STATISTICAL MODELS AND METHODS ARTICLE

Statistical Comparison of Carcinogenic Effects and Dose–Response Relationships in Rats and Mice for 2,4-Toluene Diamine to those Ascribed to Toluene Diisocyanate

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ABSTRACT

The U.S. National Toxicology Program (NTP) conducted 2-year bioassays of commercial grade toluene diisocyanate (TDI) (80% 2,4-TDI and 20% 2,6-TDI) and 2,4-toluene diamine (TDA) and concluded that both were carcinogenic in rodents. In the TDI study, there was an unproven but likely formation of TDA either because of flawed test-substance handling and storage conditions and/or the atypical exposure conditions employed. Although the carcinogenic responses in both studies were qualitatively similar, several statistical analyses were performed to substantiate this possibility more rigorously. Seven different statistical approaches combine to yield a robust and consistent conclusion that, if only a small fraction (approximately 5%) of the dose of TDI were hydrolyzed to TDA in the TDI study, then that would be sufficient to explain the observed carcinogenic responses in the TDI study.

Key Words: toluene diisocyanate (TDI), toluene diamine (TDA), tumorigenic responses in rats and mice, hydrolyze TDI to TDA, flawed test-substance handling and storage conditions, atypical (gavage) exposure conditions.

INTRODUCTION

Results of the U.S. National Toxicology Program's (NTP 1986) gavage study of toluene diisocyanate (TDI) have served as the basis for the classification of TDI

Received 5 May 2011; revised manuscript accepted 27 September 2011.

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as a reasonably anticipated human carcinogen by the NTP (2005) and as possibly carcinogenic to humans (2B) by the International Agency for Research on Cancer (IARC 1986). In contrast, a maximum tolerated dose (MTD) of TDI was not carcinogenic in 2-year bioassays of rats and mice exposed via inhalation (Löser 1983), the primary and physiologically relevant route of human exposure. While the Löser (1983) publication reported that inhalation of TDI caused respiratory tract irritation in mice, but only minor effects in rats, it also indicated that a histopathological examination of the nasal tissues had not yet been performed. That investigation in rats was reported separately by Owen (1984). It revealed a dose-related increased incidence of rhinitis, generally characterized by squamous metaplasia/hyperplasia of the respiratory mucosa, with or without exudate in the lumen, and leucocyte infiltration in the anterior nasal cavity. Thus, repeated inhalation exposures of rats and mice by Löser (1983) to an MTD of 0.15 ppm TDI (*i.e.*, 0.12 mg/kg/day in rats and 0.25 mg/kg/day in mice per USEPA [1988]) produced respiratory tract lesions but no carcinogenic effect.

The discrepancy between the TDI classifications (by NTP 2005 and IARC 1986) and the implications of the Löser (1983) study may be related to the fact that the artificial introduction of TDI directly into the acidic milieu of the stomach favors the formation of toluene diamine (TDA), while deposition of TDI in the pH-neutral and macromolecule-rich milieu of the lung favors the reaction of TDI with biomolecules and with itself to form ureas and polyureas. (It is only the 2,4-TDI isomer in the commercial product that is being converted to 2,4-TDA, a known animal carcinogen.) In support of this explanation, a qualitative inspection of the NTP TDI (1986) and TDA (1979) studies reveals a striking similarity in the tumor patterns and species affected (*e.g.*, male rats as well as female rats and mice, but not male mice). This similarity was also noted by the NTP (1986). Understanding whether TDI itself is carcinogenic or only when it forms TDA is an important distinction because TDI does not form TDA under typical use and exposure conditions.

In the workplace, dermal and inhalation exposures to TDA formed in stored TDI are extremely unlikely. For practical as well as safety concerns, TDI is stored under nitrogen or dry air in a temperature controlled area to prevent its potential reaction with moisture in the air. If water were present during storage, the favored reaction is the formation of polyureas, not TDA. If present, water will react with one of the TDI NCO groups to form an intermediate carbamic acid group, which is subject to two competing reactions. In the first (fast), the carbamic acid groups react with free TDI to form carbamic acid esters, which in turn release CO₂ to form ureas, which subsequently polymerize to form polyureas. In the second reaction (slow), the carbamic acid group releases CO₂ to form an aminoisocyanate. The amino group of the aminoisocyanate reacts with free TDI to form a urea and subsequently a polyurea. This reaction occurs at a rate orders of magnitude faster than that of the remaining NCO group with water, which (if it occurred) would result in the formation of TDA. This kinetic difference leads to the consumption of aminoisocyanates and is the fundamental reason why contact between TDI and water leads predominately to the formation of polyurea, and why the free TDA concentration is negligible under typical (pH neutral) conditions. However, if the aminoisocyanate reacts with water

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under low pH conditions (*e.g.*, NTP gavage study), this kinetic difference is lessened and TDA can be formed.

To date, there is no convincing evidence that physiological TDI exposures in rodents and humans (*i.e.*, inhalation and dermal) result in the formation of free TDA. While inhalation exposures at the MTD will not result in total exposures approaching those that can be administered via gavage, data in rats show that a lifetime inhalation exposure to 0.15 ppm TDI vapor, a concentration 30-fold greater than the TDI TLV (5 ppb), does not cause a carcinogenic effect (Löser 1983) and that inhalation of 2 ppm TDI vapor, a concentration 400-fold greater than the TDI TLV, does not result in the formation of free TDA (Timchalk *et al.* 1994).

Concern is sometimes expressed that potential sensory irritation associated with the high exposure concentration (0.15 ppm) used by the Löser study may have resulted in the Sprague Dawley rats receiving a lower dose than assumed based on exposure concentration. However, an acute 3-hour sensory irritation study conducted in the same rat strain indicated a NOEL of 0.36 ppm and an RD50 of 2.1 ppm (Shiotsuka 1987). Additionally, when Fischer 344 rats were exposed to 2 ppm radiolabeled TDI vapor for 4 hours (Timchalk *et al.* 1994), rats received an inhalation dose of ~4 mg/kg based on the mass of radiolabel recovered. The authors estimated this dose to be ~1.3-fold greater than that predicted based on rat minute volume (0.236 ml/min); they attributed the greater dose to an increase in minute volume due to stress. This information suggests that sensory irritation did not play a significant role in influencing the respiratory physiology and consequently the dose received by rats in the chronic study.

The purpose of this work is to evaluate, from a statistical perspective, the possibility that the carcinogenic effects noted in the NTP (1986) TDI study were due to the formation of TDA either because of flawed test-substance handling and storage conditions and/or the atypical exposure conditions used in the TDI study (*i.e.*, gavage administration and the route-specific sequela are unlikely events during real world human exposures). Statistical analyses of the carcinogenic effects in both sexes of rats and mice orally exposed to either TDI (gavage) or TDA (diet) have been performed herein. These analyses focus on the dose-response relationships for the carcinogenic effects reported by the study authors for TDI and TDA and the likelihood that the carcinogenic effects ascribed to TDI are the same as those that would be predicted based on the dose-response relationship for TDA.

BIOLOGICAL AND CHEMICAL PLAUSIBILITY

In recognition of TDI being unstable in water and feed, the NTP prepared TDI gavage solutions in corn oil of normal moisture content (0.05%) and stored them outside a dessicator for up to 7 days. The stability of TDI in normal moisture and dried (0.005% water) corn oil was measured over the 7-day period (Appendix I of 1986 NTP study). The results indicated that TDI levels in three dosing solutions (*i.e.*, 9, 36, and 72 mg TDI/ml corn oil) declined over the 7-day period. For normal moisture corn oil, TDI recoveries were 98–99% (@ 0 days), 74–88% (@ 1 day), and 20–73% (@ 7 days); for dried corn oil, TDI recoveries were 100% (@ 0 days), 86–93%

(@ 1 day), and 48–81% (@ 7 days). While the NTP made no attempt to measure TDA in the dosing solutions, the NTP did conduct a literature survey and listed two possible products for the reaction between TDI and water: aromatic amines (*e.g.*, TDA) and disubstituted urea. Based on theoretical grounds, the NTP believed 12% to 98% of the TDI losses seen with normal moisture corn oil were due to the reaction of TDI with water, while 90% of the losses seen with dried corn oil were caused by reactions of TDI with corn oil components other than water. Thus, based on NTP's dosing solution analyses and theoretical considerations, dosing solutions may have contained at least 1% TDA if one assumes the average loss of TDI in the dosing solutions prepared each week was 12% (range 12%–80%) and that 12% of that loss was due to reactions with water (range 12% and 98%) to produce TDA. The presence of free TDA is also consistent with the more recent observation (Seel *et al.* 1999) that TDI mixed with DMSO containing 0.04% water rapidly degraded to form significant amounts of TDI- and TDA-urea as well as trace levels of TDA. In this study, TDA was detected within 15 min of mixing with DMSO, rose to 0.8% by 30 min, and reached a maximum of 1.2% at 4 h.

Several lines of evidence indicate that the conversion of TDI to TDA due to improper test sample handling would be augmented if the degraded test sample was subsequently inserted into the acidic environment of the stomach. After gavage with high doses of ^{14}C -2,4-TDI, Jeffcoat (1988) observed that much of the isocyanate polymerized in the stomach and was excreted in the feces. However, he also noted that the fraction of radiolabel excreted in the urine increased with decreasing dose, being 3.5% at 700 mg/kg, 6.3% at 70 mg/kg, and 16% at 7 mg/kg. Urinary metabolic profiles following gavage with ^{14}C -2,4-TDI (7 mg/kg) were qualitatively similar to those following an i.v. exposure to ^{14}C -2,4-TDA (~50 mg/kg). Six 2,4-TDI metabolites (83% of radiolabeled metabolites) co-chromatographed with those seen with i.v. 2,4-TDA. Two of the 2,4-TDI metabolites exhibited similar chromatographic retention times as those for 2,4-TDA (9%) and diacetyl TDA (4%). In another study, Kennedy and Brown (1999) evaluated the *in vitro* interactions of ^{14}C -TDI with biological matrices. Solutions of either rat serum albumin (RSA) or simulated gastric secretion containing pepsin were exposed to radiolabeled TDI vapor. At pH 7.4, virtually all of the TDI (>99%) became conjugated to RSA; TDA was not detected demonstrating successful competition between the protein's reactive groups and the hydrolytic reaction pathway to TDA. In contrast, exposure of either RSA or simulated gastric secretion to TDI vapor at pH 2.3 resulted in a dramatic decrease in TDI-protein conjugate formation and the appearance of detectable concentrations of TDA and oligoureas from the hydrolytic reaction. Finally, it should be noted that the ability of an acidic, *ex vivo* environment to convert TDI/TDA conjugates to free TDA is a widely used biomonitoring method (*e.g.*, Brorson *et al.* 1991; Persson *et al.* 1993; Austin 2007; and Kaaria *et al.* 2001). Thus, it is reasonable to conclude that the acidic environment of the stomach favors the conversion of TDI to TDA.

The latter biomonitoring references indicate that TDA can be detected in “acid-hydrolyzed” blood and urine following inhalation of and/or dermal contact with TDI by humans. The fact that none of the investigators indicated that TDA could be detected in the unhydrolyzed test specimens is consistent with the observation

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that free TDA was not detected in the non-hydrolyzed urine of workers exposed to TDI (Sennbro *et al.* 2003; Skarping *et al.* 1994). The acid-hydrolysis methodology measures free TDA in the processed specimens but cannot identify the source of the TDA (*e.g.*, free TDA or TDI/TDA conjugates). Thus, one cannot state unequivocally that free TDA is formed in biological fluids following physiological exposures of humans to TDI since the source of the hydrolyzed TDA could simply be conjugates of TDI and/or TDA. The absence of free TDA in the non-hydrolyzed specimens of humans exposed under biologically relevant conditions is consistent with studies in rats, demonstrating that the route of exposure affects the form of TDI/TDA appearing in urine. Free TDA is detected in urine following gavage with TDI but not after inhalation (Timchalk *et al.* 1994) or dermal (Rosenberg and Savolainen 1985) exposures to TDI. The absence of free TDA in non-hydrolyzed human specimens is also consistent with results from three epidemiological studies with updates, representing the combined long-term mortality experience of more than 17,000 PU foam (primary use of TDI) production workers. These studies failed to find an association between occupational exposure to diisocyanates and an increased risk of cancer (Schnorr *et al.* 1996; Hagmar *et al.* 1993a,b, updated by Mikoczy *et al.* 2004; and Sorahan and Pope 1993, updated by Sorohan and Nichols 2002).

Additional support for the potential *in vivo* conversion of TDI to TDA comes from a study by Timchalk and coworkers (1994) in which male rats were gavaged with (a) 60 mg/kg TDI in dried corn oil, the same dose used in the NTP bioassay (Dieter *et al.* 1990), and (b) 3 mg/kg TDA. Urine collected from 0–12 h was analyzed for free TDA, acetylated TDA, and acid-labile TDI/TDA conjugates. Following gavage with 60 mg/kg TDI, the amount of metabolite ($\mu\text{g eq TDA}$) found in urine was: 2.08 (free TDA), 13.3 (acetylated TDA), and 44.5 (acid-labile TDI/TDA conjugate); following gavage with 3 mg/kg TDA, these urinary fractions were: 3.93 (free TDA), 16.2 (acetylated TDA), and 24.1 (acid-labile TDA conjugate). The comparable urinary excretion profiles are consistent with ~5% (*i.e.*, 3 mg/kg $\div$ 60 mg/kg) of the TDI gavage dose being converted to TDA. If some of the TDI in the Timchalk study was converted to TDA prior to dosing, as likely occurred in the NTP gavage study, one would expect the TDA metabolite fractions reported by Timchalk to have been greater than ~5%.

The NTP TDI study was flawed because it (a) mishandled the TDI test samples, and (b) placed the degraded test samples in an acidic environment (stomach). Both actions favored the conversion of TDI to TDA. The NTP investigators dismissed these concerns (Dieter *et al.* 1990) “since similar degradation of TDI would immediately occur if exposure was by inhalation.” This misconception still exists in the scientific community but is not supported by the available data. The free TDA that has been detected in human biological specimens (*e.g.*, plasma, urine) is typically found only after *ex vivo* acid hydrolysis and release from TDI/TDA conjugates. There have been a few studies indicating the presence of TDA after treatment with a weak base but it remains unclear if this represents the presence of free TDA. Studies in animals support the position that free TDA is not found systemically after physiological exposures. Free TDA can be detected in urine from rats after gavage (Jeffcoat 1988; Timchalk *et al.* 1994) but not after physiological exposures such as inhalation

(Timchalk *et al.* 1994) or dermal contact (Rosenberg and Savolainen 1985). The pH-neutral environment associated with physiological exposures favors reaction of TDI with macromolecules; free TDA is not detected (Kennedy and Brown 1999). In an acidic environment, however, these authors noted that macromolecular conjugation is reduced by about 90% and free TDA can be detected. Thus, it is reasonable that TDA, a known animal carcinogen, present as both a degradation product in the TDI dosing solution prior to gavage and as a metabolite following gavage into the acidic environment of the stomach, may be responsible for the carcinogenic activity observed with TDI. The same conclusion was offered by the NTP study authors (Dieter *et al.* 1990) based on qualitative considerations.

DOSE-RESPONSE DATA

The dose-response data to be analyzed are from the NTP (1986) gavage study of TDI and the NTP (1979) study of TDA in feed. The abstract for the TDI study concludes on page 8 that:

Under the conditions of these gavage studies, commercial grade toluene diisocyanate in corn oil was carcinogenic for F344/ N rats, causing subcutaneous fibromas and fibrosarcomas (combined) in males and females, pancreatic acinar cell adenomas in males, and pancreatic islet cell adenomas, neoplastic nodules of the liver, and mammary gland fibroadenomas in females. Toluene diisocyanate was not carcinogenic for male B6C3F1 mice. TDI was carcinogenic for female B6C3F1 mice, causing hemangiomas or hemangiosarcomas (combined), as well as hepatocellular adenomas.

The summary for the TDA study concludes on page vi that:

In male rats, fibromas of the subcutaneous tissue occurred at incidences that were dose related ($P = 0.004$) and in direct comparisons were higher in the dosed groups (P less than or equal to 0.020) than in the control group (controls 0/20, low-dose 15/30, high-dose 19/50).

and

Under the conditions of this bioassay, 2,4-diaminotoluene was carcinogenic for F344 rats, inducing hepatocellular carcinomas or neoplastic nodules in both males and females and carcinomas or adenomas of the mammary gland in females. The test chemical was also carcinogenic for female B6C3F1 mice, inducing hepatocellular carcinomas. The incidence of lymphomas in the female mice suggested that these tumors also may have been related to administration of the test chemical.

From the conclusions of the two studies, the carcinogenic responses of primary interest to the study authors were as listed below. Available dose-response data for each of these responses are indicated in Table 1. The eight endpoints (combinations of carcinogenic response and gender) analyzed in this report are identified below along with a discussion of their corresponding carcinogenic response and the available data. Except as noted, all of the carcinogenic responses in Table 1 for which there are data from both the TDI and TDA studies are analyzed herein.

Table 1. Dose-response data from the NTP(1979) study of TDA in feed and the NTP (1986) gavage study of TDI.

Organ	Response	TDA										TDI									
		#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%				
		resp. ¹	risk ¹	resp.	risk	resp.	risk	resp.	risk	resp.	risk	resp.	risk	resp.	risk	resp.	risk				
		Male Rats																			
	Reported Dose ² mg/kg/day	0		79		176		0		30		60		42.9		60					
		0		3.95		8.80		0		21.4		42.9		42.9		42.9					
Subcutaneous	Fibromas	1	20	5%	15	50	30%	19	50	38%	3	50	6%	3	50	6%	9	50	18%		
Subcutaneous	Fibrosarcomas	1	20	5%	1	50	2%	3	50	6%	0	50	0%	3	50	6%	3	50	6%		
Pancreas	Acinar cell adenomas	2	19	10.5%	10	42	23.8%	10	44	22.7%	1	47	2.1%	3	47	6.4%	7	49	14.3%		
Liver	Carcinomas or neoplastic nodules ³	0	20	0%	5	49	10.2%	10	50	20%	7	50	14%	3	50	6%	4	50	8%		
		Female Rats																			
	Reported Dose	0		79		171		0		60		120		85.7		60					
		0		3.95		8.55		0		42.9		85.7		42.9		85.7					
mg/kg/day		0	20	0%	4	50	8%	10	50	20%	0	50	0%	1	50	2%	3	50	6%		
Subcutaneous	Fibromas	0	20	0%	4	50	8%	0	50	0%	2	50	4%	0	50	0%	2	50	4%		
Subcutaneous	Fibrosarcomas	0	20	0%	4	50	8%	0	50	0%	2	50	4%	0	50	0%	2	50	4%		
Pancreas	Islet cell adenomas	No Data Available from TDA Study																			
		0	20	0%	0	50	0%	3	49	6.1%	3	50	6%	8	50	16%	8	48	16.7%		
Liver	Neoplastic nodules	1	20	5%	26	50	52%	29	50	58%	15	50	30%	21	50	42%	18	50	36%		
Mammary	Fibroadenomas																				
		Female Mice																			
	Reported Dose	0		100		200		0		60		120		85.7		60					
		0		13		26		0		42.9		85.7		42.9		85.7					
mg/kg/day		0	19	0%	13	47	27.7%	18	46	39.1%	2	50	4%	2	50	4%	3	50	6%		
Liver	Hepatocellular carcinomas	0	19	0%	5	47	10.6%	3	46	6.5%	0	50	0%	1	50	2%	5	50	10%		
Circulatory system	Hemangiomas or hemangiosarcomas	0	19	0%	5	47	10.6%	3	46	6.5%	0	50	0%	1	50	2%	5	50	10%		
Hematopoietic	Lymphomas or leukemias	2	19	10.5%	29	47	61.7%	11	46	23.9%	13	50	26%	17	50	34%	16	50	32%		

¹Number of animals with the specified response, number of animals at risk (number of animals examined), and percent of animals with the specified response.

²Reported dose is ppm (TWA) for TDA and mg/kg for 5 days/week for TDI.

³Assumes that the # of male rats with hepatocellular carcinomas or neoplastic nodules is the sum of the # of male rats with hepatocellular carcinomas plus the # of male rats with neoplastic nodules.

Male Rats

Subcutaneous fibromas and fibrosarcomas (combined) were identified as a carcinogenic response for TDI. Fibromas were identified as a carcinogenic response for TDA. Combined data for fibromas and fibrosarcomas are not provided for TDA. Data for fibromas are provided for both TDI and TDA. Endpoint 1 is subcutaneous fibromas in male rats. As indicated in Table 1, the frequency of subcutaneous fibrosarcomas is small in both the TDI and TDA studies and noticeably non-monotonic (that is, not consistently increasing or consistently decreasing with dose) in the TDA study. Since combined data were not provided for TDA, this endpoint focused on the stronger response overall to both studies. The exclusion of subcutaneous fibrosarcomas is unlikely to significantly affect our conclusions.

Pancreatic acinar cell adenomas were identified as a carcinogenic response for TDI. This response was not discussed as a carcinogenic response for TDA. Data are provided for both TDI and TDA. Endpoint 2 is pancreatic acinar-cell adenomas in male rats.

Hepatocellular carcinomas or neoplastic nodules were not discussed as a carcinogenic response for TDI but were identified as a carcinogenic response for TDA. Data are provided for both TDI and TDA. Endpoint 3 is hepatocellular carcinomas or neoplastic nodules in male rats.

Female Rats

Subcutaneous fibromas and fibrosarcomas (combined) were identified as a carcinogenic response for TDI but were not discussed as a carcinogenic response for TDA. Combined data for fibromas and fibrosarcomas are provided for TDI but not provided for TDA. Endpoint 4 is subcutaneous fibromas in female rats. As indicated in Table 1, the frequency of subcutaneous fibrosarcomas is small in both the TDI and TDA studies and noticeably non-monotonic (*i.e.*, not consistently increasing or consistently decreasing with dose) in the TDA study. Since combined data were not provided for TDA, this endpoint focused on the stronger response overall to both studies. The exclusion of subcutaneous fibrosarcomas is unlikely to significantly affect our conclusions.

Pancreatic islet cell adenomas were identified as a carcinogenic response for TDI but were not discussed as a carcinogenic response for TDA. Data are provided for TDI but not for TDA. As indicated in Table 1, the observed dose-response relationship for pancreatic islet cell adenomas in the TDI study is non-monotonic and very uncertain given that it decreased appreciably at the high dose. These observations suggest that the reported incidences may be due to chance and not indicative of a truly tumorigenic response.

With respect to hepatocellular carcinomas or neoplastic nodules, neoplastic nodules were identified as a carcinogenic response for TDI and for TDA. Only data for neoplastic nodules (not hepatocellular carcinomas) are provided for both TDI and TDA. As indicated in Table 1, the frequency (6%) of hepatocellular carcinomas or neoplastic nodules in the control female rats in the TDI study nearly exceeds the liver response frequencies in the TDA study (0% in controls, 0% at 3.95 mg/kg/day, and 6.1% at 8.55 mg/kg/day). This unexplained high background rate of liver

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tumors in the TDI study effectively eliminates this endpoint as a viable point of dose–response comparison between the TDI and TDA studies.

With respect to mammary gland tumors, fibroadenomas are identified as a carcinogenic response for TDI. Carcinomas or adenomas were identified as a carcinogenic response for TDA. No carcinomas and one adenoma are reported for TDI. Fibroadenomas data are provided for both TDI and TDA. Endpoint 5 is mammary fibroadenomas in female rats. Although results for mammary carcinomas or adenomas (namely, identified as a carcinogenic response for TDA, and no carcinomas and one adenoma reported for TDI) are not subjected to dose–response modeling, the data support the conclusion that if only a small fraction of the supposed TDI dose in the NTP (1986) gavage study were actually TDA, then that TDA would be sufficient to explain the observed cancer responses in the TDI study.

Male Mice

Both study reports stated that no tumors occurred at significantly increased incidences in the male mice.

Female Mice

With respect to hepatocellular tumors, adenomas were identified as a carcinogenic response for TDI, and carcinomas were identified as a carcinogenic response for TDA. Adenomas data are provided for TDI but not for TDA. Carcinomas data are provided for both TDI and TDA. Endpoint 6 is hepatocellular carcinomas in female mice.

Hemangiomas or hemangiosarcomas (combined) of the circulatory system are identified as a carcinogenic response for TDI but are not discussed as a carcinogenic response for TDA. However, greater increases with dose were observed in the TDA study than in the TDI study. Data are provided for both TDI and TDA. Endpoint 7 is circulatory-system hemangiomas or hemangiosarcomas in female mice.

Lymphomas or leukemias were not discussed as a carcinogenic response for TDI. Lymphomas were identified as a carcinogenic response for TDA. Lymphomas or leukemias data are provided for both TDI and TDA. Endpoint 8 is lymphomas or leukemias in female mice.

METHODS

The cancer dose–response data from the two NTP studies (TDI in the NTP (1986) gavage study and TDA in the NTP (1979) feeding study) were reviewed. It was noted that the studies were different not only in their conduct and experimental dosing but also in what tumor information was collected.

The reported dose levels for the NTP (1986) gavage study of TDI are mg/kg for 5 days per week. These are converted herein to average mg/kg/day by multiplying the specified values by 5/7. The specified dose levels for the NTP(1979) study of TDA in feed are the time-weighted average (TWA) ppm in the diet. Because these average ppm levels were not converted to mg/kg/day in NTP (1979), we converted them using the USEPA default fraction of bodyweight consumed as food (0.05 and

Table 2. The estimated coefficients (α_0 , α_1 , α_2) in the fitted multistage dose–response model for TDA in the NTP (1979) study of TDA in feed.

Endpoint	Using Multistage Model for TDA with Unrestricted Parameters			Using Multistage Model for TDA with Restricted Parameters		
	α_0	α_1	α_2	α_0	α_1	α_2
1. Male Rat, Subcutaneous fibromas	0.05	0.10	−0.0059	0.071	0.054	0
2. Male Rat, Pancreatic acinar-cell adenomas	0.11	0.060	−0.0050	0.14	0.015	0
3. Male Rat, Hepatocellular carcinomas or neoplastic nodules	0	0.029	−0.00039	0	0.026	0
4. Female Rat, Subcutaneous fibromas	0	0.017	0.0011	0	0.017	0.0011
5. Female Rat, Mammary fibroadenomas	0.05	0.24	−0.017	0.076	0.12	0
6. Female Mouse, Hepatocellular carcinomas	0	0.031	−0.00045	0	0.021	0
7. Female Mouse, Circulatory-system hemanogiomias or hemangiosarcomas	0	0.015	−0.00047	0.0035	0.0044	0
8. Female Mouse, Lymphomas or leukemias	0.11	0.12	−0.0045	0.375	0	0

0.13 kg for rats and mice, respectively) (USEPA 1988). The conversion is ppm $\times$ (fraction of bodyweight consumed as food). There was a TDA dose reduction for rats after 40 weeks that was considered by the NTP in its calculation of the TWA TDA dose (Table 2; NTP 1979).

The NTP TDA report was accepted as written by the Data Evaluation/Risk Assessment Subgroup of the Clearinghouse on Environmental Carcinogens in 1978 (p 121 of NTP TDA Report) and was subsequently used to classify TDA as “reasonably anticipated to be a human carcinogen” by the NTP (2005) and as “possibly carcinogenic to humans (Group 2B),” based on sufficient evidence for carcinogenicity in experimental animals by IARC (1986). Similarly, the TDI study was judged of sufficient scientific merit for cancer classification purposes. Despite the differences between the TDI study and the earlier TDA study, it is possible to develop some measure of statistical comparability of the tumor results.

For each of the eight specified carcinogenic endpoints, the standard regulatory quantal dose–response model (the multistage model) was fit to the response frequencies in the TDA study using USEPA’s BenchMark Dose Software (BMDS) Version 2.1.2.60. (Initially, the quadratic multistage model is fit and discussed with unrestricted parameters; subsequently, the similar results obtained when the quadratic multistage model and the linear multistage model are used with the parameters restricted to be non-negative are discussed.) For each of the eight specified endpoints, the observed response frequencies at the two non-control doses in the TDI study were compared to estimated response frequencies in the TDA fitted multistage model. For each such comparison, the dose in the TDA fitted multistage that would have resulted in the TDI observed response frequency was determined.

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The quadratic multistage model had the following form:

Probability of the specified cancer response at dose d

$$= P(d) = 1 - \exp(-\alpha_0 - \alpha_1 \times d - \alpha_2 \times d^2)$$

The parameters (α_0 , α_1 , and α_2) in the multistage model were initially fit assuming that they were unrestricted and then subsequently assuming that they were restricted to be non-negative.

The statistical analyses focus on the observed carcinogenic effects for the eight endpoints listed in the Dose–Response Data section and the likelihood that the carcinogenic effects ascribed to TDI are the same as those that would be expected for TDA. Seven different statistical approaches are used.

The methodology in the first approach is illustrated for Endpoint 1 (subcutaneous fibromas in male rats) in Figure 1. The two panels in Figure 1 correspond to the multistage model parameters being unrestricted and then restricted, respectively. Shown in Figure 1 are the best fits of the multistage model to the dose–response data (Table 1) for TDA, and the question “If a fraction of the TDI dose was actually TDA, then what would those fractions have been in order to account for all of the observed responses ascribed to TDI?” is answered. Specifically, the fractions g_2 and g_3 are identified where

the fraction g_2 is such that the predicted probability of a response in the fitted dose

–response model for TDA at a dose of TDA equal to

$$\text{TDA dose } d_2 = g_2 \times \text{TDI dose } d_2$$

is equal to the observed response in the TDI data at TDI dose d_2

and

the fraction g_3 is such that the predicted probability of a response in the fitted dose

–response model for TDA at a dose of TDA equal to

$$\text{TDA dose } d_3 = g_3 \times \text{TDI dose } d_3$$

is equal to the observed response in the TDI data at TDI dose d_3 .

For Endpoint 1, the unrestricted multistage model fit to the following TDA dose–response data from the NTP (1979) study of TDA in the feed is

Dose (mg/kg/day)	# of Responses (subcutaneous fibromas in male rats)	# of Animals at Risk
$d_1 = 0.00$	1	20
$d_2 = 3.95$	15	50
$d_3 = 8.80$	19	50

$$P(d) = 1 - \exp(-\alpha_0 - \alpha_1 \times d - \alpha_2 \times d^2) = 1 - \exp(-0.05 - 0.10 \times d + 0.0059 \times d^2) \quad (1)$$

For Endpoint 1, the dose–response data from the NTP (1986) gavage study of TDI are

Multistage model (MS) with unrestricted parameters.

Multistage model (MS) with parameters restricted to be non-negative.

Figure 1. Example of the estimated fractions g_2 and g_3 for the low and high TDI doses, respectively, for animal endpoint #1 (subcutaneous fibromas in male rats). This comparison answers the question “If a fraction of the TDI dose was actually TDA, then what would those fractions have been in order to account for all of the observed responses ascribed to TDI?” (Color figure available online.)

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Dose (mg/kg/day)	# of Responses (subcutaneous fibromas in male rats)	# of Animals at Risk
$d_1 = 0.00$	3	50
$d_2 = 21.4$	3	50
$d_3 = 42.9$	9	50

If $g_2 = 0.005$ and $g_3 = 0.038$, then using Eq. (1) (*i.e.*, the unrestricted multistage model fitted to the TDA dose–response data)

$$\begin{aligned} P([d_2 = 21.4] \times [g_2 = 0.005]) &= 0.107 \\ &= 1 - \exp(-0.05 - 0.10 \times 0.107 + 0.0059 \times 0.107^2) = 0.06 = 3/50 \end{aligned}$$

which is the observed response frequency at the low dose in the TDI study and

$$\begin{aligned} P([d_3 = 42.9] \times [g_3 = 0.038]) &= 1.63 \\ &= 1 - \exp(-0.05 - 0.10 \times 1.63 + 0.0059 \times 1.63^2) = 0.18 = 9/50 \end{aligned}$$

which is the observed response frequency at the high dose in the TDI study.

Seven different approaches are included in the statistical analyses. Approach 1 is to estimate the fractions of TDI that were actually TDA separately for the low and high dose and separately for each of the eight endpoints (*i.e.*, estimate eight pairs g_2 and g_3). Approach 2 is to estimate a common fraction (g_{common}) for both the low and high doses and do this separately for each of the eight endpoints (*i.e.*, estimate eight endpoint-specific values of g_{common}). Approaches 1 and 2 are implemented first using unrestricted multistage models and then a second time using restricted multistage models. Approach 3 is to characterize the behavior of g_2 and g_3 in a Monte Carlo study varying the observed response frequencies in both the TDA and TDI studies. Approach 4 is to estimate a single overall value of g_{common} instead of eight endpoint-specific values of g_{common} . Approach 5 is to characterize the behavior of the overall g_{common} in a Monte Carlo study varying the observed response frequencies in both the TDA and TDI studies. Whereas Approaches 1 to 5 start with a multistage model fit to the response frequencies in the TDA study data and then subsequently consider the response frequencies in the TDI study, Approach 6 estimates the overall g_{common} , and the multistage models for both the TDA and TDI studies simultaneously. Approach 7 is to compare the slopes in the restricted linear multistage models for the TDA data to the slopes in the restricted linear multistage models for the TDI data.

Monte Carlo and bootstrap simulations were implemented in FORTRAN codes with some cross-checking and follow-up analyses done in Microsoft Office Excel 2007. Bootstrap simulations were nonparametric bootstraps using sampling with replacement.

RESULTS

The estimated coefficients ($\alpha_0, \alpha_1, \alpha_2$) in the fitted multistage dose–response models for the NTP (1979) study of TDA in feed that are used in Approaches 1, 2, and 4 are shown in Table 2 for all eight endpoints.

The estimated values for g_2 and g_3 for all eight endpoints using Approach 1 are shown in Table 3. Also shown in Table 3 are the eight endpoint-specific values in Approach 2 if a single fraction g_{common} is estimated instead of the two separate fractions g_2 and g_3 . In other words, in Table 3 is shown the fraction (g_{common}) such that, if the same fraction (g_{common}) of each TDI dose (*i.e.*, both the low TDI dose and the high TDI dose) were TDA, then that amount of TDA would be sufficient to explain the observed cancer responses in NTP (1986). The estimated value of g_{common} is obtained by using the multistage model fitted to the TDA dose–response data and then maximizing (with respect to g_{common}) the likelihood of the observed responses in the TDI (1986) study. The estimated values for g_2 , g_3 , and g_{common} in Table 3 are all relatively small and suggest that if only a small fraction (approximately 5%) of the TDI dose in the NTP (1986) gavage study of TDI were TDA, then that TDA would be sufficient to explain the observed cancer responses in that study. The average values of g_2 , g_3 , and g_{common} for these endpoints are all about 0.05 (Table 3).

Naturally, the frequencies of the responses in the NTP studies (1979; 1986) are random variables (that is, they vary about their underlying expected values). (This is a general property of all such experiments.) Hence, a Monte Carlo study was done in Approach 3 for each of the eight endpoints to see how sensitive the estimated values of g_2 and g_3 were with respect to this random variability in the response frequencies. For each of 100,000 Monte Carlo trials per endpoint, the response frequencies

Table 3. Answers to the question “If a fraction of the TDI dose was actually TDA, then what would those fractions have been in order to account for all of the observed responses ascribed to TDI?”

Endpoint	Using Multistage Model for TDA with Unrestricted Parameters			Using Multistage Model for TDA with Restricted Parameters		
	g_2	g_3	g_{common}	g_2	g_3	g_{common}
1. Male Rat, Subcutaneous fibromas	0.005	0.038	0.027	−0.0079	0.055	0.038
2. Male Rat, Pancreatic acinar-cell adenomas	−0.033	0.018	0.006	−0.23	0.022	−0.026
3. Male Rat, Hepatocellular carcinomas or neoplastic nodules	0.103	0.070	0.082	0.111	0.075	0.087
4. Female Rat, Subcutaneous fibromas	0.026	0.036	0.033	0.026	0.036	0.033
5. Female Rat, Mammary fibroadenomas	0.058	0.022	0.036	0.091	0.036	0.053
6. Female Mouse, Hepatocellular carcinomas	0.032	0.024	0.027	0.045	0.034	0.038
7. Female Mouse, Circulatory-system hemangiomas or hemangio-sarcomas	0.034	0.128	0.077	0.088	0.270	0.209
8. Female Mouse, Lymphomas or leukemias	0.063	0.028	0.040	— ¹	— ¹	— ¹
Average	0.037	0.046	0.041	0.015	0.066	0.054

¹The fitted multistage model with restricted parameters does not increase with dose.

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in the NTP (1979) and NTP (1986) studies were simulated using nonparametric bootstrap sampling with replacement that mimics the observed data closely but treats observed response frequencies of zero as fixed at zero. In each of these simulated trials, the values of g_2 and g_3 were estimated. In Figures 2 and 3 are shown the resulting distributions of 100,000 estimated g_2 and g_3 values, respectively. Numerical comparisons between estimated g_2 and g_3 values and average simulated values are provided in Table 4. In these figures the distributions for each of the endpoints are quite similar across endpoints. Also, most of the distribution is concentrated below approximately 0.10 (*i.e.*, $g_2 \leq 0.10$ and $g_3 \leq 0.10$). These figures support the conclusions drawn from Table 3; namely, that the values of g_2 and g_3 are small, and they are quite similar across endpoints.

Approach 4 is a potentially stronger statistical approach that can also be used to show that not only are the fractions (g_2 , g_3 , and g_{common}) the same for all doses at each endpoint, but also there is a single overall fraction across all eight endpoints. In Approach 4 the eight endpoint-specific estimates of g_{common} are replaced by a single overall value of g_{common} . The maximum likelihood estimate of this overall fraction is 0.037 in the unrestricted case and 0.051 in the restricted case, when a quadratic multistage model is used. Furthermore, the likelihood for all eight endpoints combined

Figure 2. Similarity of the frequency plots of 100,000 estimated fractions, g_2 , in 100,000 Monte Carlo bootstrap simulations comparing eight animal responses in the NTP studies from 1979 (TDA in feed) and 1986 (TDI via gavage). (Color figure available online.)

Figure 3. Similarity of the frequency plots of 100,000 estimated fractions, g_3 , in 100,000 Monte Carlo bootstrap simulations comparing eight animal responses in the NTP studies from 1979 (TDA in feed) and 1986 (TDI via gavage). (Color figure available online.)

evaluated using one overall fraction (0.037 in the unrestricted case and 0.051 in the restricted case) is not significantly different than the corresponding likelihood using eight pairs of g_2 and g_3 fractions even when the g_2 and g_3 within a pair can be different. That is, looking at all eight endpoints simultaneously and the corresponding sixteen binomial probabilities of the observed response frequencies at the two non-control doses in the TDI study, the likelihood ratio test with a p -value of .18 in the unrestricted case and .09 in the restricted case implies that, based on the response frequencies for the eight specified endpoints in the TDI study and the fitted dose–response relationships for these endpoints in the TDA study, the hypothesis of a common fraction of TDI being TDA cannot be rejected.

Approach 5 is a related bootstrap simulation that shows that a 95% confidence interval on the overall common fraction is [0.030, 0.045]. This simulation was done using the animal and all of its associated carcinogenic responses as the sampling unit. In the simulation there were 20 trials. In each trial, a bootstrap sample of the 50 animals put on test at each dose in the TDI study was determined, and each animal's observed response or non-response for each of the eight endpoints taken to be that for that animal in the TDI study. Then, the maximum likelihood estimate of the overall common fraction was determined for this simulated experimental outcome.

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Table 4. Monte Carlo corroboration of the answers to the question “If a fraction of the TDI dose was actually TDA, then what would those fractions have been in order to account for all of the observed responses ascribed to TDI?”: Monte Carlo estimates of the fraction g_2 of the low TDI dose and the fraction g_3 of the high TDI dose.

Endpoint	Estimates Based on Observed Data Set		Estimates from 100,000 Monte Carlo Simulated Data Sets	
	Estimated g_2	Estimated g_3	Average of 100,000 Simulated Estimates of g_2	Average of 100,000 Simulated Estimates of g_3
1. Male Rat, Subcutaneous fibromas	0.005	0.038	0.016	0.043
2. Male Rat, Pancreatic acinar-cell adenomas	-0.033	0.018	0.013	0.032
3. Male Rat, Hepatocellular carcinomas or neoplastic nodules	0.103	0.070	0.117	0.073
4. Female Rat, Subcutaneous fibromas	0.026	0.036	0.031	0.036
5. Female Rat, Mammary fibroadenomas	0.058	0.022	0.062	0.024
6. Female Mouse, Hepatocellular carcinomas	0.032	0.024	0.040	0.030
7. Female Mouse, Circulatory-system hemanogiosarcomas or hemangiosarcomas	0.034	0.128	0.054	0.055
8. Female Mouse, Lymphomas or leukemias	0.063	0.028	0.071	0.033
Average	0.046	0.046	0.051	0.041

The corresponding 20 maximum likelihood estimates of the overall common fraction (not including the original experiment with estimated common fraction being 0.037) ranged between 0.025 and 0.040, had mean and standard deviation equal to 0.033 and 0.0036, respectively, and a two-sided 95% confidence interval of [0.030, 0.045] based on a Student- t distribution with 19 degrees of freedom.

In Approaches 1 to 5, the multistage models have been fit to the TDA data, and then the values of g_2 , g_3 , and g_{common} estimated by relating the observed TDI response frequencies to those fitted TDA models. Approach 6 is possibly the strongest approach to estimating an overall common value for g_{common} (common over the low and high doses as well as common over the eight endpoints) is to fit the combined TDA and TDI response data simultaneously. In Approach 6 the fit to the TDA data is impacted by the TDI data and the proposed overall value of g_{common} . Here, the probability of an animal at dose d developing a response corresponding to the i -th endpoint ($i = 1, 2, \dots, 8$) in the j -th study ($j = 1$ for the TDA study and $j = 2$ for

the TDI study) is as follows:

$$1 - \exp(-\alpha_0(i, 1) - \alpha_1(i) \times d - \alpha_2(i) \times d^2) \text{ for the TDA study, and}$$

$$1 - \exp(-\alpha_0(i, 2) - \alpha_1(i) \times d \times g_{\text{common}} - \alpha_2(i) \times [d \times g_{\text{common}}]^2) \text{ for the TDI study}$$

In these probabilities, the intercepts $\alpha_0(i, 1)$ and $\alpha_0(i, 2)$ are allowed to differ between studies, but the shape parameters ($\alpha_1(i)$ and $\alpha_2(i)$) are required to be the same for both studies. That is, except for the background probabilities, the dose-response relationships for TDA and TDI differ only by g_{common} . There are 33 estimated parameters in this approach (16 α_0 's, 8 α_1 's, 8 α_2 's, and 1 g_{common}). Using the Solver function in Microsoft Excel, the maximum likelihood estimate of g_{common} in Approach 6 was 0.015 using a quadratic multistage model with unrestricted parameters, and the maximum likelihood estimate of g_{common} was 0.022 using a linear multistage model with restricted parameters. The estimates of g_{common} were similar using least squares instead of maximum likelihood and were fairly robust in limited sensitivity analyses.

Shown in Table 2 are the unrestricted estimated parameters (*i.e.*, not required to be non-negative) in the fitted quadratic multistage dose-response models. These are saturated models in the sense that have sufficient flexibility to pass exactly through the observed response frequencies at the three doses. Saturated models (rather than simpler linear multistage models) were used in conjunction with the graphical projection method of estimating the g 's to enable the fitted TDA models to represent the observed TDA data as closely as possible and minimize the impact on the estimated g 's of the choice of the model used to fit the TDA data. In Approach 7, instead of these unrestricted quadratic multistage dose-response models, USEPA's default multistage modeling approach is used (namely, a linear multistage model with restricted estimated coefficients). Here, the multistage models can be denoted by

$$\text{TDA : } P(d) = 1 - \exp(-\alpha_0 - \alpha_1 \times d) \text{ and}$$

$$\text{TDI : } P(d^*) = 1 - \exp(-\beta_0 - \beta_1 \times d^*)].$$

In order for these models to be approximately parallel (*i.e.*, $g \times d^*$ acts like d or, equivalently, d^* acts like d/g), it would be true that $\beta_1 \times d^* = \beta_1 \times d/g = (\beta_1/g) \times d$ or $\alpha_1 = \beta_1/g$ and $g = \beta_1/\alpha_1$. As shown in Table 5, the median (over the eight endpoints) of Approach 7's estimate of g (*i.e.*, the median of β_1/α_1) is approximately 0.05, which is similar to the other estimates of the relationship between the doses in the TDI and TDA studies.

DISCUSSION

In the NTP TDA study, the low and high doses for rats were lowered after 40 weeks due to excessive toxicity. Although the low dose rats were carried to term (103 weeks), the high dose male (79 weeks) and female (84 weeks) were terminated earlier due to excessive mortality. Thus, the cancer incidence for the male and female high dose rats might be biased low, which could result in a lower g fraction for five of the 16 cancer endpoints (g_2 and g_3) assessed. However, the five high-dose rat values (g_3) in question are comparable to the five low-dose values (g_2) for the

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Table 5. Estimated parameters and ratios in linear multistage models (with parameters restricted to be non-negative).

Endpoint	TDA Study		TDI Study		Ratio of Slopes: β_1/α_1
	α_0	α_1	β_0	β_1	
1. Male Rat, Subcutaneous fibromas	0.071	0.054	0.049	0.0030	0.056
2. Male Rat, Pancreatic acinar-cell adenomas	0.14	0.015	0.020	0.0028	0.187
3. Male Rat, Hepatocellular carcinomas or neoplastic nodules ¹	0	0.026	0.040	0.0010	0.038
4. Female Rat, Subcutaneous fibromas	0	0.024	0	0.00064	0.027
5. Female Rat, Mammary fibroadenomas	0.076	0.12	0.33	0.0012	0.010
6. Female Mouse, Hepatocellular carcinomas	0	0.021	0.037	0.00023	0.011
7. Female Mouse, Circulatory-system hemanogiomias or hemangiosarcomas	0.0035	0.0044	0	0.00097	0.220
8. Female Mouse, Lymphomas or leukemias	0.375	0	0.27	0.0011	infinite
Median					0.047

¹Slope for TDI study based on 6% and 8% observed response frequency at low and high dose, respectively, excluding the abnormally high (14%) observed response frequency in the controls.

same cancer endpoints as well as the five high- and low-dose values (g_2 and g_3) for different cancer endpoints in mice (Table 3). Thus, although the chronic exposure to high-dose rats was $\sim 20\%$ shorter than desired, it does not dramatically affect the conclusion that conversion of a small fraction of the TDI dose to TDA provides a consistent explanation for the NTP TDI results across multiple cancer endpoints.

For each tumor type, there is binomial variability in the number of tumors observed at a dose. Thus, the *estimated* dose–response relationship is different for each repetition of a study. Therefore, any comparison between estimated dose–response relationships in two studies is expected to vary for different repetitions of the studies. As an illustration of this variability, a Monte Carlo simulation of a comparison between two studies was done. Both studies had three doses (including the control), 50 animals per dose, and the same underlying linear multistage dose–response relationship. The comparison (the ratio equal to the estimated slope in the second study divided by the estimated slope in the first study) was computed for 1000 simulated pairs of study outcomes. Although the median of the ratio was near 1 (as expected), the distribution of ratios was spread out over a substantial range. For example, the 25th and 75th percentiles of the ratio were 0.80 and 1.29 when the expected response frequencies in the underlying dose–response relationship were approximately 5%, 22%, and 36% at the three doses, respectively, (a substantial increase with dose). Analogously, the 25th and 75th percentiles of the ratio were 0.25 and 76,000 when the expected response frequencies in the underlying dose–response

relationship were approximately 5%, 7%, and 9% at the three doses, respectively (a smaller increase with dose). Therefore, some variability in comparisons between the same endpoint in two studies is expected. That is, it would be unrealistic to expect that the estimated g 's in the analyses herein to not vary somewhat from endpoint to endpoint.

The above simulations were repeated for the situation where the doses in the second study were only 5% of the doses in the first study. In these simulations, the ratios were understandably much greater. For example, when the expected response frequencies in the underlying dose-response relationship were approximately 5%, 22%, and 36% at the three doses in the first study, respectively, (a substantial increase with dose), they were only slightly greater than 5% at the two highest doses in the second study (a very slight increase with dose). Then, the ratio (estimated slope in the second study divided by the estimated slope in the first study) had 50th, 55th, 60th, 65th, 70th, and 75th percentiles of 5, 8, 11, 20, 33, and 23,000 respectively. Thus, finding one response (response #7, circulatory-system hemangiomas or hemangiosarcomas in female mice) with estimated g 's slightly larger than the other responses is not unexpected. Nor is it unexpected to find one response (response #2, pancreatic acinar-cell adenomas in male rats) where a non-monotonic TDI-induced tumor response is observed.

CONCLUSION

The average values of g_2 , g_3 , and g_{common} all approximate 0.05 using either unrestricted or restricted multistage models in Approaches 1 and 2. Related Monte Carlo simulations in Approach 3 suggested values of g_2 and g_3 less than 0.10. The estimates of an overall common fraction were 0.037 and 0.051 using unrestricted and restricted models, respectively, in Approach 4 with a related bootstrap simulation in Approach 5 resulting in a 95% confidence interval on the overall common fraction of [0.030, 0.045]. Estimating the multistage models for TDA and TDI and the overall common fraction simultaneously in Approach 6 resulted in estimated values for the overall common fraction less than 0.025. Finally, in Approach 7, the ratio of the slopes in the estimated restricted linear multistage models for TDA and TDI had a median of 0.047. The consistency of these statistical results across the eight tumor endpoints and the seven different statistical approaches strongly supports the robust conclusion that if only a small fraction (approximately 5%) of the supposed TDI dose in the NTP (1986) gavage study were actually TDA, then that TDA would be sufficient to explain the observed cancer responses in the TDI study.

Our results and those of others reported here suggest that TDI under typical exposure conditions is not carcinogenic. TDI produces tumors only under atypical exposure conditions (*e.g.*, gavage) that results in the formation of TDA. While a detailed mode of action (MOA) evaluation is beyond the scope of this article, available information meets many of the modified Hill criteria (USEPA 2005). As discussed earlier, the conversion of a small fraction of the administered TDI dose to TDA both prior to and after gavage administration into the acidic environment of the stomach is biologically and chemically plausible and consistent with the observations that free TDA can be detected systemically after tumorigenic doses of TDI administered via

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gavage (Timchalk *et al.* 1994) but not after non-tumorigenic, biologically relevant exposures (*i.e.*, inhalation and dermal contact) in animals (Löser 1983; Timchalk *et al.* 1994; Rosenberg and Savolainen 1985) and humans (Sennbro *et al.* 2003; Skarping *et al.* 1994). In addition, our statistical comparison of the tumors caused by oral exposures to TDI (NTP 1986) and TDA (NTP 1979) show a strong, consistent, and specific dose–response relationship across multiple tumor endpoints, species, and sexes.

The fact that both 2,4- and 2,6-TDI (Zeiger *et al.* 1987), like both 2,4- and 2,6-TDA (Cheung *et al.* 1996), are mutagenic in the Ames assay is seemingly inconsistent with the possibility that free TDA plays a central role in the tumorigenic MOA seen after gavage exposures to commercial grade TDI. However, this apparent inconsistency can be reconciled by the observations that TDI rapidly forms TDA within minutes when TDI is dissolved in solvents with the low residual water levels (*e.g.*, 0.04%) or within seconds when TDI is introduced into the aqueous Ames test system (Seel *et al.* 1999). This rapid formation of TDA from TDI in conventional *in vitro* mutagenicity assays is consistent with the observations that the *in vitro* mutagenicity of TDA and TDI isomers requires metabolic activation, apparently by a cytochrome P4501A mediated process (Cheung *et al.* 1996). Another seeming anomaly is that, in contrast to *in vitro* mutagenicity results, only 2,4-TDA is tumorigenic in rodents (NTP 1979); 2,6-TDA is not (NTP 1980). This discrepancy may be explained by the observations that only the 2,4-TDA isomer binds to the cytosolic Ah receptor, resulting in CYP1A activation, the subsequent metabolic activation of 2,4-TDA, and the eventual promotion of mutated cells via enhanced cellular proliferation (Cheung *et al.* 1996; Cunningham *et al.* 1991). By itself, the 2,6-TDA isomer is insufficient for these purposes, although it may participate in the tumorigenic response in the presence of an Ah receptor agonist. While the contribution of other forms of TDA (*e.g.*, conjugates) to this process *in vivo* cannot be excluded, available data demonstrate that free 2,4-TDA likely plays a central role in the carcinogenic response to 2,4-TDA, possibly via a non-genotoxic mechanism.

Our statistical findings combined with apparently central role played by TDA in the tumorigenic response to atypical TDI exposures may impact the carcinogenic classification of TDI by the NTP and IARC since TDI is not carcinogenic under physiological exposure conditions (Löser 1983) where little if any TDA is formed (Timchalk *et al.* 1994).

ACKNOWLEDGMENTS

This work was sponsored by the International Isocyanate Institute. The conclusions are those of the authors.

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