The Future of Uncertainty Factors With In Vitro Studies Using Human Cells

Michael Dourson,* Lorna Ewart †, Suzanne C. Fitzpatrick,‡ Silvia B. M. Barros,§ Brinda Mahadevan,¶ and A. Wallace Hayes‖,1

*TERA, Cincinnati, Ohio 45223, USA, †Emulate Inc., Boston, Massachusetts 02210, USA, ‡CFSAN, U.S. Food and Drug Administration, College Park, Maryland 20740, USA, §University of São Paulo, São Paulo, Brazil, ¶Brincor Associates, LLC, New Albany, Ohio 43054, USA, and ‖College of Public Health, University of South Florida, Tampa, Florida 33612, USA

1To whom correspondence should be addressed. E-mail: awallacehayes@comcast.net.

ABSTRACT

New approach methodologies (NAMs), including in vitro toxicology methods such as human cells from simple cell cultures to 3D and organ-on-a-chip models of human lung, intestine, liver, and other organs, are challenging the traditional “norm” of current regulatory risk assessments. Uncertainty Factors continue to be used by regulatory agencies to account for perceived deficits in toxicology data. With the expanded use of human cell NAMs, the question “Are uncertainty factors needed when human cells are used?” becomes a key topic in the development of 21st-century regulatory risk assessment. M.D., PhD, the coauthor of an article detailing uncertainty factors within the U.S. EPA, and L.E., PhD., Executive Vice President, Science, Emulate, who is involved in developing organ-on-a-chip models, debated the topic. One important outcome of the debate was that in the case of in vitro human cells on a chip, the interspecies (animal to human) uncertainty factor of 10 could be eliminated. However, in the case of the intraspecies (average human to sensitive human), the uncertainty factor of 10, additional toxicokinetic and/or toxicodynamic data or related information will be needed to reduce much less eliminate this factor. In the case of other currently used uncertainty factors, such as lowest observable adverse effect level to no-observed adverse effect level extrapolation, missing important toxicity studies, and acute/subchronic to chronic exposure extrapolation, additional data might be needed even when using in vitro human cells. Collaboration between traditional risk assessors with decades of experience with in vivo data and risk assessors working with modern technologies like organ chips is needed to find a way forward.

Key words: alternatives; new approach methodologies; uncertainty factors; organ-on-a-chip.
The use of uncertainty factors by regulatory agencies to account for perceived deficits in toxicology data using animals led to the question at the center of the roundtable session at the 45th Annual Meeting of the Society of Toxicology “Are uncertainty factors needed when human cells are used?” The introduction of factors interchangeably referred to as safety, uncertainty, correction, assessment, adjustment, or extrapolation factors cannot be separated from the need to derive safe levels of additives or contaminants in food or other areas of regulatory toxicology. These factors originate from the emergence of the acceptable daily intake (ADI), a concept widely credited to European and American toxicologists including the late French toxicologist Professor Truhaut in the early 1950s (Hayes and Kruger, 2014). These uncertainty factors were introduced to extrapolate toxicological data from animal experiments to humans. A safety factor of 100 was originally proposed by Lehman and Fizhugh (1954) of the U.S. Food and Drug Administration. A safety factor of 100 was arbitrarily set but was originally intended to account for interspecies (animal-to-human) variability and interindividual (human-to-human) variability, which allowed sensitive human populations to be compared with healthy experimental animals. The further subdivision of the conventional 10-fold safety factors for each experimental animal to human extrapolation and within human variability into toxicokinetics and toxicodynamics subfactors, was proposed independently by Renwick (1991) and the U.S. EPA (1994), and further expanded by Dorne and Renwick (2005).

For environmental chemicals, several additional factors beyond the initial 100 safety factor arbitrarily suggested by Lehman and Fizhugh were proposed to account for various shortcomings in the experimental data, such as inappropriate study design, using acute or subchronic rather than chronic data (since the objective was to determine a lifetime ADI), or relying on a lowest observable adverse effect level (LOAEL) rather than a no-observed adverse effect level (NOAEL), as well as other factors such as severe or irreversible effects (Dourson and Stara, 1983). With the passage of the Food Quality Protection Act (FQPA) in 1996, an additional factor of 10 must be considered for children and other hypersensitive populations by the U.S. Environmental Protection Agency (U.S. EPA) unless the science supports otherwise [EPA/FQPA. Public Law 104–170, Environmental Protection Act. Washington, DC: Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office.]. The U.S. EPA (2002) has opined on how this factor is to be interpreted and used.

Dr. M.D. initiated the debate by reminding us that uncertainty factors are considered necessary adjustments in the experimental animal in vivo dose showing no adverse effects to the expected in vivo no-effect dose for a sensitive subgroup of humans and that these factors will be needed even with studies using human cells in vitro. This no-effect dose in a sensitive subgroup is synonymous with an ADI or other similar safe or acceptable dose concepts, described in Figure 1 and more extensively by Dourson and Stara (1983), Dourson and DeRosa (1991), Dourson et al. (1996), Dourson and Parker (2007), and Dankovic et al. (2015).

In the development of safe doses with in vivo data, the use of different uncertainty factors for different chemicals is often necessary, because the underlying experimental data are not always uniform. The use of different uncertainty factors is currently a routine part of safe dose assessment because of these differing databases. Typical uncertainty factor used by the U.S. EPA is shown in Figure 2, where 2 of these factors are seen to reduce the projected risk (UFH and UFL) and the other 3 factors are seen to move from 1 dose-response curve to another without any risk reduction. Other regulatory authorities often use a similar uncertainty factor construct.

Although some consider uncertainty factors as arbitrary, this is a misconception as suggested by a quotation from a famous historical figure:

> It is the mark of an instructed mind to rest satisfied with the degree of precision which the nature of the subject permits and not to seek an exactness where only an approximation of the truth is possible.

---

**Aristotle**

Rather than being arbitrary, uncertainty factors are imprecise. This is because the underlying biology is imprecise as readily demonstrated by innumerable biological measurements. Now if someone were to recommend the use of an uncertainty factor for the experimental animal to human extrapolation when starting with human data, that would be arbitrary.

Yet, another misconception of uncertainty factors, is that the default uncertainty factor for addressing human variability is too small.

- **Supposition:** Human variability in a toxic response is often well beyond 10-fold in response to drugs and unintended chemical exposures.
- **Supposition:** This variability is easily demonstrated in clinical trials, human observational studies, and in vitro systems that use cells or organelles from different human populations.
- **Therefore:** The usual default uncertainty factor of 10-fold to estimate a safe dose from human data can be seen as not near enough in many cases.

Really? No, not really.

Human variability is indeed diverse, sometimes reaching hundreds and perhaps even thousands fold. But uncertainty factors, and specifically the one for human variability, never start with the most resistant individual, but rather from an individual or group in the more sensitive area of the dose-response curve as shown in Figure 3. Thus, the usual 10-fold uncertainty factor for this area of extrapolation accounts for larger variability in the human population, when used correctly.

So what about the future? The plethora of in vitro data from human cells will eliminate the need for at least one of the traditional factors… that of experimental animal to human extrapolation. But like in vivo data, in vitro databases may also not likely be uniform amongst chemicals. Thus, uncertainty factors for human variability, subchronic to chronic exposure, and LOAEL to NOAEL, will most certainly be needed.
Furthermore, additional uncertainties can be envisioned. For example, uncertainties in determining the critical effect of a chemical will also increase with in vitro data, since not all inter-organ, or even intraorgan, interactions are testable in vitro. In addition, one of the more common critical effects is the loss of body weight. Do we have an organ chip for that? Well, perhaps not yet.

Moreover, the use of such in vitro data will introduce additional uncertainties, not even ones traditionally considered, such as extrapolation from in vitro concentration to in vivo exposure.

Insofar as in vitro data can address these concerns, fewer uncertainty factors might be needed in the future. We look forward to working with colleagues to incorporate these new data into future risk assessments.

Dr. L.E. countered by asserting that advanced in vitro technologies can reduce the need for uncertainty or safety factors. Toxicology is not new. Indeed, it can be traced back to the work of the physician Paracelsus in the 15th century. And what is interesting about this infamous quote from Paracelsus is that the concept of dose, and thus exposure, was central to the determination of response. Now in the 21st century, exposure assessment remains central to the risk assessment paradigm, but decades of research have also pointed to the need to integrate these data with that identifying the hazard and the characterization of the hazard. Given the advances in metrology, we can now measure substances and their subsequent response at increasingly greater degrees of sensitivity. As such detailed databases can be built that contribute to the overall risk assessment process. However, even with advances in science and technology, hazard assessment remains typically based on data from animal models which is then extrapolated to humans, a process most toxicologists agree is subject to degrees of uncertainty. But, the human population is also more diverse than the average inbred laboratory animal and with data from deeper genetic profiling together with knowledge on the role environmental factors can play, further uncertainty creeps into the risk assessment paradigm when considering the impact of diversity. At the end of the day risk assessment is a conservative endeavor although in today’s modern world it is warranted to consider alternative or complementary approaches to achieve the same overall goal.

So, how can greater certainty be brought to risk assessment? Toxicologists have long since recognized that there are many steps between the “dose” of a substance and the resultant toxic response. By breaking this big step down into a series of smaller ones, we can start to discover potential opportunities for increasing certainty and potentially reducing the need for

---

Figure 2. Areas of uncertainty to consider in non-cancer dose response assessment.

Figure 3. Hypothetical response as a function of dose for humans of different sensitivities.
Figure 4. Central to Organ-Chip models is the recreation of the tissue-tissue interface which is enabled by culturing organ-specific cells in 2 independent, parallel microfluidic channels.
Most in vitro models involve bathing cells in a medium containing drug (or toxicant) which is not representative of the in vivo response. This is 1 reason why animal models are favored in hazard characterization because the toxicokinetics can be measured. Owing to the microfluidics, exposure dynamics can be recreated in organ chips offering a further advantage to their use in the risk assessment paradigm. Staying with the bone marrow chip, Chou et al. (2020) were able to exemplify how organ chips can reproduce clinical exposure profiles and how these profiles are connected to the clinical response. AZD2811, a drug in development within AstraZeneca’s oncology therapy area, was assessed within the bone marrow chip. As is common with oncology therapeutics, the bone marrow is a target organ for toxicity. In clinical trials, patients received a 2-h infusion of AZD2811 which resulted in anemia (Boss et al., 2011), and a reformulation of the drug tested in a subsequent clinical trial over a 48-h infusion resulted in neutropenia (Schwartz et al., 2013). The human bone marrow chip was able to reproduce this complex concentration-effect-time relationship. Furthermore, by measuring the impact on the myeloid and erythroid populations, the bone marrow chip reproduced the clinical responses that were reported. Taken together, data such as these illustrate the value that organ chips can bring to human risk assessment.

Since extrapolation between species introduces uncertainty into the risk assessment process, can organ chip technology address this uncertainty? Because organ chips are agonistic to cell sources, cells from nonhuman animals and humans can be seeded within the chip enabling scientists to get closer to the goal of predicting human response using surrogate models that show concordance. Jang et al. (2019) created liver chips using human, rat, or dog cells. It was shown that each of the species liver chips maintained albumin and urea production over 14 days in culture with the production of these markers being higher, and within the expected physiological range, compared with conventional cell culture. Because the metabolism of xenobiotics and other toxic substances is a key function of the liver, the study also demonstrated that liver chips with human, rat, or dog cells displayed activity across 3 major P450 families, CYP1A, 2B, and 3A. In all cases, the chip also outperformed the conventional 2D sandwich and metabolic competency was maintained in liver chips for 14 days. In some cases, this competency was comparable to activity seen in freshly isolated hepatocytes. These data provide evidence that chips can not only be used to measure functionality in nonclinical species but that organ chips can also contribute to the acquisition of data to understand the effects that target organ metabolism can have on the assessment of risk. Finally, this seminal work also showed that the species liver chips were able to discriminate species differences to the endothelin receptor antagonist Bosentan. Bosentan inhibits the bile salt export pump (BSEP) which is involved in the elimination of bile salts from the hepatocyte. Inhibition of BSEP results in accumulation of bile salts which drives a cholestatic liver injury. The accumulation of bile salts was measured in chips using the fluorescent molecule choly-l-lysyl-fluorescein which is a substrate for BSEP. In the presence of Bosentan, there was greater fluorescence confirming transporter inhibition. Humans and dogs are sensitive to the effects of Bosentan on the BSEP transporter whereas rats are not. By measuring albumin production and calculating IC50, it was shown that human chips were the most sensitive to Bosentan, with the hepatotoxicity occurring at an in vivo relevant dose. This concordance between nonhuman animals and animals in vitro and in vivo data sets increases the confidence that organ chip technology can predict animal and human response.

Finally, how can organ chips represent human population diversity? This is arguably the greatest challenge and will require careful selection of multiple cell donors, but it remains to be determined how many donors are needed to give confidence that population variability is being correctly represented. For the development of Chemical-Specific Adjustment Factors, the IPCS (International Programme on Chemical Safety) (2005) suggests that the number of subjects within the population, or within the major subgroup if there are 2 or more groups, should be sufficient to provide an accurate measure of the central tendency. As a guide, the standard error (SD of the sample divided by the square root of the sample size) should be less than approximately 20% of the mean. Based on available data, this would normally involve a minimum number of approximately 5 subjects or samples from 5 individuals, unless the variability is very low (ie, small coefficient of variability).

Both presenters provided a short rebuttal that following their original arguments before the Q&A session. Two panel members, Dr S.B.M.B. and Dr B.M. put forth interesting questions. Dr S.B.M.B.’s question was addressed to Dr L.E.: How can chronic in vivo exposure studies be modeled on organ chips? Dr L.E. explained that cellular functionality has been studied for up to 28 days on organ chips. However, the chronicity question can only be answered through a combination of 28 days of good, solid data and building a mathematical model around that information to understand chronic exposure.

Dr B.M. question was addressed to Dr M.D.: Considering that animal data are obtained from high-dose studies and then moving on to low doses, how can this complement what is done on organ chips? Dr M.D. explained that the advantage of in vitro systems is that mechanisms of toxicity are better understood in vitro than in vivo, that there are limitations of observing critical effects in vivo, and it may be that in vitro data can provide answers in such cases. Additionally, mixture assessments can be assessed a lot quicker through in vitro systems than through in vivo approaches.

One important outcome of the workshop debate was that in the case of in vitro human cells on a chip, the interspecies (animal to human) uncertainty factor of 10 could be eliminated. However, in the case of the intraspecies (average human to sensitive human), the uncertainty factor of 10, additional toxicokinetic and/or toxicodynamic data or related information will be needed to reduce much less eliminate this factor. In the case of other currently used uncertainty factors, such as LOAEL to NOAEL extrapolation, missing important toxicity studies, and acute/subchronic to chronic exposure extrapolation, additional data might be needed even when using in vitro human cells. The Roundtable session concluded with the panel members agreeing that collaboration is needed between traditional risk assessors with decades of experience with in vivo data and risk assessors working with modern technologies like organ chips to find a way forward.

DEVELOPMENT OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

Apostolou, A., Panchakshari, R. A., Banerjee, A., Manatakis, D. V., Paraskevopoulou, M. D., Luc, R., Abu-Ali, G., Dimitriou, A., Lucchesi, C., Kulkami, G., et al. (2021). A novel
microphysiological colon platform to decipher mechanisms driving human intestinal permeability. Cell Mol. Gastroentrol. Hepatol. 12, 1719–1741.

Boss, D. S., Witteveen, P. O., van der Sar, J., Lolkema, M. P., Voest, E. E., Stockman, P. K., Ataman, O., Wilson, D., Das, S., Schellens, J. H., et al. (2011). Clinical evaluation of AZD1152, an i.v. inhibitor of Aurora B kinase, in patients with solid malignant tumors. Ann. Oncol. 22, 431–437.

Chou, D. B., Frismantas, V., Milton, Y., David, R., Pop-Damkov, P., Ferguson, D., MacDonald, A., Vargel-Bolukbasi, O., Joyce, C. E., Moreira-Teixeira, L. S., et al. (2020). On-chip recapitulation of clinical bone marrow toxicities and patient-specific pathophysiology. Nat. Biomed. Eng. 4, 394–406.

Dankovic, D. A., Naumann, B. D., Maier, A., Dourson, M. L., and Levy, L. S. (2015). The scientific basis of uncertainty factors used in setting occupational exposure limits. J. Occup. Environ. Hyg. 12(Suppl. 1), S55–S68.

Dorne, J. L. C. M., and Renwick, A. G. (2005). The refinement of uncertainty/safety factors in risk assessment by the incorporation of data on toxicokinetic variability in humans. Tox. Sci. 86, 20–26.

Dourson, M. L., and DeRosa, C. T. (1991). Uncertainty factors in establishing “safe” levels of exposure. In Statistics in Toxicology (D. Krewski, and C. Franklin, Eds.), pp. 613–627. Gordon and Breach Science Publishers, New York, NY.

Dourson, M. L., Felter, S. P., and Robinson, D. (1996). Evolution of science-based uncertainty factors in noncancer risk assessment. Regul. Toxicol. Pharmacol. 24, 108–120.

Dourson, M. L., and Parker, A. (2007). Past and future use of default assumptions and uncertainty factors: Default assumptions, misunderstandings, and new concepts. Hum. Ecol. Risk Assess. 13, 82–88.

Dourson, M. L., and Staru, J. F. (1983). Regulatory history and experimental support of uncertainty safety factors. Regul. Toxicol. Pharmacol. 3, 224–238.

Hassell, B. A., Goyal, G., Lee, E., Sontheimer-Phelps, A., Levy, O., Chen, C. S., and Ingber, D. E. (2017). Human Organ Chip models recapitulate orthotopic lung cancer growth, therapeutic responses and tumor dormancy in vitro. Cell Rep. 21, 508–516.

Hayes, A. W., and Kruger, C. L. (2014). Hayes’ Principles and Methods of Toxicology, 6th ed. Taylor and Francis, London.

Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y., and Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. Science 328, 1662–1668.

IPCS (International Programme on Chemical Safety). (2005). Chemical-specific adjustment factors for Interspecies differences and human variability: Guidance document for use of data in dose/concentration-response assessment. Geneva, Switzerland. Available at: www.who.int/ipcs/methods/harmonization/areas/uncertainty/en/index.html. Accessed August 2021.

Jang, K.-J., Otieno, M. A., Ronxhi, J., Lim, H.-K., Ewart, L., Kodella, K., Petropolis, D., Kulkarni, G., Rubins, J. E., Conegliano, D., et al. (2019). Liver-Chip: Reproducing human and cross-species toxicities. Sci. Transl. Med. 11, eaax5516.

Lehman, A. J., and Fitzhugh, O. F. (1954). 100-fold margin of safety. Assoc. Food Drug Off. U.S. Q. Bull. 18, 33–35.

Jalili-Firoozinezhad, S., Gazzaniga, F. S., Calamari, E. L., Camacho, D. M., Fadel, C. W., Nestor, B., Cronce, M. J., Tovaglieri, A., Levy, O., Gregory, K. E., et al. (2019). A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. Nat. Biomed. Eng. 3, 520–531.

Jalili-Firoozinezhad, S., Prantil-Baun, R., Jiang, A., Potla, R., Mammoto, T., Weaver, J. C., Ferrante, T. C., Kim, H. J., Cabral, J. M. S., Levy, O., et al. (2018). Modeling radiation injury-induced cell death and countermeasure drug responses in a human Gut-on-a-Chip. Cell Death Dis. 9, 223.

Renwick, A. G. (1991). Safety factors and establishment of acceptable daily intakes. Food Addit. Contam. 8, 135–149.

Schwartz, G. K., Carvajal, R. D., Midgley, R., Rodig, S. J., Stockman, P. K., Ataman, O., Wilson, D., Das, S., and Shapiro, G. I. (2013). Phase I study of barasertib (AZD1152), a selective inhibitor of Aurora B kinase, in patients with advanced solid tumors. Invest. New Drugs 31, 370–380.

U.S. EPA. (1994). Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry. U.S. EPA, Washington, DC. EPA/600/8-90-066F. October.

U.S. EPA. (2002). Determination of the Appropriate FQPA Safety Factor(s) in Tolerance Assessment. U.S. EPA, Washington, DC, February 28.