34025.

USEPA 1988. Technical Support Document on Risk Assessment of Chemical Mixtures. EPA/600/8-90/064, November 1988.

USEPA 2000. Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures EPA/630/R-00/002, August 2000.

Villeneuve, D.C., Valli, V.E., Norstrom, R.J., Freeman, H., Sanglang, G.B., Ritter, L., Becking, G.C., 1981. Toxicological response of rats fed Lake Ontario or Pacific Coho salmon for 28 days. J. Environ. Sci. Health B16, 649–689.

Waring, J.F., Ciurlionis, R., Jolly, R.A., Heindel, M., Ulrich, R.G., 2001a. Microarray analysis of hepatotoxins in vitro reveals a correlation between gene expression profiles and mechanisms of toxicity. Toxicol. Lett. 120, 359–368.

Waring, J.F., Jolly, R.A., Ciurlionis, R., Lum, P.Y., Praestgaard, J.T., Morfitt, D.C., Buratto, B., Roberts, C., Schadt, E., Ulrich, R.G., 2001b. Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. Toxicol. Appl. Pharmacol. 175, 28–42.

Webster, P.W., van Der Heijden, C.A., Bisschop, A., van Esch, G.J., Wegman, R.C.C., De Vries, T., 1985. Carcinogenicity study in rats with a mixture of eleven volatile halogenated hydrocarbon drinking water contaminants. Sci. Total Environ. 47, 427.

Wyzga, R.E., Goldstein, L.S., 1994. Assessing the carcinogenic potency of environmental coal tars, by-products of coal gasification. In: Yang,

R.S.H. (Ed.), Toxicology of Chemical Mixtures: Case Studies, Mechanisms, and Novel Approaches. Academic Press, San Diego, CA, pp. 83–97.

Yang, R.S.H., 1994a. Toxicology of Chemical Mixtures: Case Studies, Mechanisms, and Novel Approaches. Academic Press, San Diego, CA, p. 720.

Yang, R.S.H., 1994b. Introduction to the toxicology of chemical mixtures. In: Yang, R.S.H. (Ed.), Toxicology of Chemical Mixtures: Case Studies, Mechanisms, and Novel Approaches. Academic Press, San Diego, CA, pp. 1–10.

Yang, R.S.H., 1997. Toxicologic interactions of chemical mixtures. In: Bond, J. (Ed.), Comprehensive Toxicology. General Principles, Toxicokinetics, and Mechanisms of Toxicity, vol.1. Elsevier Science Ltd., Oxford, UK, pp. 189–203.

Yang, R.S.H., 1998. Some critical issues and concerns related to research advances on the toxicology of chemical mixtures. Environ. Health Perspect. 106 (Suppl. 4), 1059–1063.

Yang, R.S.H., Goehl, T., Brown, R., Chatham, A., Arneson, D., Buchanan, R., Harris, R., 1989. Toxicology studies of a chemical mixture of 25 groundwater contaminants: (I) chemistry development. Fundam. Appl. Toxicol. 13, 366–376.

Yang, R.S.H., Rauckman, E.J., 1987. Toxicological studies of chemical mixtures of environmental concern at the National Toxicology Program: health effects of ground water contaminants. Toxicology 47, 15–34.

Yang, R.S.H., Suk, W.A., 1998. Current issues on chemical mixtures. Environ. Health Perspect. 106 (Suppl. 6), 1261–1448.

Yang, R.S.H., Suk, W.A., 2002. Application of technology to chemical mixture research. Environ. Health Perspect. 110 (Suppl. 6), 891–1036.

Yang, R.S.H., Liao, K.H., Reisfeld, B., in press. The integration of computer modeling and experimental toxicology for the study of chemical mixtures and multiple stressors. Arch. Complex Environ. Factors.