High-dose exposure to synthetic chemicals, hormones, or homeostatic substances in experimental animals or humans can induce artefactual pathology

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Abstract
The maximum tolerated dose (MTD) provides the highest probability of a positive result in a toxicology bioassay. The assumption underlying the MTD in animal bioassays is that adverse effects at very high doses are qualitatively the same as those occurring at low doses. In contrast with the MTD, the optimal top dose in a toxicology animal study is the highest dose that does not produce a pathological end point that presents no risk at lower doses, for example, the dose below which cytotoxicity induces tumors in the absence of genotoxicity or other carcinogenic mechanisms. Normal concentrations or biological activity levels of many substances necessary for normal physiological function induce pathology when found at high levels. For example, the demonstration that ingestion of abnormally high levels of certain dietary fats can cause or exacerbate atherosclerosis in relevant animal models like rhesus macaques does not demonstrate that normal levels of these fats should be considered as toxic. Excessive estrogenic stimulation is associated with breast, ovarian, and endometrial cancers. This does not imply that normal age-appropriate levels of estrogen are toxic. Normal wound healing is associated with transforming growth factors beta 1 and 2. Excessive stimulation of fibroblasts by these growth factors results in hypertrophic scarring and keloid formation. An understanding of the mode of action of a test substance can facilitate the selection of dose levels much higher than those expected to be experienced by humans, but not beyond a dose level at which pathology is an experimental artefact of the high-dose level.

Keywords
Maximum tolerated dose, pathology, artefacts, cytotoxicity, supra-physiological levels

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History of the maximum tolerated dose
The use of the maximum tolerated dose (MTD), especially in animal carcinogenicity studies, remains controversial today despite its long history.1,2 The development of the MTD occurred in evolutionary steps over a period of years.3 By the 1950s, rodents were in wide use in comparatively short-term studies wherein exposures much higher than those expected in exposed humans were employed. The LD50 (dose at which 50% of the test animals expired) was established during this decade.3 President Lyndon Johnson’s “War on Cancer” provided funding to the National Cancer Institute (NCI) during the 1960s that led to extensive carcinogenicity testing of chemicals in rodents usually administered at doses comparable to today’s

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MTD. In 1976, Sontag et al. at the NCI published “Guidelines for Carcinogen Bioassay in Small Rodents.” This publication defined the MTD as “…the highest dose of the test agent during the chronic study that can be predicted not to alter the animals’ longevity from effects other than carcinogenicity.” Sontag et al. further stated the following: the dose should cause no more than a 10% weight decrement as compared with control groups; not produce mortality, clinical signs of toxicity, or pathologic lesions not related to the neoplastic response that could shorten the test animal’s life span.

In the intervening years since the publication of Sontag et al., the understanding of the MTD has been clarified further. Under current guidelines, the primary consideration for selection of the MTD is histopathological appearance with adverse effects on weight gain assuming a secondary role. Numerous regulatory organizations and advisory bodies support this approach including the International Agency for Research on Cancer (IARC), the United States Environmental Protection Agency (US EPA), the Report of the National Toxicology Program (NTP) Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation, the International Life Sciences Institute (ILSI), and Office of Science and Technology Policy (OSTP).

**Proposed alternatives to the MTD approach**

The pharmacokinetic (PK) profile of a test chemical includes measured values indicating absorption, distribution, metabolism, and excretion. These measurements can be used to select the highest dose in cancer or developmental toxicology bioassays. The PK approach attempts to establish a dose of test chemical that approximates the maximum steady state, that is, saturation, concentration for a particular species, and sex that does not exceed rate-limiting processes. In some cases, the dose established via PK can be significantly lower than the MTD. PK dose modelers have posited that doses exceeding saturation levels for selection of the MTD is histopathological appearance with adverse effects on weight gain assuming a secondary role. Numerous regulatory organizations and advisory bodies support this approach including the International Agency for Research on Cancer (IARC), the United States Environmental Protection Agency (US EPA), the Report of the National Toxicology Program (NTP) Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation, the International Life Sciences Institute (ILSI), and Office of Science and Technology Policy (OSTP).

Currently employed protocols regarding high-dose selection in rodent bioassays date from the late 1980s. Although not widely implemented, a number of modifications have been proposed. In 2014, the European Medicines Agency (EMA) considered Bayesian model-based methods to be potentially useful. The goal of the EMA was to optimize the therapeutic index of new drugs during early clinical development. Toward that goal, Bayesian model-based methods are coupled with PK/pharmacodynamics (modeling to identify the biologically effective dose from preclinical and emerging clinical data. In addition, EMA noted that the MTD still predominates in selection of the phase II clinical trial dose for oncology products. In situations where the dose administration is continuous, EMA noted that the MTD might not always represent the optimal dose.

**Mitogenesis causes mutagenesis at doses at or near the MTD: Artefactual induction of rodent tumors**

In the 1980s, Cohen et al. conducted a series of studies demonstrating that high doses of nongenotoxic chemicals can induce cytotoxicity leading to increased cellular proliferation that amplifies the background mutation rate thereby increasing tumor formation in experimental animals. The induction of bladder cancer in male rats via high-dose administration of the artificial sweetener saccharin was shown by these authors to occur via this cytotoxicity-proliferation mechanism. The studies of Moolgavkar and Knudson also played an important role during this era.

Throughout the 1990s, Bruce Ames, Tom Sloane, and Lois Gold incorporated these new findings into their thinking. These investigators set up the Carcinogenic Potency Database (CPDB), which compiled the results from 6540 long-term animal cancer tests conducted on 1547 chemicals. These 6540 tests included both positive and negative results published over the past 50 years in the general literature up through 2001. The CPDB also included studies conducted by the NTP up through 2004. Ames et al. qualitatively and quantitatively analyzed this huge data set with their interpretative analyses resulting in over 100 scientific publications. Surprisingly, over 50% of the all the chemicals ever tested, whether synthetic or naturally occurring, induced a tumor in rodents.

Based upon the earlier findings of Cohen and Ellwein, Cohen et al., Moolgavkar and Knudson, and Ames and Gold and their colleagues our research group throughout 2017–2018 statistically and mechanistically analyzed the entire 594-study NTP 2-year rodent cancer database as published up through 2018. Of the 594 2-year studies, the results for 470 chemicals were of sufficient technical quality to result in the production of a final technical report. Since 2018, several additional NTP 2-year
rodent studies have been completed by the NTP for a total of 598. No additional NTP reports published as of May 2020 met our previous criteria for analysis and thus are not included here.

Our analyses from 2017 to 2018 validated the contentions proffered by these earlier investigators in a number of ways. First, across different routes of administration, relatively phylogenetically similar rats and mice were nonetheless discordant for the development of tumors at similar organ sites. Tumor site concordance across sex within species was higher than tumor site concordance across species. Second, many chemicals negative in the Ames test nonetheless induced tumors in either rats or mice. Of the 482 chemicals tested by NTP (479 from the 470 NTP reports and three from two Report on Carcinogens [RoC] reports) that resulted in the writing of a final report or were described in the two additional RoC reports, 331 were negative in the Ames Salmonella mutagenicity test. Two hundred and four of these 331 Ames-negative chemicals induced tumors in either rats or mice (204/331, 62%); third, 11 out of 58 chemicals tested by the inhalation route induce lung tumors in mice and not rats, are not only negative in the Ames test, but also exhibit hyperplasia. If NTP’s contention that no battery of in vitro mutagenicity tests is better than just the Ames Salmonella mutagenicity assay alone in predicting tumorigenicity in rats and mice, then the high level of tumor induction by Ames-negative chemicals is consistent with a significant rate of nongenotoxic cytotoxicity-induced cellular proliferation leading to tumor formation. In addition to the large number of Ames-negative chemicals that nonetheless induce at least one rodent tumor, of the 331 chemicals that induced at least one tumor, 54 chemicals did not possess a structural alert of carcinogenicity resulting in a false-negative rate of 54/331 (16.3%). Similarly, Oncologic (Oncologic™) (a computer program developed by the US EPA to predict carcinogenic potential) predicted that 50 of 331 chemicals with either low or inactive carcinogenic potential for a false-negative rate of 50/331 (15.1%). If marginal ratings are included, the false-negative rate predicted by Oncologic is 95/331 (28.7%). However, it should be noted that the relatively low false-negative rates predicted by structural alerts of carcinogenicity and Oncologic are depressed by the high false-positive rates of 40% for structural alerts and 43% for Oncologic. In summary, our more recent analysis, and the seminal results from earlier researchers previously noted, both support that the interactive effects of mitogenesis and mutagenesis occurs across a spectrum rather than in discrete categories. At one end of the spectrum, a completely nongenotoxic but cytotoxic chemical can induce tumors. However, many chemicals are both genotoxic and cytotoxic to some degree and the impact on tumorigenicity will be some nonlinear combination based on the quantity and qualitative characteristics of the induced mutations and the robustness of the proliferative response.

**Induction of murine bronchioalveolar lung tumors as an artefact of high doses**

In 2-year inhalation studies using rats and mice, when pulmonary tumors are seen in only male or female mice or both, but not in either sex of rat, there is a high probability that the murine pulmonary tumor has been produced via Clara cell or club cell (CC) metabolism of the inhaled chemical to a cytotoxic metabolite. Cytotoxicity-induced mitogenesis increases mutagenesis via amplification of the background mutation rate. If the chemical being tested is also negative in the Ames Salmonella mutagenicity assay, and only mouse pulmonary tumors are induced, the probability that this pulmonary tumor is not relevant to human lung cancer risk goes even higher. In humans, the respiratory bronchioles form the margin between the larger conducting airways and the distal respiratory zone (alveolar ducts and sacs) where gases are exchanged. Five different cell types are found in the respiratory bronchioles: ciliated cells (most common), microvillar cells (few), small granule cells (few), and bronchiolar cells known as club cells or Clara cells. In the epithelium of the alveoli, there are four different cell types including type I pneumocytes, type II pneumocytes, type III pneumocytes (rare), and numerous pulmonary macrophages. CCs are also known as bronchiolar cells or nonciliated non-mucus secretory cells of the bronchiolar epithelium. The presence of a large number of mitochondria facilitates a high level of metabolic activity in CCs. Each CC usually contains six, approximately 0.3 mm diameter dense granules located near the basement membrane. The composition of the granule contents includes proteins, glycoproteins, and lipids. CCs contain substantial amounts of cytochrome P450 enzymes and mixed-function oxidases working collaboratively. In both an apocrine (membrane budding) and merocrine (secretion) manner. CCs produce and secrete a number of different substances. Innervating adrenergic fibers stimulate the secretory activity of CCs.

Nonhuman primates are much closer phylogenetically to humans than are rodents, for example, the rhesus macaque monkey and humans shared a common ancestor only approximately 25 million years ago, as compared with 80 million years ago for the lineal divergence of rodents and humans. Differences in pulmonary anatomy and physiology between even monkeys and humans are sufficiently large that Boers et al. stated that “Even species with an extensive branching of respiratory bronchioles, e.g. nonhuman primates, are far from an ideal model for the human lung, in contrast to the speculation of Plopper and colleagues.”

Airway organization, epithelial cell composition, and CC ultrastructure differ between humans and all other animals. Based on an autopsy study on seven healthy human lungs, CCs comprise approximately 9% of the total population of airway epithelial cells. CCs are almost absent in...
the mucus membranes of the proximal segments of the bronchial tree including the trachea, primary bronchi (first branch), lobar bronchi (narrower secondary bronchi), and segmental bronchi (narrower tertiary and further branching bronchi). Approximately 11–22% of CCs (0.99–1.98% of total lung epithelial cells) are located in the terminal bronchioles. Despite their scarcity, under physiological conditions CCs constitute approximately 15–44% of all proliferating cells in the terminal bronchioles.\(^5,\)\(^6\) Possibly due to the difference in CC numbers, the proliferation percentage in the respiratory bronchioles (44%) is higher than in the terminal bronchioles (15%).

Direct experimental evidence on the proliferative response of the human lung to injury cannot be collected under the clinical exigencies of treating such an injury. Circumstantial evidence suggests that chronic pulmonary injury in humans might reduce rather than increase CC numbers. CC numbers are reduced in cigarette smokers.\(^5,\)\(^4\)\(^–\)\(^6\) In addition, CC secretory proteins CC10 and P1 detected in serum and bronchoalveolar lavage fluid are decreased in smokers,\(^5\) patients with bacterial pneumonia,\(^3\) and patients with COPD and lung cancer.\(^5\) In contrast, in rodents CCs clearly proliferate as a response to lung injury. Results from oxidant gas pulse-chase exposure studies suggest that the majority if not all rabbit and rat CCs proliferate in response to injury.\(^5\)\(^0\) CCs participate in cell renewal in hamster bronchial epithelium.\(^6\) CC division is the predominant contributor to the proliferative response of the bronchiolar epithelium after exposure of rats to NO\(_2\) or O\(_3\).\(^6\)\(^2\)

In an elegant series of publications, Cruzan et al. have demonstrated that the mode of action (MOA) of the mouse specific lung carcinogen styrene is neither quantitatively nor qualitatively relevant to humans.\(^6\)\(^3\) The pulmonary CCs in mice contain high levels of the metabolic enzyme CYP2F2 which hydroxylates the aromatic ring of styrene producing 4-hydroxystyrene, 3,4-dihydroxystyrene, and 4-hydroxystyrene-7,8-oxide as metabolites.\(^6\) The hydroxylation of aromatic rings in the synthesis of Coenzyme Q is thought to be the normal function of CYP2F2.\(^6\) In addition to styrene, the tumorigenicity of naphthalene and coumarin in mouse lung also results from a similar metabolism of these chemicals by high levels of CC CYP2F2.\(^6\) Rats do not develop lung tumors from inhaling styrene, naphthalene, and coumarin because although rat CYP2F4 appears to be equally active to mouse CYP2F2 in metabolizing these chemicals, CYP2F4 occurs at a much lower concentration in rat CCs with cytotoxic metabolite production insufficient to cause reparative cell proliferation and tumor development. The difference between humans and mice is dramatically greater than between rats and mice. Human lungs contain very few CCs as compared with rats and especially mice. Human lung microsomes only marginally or do not metabolize styrene, naphthalene, and coumarin. In addition, morphological differences between human CCs and mouse CCs render the mouse cells as much more sensitive to damage via reactive metabolites.\(^6\)\(^6\)

Up through 2017, the NTP had tested a total of 60 single agents or mixtures in 2-year inhalation studies in both rats and mice. Fifty-eight of the 60 inhalation studies were amenable to statistical analysis. Eleven out of 58 agents tested in the NTP inhalation studies using rats and mice were negative in the Ames assay and showed lung tumors in mice only.\(^3\)\(^\)\(^1\) Ten of the 11 chemicals (90.9%) are insoluble or slightly soluble in water, soluble in organic solvents, and have moderately hydrophobic log base 10 octanol–water partition coefficients: nitrobenzene, CAS No. 98-95-3, slightly soluble in water, soluble in organic solvents. Log \(p = 1.85\); trichloroethylene, CAS No. 79-01-6, slightly soluble in water, soluble in ethanol, acetone, diethyl ether, and chloroform, and miscible in oil. Log \(p = 2.61\); vinylidene chloride, CAS No. 75-35-4, clear volatile liquid, insoluble in water but miscible with most organic solvents. Log \(p = 2.13\); 1-bromopropane, CAS No. 106-94-5, slightly soluble in water, soluble in most organic solvents. Log \(p = 2.10\); cumene, CAS No. 98-82-8, alkylated benzene volatile at room temperature. Log \(p = 3.66\); divinylbenzene-HP, CAS No. 1321-74-0, insoluble in water and soluble in methanol and ether. Log \(p = 3.8\); naphthalene, CAS No. 91-20-3, not soluble in water, soluble in organic solvents. Log \(p = 3.3\); chloroprene, CAS No. 126-99-8, practically insoluble in water, soluble in alcohol, and miscible with acetone, benzene, and ethyl ether. Log \(p = 2.53\); ethylbenzene, CAS No. 100-41-4, practically insoluble in water but soluble in most organic solvents. Log \(p = 3.15\); nitromethane, CAS No. 75-52-5, soluble in water, alcohol, ether, acetone, and dimethylformamide. Log \(p = 0.17\); isopropene, CAS No. 78-79-5, log \(p = 2.42\). These moderate log \(p\) (log Kow) values of 0.17, 1.85, 2.10, 2.13, 2.42, 2.53, 2.61, 3.15, 3.30, 3.66, and 3.80 are near the optimum values for penetrating the lipid bilayer membranes of cells.\(^6\)\(^7\)

These chemicals induce hyperplasia in the airways of mice. Of these 11 chemicals, the nongenotoxic mechanism of mouse lung tumor induction described above by Cruzan et al.\(^6\)\(^8\) has been elucidated for cumene and naphthalene. A third member of the list of 11 chemicals, trichloroethylene is both acutely toxic and carcinogenic to the mouse lung via the production of reactive metabolites by CCs following exposure via inhalation. As in the cases of styrene, cumene, and naphthalene, the absence of metabolic capacity in human lung suggests that the risk of human lung cancer from trichloroethylene is minimal.\(^6\)\(^8\)

The induction of pulmonary tumors via CC metabolism of a chemical to a cytotoxic metabolite that elicits cellular proliferation is not limited to mice and rats. Hukkainen et al.\(^6\)\(^9\) describe the following species as capable of metabolizing the listed chemical to a pneumotoxic metabolite(s): 3-methylindole metabolized via CYP2F and CYP4B1 by the CCs of cow, goat, mouse, and rat; 4-ipomeanol metabolized via CYP4B1 by the CCs of the
rabit; coumarin metabolized via CYP2B subfamily by the CCs of the mouse; dichloroethylene metabolized via CYP2E1 by the CCs of the mouse; and ethyl carbamate and vinyl carbamate metabolized by CYP2E1 by the CCs of the rat and mouse. In summary, the discovery that a chemical is metabolized to a pneumotoxic metabolite in an animal, especially a mouse, but not in a human is a common finding. The differential anatomic distribution between the few CCs in human airways and the many CCs in murine airways probably affects the absorption and metabolism of hydrophobic chemicals. Many inhaled, highly lipophilic compounds, for example, polycyclic aromatic hydrocarbons (PAHs), have longer retention times with resultant higher local doses in bronchial and bronchiolar epithelium than less lipophilic compounds. Gerde et al. have constructed a dosimetric model for inhaled PAHs in which a larger fraction of inhaled PAHs is deposited in the alveolar region, that is, respiratory bronchioles. PAHs depositing in this region absorb into circulating blood at such a rapid rate that there is little time for local metabolism. Only 5% of human lung cancers develop in this region. In humans, this region of low metabolism is the same area where the few CCs that are proliferating are found, that is, proliferation percentage in human respiratory bronchioles (44%) is higher than in the terminal bronchioles (15%). Results from Kiraly et al. illustrate that the coincidence of exposure to mutagens in the presence of cellular proliferation is of special concern to elevation of cancer risk. The relative scarcity of human lung tumor development in this alveolar region is consistent with a lack of chemical metabolism to a carcinogenic metabolite being geographically coincident with the vast majority of the CCs that are proliferating, although the absolute number of CCs in human epithelium is small. In stark contrast with the distribution of proliferating CCs in the human lung, both the proximal intrapulmonary epithelium and the terminal bronchiolar epithelium in the mouse are predominately lined with CCs, 59–61% and 60–80%, respectively.

Noncancer pathologies occurring at only high concentrations

Most of the discussion regarding the appropriateness of the MTD has historically centered on rodent cancer bioassays. The assumption underlying the wide application of the MTD is that very high doses of a test agent administered over the relatively short lifespan of rodents can be extrapolated to much lower levels of exposure experienced by humans over a much longer time period. Toward determination of a potential dose-response relationship, at least three dose levels are usually selected with a 10-fold increase between dose levels being a common choice. Even the lowest dose selected under this protocol is usually much higher than exposures expected to be experienced by humans, especially in nonoccupational cohorts. The situation wherein adverse effects are only seen at the highest dose level is particularly problematic regarding interpretability. In common practice, adverse effects seen at only the highest dose level are considered to be indicative of processes also occurring at the lower dose levels, but at levels below the detection sensitivity of the experimental design. While this assumption might sometimes be true, although difficult to validate, numerous examples illustrate that many common metabolic precursors, small and large molecules intended for use as therapeutics, chemical constituents of food, and industrial chemicals are toxic at high doses, and not toxic at lower doses. In the following text, several examples are provided that illustrate the principle that pathology seen at very high doses is frequently not seen at lower doses.

The difficulty of validating the assumption that adverse high-dose effects are exaggerated versions of low dose effects can be illustrated by the case of low-dose daily aspirin administration. Aspirin is an irreversible inhibitor of platelet aggregation and has been shown to reduce the risk for recurrence of myocardial infarction. The most commonly prescribed aspirin dose for cardiovascular event prophylaxis is an 81 mg per day baby aspirin, as compared with the standard 325 mg per day adult aspirin dose. A recently published meta-analysis illustrates the large number of subjects required to detect relatively rare adverse events, for example, intracranial hemorrhage. These authors analyzed the results from 13 randomized clinical trials of low-dose (<100 mg per day) aspirin use for primary prevention, with a total enrollment of 134,446 patients. Using this extremely large number of patients, the authors were able to detect two additional intracranial hemorrhages per every 1000 patients. In this case, an adverse effect sometimes seen at the 325 mg per day dose was also detectable for the 81 mg per day dose, but 134,446 patients required examination to pick up just two additional cases per 1000 patients.

In contrast with the technical difficulties inherent in validating the clinical or biological relevance to humans of adverse effects induced at high doses in rodent bioassays, numerous examples illustrate that many substances toxic at high doses are not only not toxic at lower doses, but are essential to homeostasis or health. These substances include common metabolic precursors, small and large molecules intended for use as therapeutics, chemical constituents of food, and industrial chemicals. The demonstration that ingestion of abnormally high levels of saturated or trans fats can cause or exacerbate atherosclerosis in relevant animal models like rhesus macaques does not demonstrate that normal levels of these dietary fats should be considered as toxic. Excessive estrogenic stimulation is associated with breast, ovarian, and endometrial cancers. This does not imply that normal age-appropriate levels of estrogen are toxic. Normal wound healing is associated with transforming growth factors beta 1 and 2. Excessive stimulation of fibroblasts by these growth factors results in
hypertrophic scarring and keloid formation.\textsuperscript{91} Excessively high levels of many essential minerals and vitamins are toxic including vitamin A,\textsuperscript{92} pyridoxine (vitamin B\textsubscript{6}),\textsuperscript{93} niacin,\textsuperscript{94} and selenium.\textsuperscript{95} In summary, normal concentrations or biological activity levels of these and an extensive number of other substances are necessary for normal physiological function, while excesses or deficiencies are associated with pathology.

**Conclusions**

Over the last several years, the Kell group at the University of Liverpool and others have demonstrated that both pharmacological agents and toxicants enter and exit cells via substance-specific carrier proteins with a negligible contribution from passive diffusion.\textsuperscript{96} This surprising observation of an additional level of specificity adds to the weight of evidence that the maximum relevant dose in a toxicity study will vary on a substance to substance basis, and with the specific biological end point. The optimal top dose in a toxicology study employing animals is the highest dose that does not produce a pathological end point that presents no risk at lower doses, for example, the dose below which cytotoxicity induces tumors in the absence of genotoxicity or other carcinogenic mechanisms.

In conclusion, the MTD will provide the highest probability of a positive result in a toxicology assay or animal study. However, in the absence of an understanding of the MOA of the test substance, the relevance of these positive test results is unclear. An understanding of the MOA of a test substance can facilitate the selection of dose levels much higher than those expected to be experienced by humans, but not beyond a dose level at which pathology becomes an experimental artefact of the high-dose level. As noted by Heringa et al.,\textsuperscript{1} under-classification of substance hazard is highly undesirable. However, over-classification of hazard can also be problematic as replacing an important chemical based on artefactual rodent results with another chemical could be disadvantageous economically and also from a human and environmental health perspective.

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