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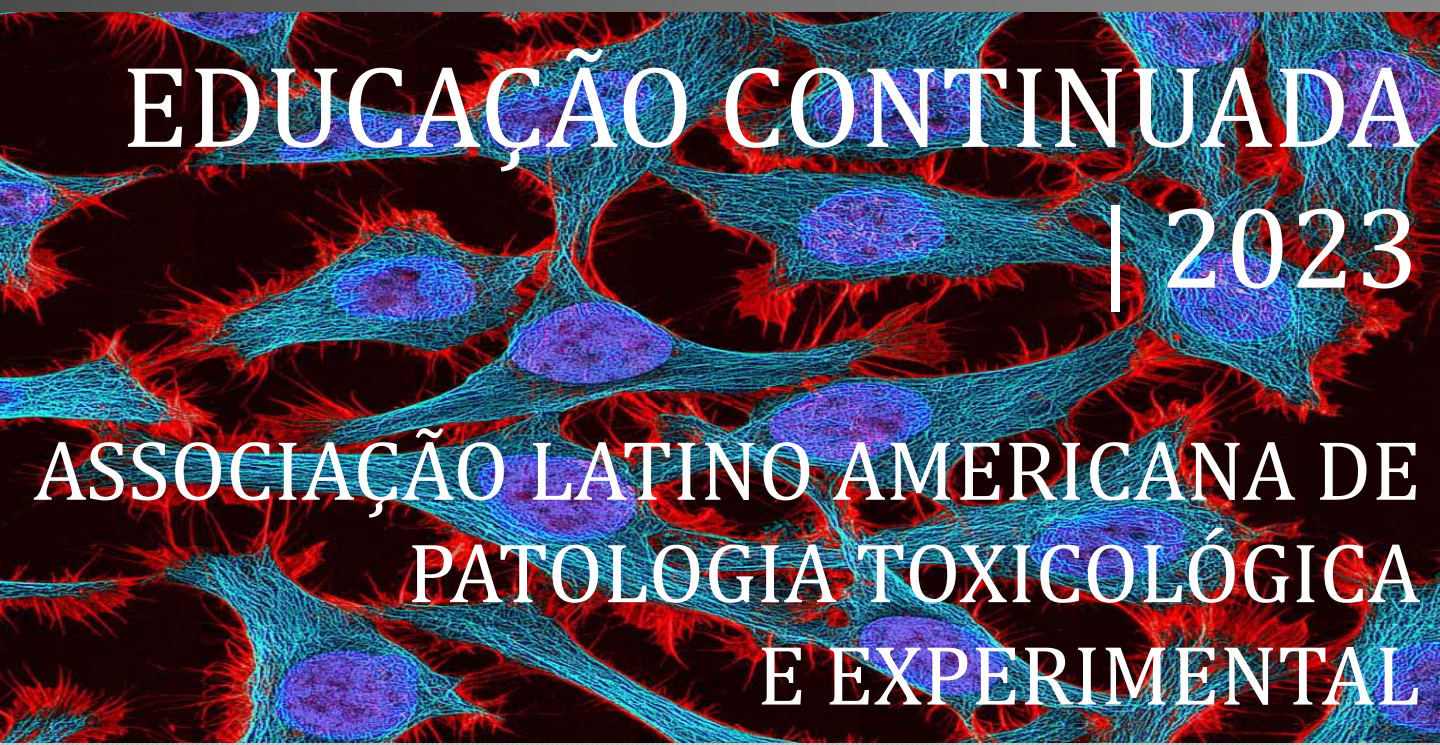
ALAPTE



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10% Body weight (gain) change as criterion for the maximum tolerated dose: A critical analysis

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ABSTRACT

The concept of the Maximum Tolerated Dose (MTD) was introduced in the seventies for carcinogenicity testing and was defined as the highest dose inducing clear toxicity, but not mortality by causes other than cancer. As estimation of the MTD in a carcinogenicity study, the highest dose that causes a 10% decrease in body weight compared to control animals over the course of a 90-day study, was formulated as a suitable criterion. This criterion was not seen as indicator of excessive toxicity but as a means to avoid false negative outcomes in a carcinogenicity study, as tumor formation may be reduced when body weight is significantly decreased. The body weight-based MTD criterion, however, turned up in carcinogenicity test guidelines and guidance (e.g., from OECD) as the highest dose that causes a 10% decrease in body weight *gain* relative to controls. Moreover, the 10% decrease in body weight *gain* criterion for MTD also ended up in test guidelines and guidances for toxicity endpoints other than carcinogenicity, so outside the context it was intended for. A 10% decrease in body weight *gain* relative to controls is however not a biologically relevant effect as it corresponds to less than 3% body weight reduction relative to controls in a 90-day study, which is within the normal variation in body weight. It therefore should certainly not be considered as a condition of excessive toxicity. Using the 10% lower weight *gain* criterion and incorrectly associating it with excessive toxicity has major implications for top dose selection in regulatory safety studies, resulting in tests performed at doses too low to elicit toxicity. This negatively impacts the reliability of studies and their regulatory usability; moreover, it results in a waste of experimental animals, which is ethically highly undesirable. Hence, our plea is to remove this MTD criterion for top dose selection in test guidelines and guidances for toxicity endpoints other than carcinogenicity and to reinstall the original 10% decrease in body weight criterion in test guidelines and guidances for carcinogenicity.

1. Introduction

As long as robust non-animal methods are not available for several regulatory endpoints, it is crucial to make the most of animal studies, to ensure that they provide the information needed for toxicological assessment of potentially hazardous substances.

Dose-setting is an important aspect of study design, and the selection of the high dose in particular has been subject to extensive discussion, especially in relation to the anticipated level of toxicity. On the one hand, the dose must be high enough to induce clear toxicity, in order to draw meaningful conclusions on the hazardous properties of the test substance and to allow for regulatory follow up. On the other hand, the dose should not be excessively high, in order to avoid death or severe animal suffering. For some substances that are of low toxicity, the limit dose concept as introduced in most OECD test guidelines (i.e. testing at a top dose of 1000 mg/kg bw/day, unless human exposure indicates the need for a higher dose level to be used) may be useful. For other substances, however, this limit dose is too toxic and other concepts will have to be used to establish an appropriate top dose.

One such concept is the Maximum Tolerated Dose (MTD), an approach originally used to set the high dose in carcinogenicity studies, but which has also found its way into other regulatory toxicology guidance documents and guidelines (see section 2.3). The MTD concept was famously defined by National Cancer Institute researchers James Sontag, Norbert Page and Umberto Saffiotti in their 1976 "Guidelines for Carcinogen Bioassay in Small Rodents" (*Sontag* et al., *1976*) as:

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"*The MTD is defined as the highest dose of the test agent given during the chronic study that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity*."

This is very close to the MTD defined by the National Toxicology Program (NTP; *NTP/DHHS, 1984*): "*The MTD is defined by the National Toxicology Program (NTP) as "that dose which, when given for the duration of the chronic study as the highest dose, will not shorten the treated animals' longevity from any toxic effects other than the induction of neoplasms*". In the FDA guidance for carcinogenicity studies the MTD is defined similarly as: "*should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study*" (US FDA, 2006).

In contrast, the US EPA defines the MTD as a dose that is "minimally toxic" (US EPA, 2005), while confusingly a large, often-cited review from 2007 distinguishes between several top doses, including the MTD and what is referred to as the "minimally toxic dose" (*Rhomberg* et al., *2007*). What is generally agreed upon though, is that the MTD should be defined based on the results from a sub-chronic, 90-day study that is considered as a screening/pilot study for the carcinogenicity study.

Several pieces of information (e.g. various toxicity parameters such as mortality, pathology and clinical signs, kinetic data, etc.) should be considered in dose-setting and MTD selection. One parameter that is generally considered useful for MTD selection is body weight change. In Sontag et al. (1976), this is described as:

"*The MTD is estimated after a review of the subchronic data. The MTD should be the highest dose that causes no more than a 10% weight decrement, as compared to the appropriate control groups; and does not produce mortality, clinical signs of toxicity, or pathologic lesions (other than those that may be related to a neoplastic response) that would be predicted to shorten the animal's natural life span.*"

Thus, a 10% lower body weight compared to control animals at the end of a 90-day sub-chronic study was considered as a possible basis for the top dose selection in a carcinogenicity study. Remarkably however, the original criterion of a 10% body weight change has found its way in several test guidelines and guidance documents (including those of the Organization for Economic Co-operation and Development (OECD) and the ECHA guidance on application of the CLP criteria (*ECHA, 2017*)), as a 10% body weight ***gain*** change. This might seem like a minor adaptation, but upon closer examination there could be a devil in this particular detail. To the best of our knowledge, the earliest literature source where the term *gain* was added to body weight change in relation to the MTD is a 1997 publication from the International Life Sciences Institute on dose selection in chronic rodent bioassays (*ILSI, 1997*). The current commentary will analyze and discuss the implications of this adaptation for regulatory toxicity testing.

Throughout the paper, the following descriptors of reduced growth/body weight are distinguished:

I. **10% Body weight change relative to controls**: referring to the mean body weight in an animal study group receiving a certain compound/substance compared to the mean body weight in the control group. This is determined at a certain time point during the animal study.
II. **10% Body weight *gain* change relative to controls**: this refers to the mean body weight gain in an experimental group over a certain period of time (body weight gain may assessed over a few days, a week or over the complete duration of a 90-day toxicity study), compared to the mean body weight gain in control animals over the same period.
III. **Body weight loss**: this refers to the situation where an individual animal or the mean weight of a group of animals decreases over time, i.e., at time point X+1 the body weight is lower than at time point X. Body weight loss is one of the criteria (i.e., 25% BW loss in 7 days) mentioned in OECD Guidance Document 19 as an indicator for excessive toxicity/animal suffering.

Please note that our commentary is only on body weight (gain) change as parameter for MTD selection, i.e. descriptors I. and II. Body weight loss (i.e. descriptor III) is mentioned only for the purpose of comparison/to provide context. As described above, other parameters than those based on body weight should also be considered when selecting an appropriate MTD, but these are not within the scope of this commentary.

1.1. Inclusion of an MTD based on reduced body weight (gain) in OECD guidance for carcinogenicity studies

In OECD Guidance Document 116 (*2012*) *on the Conduct and Design of Chronic Toxicity and Carcinogenicity Studies,* Supporting *Test Guidelines 451, 452 and 453*, paragraph 88 mentions that "*the Maximum Tolerated Dose (MTD)* [is] *conventionally defined as the highest dose to produce toxic effects without causing death and to decrease body weight gain by no more than 10% relative to controls*". A similar phrase is found in paragraph 90 of OECD GD 116, and the reference provided is OECD Guidance Notes 35 (*2002*) *for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies*. However, OECD GN 35 only refers to a decrease in body weight, not a decrease in body weight *gain*; in paragraph 1.2.1 it is stated that the largest administered dose should, amongst others, not [emphasis added by current authors]:

- "*in a chronic study, exceed the maximum tolerated dose (or MTD) defined as the highest dose to produce toxic effects without causing death and to decrease body weight by no more than 10% relative to controls* (*Derelanko, 2000*); "
- "*in a carcinogenicity study, significantly affect the survival rate except through tumor production, or cause a body weight decrement greater than 10–12% of concurrent control values, because larger decreases can mask, reduce, delay or prevent the development of tumors* (*DeGeorge, 1999*)."

From this it can be concluded that OECD GN 35 is improperly referenced in OECD GD 116 and that the criterion of a 10% reduced body weight *gain* (relative to controls) in OECD GD 116 for MTD, and the subsequent inclusion thereof in the OECD test guidelines for carcinogenicity and chronic toxicity (OECD TG nos. 451, 452 and 453), is incorrect.

The reason to include a body weight (gain) decrement criterion for top dose selection is that a significant decrease in body weight (gain) could reduce the animal's response to substance-induced toxicity, including cancer, resulting in lower cancer incidence. Indeed, dietary restriction has been shown to improve survival rate and to lower the susceptibility to tumor development (*Keenan, 1996*; *Keenan* et al., *1997*; *Weindruch and Walford, 1988*).

The MTD criterion based on body weight change is thus intended to avoid false *negative*, and not false *positive,* results in a carcinogenicity study. Consequently, the finding of an increase in tumor incidence at or above such an MTD cannot be simply discarded as being false *positive* results, as is often argued.

1.2. MTD based on reduced body weight (gain) for genotoxic vs. non-genotoxic carcinogens

In paragraph 92 of OECD GD 116, it is indicated that the MTD consideration based on body weight only applies for substances that are (or potentially are) genotoxic. For substances that are not genotoxic, the top dose should be informed by considerations of mode of action of the substance. Despite this, it is current practice to also apply the MTD criterion based on 10% body weight gain change for testing non-genotoxic carcinogens.

First indications that a substance is a genotoxic carcinogen can be

found using short-term *in vivo* and *in vitro* methods, but non-genotoxic carcinogens are usually only identified using a rodent carcinogenicity study (*Luijten* et al., *2020*). Whereas selection of a low top dose would be expected to be less of an issue for genotoxic carcinogens (which are assumed not to have a threshold dose), for non-genotoxic carcinogens this can be much more problematic. The various mechanisms known for non-genotoxic carcinogenicity (including hormonal effects, cytotoxicity, chronic inflammation, cell proliferation and epigenetic changes (e.g., *Nohmi, 2018*)) are generally considered to have a threshold dose for effects to occur. So, an MTD selected below the threshold dose for a non-genotoxic carcinogen could result in a false negative outcome. This impairs hazard and risk assessment and likely results in misclassification.

1.3. Adoption of the MTD based on reduced body weight gain in other guidances

As illustrated in the preceding sections, the MTD criterion mentioned in OECD GD 116 and OECD GN 35 only applies to *carcinogenicity* studies, more specifically to carcinogenicity studies assessing *(potentially) genotoxic* substances. The MTD concept and the body weight *gain* criterion have however been extrapolated to test guidelines and guidance documents intended for endpoints other than carcinogenicity. For instance, in recent ECHA/EFSA guidance for substances with endocrine disrupting properties, the following text is found (ECHA/EFSA, 2018):

- "*The aim of the MTD is to produce a minimum toxic effect over the course of the study. Elements to consider are alterations in physiological function, including: no more than 10% decrease in body weight gain relative to control, target organ toxicity and alterations in clinical pathological parameters. … Elements which indicate that the MTD has been exceeded are reported in the OECD Guidance on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation* (*OECD GD 19, 2000*)."

The OECD Guidance mentioned in the text above refers to OECD GD 19 on humane endpoints (see also section 2.4); however, OECD GD 19 does not address/mention an MTD, so besides the incorrect extrapolation of the MTD to a non-carcinogenic endpoint, the reference given is incorrect.

Similarly, in OECD TG 426 (Developmental Neurotoxicity Study) a top dose is suggested to be based on a 10% reduced body weight gain relative to controls.

From a scientific and regulatory point of view, this extrapolation to other endpoints is highly problematic, because the 10% body weight decrement criterion relative to controls (let alone the 10% body weight *gain* decrement criterion) is incorrectly interpreted as an indicator of excessive toxicity/animal suffering, which it is not (see section 2.5).

1.4. Body weight effects that indicate severe/excessive toxicity

What is considered a clear indicator for health problems and excessive toxicity is weight *loss*. Weight loss differs from reduced growth/weight gain in that the body weight actually decreases (compared to an animal's body weight at an earlier time point), whereas in reduced growth the body weight still increases, but less so than in control animals. Weight loss is one of the humane endpoints described in OECD GD 19 (2000) on *the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation*. Here, *rapid* and/or *excessive* weight loss is a sign of serious discomfort that warrants consideration of sacrifice of affected animals. In Annex 3 to this document this is made quantitative [emphasis added by current authors]:

- "*Body weight loss or emaciation: Particularly when bodyweight has decreased by more than 20% compared with control animals, or bodyweight has decreased by more than 25% over a period of 7 days or more.*"

In contrast to OECD GD 116 and OECD GN 35, OECD GD 19 is applicable for animal studies in general and provides guidance on what is to be considered excessive toxicity/suffering, and thus what is maximal acceptable toxicity in experimental animals. Of further note, the MTD is not specifically referred to in OECD GD 19.

1.5. Impact of selecting an MTD based on body weight change vs. body weight gain change

Dose selection for a carcinogenicity study (a higher tier study), is guided by the outcome of sub-chronic studies (typically 90-day duration). Originally, an effect/dose to look for was a 10% decrease in body weight relative to controls at the end of a 90-day study: not as a sign of excessive toxicity, but as a good indicator for a hypothesized MTD in a carcinogenicity study. As noted before, this was later changed into a 10% decrease in body weight *gain* relative to controls, without argumentation being provided.

The impact of selecting body weight *gain* change instead of body weight change can be very large: for instance, let us consider an OECD TG 413 study (90-day inhalation). Rats are 7–9 weeks old at the day of randomization and need to be acclimatized in their cages for at least 5 days prior to exposure. This means that rats are around 8–10 weeks old at the start of the 13 week exposure, and 21–23 weeks old at the end of exposure (without consideration of a recovery period). For male Wistar rats (a commonly used strain for regulatory studies), the average body weight is around 250 g at 9 weeks of age (see Fig. 1). At 23 weeks of age, this would be around 350 g (interpolated from Fig. 3 in *Nistiar* et al.,

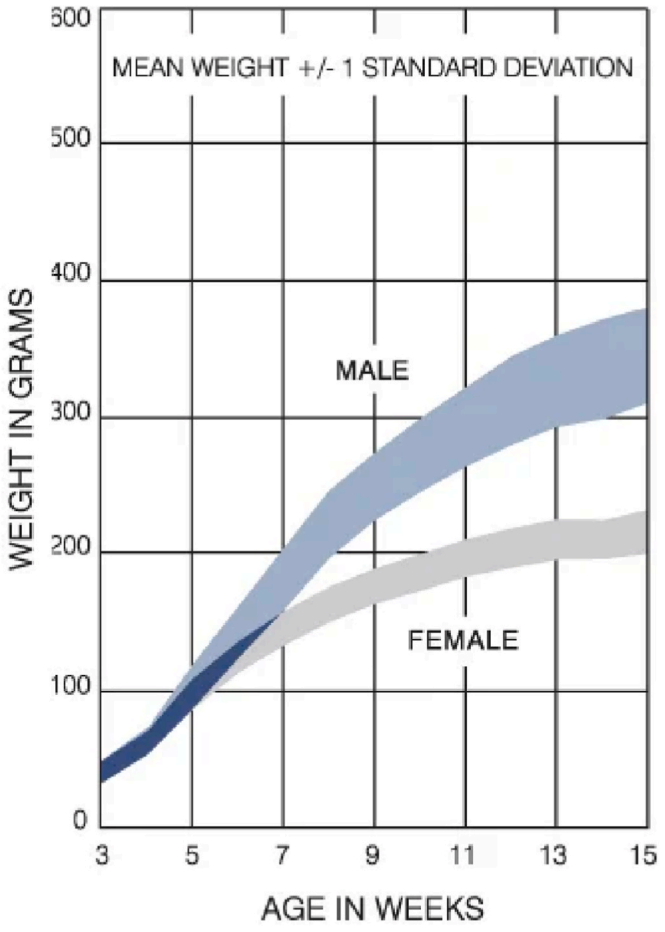

Fig. 1. Example of weight development in a commonly used rat strain for regulatory toxicity testing (Wistar Han IGS), mean ± 1SD – source: Charles River laboratories website, as accessed on Oct 22, 2021 *(https://www.criver.com/products-services/find-model/wistar-han-igs-rat?region=3651)*.

2012). A 10% decrease in body weight in the top dose group after 90 days would mean that the average body weight would be 315 g. A 10% lower body weight gain would mean that the average weight at the end of the 90-study would be 340 g, corresponding to a 2.9% lower body weight. This clearly is a major difference; one must question whether a weight difference of less than 3% between groups would be inside the normal variation observed in the dose groups of either a sub-chronic toxicity or a carcinogenicity study. In Fig. 1 it can be seen that the body weight of male Wistar Han rats at 15 weeks of age is around 310 (mean – 1SD) to 380 (mean + 1SD) grams, which means that the SD is around 35, i.e. >10% different from the mean value. Hence, a 10% lower body weight, and certainly a 10% lower body weight *gain*, cannot be considered as excessive toxicity, as is often argued in more recent studies and toxicological assessments.

The differential impact on final body weight in a 90-day repeated toxicity study of the various recommendations specified in OECD guidance documents is visualized in Fig. 2 by using the earlier example of the 90-day study where animals in the control group weighed 350 g on average at the end of exposure.

When selecting a top dose based on body weight effects in a 90-day toxicity study, one can come to very different results when following various OECD guidance documents:

- Based on OECD GD 19, exposed animals with a body weight of 280 g or less at the end of exposure would be candidate for application of the humane endpoint, i.e. for sacrifice due to excessive toxicity. Whether the animal will be sacrificed also depends on whether other clinical signs are observed and whether food intake is decreased or absent. (Note: the other criterion mentioned in OECD GD 19, i.e. a 25% weight *loss* in 7 days (so from e.g. 250 g–187.5 g), is not depicted in Fig. 2).
- Based on OECD GN 35, a top dose group with an average weight of 315 g, vs. 350 g in the control group, is the highest dose that can be used in a 2-year carcinogenicity study.
- Based on OECD GD 116, a top dose group with an average weight of 340 g, vs. 350 g in the control group, is the highest dose that can be used in a 2-year carcinogenicity study.

Thus, all three guidances come to very different recommendations on the top dose in a carcinogenicity study, with serious consequences for its regulatory usability.

It is evident that, relative to controls, a 10% reduction in body weight, and certainly a 10% reduction in body weight *gain* (corresponding to <3% body weight reduction relative to controls), are not to be considered a condition of excessive toxicity. In fact, one could question whether a 10% reduction in body weight gain relative to controls is a biologically relevant (toxic) effect *at all*. What is to be realized when interpreting such body weight effects is firstly that control animals in a regulatory toxicity study are allowed to feed *ad libitum*; they are well-fed and possess significant reserves. The fat percentage of the three most common rat strains used for regulatory toxicity testing (the Sprague Dawley, Fischer F344, and Hannover Wistar strains), fed *ad libitum* with a standard feed, is around 20% (*Reed* et al., *2011*). Secondly, natural variation in body weight can be significant; as shown in Fig. 1, it is roughly 20% for male Wistar rats aged 15 weeks. Moreover, guidelines for most regulatory toxicity studies consider a body weight variation of up to maximally 20% in a study group at the start of the study as acceptable. Thirdly, dietary restriction markedly affects lifespan in rats, not lowering but typically extending it with 14–45%, showing that lower body weight to some extent actually *benefits* animals health (*Swindell, 2012*).

Evidently, when 1) extrapolating the MTD concept based on 10% body weight change relative to controls to endpoints other than carcinogenicity and when 2) incorrectly interpreting the 10% body weight (gain) decrement criteria as indicators of excessive toxicity, this results in studies with top doses that are too low. As a consequence, no or only minimal toxicity is observed in such toxicity studies. This seriously impacts human health hazard and risk assessment and subsequent risk management options, as discussed and illustrated by *Woutersen et al. (2020)*. From a regulatory perspective this is of great concern, which has already led to incorporation of a statement in the recently amended information requirements under REACH that "the chosen study design shall ensure that the data generated are adequate for hazard identification and risk assessment. To this end, testing shall be performed at appropriately high dose levels." (*European Commission, 2021*).

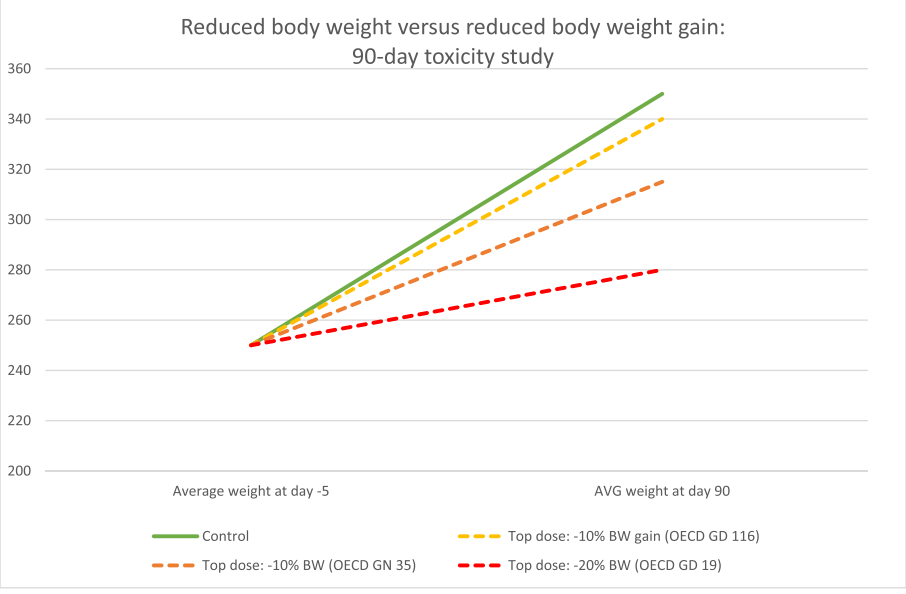

Fig. 2. Actual body weight of male rats (y-axis, in grams) corresponding with the different body weight change standards described in OECD GD nos. 19 and 116 and OECD GN 35.

2. Conclusions

This commentary relates to the use of the *10% body weight (gain) change relative to controls* (i.e., not body weight loss) as parameter for MTD selection and the consequences for the regulatory usability of toxicity studies. Other parameters, while important, are outside the scope of this manuscript.

The main conclusions can be listed as follows:

Conclusion 1: At present, there is no universal definition of the MTD, which creates confusion. The MTD as defined by Sontag et al. (1976) should not be seen as a "minimally toxic dose", which is a different concept altogether. In this article MTD is used as abbreviation of "maximum tolerable dose", but also this definition has various interpretations.

Conclusion 2: Neither a 10% lower body weight relative to controls nor a 10% lower body weight *gain* relative to controls should be interpreted as an indication of excessive toxicity. It is even questionable whether a 10% lower body weight *gain* relative to controls, which corresponds to <3% body weight reduction at the end of a 90-day study, is a biologically relevant effect at all, given that a 3% difference is within normal biological variation of body weight. Body weight change in the context of MTD selection should be interpreted with consideration of the fact that controls animal in a regulatory toxicity study are usually allowed to feed *ad libitum*, resulting in a well-fed animal with significant reserves.

Conclusion 3: The dose associated with a 10% lower body weight relative to controls (i.e., without "gain") in a 90-day study can be taken as an indicator of the MTD in a carcinogenicity study, when assessing (potentially) genotoxic carcinogens. This is in line with how it was originally intended, to prevent false negative results.

Conclusion 4: The 10% lower body weight *gain* (relative to controls) criterion for MTD in OECD guidance documents and test guidelines for carcinogenicity testing of (potentially) genotoxic substances needs to be changed to the original 10% lower body weight (relative to controls) criterion (i.e., without "gain"). The addition of "gain" is not explained anywhere, whereas it is clear that it can easily lead to poor design of regulatory carcinogenicity studies, with no or only minimal toxicity as outcome and serious impact on human health hazard and risk assessment/management.

Conclusion 5: The MTD criterion based on 10% body weight change relative to controls is intended to avoid false *negative* results in a carcinogenicity study. Consequently, the finding of an increase in tumor incidence at or above such an MTD (and even more so on a 10% body weight *gain* change) cannot be simply discarded as being false *positive* results, as is often argued.

Conclusion 6: The MTD criterion based on a 10% lower body weight (gain) relative to controls included in test guidelines/guidances for endpoints other than carcinogenicity needs to be taken out altogether. It is not conform the original intention and most importantly, as noted before, a 10% lower body weight *gain* compared to the control group is not to be interpreted as an indication of excessive toxicity, nor is a 10% lower body weight relative to controls. This criterion may easily result in too low dosing in studies used for regulatory decision making. Whereas this may seem animal-friendly (the lower the dose, the less suffering/discomfort), it actually leads to a waste of animals and thus it is unethical to conduct animal studies that do not fulfil their purpose, i.e. to reveal the potential hazardous properties of the substance studied.

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CRediT authorship contribution statement

Damiën van Berlo: Writing – original draft, Conceptualization, Investigation. **Marjolijn Woutersen:** Writing – review & editing. **Andre Muller:** Writing – review & editing. **Marja Pronk:** Writing – review & editing. **Jelle Vriend:** Writing – review & editing, Investigation. **Betty Hakkert:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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