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REVIEW

Impact of sampling time on the detection of mutations in rapidly proliferating tissues using transgenic rodent gene mutation models: A review

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Abstract

The OECD Test Guideline 488 (TG 488) for the Transgenic Rodent Gene Mutation Assay has undergone several revisions to update the recommended design for studying mutations in somatic tissues and male germ cells. The recently revised TG recommends a single sampling time of 28 days following 28 days of exposure (i.e., 28 + 28 days) for all tissues, irrespective of proliferation rates. An alternative design (i.e., 28 + 3 days) is appropriate when germ cell data is not required, nor considered. While the 28 + 28 days design is clearly preferable for slowly proliferating somatic tissues and germ cells, there is still uncertainty about the impact of extending the sampling time to 28 days for rapidly somatic tissues. Here, we searched the available literature for evidence supporting the applicability and utility of the 28 + 28 days design for rapidly proliferating tissues. A total of 79 tests were identified. When directly comparing results from both designs in the same study, there was no evidence that the 28 + 28 days regimen resulted in a qualitatively different outcome from the 28 + 3 days design. Studies with a diverse range of agents that employed only a 28 + 28 days protocol provide further evidence that this design is appropriate for rapidly proliferating tissues. Benchmark dose analyses demonstrate high quantitative concordance between the 28 + 3 and 28 + 28 days designs for rapidly proliferating tissues. Accordingly, our review confirms that the 28 + 28 days design is appropriate to assess mutagenicity in both slowly and rapidly proliferating somatic tissues, and germ cells, and provides further support for the recommended design in the recently adopted TG 488.

KEYWORDS
OECD test guideline, optimal sampling time, transgenic mutation assay

1 | INTRODUCTION

Cellular proliferation is considered essential to convert unrepaired DNA lesions into stable mutations (White et al., 2017). Accordingly, gene mutation studies normally include a period of time following an exposure, known as "manifestation time," "expression time," or "sampling time" (Thybaud et al., 2003), to allow for mutation manifestation (Heddle, 1999). Because proliferation rate varies among tissues, a different optimal "sampling time" might be expected for every tissue when performing a transgenic rodent (TGR) gene mutation assay (Organization for Economic Co-operation and Development [OECD], 2009). However, in regulatory testing, different sampling times are not practical in the same study, or desirable from a "3Rs" (i.e., Replace, Reduce, and Refine) perspective. As a compromise for the measurement of TGR mutant frequencies in both rapidly and slowly proliferating tissues, a regimen of 28 consecutive daily treatments followed by a 3-day sampling time (i.e., 28 + 3 days) was originally established for OECD Test Guideline 488 (OECD, 2011). This compromise was based not only on the ability to detect mutations in both slowly and rapidly proliferating somatic tissues, but also to achieve a refinement in animal use and reduction in animal care costs. However, the consequence is that while a gene mutation test performed using the 28 + 3 days design may be favorable for rapidly proliferating tissues, it is demonstrably not optimal for slowly proliferating tissues (Heddle et al., 2003; Thybaud et al., 2003).

An additional issue with the 28 + 3 days design was its ineffectiveness for assessing mutations in germ cells. A review of the available germ cell data has demonstrated that analysis of mutations in developing germ cells from seminiferous tubules, or sperm, collected at 28 + 3 days is unreliable (Marchetti et al., 2018b). This review, coupled with a modeling of mammalian spermatogenesis (Marchetti et al., 2018a), showed further that a 28 + 28 days sampling regimen is significantly better for the assessment of germ cell mutagenicity for both male mice and rats. This regimen enables the evaluation of mutations in a seminiferous tubule cell population that received most of its 28-day mutagen exposure during the proliferative phase of spermatogenesis. Subsequently, the 28 + 28 days regimen was adopted as the preferred OECD design for germ cell testing in a previous revision of TG 488 (OECD, 2020).

Having distinct experimental designs requiring different sampling times for somatic (28 + 3 days) and germ cell (28 + 28 days) mutation analysis could lead to significant confusion. Requiring two sampling times would also lead to the excess use of animals, which is counter to the "3Rs" principle respecting animal use, and has been a source of criticism during the regulatory review process. In order to avoid the problem of multiple sampling times, the 2020 Test Guideline suggests that when both somatic and germ cells need to be collected and/or tested, a single 28 + 28 days sampling regimen permits an assessment of mutagenicity in both somatic tissues and male tubule germ cells.

Although the 2020 version of TG 488 already recognized the 28 + 28 days design as appropriate for slowly proliferating somatic tissues such as the liver, before this design could also be used as the default recommendation for rapidly proliferating tissues, it was necessary to determine if chemicals that are positive in rapidly proliferating tissues (such as bone marrow, spleen, small intestine, and colon) with a 28 + 3 days design would still be positive with a 28 + 28 days design. Recently, Marchetti et al. (2021) showed experimentally that increasing the sampling time from 3 to 28 days after 28 days of dosing does not affect the qualitative or quantitative detection of mutations in MutaMouse bone marrow for four diverse-acting chemical mutagens (benzo[a]pyrene [BaP], procarbazine [PRC], isopropyl methanesulfonate [iPMS], and triethylenemelamine [TEM]; Marchetti et al., 2021). These experiments further demonstrated that mutant frequencies remain stable for over 2 months after the end of exposure, when strong mutagens are used. However, for the weak mutagen TEM, sampling >28 days produced false negative results. Accordingly, these results provide convincing empirical evidence that the 28 + 28 days study design can be a unifying regimen for simultaneously assessing in vivo mutagenesis in both rapidly and slowly proliferating somatic tissues, as well as male seminiferous tubule germ cells.

In order to expand the data supporting the use of a common 28 + 28 days design to include additional chemicals tested in a variety of rapidly proliferating tissues, a subgroup of the Genetic Toxicology Technical Committee (GTTC), Health and Environmental Sciences Institute (HESI) conducted a review of the available TGR literature for which there are mutation studies on rapidly proliferating tissues involving various combinations of administration and sampling times. The results of this review demonstrated the suitability of the 28 + 28 days design for a variety of rapidly proliferating tissues and mutagens and that this regimen permits the testing of mutations in somatic tissues and tubule germ cells from the same animals. Thus, our results provide further support for the latest version of TG488 (OECD, 2022) which recommends a common 28 + 28 days design for both rapidly and slowly proliiferating somatic tissues when both somatic and germ cells need to be collected and/or tested, based on regulatory requirements, or toxicological information.

2 | MATERIALS AND METHODS

The principal question posed in this review is: "are chemicals that are positive in rapidly proliferating tissues using the 28 + 3 days testing design also positive using the 28 + 28 days design?" To address this question, the Transgenic Rodent Assays Information Database (OECD, 2009) and unpublished updates, together with a search of more recent publications were queried for TGR mutation studies in certain rapidly proliferating tissues (i.e., bone marrow, small intestine, spleen, and colon). Other, more slowly proliferating tissues were excluded since the issue under study concerned specifically the performance of rapidly proliferating tissues at longer sampling times. The resulting data were stratified in various categories covering long exposure (≥28 days), acute or short exposure (1–5 days), early sampling time (3–5 days), late sampling time (≥28 days), and tabulated according to the combination of these

TABLE 1 Studies on chemicals that are mutagenic in rapidly proliferating tissues using Long administration periods with both short and Long sampling times

No.	Chemical or agent	TGR model	Tissue	Admin. period: days	Early sampling time: days	Late sampling time: days	Vehicle control MF ($\times 10^{-5}$)	Maximum MF ($\times 10^{-5}$)	Fold change	Response	References
1	3-Nitrobenzanthrone	MutaMouse	Bone marrow	28	3		4.9	14.6	3.0	+	Chen et al. (2008)
	3-Nitrobenzanthrone	MutaMouse	Bone marrow	28		28	6.4	14.8	2.3	+	Arlt et al. (2008)
2	Acrylamide	MutaMouse	Bone marrow	28	3		4.4	16.4	3.8	+	Thybaud et al. (2003)
	Acrylamide	MutaMouse	Bone marrow	28		28	5.5	9.4	1.7	+	Thybaud et al. (2003)
3	BaP	lacZ plasmid mouse, wild type ($XPA^{+/+}$)	Spleen	35 (15 doses)	3		3.8	26.5	7.0	+	de Vries et al. (1997)
	BaP	lacZ plasmid mouse, wild type ($XPC^{+/+}$)	Spleen	42 (18 doses)		42	12.7	55.0	4.3	+	Verhofstad et al. (2010)
	BaP	MutaMouse	Bone marrow	28	3		4.4	716.7	162.9	+	Marchetti et al. (2021)
	BaP	MutaMouse	Bone marrow	28		28	4.4	1204.1	273.7	+	Marchetti et al. (2021)
	BaP	MutaMouse	Bone marrow	28		42	5.1	885.8	173.7	+	Marchetti et al. (2021)
	BaP	MutaMouse	Bone marrow	28		70	5.1	1066.3	209.1	+	Marchetti et al. (2021)
4	EC (urethane)	MutaMouse	Bone marrow	28	3		5.4	9.3	1.7	+	Singer (2006)
	EC (urethane)	MutaMouse	Bone marrow	28		28	4.7	10.0	2.1	+	Singer (2006)
	EC (urethane)	MutaMouse	Small Intestine	28	3		9.3	16.8	1.8	+	Singer (2006)
	EC (Urethane)	MutaMouse	Small Intestine	28		28	9.3	19.7	2.1	+	Singer (2006)
5	iPMS	MutaMouse	Bone marrow	28	3		4.6	21.8	4.7	+	Marchetti et al. (2021)
	iPMS	MutaMouse	Bone marrow	28		28	4.6	17.3	3.8	+	Marchetti et al. (2021)
	iPMS	MutaMouse	Bone marrow	28		42	5.9	18.9	3.2	+	Marchetti et al. (2021)
	iPMS	MutaMouse	Bone marrow	28		70	5.9	17.2	2.9	+	Marchetti et al. (2021)
6	ENU	MutaMouse	Bone marrow	28	3		4.9	39.8	8.1	+	Marchetti (2018, unpublished)
	ENU	MutaMouse	Bone marrow	28		70	3.0	39.2	13.1	+	Marchetti (2018, unpublished)
	ENU	BigBlue Mouse	Bone marrow	28	3		4.8	71.1	14.9	+	Young et al. (2014)
	ENU	BigBlue Mouse	Bone marrow	28		49	3.2	54.3	17.0	+	Young et al. (2014)
7	PRC	MutaMouse	Bone marrow	28	3		6.4	42.0	6.6	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	28		28	6.4	29.1	4.5	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	28		42	7.2	43.8	6.1	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	28		70	7.2	34.2	4.8	+	Marchetti et al. (2021)
8	TEM	MutaMouse	Bone marrow	28	3		4.3	11.9	2.8	+	Marchetti et al. (2021)
	TEM	MutaMouse	Bone marrow	28		28	4.3	8.3	1.9	+	Marchetti et al. (2021)
	TEM	MutaMouse	Bone marrow	28		42	6.0	8.1	1.4	–	Marchetti et al. (2021)
	TEM	MutaMouse	Bone marrow	28		70	6.0	9.4	1.6	–	Marchetti et al. (2021)

Abbreviations: BaP, Benzo(a)pyrene; ENU, *N*-ethyl-*N*-nitrosourea; iPMS, isopropyl methanesulfonate; PRC, procarbazine; TEM, triethylenemelamine; TGR, transgenic rodent.

parameters used within each study. The authors' conclusions regarding positive and negative outcomes were taken as they described. Three separate data sets of studies were derived from the database search.

- Category 1: studies that used a 28-day administration period with both a 3-day and ≥ 28-day sampling time in which either study design, or both, were positive in the same tissue;
- Category 2: studies that used an ~28-day administration period with a late (21–112 days) sampling time that were positive; and
- Category 3: studies that used a short administration period (1–5 days) and a late sampling time (25–182 days) that were positive.

Data in Categories 2 and 3 above were collected with the premise that if a chemical was positive after a late sampling time in rapidly proliferating tissues, it likely would also have been positive at early sampling times (subject to consideration of mode of administration, deposition, and metabolism), as was observed for Category 1 chemicals (Table 1).

Benchmark dose (BMD) analysis was used to compare the dose response kinetics between early and late sampling times for chemicals in Table 1. Analysis was conducted on two chemicals, ethyl carbamate (EC) and *N*-ethyl-*N*-nitrosourea (ENU), for which published BMD analyses were not already available. Dose response modeling was conducted on the mutant frequency values calculated for each individual animal using the U.S. Environmental Protection Agency's (EPA) Benchmark Dose Software v3.2 (U.S. Environmental Protection Agency and National Institute for Occupational Safety and Health, 2020). The likelihood ratio tests (LRT) built into BMDS were used to select between homogeneous or non-constant variance models. Based on these tests, a constant variance model was deemed appropriate for the EC data set, and a nonconstant variance model was used for the ENU data set. Best models were selected based on the lowest Akaike Information Criterion score. BMDs were determined based on a benchmark response of 50% increase over the modeled controls. The confidence intervals between the lower and upper 95% confidence limits (BMDL and BMDU) were determined; overlapping confidence intervals demonstrate that dose responses are not statistically different (Wills et al., 2016). Published BMD analyses on four additional chemicals (BaP, PRC, iPMS, and TEM; Marchetti et al., 2021) from Table 1 were also used in this analysis.

3 | RESULTS

The chemicals and agents assessed in this review include a wide selection of molecular structures and mutagenic potencies with associated mechanisms of mutagenic action from simple alkylating agents to chemicals forming bulky DNA adducts, including both direct acting, and chemicals requiring metabolic activation. Accordingly, they are a representative sample of the diverse population of available mutagens.

3.1 | Studies using Long administration periods with both early and late sampling times in the same study

Data from studies that include a 28-day (or longer) administration period with both early and late sampling times are the most complete and informative in demonstrating that there is no qualitative change in mutagenic response by increasing the sampling time from 3 to ≥28 days for rapidly proliferating tissues (Table 1). In this regard, there were 30 tests on 8 chemicals that used both 28 + 3 and 28 + ≥28 days regimens in rapidly proliferating tissues in the same study (Table 1; 3-nitrobenzanthrone, acrylamide, EC, BaP, ENU, iPMS, PRC, and TEM). The majority of these tests were conducted in bone marrow; however, data in small intestine, spleen, were also available. In all of these cases, there were no qualitative differences in study outcomes (i.e., ability to detect a positive response) between the two sampling times.

In addition, there were two examples (BaP/spleen and 3-nitrobenzanthrone/bone marrow) where the results after early and late sampling times came from separate studies. The results from these studies agree with those in which both early and late sampling times were from the same experiment, i.e., these chemicals were positive for mutagenicity with both the early and late sampling time study designs.

Overall, the studies shown in Table 1 demonstrate that a ≥ 28 days sampling time results in qualitatively similar positive results to those obtained for a 3 days sampling time. However, there is one example demonstrating that sampling times >28 days (in this case, 42 and 70 days sampling time) may result in false negative

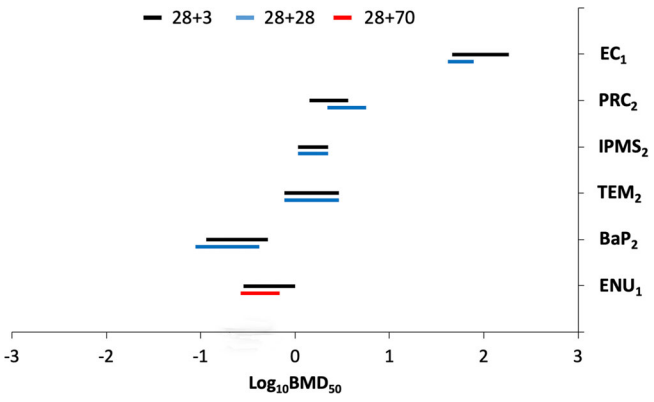

FIGURE 1 BMD modeling of the effect of sampling time on point of departure kinetics (i.e., mutagenic potency/). The horizontal bars show 95% confidence intervals for BMD50 estimates for the indicated testing regimens for the named chemicals. Overlapping confidence intervals for a chemical indicates that mutagenic potencies are not statistically different. EC, ethyl carbamate; PRC, procarbazine; iPMS, isopropyl methanesulfonate; TEM, triethylenemelamine; BaP, benzo(a)pyrene; ENU, *N*-ethyl-*N*-nitrosourea (underlying data are available in Supplementary Data S1). 1BMD data calculated as per methods and materials in this review. 2BMD data were published previously in Marchetti et al. (2021)

outcomes when a weak mutagen, such as TEM, is used (Marchetti et al., 2021). Nevertheless, it is important to note that TEM was positive with a 28 + 28 days design.

We also evaluated whether later sampling times impacted the quantitative response. For most chemicals in Table 1 for which there are rapidly proliferating tissue data for both early and late sampling times, the maximum fold change at late sampling times was about the same or slightly less than for early sampling time.

In order to assess further the impact of sampling time on the quantitative response kinetics, BMD modeling was conducted to determine points of departure (BMD_{50}) and these were compared at early and late sampling times for selected data in Table 1. The EC study used only two dose groups plus historical control group, and modeling analysis of the data showed that an exponential model was slightly better than linear (see Supplementary Data S1). The ENU study used three dose groups plus control group and fitted an exponential model (see Supplementary Data S2). There were four additional chemicals in Table 1 for which BMD analyses were previously conducted and published (Marchetti et al., 2021). The BMDL–BMDU confidence intervals for early and late sampling times were plotted for these six chemicals (Figure 1). While these chemicals cover a wide range of potencies, as depicted by the wide dispersion of BMD_{50} confidence intervals, the different sampling times for individual chemicals have highly overlapping confidence intervals indicating that the BMD_{50} sampling time values for each chemical were not significantly different. Taken together, these analyses show that, over the sampling times investigated, chemical potency values (i.e., the BMDs in Figure 1) were not significantly affected by sampling time.

3.2 | Studies using only long administration period and late sampling times

There were very few chemicals with negative results from late sampling times and these lacked data from early sampling times. In the absence of comparable results at early sampling times, these negative results were not informative for the purpose of this study. Accordingly, only studies with positive results and a late sampling times were considered (Table 2).

Only 13 tests involving 8 chemicals that used long administration periods (12–182 days) and late sampling times (21–112 days; Table 2) were observed, and for which there were no data from early and late sampling times in the same study. Some administration periods in this table included fewer actual dose administrations than the indicated total number of days of administration period per se because animals were not treated daily. For the eight chemicals in Table 2, there are data for EC and ENU with early sampling times from different studies than seen in Table 1. Since these later sampling times also resulted in the same positive outcomes as the early sampling times seen in Table 1, they provide further support for the general applicability of using a late sampling time in rapidly proliferating tissues for assessing mutation induction.

TABLE 2 TGR studies on chemicals (+ radiation) that are mutagenic in rapidly proliferating tissues using Long administration periods and late sampling time

No.	Chemical or agent	TGR model	Tissue	Admin. period: days	Late sampling time: days	Vehicle control MF ($\times 10^5$)	Maximum MF ($\times 10^5$)	Fold change	Response	References
1	1,8-Dinitropyrene	MutaMouse	Bone marrow	56 (8 doses)	29	4.1	7.6	1.9	+	OECD (2009)
2	3-Aminobenzanthrone	MutaMouse	Bone marrow	28	28	3.7	9.7	2.6	+	Arlt et al. (2008)
3	E (urethane)	Big blue mouse	Spleen	28	28	4.8	11.0	2.3	+	Chang et al. (2003)
4	ENU	lacz plasmid mouse	Spleen	42 (18 doses)	42	12.2	55.1	4.5	+	Verhofstad et al. (2010)
	ENU	MutaMouse	Spleen	30	21	17.8	178.9	10.0	+	Cosentino & Heddle (2000)
	ENU	MutaMouse	Spleen	30	21	33.4	331.4	9.9	+	Cosentino & Heddle (2000)
	ENU	MutaMouse	Splenic lymphocytes	28	49	6.5	113.2	17.4	+	Walker et al. (2020)
	ENU	MutaMouse	Bone marrow	28	49	5.4	112.3	20.8	+	Walker et al. (2020)
5	*N*-hydroxy-2-AAF	Big Blue rat	Spleen	12 (4 doses)	28	2.0	5.6	2.7	+	Chen et al. (2001)
6	PhIP	BC-1 mouse (lacI/Msh2$^{+/+}$)	Colon (caecum)	28 (4 doses)	84	5.3	14.9	2.8	+	Zhang et al. (2001)
	PhIP	BC-1 mouse (lacI/Msh2$^{+/-}$)	Colon (proximal)	28 (4 doses)	84	6.8	12.6	1.9	+	Zhang et al. (2001)
	PhIP	BC-1 mouse (lacI/Msh2$^{-/-}$)	Colon (distal)	28 (4 doses)	84	44.0	70.6	1.6	+	Zhang et al. (2001)
7	X-rays	MutaMouse	Spleen	182	112	6.9	21.4	3.1	+	Ono et al. (1997)

Abbreviations: EC, ethyl carbamate; ENU, *N*-ethyl-*N*-nitrosourea; PhIP, 2-amino-1-methy1-6-phenolimidazo [4,5-b] pyridine; TGR, transgenic rodent.

TABLE 3 TGR studies on chemicals that are mutagenic in rapidly proliferating tissues using short administration periods and mostly late sampling times

No.	Chemical or agent	TGR model	Tissue	Admin. period: days	Early sampling time: days	Late sampling time: days	Vehicle control MF ($\times 10^{-5}$)	Maximum MF ($\times^{-5}$)	Fold change	Response	References
1	3-Nitrobenxanthrone	MutaMouse	Bone marrow	1		28	4.9	14.6	3.0	+	Chen et al. (2008)
2	4 NQO	MutaMouse	Bone marrow	1		28	10.9	80.8	7.4	+	Nakajima et al. (1999)
3	R7000	Big Blue mouse	Small Intestine	4		28	8.2	26.1	3.2	+	Quillardet et al. (2000)
	R7000	Big Blue mouse	Colon (caecum)	4		28	4.4	17.5	4.0	+	Quillardet et al. (2000)
	R7000	Big Blue mouse	Colon	4		28	5.5	12.3	2.2	+	Quillardet et al. (2000)
	R7000	Big Blue mouse	Spleen	4		28	3.8	10.04	2.6	+	Quillardet et al. (2000)
4	7,12-Dimethylbenzanthracene	MutaMouse	Bone marrow	1		28	3.6	70.0	19.4	+	Hachiya et al. (1999)
	7,12-Dimethylbenzanthracene	Big Blue rat	Bone marrow	1		42	1.8	21.1	11.9	+	Shelton et al. (2000)
	7,12-Dimethylbenzanthracene	Big Blue rat	Spleen	1		42	2.5	34.1	13.6	+	Manjanatha et al. (1998)
	7,12-Dimethylbenzanthracene	MutaMouse	Colon	1		28	5.5	18.8	3.4	+	Hachiya et al. (1999)
	7,12-Dimethylbenzanthracene	MutaMouse	Bone marrow	1		28	3.1	7.0	2.3	+	Hachiya et al. (1999)
5	Azathioprine	MutaMouse	Bone marrow	5		25	5.3	14.8	2.0	+	Smith et al. (1999)
6	Benzo(a)pyrene	MutaMouse	Spleen	5		182	5.8	89.0	15.3	+	Hakura et al. (1999)
	Benzo(a)pyrene	MutaMouse	Colon	5		182	24.7	391.0	15.8	+	Hakura et al. (1999)
	Benzo(a)pyrene	Gpt delta mouse (spi)	Bone marrow	1		49	0.3	4.5	15.9	+	Horibata et al. (2013)
	Benzo(a)pyrene	Big Blue mouse (lacI)	Spleen	1		21	11.8	15.6	8.7	+	Monroe et al. (1998)
7	Chlorambucil	MutaMouse	Bone marrow	5		25	7.3	15.5	2.1	+	Smith et al. (1999)
8	DMN	MutaMouse	Spleen	1		28	4.0	8.3	1.6	+	Souliotis et al. (1998)
9	ENU	Gpt delta mouse (spi)	Bone marrow	1		49	0.3	2.5	8.7	+	Horibata et al. (2013)
10	MNU	Big Blue mouse (lacI)	Spleen	1		21	2.7	7.8	2.9	+	Monroe et al. (1998)
	MNU	SWR x MutaMouse F1 (lacZ)	Colon	1		35	5.2	67.0	12.9	+	Shima et al. (2000)
	MNU	SWR x MutaMouse F1 (lacZ)	Bone marrow	1		35	6.1	20.1	3.3	+	Shima et al. (2000)
	MNU	SWR x MutaMouse F1 (lacZ)	Small Intestine	1		35	4.4	106.0	24.1	+	Shima et al. (2000)

(Continues)

TABLE 3 (Continued)

No.	Chemical or agent	TGR model	Tissue	Admin. period: days	Early sampling time: days	Late sampling time: days	Vehicle control MF ($\times 10^{-5}$)	Maximum MF ($\times^{-5}$)	Fold change	Response	References
	MNU	SWR x MutaMouse F1 (cll)	Small Intestine	1		35	4.1	61.8	15.1	+	Shima et al. (2000)
11	ENU	MutaMouse	Bone marrow	1		28	2.5	19.0	7.7	+	Itoh et al. (1999)
	ENU	MutaMouse	Spleen	1		28	9.0	31.2	3.5	+	Itoh et al. (1999)
12	o-Aminozotoluene	MutaMouse cll	Colon	1		28	5.5	51.1	9.3	+	Kohara et al. (2001)
	o-Aminozotoluene	MutaMouse	Colon	1		28	10.0	33.5	3.4	+	Ohsawa et al. (2000)
13	PRC	MutaMouse	Bone marrow	1 (50 mg/kg)		28	3.8	4.2	1.1	–	Suzuki et al. (1999)
	PRC	MutaMouse	Bone marrow	5 (150 mg/kg)		28	3.8*	29.4	7.7	+	Suzuki et al. (1999)
	PRC	MutaMouse	Spleen	1 (50 mg/kg)		28	2.1	2.7	1.3	–	Suzuki et al. (1999)
	PRC	MutaMouse	Spleen	5 (50 mg/kg)		28	4.3	25.1	5.8	+	Suzuki et al. (1999)
14	Proton radiation	lacZ plasmid	Spleen	1 (0.5 Gy)	7		4.2	5.5	1.3	+?	Chang et al. (2005)
	Proton radiation	lacZ plasmid	Spleen	1 (0.5 Gy)		56	5.8	11.3	1.9	+	Chang et al. (2005)
15	Vinyl carbamate	Big Blue mouse (cll)	Small intestine	1		28	2.6	9.1	3.5	+	Hernandez and Forkert (2007)
16	X-rays	MutaMouse	Spleen	1		112	6.9	21.4	3.1	+	Ono et al. (1997)

[a]Vehicle control from Suzuki et al. (1999), 1× treatment.

Abbreviations: 4 NQO, 4-Nitroquinoline-1-oxide; DMN, dimethylnitrosamine; ENU, *N*-ethyl-*N*-nitrosourea; MNU, *N*-methyl-*N*-nitrosourea; R7000, 7-Methoxy-2-nitronaphtho[2,1-b]furan; PRC, procarbazine; TGR, transgenic rodent.

TABLE 4 Compilation of chemicals appearing in more than one table

No.	Chemical or agent	TGR model	Tissue	Admin. period: days	Early sampling time: days	Late sampling time: days	Vehicle control MF ($\times 10^{-5}$)	Maximum MF ($\times 10^{-5}$)	Fold change	Table no.	Response	References
1	3-Nitrobenzanthrone	MutaMouse	Bone marrow	28	3		4.9	14.6	3.0	I	+	Chen et al. (2008)
	3-Nitrobenzanthrone	MutaMouse	Bone marrow	28		28	6.4	14.8	2.3	I	+	Arlt et al. (2008)
	3-Nitrobenzanthrone	MutaMouse	Bone marrow	1	28		4.9	14.6	3.0	III	+	Chen et al. (2008)
2	Benzo(a)pyrene	lacZ plasmid mouse ($XPA^{+/+}$)	Spleen	35 (15 doses)	3		3.8	26.5	7.0	I	+	de Vries et al. (1997)
	Benzo(a)pyrene	lacZ plasmid mouse ($XPA^{+/+}$)	Spleen	42 (18 doses)		42	12.7	55.0	4.3	I	+	Verhofstad et al. (2010)
	Benzo(a)pyrene	MutaMouse	Bone marrow	28	3		4.4	716.7	162.9	I	+	Marchetti et al. (2021)
	Benzo(a)pyrene	MutaMouse	Bone marrow	28		28	4.4	1204.1	273.7	I	+	Marchetti et al. (2021)
	Benzo(a)pyrene	MutaMouse	Bone marrow	28		42	5.1	885.8	173.7	I	+	Marchetti et al. (2021)
	Benzo(a)pyrene	MutaMouse	Bone marrow	28		70	5.1	1066.3	209.1	I	+	Marchetti et al. (2021)
	Benzo(a)pyrene	MutaMouse	Spleen	5	14		3.3	84.0	25.5	III	+	Hakura et al. (1999)
	Benzo(a)pyrene	MutaMouse	Spleen	5		182	5.8	89.0	15.3	III	+	Hakura et al. (1999)
	Benzo(a)pyrene	MutaMouse	Colon	5	14		7.4	275.0	37.2	III	+	Hakura et al. (1999)
	Benzo(a)pyrene	MutaMouse	Colon	5		182	24.7	391.0	15.8	III	+	Hakura et al. (1999)
	Benzo(a)pyrene	gpt delta mouse (spi)	Bone marrow	1		49	0.3	4.5	15.9	III	+	Horibata et al. (2013)
3	EC (urethane)	MutaMouse	Bone marrow	28	3		5.4	9.3	1.7	I	+	Singer (2006)
	EC (urethane)	MutaMouse	Bone marrow	28		28	4.7	10.0	2.1	I	+	Singer (2006)
	EC (urethane)	MutaMouse	Small Intestine	28	3		9.3	16.8	1.8	I	+	Singer (2006)
	EC (urethane)	MutaMouse	Small Intestine	28		28	9.3	19.7	2.1	I	+	Singer (2006)
	EC (urethane)	Big Blue mouse	Spleen	28		28	4.8	11.0	2.3	II	+	Chang et al. (2003)
4	ENU	MutaMouse	Bone marrow	28	3		4.9	39.8	8.1	I	+	Marchetti (2018, unpublished)
	ENU	MutaMouse	Bone marrow	28		70	3.0	39.2	13.1	I	+	Marchetti (2018, unpublished)
	ENU	LacZ plasmid mouse	Spleen	28 (4 doses)		42	12.2	55.1	4.5	II	+	Verhofstad et al. (2010)
	ENU	MutaMouse	Spleen	30		21	17.8	178.9	10.0	II	+	Cosentino & Heddle (2000)
	ENU	MutaMouse	Small Intestine	30		21	33.4	331.4	9.9	II	+	Cosentino & Heddle (2000)

(Continues)

TABLE 4 (Continued)

No.	Chemical or agent	TGR model	Tissue	Admin. period: days	Early sampling time: days	Late sampling time: days	Vehicle control MF ($\times 10^{-5}$)	Maximum MF ($\times 10^{-5}$)	Fold change	Table no.	Response	References
	ENU	MutaMouse	Bone marrow	28		49	5.4	113.2	21.0	II	+	Walker et al. (2020)
	ENU	MutaMouse	Splenic lymphocytes	28		49	6.5	113.2	17.4	II	+	Walker et al. (2020)
	ENU	Big Blue mouse	Bone marrow	28	3		4.8	71.1	14.9	I	+	Young et al. (2014)
	ENU	Big Blue mouse	Bone marrow	28		49	3.2	54.3	17.0	I	+	Young et al. (2014)
5	PRC	MutaMouse	Bone marrow	28	3		6.4	42.0	6.6	I	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	28		28	6.4	29.1	4.5	I	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	28		42	7.2	43.8	6.1	I	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	28		70	7.2	34.2	4.8	I	+	Marchetti et al. (2021)
	PRC	MutaMouse	Bone marrow	1 (50 mg/kg)		28	3.8	4.2	1.1	III	–	Suzuki et al. (1999)
	PRC	MutaMouse	Bone marrow	5 (150 mg/kg)		28	3.8*	29.4	7.7	III	+	Suzuki et al. (1999)
	PRC	MutaMouse	Spleen	1 (50 mg/kg)		28	2.1	2.7	1.3	III	–	Suzuki et al. (1999)
	PRC	MutaMouse	Spleen	5 (150 mg/kg)		28	4.3	25.1	5.8	III	+	Suzuki et al. (1999)
6	X-rays	MutaMouse	Spleen	182 (78 doses)		112	6.9	21.4	3.1	II	+	Ono et al. (1997)
	X-rays	MutaMouse	Spleen	1		112	6.9	21.4	3.1	III	+	Ono et al. (1997)

[a]Vehicle control from Suzuki et al. (1999) 1× treatment.

Abbreviation: EC, ethyl carbamate; ENU, *N*-ethyl-*N*-nitrosourea; PRC, procarbazine.

TABLE 5 Summary compilation of results

	Administration period (days)	Sampling time (days)	Number of chemicals/agents	Total number of tests	Tissues (No. of tests)	Highlights
Group 1	28	Both 3 and ≥ 28	8	30	Bone marrow (26) Spleen (2) Small intestine (2)	All chemicals that induced mutation at 3 days also produced measurable increases in mutation with a 28 days sampling time BMD analysis of six chemicals indicated virtually identical points of departure for 3 and 28 days sampling times
Group 2	12–182	21–112	8	13	Bone marrow (3) Spleen (7) Colon (3)	Data on two chemicals (EC, ENU) provide additional support for the findings in Table 1 Late sampling times facilitate the detection of mutagenicity in a diversity of chemicals and rapidly proliferating tissues
Group 3	1–5	25–182	16	36	Bone marrow (13) Spleen (12) Small intestine (4) Colon (7)	Five test results provide additional support for the findings in Table 1 Late sampling times facilitate the detection of mutagenicity in a diversity of chemicals and rapidly proliferating tissues
Total			32	79		

Abbreviation: BMD, benchmark dose; ENU, *N*-ethyl-*N*-nitrosourea.

3.3 | Studies using short administration periods and predominantly late sampling times

There were 36 tests on 16 agents in Table 3 that had positive results with short administration periods and late sampling times in a diversity of rapidly proliferating tissues. Most of these studies employed i.p. dose administration. As with Table 2, none of these chemicals have data from early sampling times at correspondingly short administration times. While the data in Table 3 demonstrate further that mutagens are positive in rapidly proliferating tissues after long sampling times, they do not provide information on the influence of repeated dose regimens.

There was only one agent (no. 14, proton radiation) for which there were both relatively early and late sampling times after a short administration period. This result mirrors the findings in Table 1 show that late sampling times do not lead to negative results with agents for which an early sampling time resulted in a positive test. There were four additional test results in Table 3 with B(a)P (spleen, colon, and bone marrow), which support the BaP findings of Marchetti et al. (2021) at late sampling times that are reported in Table 1.

There was one chemical, PRC (no. 13), for which a single low dose was not mutagenic after a 28-day sampling time in either bone marrow or spleen. However, a much higher dose (5× repeated) did cause a positive response in these tissues, indicating that a 28-day sampling time does not yield a qualitatively negative result for this chemical.

4 | SUMMARY OF RESULTS

The results were consolidated and summarized in Tables 4 and 5 to show the scope and depth of the data available for these agents. Table 4 shows chemicals appearing in more than one Table across the three data sets. Table 5 further summarizes the results in terms of the numbers of chemicals and observations within the three data sets analyzed. Tables 4 and 5 emphasize the quantity and diversity of data supporting the assertion that the +28 days sampling time provides qualitatively the same outcome as the +3 days sampling time in rapidly proliferating tissues. Figure 1 further illustrates the strength of this relationship by providing evidence that there is no quantitative difference among the responses of six chemicals in Table 1 for which there was sufficient data to perform point of departure (POD) BMD modeling.

5 | DISCUSSION

This review was undertaken to assess whether the available data support the contention that there would be no substantial loss of mutations in rapidly proliferating tissues by increasing the sampling time from 3 to 28 days after the end of 28 days of dosing. Since a mutation is an irreversible, heritable change in the DNA, it would persist unless a cell containing it was lost due to cytotoxicity, apoptosis, or phagocytosis. It is well known that the transgenes used in the TGR assay are not transcribed (i.e., are neutral) and are not under selective pressure

(OECD, 2009). Therefore, assuming there are no deleterious additional mutations in endogenous genes from the cells already containing a mutated transgene, or chemical-induced toxicity, loss of mutant cells from a stem cell population would not be expected. Indeed, the main finding gained from the analysis of available data is that there is no chemical identified for which a 28 days sampling time would provide a qualitatively different result from that obtained using a + 3 days sampling time; that is, no chemicals were found to be positive at a + 3 days sampling time that were negative at +28 days. This conclusion is made all the more convincing by the fact that the data include a wide variety of chemicals representing a diverse range of mutagenic mechanisms and potencies.

There is growing interest in applying BMD modeling of genotoxicity data for establishing POD estimates for health risk assessment. The present BMD analysis conducted on EC and ENU supports the clear demonstration previously provided by Marchetti et al. (2021) that there are no quantitative differences in POD kinetics among sampling times across a range of mutagenic potency. Evidence of quantitative similarity between different sampling times is also provided by the fact that the maximum fold increase at later sampling times is similar to that seen for early sampling times.

Thus, this review confirms the recent results of Marchetti et al. (2021), and provides very strong additional evidence that a 28 + 28 days regimen for the TGR assay is suitable for rapidly proliferating tissues, as previously recommended for slowly proliferating tissues in TG488 (OECD, 2020) for the detection of mutagenicity in the TGR gene mutation assay. Accordingly, the 28 + 28 days sampling regimen is clearly suitable to assess mutagenicity in both slowly and rapidly proliferating somatic tissues, as well as in male seminiferous tubule germ cells. The finding in this review confirm further the recommendation of the newly adopted revision of TG488 (OECD, 2022) that 28 + 28 days be adopted as the preferred TGR assay design, especially when germ cells are required or considered, in addition to somatic tissues.

AUTHOR CONTRIBUTIONS

Conceptualization: George R. Douglas and Francesco Marchetti; Methodology: George R. Douglas and Francesco Marchetti; formal analysis and investigation: George R. Douglas, Francesco Marchetti, and Jason M. O'Brien; writing—original draft preparation: George R. Douglas, Francesco Marchetti, and Jason M. O'Brien; writing—review and editing: all authors; funding acquisition: Francesco Marchetti; resources: Francesco Marchetti and Carole L.Yauk.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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