Review Article

Journal of Applied Toxicology

Received: 28 November 2011, Accepted: 23 December 2011 Published online in Wiley Online Library: 15 February 2012

(wileyonlinelibrary.com) DOI 10.1002/jat.2721

The genetic toxicity of methylphenidate: a review of the current literature†

Suzanne M. Morris,[a]* Dayton M. Petibone,[a] Wei-Jiun Lin,[b] James J. Chen,[b] Benedetto Vitiello,[c] Kristine L. Witt[d] and Donald R. Mattison[e]

ABSTRACT: Attention deficit/hyperactivity disorder (ADHD), a common children's behavioral disorder, is characterized by inattention, hyperactivity and impulsivity. The disorder is thought to stem from abnormalities in the catecholamine pathway and the symptoms of the disorder have been successfully treated with methylphenidate (MPH) since the FDA approved the drug in the 1950s. MPH underwent the appropriate safety testing as part of the FDA approval process; however, a publication in 2005 that reported significant increases in cytogenetic damage in the lymphocytes of MPH-treated pediatric patients caused concern for patients and their families, the pharmaceutical industry and regulatory agencies. This communication will review the many studies that were subsequently initiated worldwide to address the genetic safety of MPH in both animal models and human subjects. Animal experiments broadened the study protocols used in the 2005 investigation to include a wider dose-range, a longer treatment period and automated scoring of biological endpoints, where possible, to reduce observer bias. The human subject studies replicated the experimental design used in the 2005 study, but increased the treatment periods and the sizes of the study populations. Neither the laboratory animal nor human subject studies found an increase in any of the measures of genetic damage that were evaluated. Taken together, these new studies are consistent with the original safety evaluation of the FDA and do not support the hypothesis that MPH treatment increases the risk of genetic damage in ADHD patients. Published 2012. This article is a US Government work and is in the public domain in the USA.

Keywords: methylphenidate; ADHD; chromosome damage; mutation

INTRODUCTION

Attention deficit/hyperactivity disorder (ADHD) is one of the most common behavioral disorders in the pediatric population. Incidence rates in the USA range from 7 to 16%, depending upon the criteria used for diagnosis (Faraone *et al*., 2003). The symptoms of the disorder are highly variable from patient to patient and the diagnosis of ADHD relies on the standards set forth by the American Psychiatric Association (APA). The APA scale is presented in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edn (American Psychiatric Association, 2000), and the diagnosis of AHDH is determined by assessment of 18 behavioral symptoms (reviewed in Aguiar *et al*., 2010).

Symptoms of ADHD include inattention, hyperactivity and impulsivity, all of which are related to abnormalities in the neurochemistry of the catecholamines dopamine and norepinephrine in the prefrontal cortex and the striatum. One of the most common treatments for symptom control is the use of methylphenidate hydrochloride (MPH) to improve attention and concentration (reviewed in Tripp and Wickens, 2009). Although the neurobiochemistry of MPH action is well studied, the mechanism by which MPH ameliorates the symptoms of ADHD remains an active area of investigation. Evidence from positron emission tomography (PET) imaging indicates that MPH occupies the dopamine transporter (DAT), which blocks the reuptake of dopamine and the activation of the D1 receptor (Spencer *et al*., 2006). More recently, PET studies have demonstrated that MPH also occupies the norepinephrine transporter (NET) with a higher affinity than the DAT (Hannestad *et al*., 2010). Thus, the interaction of MPH with the DAT and the NET may contribute to the alleviation of the symptoms of ADHD.

MPH was approved for use in the treatment of ADHD in the 1950s, at which time it underwent the appropriate genetic safety testing required for approval by the US FDA. More recently, MPH underwent a thorough evaluation for carcinogenicity, genetic toxicity and reproductive toxicity by the National Toxicology Program (1995). In the assays conducted by the National Toxicology Program (NTP), negative results were found in the Ames assay (Mortelmans *et al*., 1986; National Toxicology Program, 1995), suggesting that MPH was not a mutagen. The results of two independent assays measuring induction of sister

* *Correspondence to: Suzanne M. Morris, Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, US FDA, 3900 NCTR Road, Jefferson, AR 72079, USA. E-mail: suzanne.morris@fda.hhs.gov*

† *The work presented here reflects the opinions of the authors and does not necessarily reflect the opinions or the policies of the US FDA or the US NIH.*

[a] *Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, US FDA, Jefferson, AR, USA*

[b] *Division of Personalized Nutrition and Medicine, National Center for Toxicological Research, US FDA, Jefferson, AR, USA*

[c] *Child and Adolescent Treatment and Prevention Branch, National Institute of Mental Health, NIH, Bethesda, MD, USA*

[d] *Division of the National Toxicology Program, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA*

[e] *Epidemiology Branch, Division of Epidemiology, Statistics, and Prevention Research, Eunice Kennedy Shriver National Institute for Child Health and Human Development, NIH, Bethesda, MD, USA*

Published 2012. This article is a US Government work and is in the public domain in the USA.

Journal of Applied Toxicology

chromatid exchanges (SCE) in cultured Chinese hamster ovary cells were inconsistent, with a small increase in the frequency of SCEs observed in one laboratory after treatment with MPH but not in a second laboratory (National Toxicology Program, 1995); however, results from two independent *in vitro* chromosomal aberrations assays were positive, although excessive cytotoxicity may have confounded the interpretation of the results (Galloway *et al*., 1987; National Toxicology Program, 1995). In contrast to the positive results seen in the *in vitro* cytogenetic tests, no increases in micronucleated erythrocytes (biomarkers of chromosomal damage) were seen in peripheral blood samples of male or female transgenic mice administered MPH via dosed feed for 24 weeks (National Toxicology Program, 1995).

Results of a 2-year rodent carcinogenicity study conducted by the NTP with MPH showed no evidence of carcinogenicity in rats, but a significant increase in hepatocellular neoplasms was seen in male and female B6C3F1 mice exposed to 500 ppm MPH via dosed feed (National Toxicology Program, 1995). Because of the negative results from the Ames and the micronucleus (MN) assays, and the questionable results in the *in vitro* cytogenetics tests with MPH, the NTP concluded that the increase in mouse liver tumors was probably due to altered cell proliferation rather than direct genetic damage (Dunnick and Hailey, 1995). This proposed nongenotoxic mode of action for MPH in the mouse bioassay was consistent with the negative results in bone marrow MN assays reported by Teo *et al*. (2003) and Suter *et al*. (2006). Additional support for the nongenotoxic mode of action for MPH came from the negative results seen in a 6-month bioassay using transgenic TSG™ $p53^{+/-}$ mice, a strain that is presumed to be responsive to both *in vivo* mutagens and clastogens (Storer *et al*., 2001).

In 2005, the nongenotoxicity of MPH was challenged by a publication by El-Zein *et al*. (2005) that raised the first serious concerns about the genetic safety of MPH. In this study, significant increases in the frequencies of chromosomal aberrations, SCEs and MN in peripheral blood lymphocytes were reported in each of 12 pediatric ADHD patients following three months of treatment with MPH. These findings, which were widely reported, caused great concern among ADHD patients treated with MPH, their families, the pharmaceutical industry and US regulatory agencies and other government agencies. Thus, in swift response to the El-Zein *et al*. publication, a working group was established under the direction of the US National Institutes of Health (National Institute of Child Health & Human Development (NICHD), National Institute of Mental Health (NIMH) and National Institute of Environmental Health Sciences NIEHS)) and the US Food and Drug Administration to review the study and develop a framework for additional studies designed to confirm or refute the published findings. After conducting a detailed investigation and a thorough re-evaluation of the data from the El-Zein *et al*. study that revealed several short-comings, the working group recommended specific additional studies (Preston *et al*., 2005; Jacobson-Kram *et al*., 2008), including a repeat of the original El-Zein study, but using a larger, well-characterized study population and more detailed cytogenetics evaluations. The working group was particularly troubled to learn that, for six of the 12 patients in the El-Zein study, an evaluation of 50 cells per subject revealed no SCEs. This was an unprecedented finding owing to the SCE-inducing nature of the DNA analog used in this assay to differentially label the sister chromatids (Pinkel *et al*., 1985; Tucker *et al*., 1986). Other areas of concern prompting the recommendation to repeat and expand the El-Zein *et al*. study were the small study population (12 children), the lack of a concurrent untreated control group and the critical lack of detail with regards to the methods used in blood sample processing and slide scoring (Preston *et al*., 2005; Jacobson-Kram *et al*., 2008). Other proposed modifications to the study designs that were considered included extending the treatment time and including intermediate sampling times and appropriate control groups (non-ADHD patients, age- and sex-matched ADHD patients not undergoing pharmacological intervention).

Within a year of the El-Zein *et al*. (2005) report, numerous studies were initiated to further investigate the genotoxicity of MPH. These follow-up studies were sponsored both by regulatory agencies and by the pharmaceutical industry, conducted in human subjects as well as animal models, and designed to address the specific criticisms of the El-Zein study. The purpose of this communication is to review the studies undertaken since 2005, and to provide a framework for determining if MPH has mutagenic or clastogenic properties.

STUDIES IN HUMAN SUBJECTS

Five human subject studies have been conducted since 2005 (Table 1), each one carefully designed to replicate or extend the original observations and satisfy the recommendations of Preston *et al*. (2005) and Jacobson-Kram *et al*. (2008). In the studies of Walitza *et al*. (2007, 2009), MN frequency, as determined by the microscopic analysis of binucleated cultured human lymphocyte preparations, was measured prior to the initiation of MPH treatment, and then after 1, 3, 6 and 12 months of treatment. MPH doses were within the accepted therapeutic range (5–60 mg per day) and all study subjects (age range of 5–17 years; $n = 30$) were diagnosed with ADHD according to the clinical criteria of *DSM IV* (American Psychiatric Association, 2000). Statistical analyses of MN frequencies were conducted at each time point and no significant increases in the MN frequencies of MPH-treated ADHD patients compared with nontreated ADHD patients were detected at any time point. Of interest was their observation that the MN frequency in nontreated ADHD patients was significantly higher than in nontreated non-ADHD patients. In the study reported by Ponsa *et al*. (2009), both children ($n = 12$) and adult ($n = 7$) patients (diagnosed with ADHD according to the *DSM IV* criteria) undergoing an MPH treatment regimen were observed for potential genotoxic effects. Patients were treated with either immediate or extended-release MPH at therapeutic levels for 3 months and the frequencies of MN, SCE and CA were determined in peripheral lymphocytes in each study subject. No significant effect of MPH treatment on any of the three endpoints was detected. Similarly, in a study sponsored by the US NIH, 25 children, diagnosed with ADHD according to *DSM IV* criteria and ranging in age from 6 to 12 years, were evaluated for the frequencies of MN, SCE and CA in peripheral blood lymphocytes before and after three months of treatment with normal therapeutic doses of MPH (18–54 mg per day at the end of 3 months of titration; Witt *et al*., 2008). No MPH-related increases in any of the three endpoints were detected. In the final human subjects study conducted in direct response to the El-Zein *et al*. (2005) study, MN, SCE and CA frequencies were measured in 29 children diagnosed with ADHD according to the *DSM IV* criteria; after 3 months of MPH treatment, no significant increases in any of the three endpoints were observed (Tucker *et al*., 2009). Each of the five human studies described above utilized a design similar to that employed by El-Zein *et al*. (pre-treatment assessment of MN, SCE and CA frequencies followed by re-evaluation of all

Published 2012. This article is a US Government work and is in the public domain in the USA.

Table 1. Human subject studies in which the frequency of chromosome aberrations (CA), micronuclei (MN) and sister-chromatid exchanges (SCE) in methylphenidate (MPH)-treated pediatric patients was determined in peripheral blood cells

Age (and number) of test subject	Dose	Time on Dose	ADHD	Endpoint	Outcome	Reference
>7 years (12)	20–54 mg per day	3 months	Yes	CA	Positive	El-Zein *et al.* (2005)
>7 years (12)	20–54 mg per day	3 months	Yes	MN	Positive	El-Zein *et al.* (2005)
>7 years (12)	20–54 mg per day	3 months	Yes	SCE	Positive	El-Zein *et al.* (2005)
7–14 years (12)	15–45 mg per day	3 months	Yes	CA	Negative	Ponsa *et al.* (2009)
21–58 years (7)	36–90 mg per day	3 months	Yes	CA	Negative	Ponsa *et al.* (2009)
7–14 years (12)	15–45 mg per day	3 months	Yes	MN	Negative	Ponsa *et al.* (2009)
21–58 years (7)	36–90 mg per day	3 months	Yes	MN	Negative	Ponsa *et al.* (2009)
7–14 years (12)	15–45 mg per day	3 months	Yes	SCE	Negative	Ponsa *et al.* (2009)
21–58 years (7)	36–90 mg per day	3 months	Yes	SCE	Negative	Ponsa *et al.* (2009)
4.9–17 years (30)	5–40 mg per day	1 month	Yes	MN	Negative	Walitza *et al.* (2007)
4.9–17 years (21)	15–45 mg per day	3 months	Yes	MN	Negative	Walitza *et al.* (2007)
4.9–17 years (8)	15–45 mg per day	6 months	Yes	MN	Negative	Walitza *et al.* (2007)
7.1–16 years (9)	15–60 mg per day	6–24 months	Yes	MN	Negative	Walitza *et al.* (2007)
7–17 years (23)	Control	NA	No	MN	NA	Walitza *et al.* (2009)
5–16 years (26)	Drug-naive, ADHD vs non-ADHD	NA	Yes	MN	Positive	Walitza *et al.* (2009)
7–11 years (17)	0.21–0.78 mg kg^{-1} b.w. per day	3 months	Yes	MN	negative	Walitza *et al.* (2009)
7–12 years (11)	0.22–1.35 mg kg^{-1} b.w. per day	6 months	Yes	MN	negative	Walitza *et al.* (2009)
9–16 years (21)	0.35–1.64 mg kg^{-1} b.w. per day	Chronic (12 months)	Yes	MN	negative	Walitza *et al.* (2009)
6–12 years (25)	Clinical titration to 0.33–2.78 mg kg^{-1} per day	3 months	Yes	CA	Negative	Witt *et al.* (2008)
6–12 years (25)	Clinical titration to 0.33–2.78 mg kg^{-1} per day	3 months	Yes	MN	Negative	Witt *et al.* (2008)
6–12 years (25)	Clinical titration to 0.33–2.78 mg kg^{-1} per day	3 months	Yes	SCE	Negative	Witt *et al.* (2008)
6–12 years (33)	10–60 mg per day	3 months	Yes	CA	Negative	Tucker *et al.* (2009)
6–12 years (34)	10–60 mg per day	3 months	Yes	MN	Negative	Tucker *et al.* (2009)
6–12 years (29)	10–60 mg per day	3 months	Yes	SCE	Negative	Tucker *et al.* (2009)

b.w., Body weight.

Published 2012. This article is a US Government work and is in the public domain in the USA.

endpoints after a 3-month treatment period with MPH, and utilization of the *DSM IV* criteria for ADHD diagnosis), but with the exception that more subjects were enrolled. None of the repeat studies was able to replicate the results of El-Zein *et al*. (2005), an outcome that does not support the hypothesis that MPH induces chromosomal damage in the lymphocytes of children with ADHD.

STUDIES IN ANIMAL MODELS

The animal models utilized in genetic toxicity studies with MPH included the nonhuman primate (NHP), the Wistar rat and the B6C3F1 mouse. A wider dose range of MPH was tested in these models than in the human subject studies, and the genetic toxicity measurements were evaluated in both peripheral blood and the target tissues of liver and brain (mouse and rat).

Micronucleus Assays

The MN assay was independently employed by multiple groups to test for the ability of MPH to induce chromosomal aberrations, either numerical or structural, in mice, rats, and NHPs (Table 2). Andreazza *et al*. (2007) measured MN frequencies in 25- and 60-day-old Wistar rats. The evaluation was performed by Giemsa staining of binucleated peripheral blood lymphocytes after 28 days of treatment with MPH administered by intraperitoneal injection at doses of 1, 2 or 10 mg kg^{-1}. No significant increases in MN frequencies were observed in these experiments. As follow-up to the Andreazza *et al*. study, MN frequencies in circulating reticulocytes were measured using flow cytometry (FCM) in MPH-treated Wistar rats by Witt *et al*. (2010). In this study, 60-day-old male rats were administered 2, 10 or 25 mg kg^{-1} MPH by oral gavage for 28 days and blood samples were obtained 4 h after the final dosing. The FCM methodology has the advantages of high-throughput, automated nonsubjective scoring, and direct analysis of the cells of interest, eliminating any confounding factors that may be associated with lymphocyte culture. No increases in reticulocyte MN frequencies were detected by Witt *et al*. (2010) utilizing this approach.

In the study of Morris *et al*. (2009), MN frequency was determined by FCM enumeration of micronucleated reticulocytes obtained monthly from NHP over a 20-month treatment period with MPH. Two MPH doses were employed: 5 mg kg^{-1} per day, which resulted in plasma levels approximating the therapeutic levels aimed for in pediatric patients, and 25 mg kg^{-1} per day, which gave a 10-fold increase in the pediatric-equivalent plasma levels of MPH. No significant increases in MN frequencies were detected, either at the end of the 20-month exposure period or at any of the intermediate time points. The MN studies of Manjanatha *et al*. (2008, 2009) were also conducted using the FCM high-throughput methodology and measured MN frequency in peripheral erythrocytes of B6C3F1 mice over a wide dose range and exposure time. In these studies, mice were exposed to concentrations of 50 to 4000 ppm MPH in the diet for up to 6 months. This experimental design was chosen to replicate the NTP rodent carcinogenicity studies (National Toxicology Program, 1995). No increases in MN frequencies were detected at any time point or at any dose level in these experiments (Manjanatha *et al*., 2008, 2009).

Comet Assay

The Comet assay detects a variety of DNA damage including both single- and double-strand DNA breaks in single cells and thus, is considered a biomarker of DNA damage. Two independent studies were undertaken to determine if MPH-induced DNA damage could be detected by the Comet assay in blood and brain tissues from Wistar rats (Table 3). In the study of Andreazza *et al*. (2007), an increase in cells exhibiting DNA migration was reported in peripheral blood leukocytes and cells of the hippocampus and the striatum of young (28-day-old) and mature (60-day-old) Wistar rats administered 1, 2 or 10 mg kg^{-1} MPH by intraperitoneal injection. Significant increases in DNA damage were found after acute and 28-day exposures in both age groups, but the effect was more consistent and pronounced in the older rats. When this study was repeated using doses of 2, 10 and 25 mg kg^{-1} MPH administered by gavage for 28 days to mature (60-day-old) rats by Witt *et al*. (2010), the positive results could not be confirmed. In the latter study, not only was the Comet assay negative in peripheral blood leukocytes, and the hippocampus, striatum, and frontal lobe of the brain, it was also negative in the liver which was the target organ for MPH-induced tumors in the NTP carcinogenicity study in mice. As discussed by Witt *et al*., the studies of Andreazza *et al*. (2007) were conducted using microscopic evaluation, rather than the automated, image analysis approach used by Witt *et al*. (2010). The use of the automated system may have resulted in a lower error rate than the microscopic analysis.

Mutation Studies

Although the concerns raised by the El-Zein *et al*. (2005) study were focused on the clastogenicity of MPH, and reassuringly, these effects were not replicated in numerous follow-up studies, reports of the formation of 8-hydroxyguanosine (8-OH-dG) as a result of MPH metabolism in the brain raised additional concerns about MPH mutagenicity (Martins *et al*., 2006; Gomes *et al*., 2008). The formation of 8-OH-dG in the brain is linked to the formation of oxidative lesions and single-strand breaks (Bagnyukova *et al*., 2008). 8-OH-dG is considered a promutagenic lesion and, when Big Blue/*Ogg1*$^{-/-}$ mice were exposed to low levels of ionizing radiation, an increased frequency of G:C → T:A transversion mutations was induced (Larsen *et al*., 2006). Thus, several studies designed to investigate the mutagenic potential of MPH were undertaken in both target and surrogate tissue from humans, mice, and NHP (Table 4), as well as cell culture models.

In one of the initial studies, Chovanova *et al*. (2006) noted an increase in DNA damage in peripheral lymphocytes by the Comet assay (modified to detect oxidative damage such as 8-OH-dG) in untreated ADHD patients compared with non-ADHD patients. In cell culture studies, Schmidt *et al*. (2010a) found that exposure of human SK neuroblastoma cells to MPH resulted in a decreased expression of *hOGG-1*, the glycoslyase that repairs 8-OH-dG (Larsen *et al*., 2006). Schmidt *et al*. (2010b) treated human neuroblastoma cells with MPH and found reduced levels of 8-OH-dG formation. When interpreted in light of the correlation between hOGG-1 activity and levels of oxidative DNA damage formation (Larsen *et al*., 2006; Liu *et al*., 2011; Wong *et al*., 2008) in brain tissue, Schmidt *et al*. (2010b) suggested that MPH might exert a protective effect on the cells by reducing 8-OH-dG levels. This concept is also supported by additional experiments in which cell survival increased in these cells after exposure to MPH (Schmidt *et al*., 2010b). That MPH does not increase 8-OH-dG formation is also indicated by the studies of Walitza *et al*. (2010) in which urinary 8-OH-dG level was not affected by MPH in the ADHD patients after three months of treatment.

Published 2012. This article is a US Government work and is in the public domain in the USA.

Table 2. Effect of acute and chronic exposure to methylphenidate (MPH) on the micronucleus (MN) frequency in peripheral blood cells of animal models.

Species	Strain	Age	Dose	Time point	Route	Tissue/method	Results	Reference
Nonhuman primate	Indian Origin	2–4 years	2.5, 12.5 mg kg^{-1} twice per day	M0–M20	Oral dosing solution	MN-Ret/Flow	Negative	Morris *et al* (2009)
Rat	Wistar	25 days	1, 2, 10 mg kg^{-1}	Acute	i.p.	Binucleated PBL (Giemsa)	Negative	Andreazza *et al.* (2007)
Rat	Wistar	60 days	1, 2, 10 mg kg^{-1}	Acute	i.p	Binucleated PBL (Giemsa)	Negative	Andreazza *et al.* (2007)
Rat	Wistar	25 days	1, 2, 10 mg kg^{-1}	28 days	i.p.	Binucleated PBL (Giemsa)	Negative	Andreazza *et al.* (2007)
Rat	Wistar	60 days	1, 2, 10 mg kg^{-1}	28 days	i.p.	Binucleated PBL (Giemsa)	Negative	Andreazza *et al.* (2007)
Rat	Wistar	60 days	2, 10, 25 mg kg^{-1}	28 days	Gavage	MN-Ret/Flow	Negative	Witt *et al.* (2010)
Rat	Sprague–Dawley	29 days	3 mg kg^{-1} per day for 21 days	29 days	Oral	MN-Ret/Flow	Negative	Dobrovolsky *et al.* (2010)
Rat	Sprague–Dawley	29 days	3 mg kg^{-1} per day for 21 days	50 days	Oral	MN-Ret/Flow	Negative	Dobrovolsky *et al* (2010)
Rat	Sprague–Dawley	29 days	3 mg kg^{-1} per day for 21 days	90 days	Oral	MN-Ret/Flow	Negative	Dobrovolsky *et al* (2010)
Mouse	B6C3F1	Young	62.5, 125, 250 mg kg^{-1} b.w.	16 h	Gavage	MN-PCE/ (Giemsa)	Negative	Suter *et al.* (2006)
Mouse	B6C3F1	Young	62.5, 125, 250 mg kg^{-1} b.w.	48 h	Gavage	MN-PCE/ (Giemsa)	Negative	Suter *et al.* (2006)
Mouse	B6C3F1	6 weeks	250, 500, 1000, 2000, 4000 ppm	28 days	Dietary	MN-Ret/Flow	Negative	Manjanatha *et al.* (2008)
Mouse	B6C3F1	6 weeks	50, 150, 250, 500, 1000, 1500, 2000, 4000 ppm	4 and 12 weeks (0 and 4000 ppm); 24 weeks (all doses)	Dietary	MN-Ret/Flow	Negative	Manjanatha *et al.* (2009)

Body weight (b.w.). Parts per million (ppm), peripheral blood lymphocytes (PBL), micronucleated-reticulocyte (MN-RET), polychomatic erythrocyte (PCE).

Published 2012. This article is a US Government work and is in the public domain in the USA.

Table 3. Animal studies in which the level of DNA damage induced by methylphenidate (MPH) treatment was determined in the peripheral blood cells and brain tissue of Wistar rats. Molecular weight (MW).

Species	Strain	Age (days)	Dose	Time point	Route	Tissue	Assay/method	Results	Reference
Rat	Wistar	25	1, 2, 10 mg kg^{-1}	Acute	i.p.	Hippocampus/striatum	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	60	1, 2, 10 mg kg^{-1}	Acute	i.p	Hippocampus/striatum	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	25	1, 2, 10 mg kg^{-1}	28 days	i.p.	Hippocampus/striatum	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	60	1, 2, 10 mg kg^{-1}	28 days	i.p.	Hippocampus/striatum	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	25	1, 2, 10 mg kg^{-1}	Acute	i.p.	Peripheral blood	Comet/microscopy	Negative	Andreazza *et al.* (2007)
Rat	Wistar	60	1, 2, 10 mg kg^{-1}	Acute	i.p	Peripheral blood	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	25	1, 2, 10 mg kg^{-1}	28 days	i.p.	Peripheral blood	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	60	1, 2, 10 mg kg^{-1}	28 days	i.p.	Peripheral blood	Comet/microscopy	Positive	Andreazza *et al.* (2007)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Peripheral blood	Comet/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Liver	Comet/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Frontal cortex	Comet/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Hippocampus	Comet/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Striatum	Comet/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Peripheral blood	Low MW DNA/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Liver	Low MW DNA/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Frontal cortex	Low MW DNA/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Hippocampus	Low MW DNA/automated	Negative	Witt *et al.* (2010)
Rat	Wistar	60	2, 10, 25 mg kg^{-1}	28 days	Gavage	Striatum	Low MW DNA/automated	Negative	Witt *et al.* (2010)

Published 2012. This article is a US Government work and is in the public domain in the USA.

Table 4. Effect of chronic MPH treatment on the *cII* and *Hprt* mutant frequency in B6C3F1 mice, the *HPRT* and *PIG-A* mutant frequency in nonhuman primates, and the *Pig-A* mutant frequency in Sprague–Dawley rats

Species	Age	Dose	Time point	Route	Tissue	Locus	Results	Reference
Mouse B6C3F1	6 weeks	250, 500, 1000, 2000, 4000 ppm	28 days	Dietary	Spleen lymphocyte	*Hprt*	Negative	Manjanatha *et al.* (2008)
Mouse B6C3F1	6 weeks	4000 ppm	4 weeks	Dietary	Liver	*cII*	Negative	Manjanatha *et al.* (2009)
Mouse B6C3F1	6 weeks	4000 ppm	12 weeks	Dietary	Liver	*cII*	Negative	Manjanatha *et al.* (2009)
Mouse B6C3F1	6 weeks	50, 150, 250, 500, 1000, 1500, 2000, 4000 ppm	24 weeks	Dietary	Liver	*cII*	Negative	Manjanatha *et al.* (2009)
Mouse B6C3F1	6 weeks	4000 ppm	4 weeks	Dietary	Spleen lymphocytes	*Hprt*	Negative	Manjanatha *et al.* (2009)
Mouse B6C3F1	6 weeks	4000 ppm	12 weeks	Dietary	Spleen lymphocytes	*Hprt*	Negative	Manjanatha *et al.* (2009)
Mouse B6C3F1	6 weeks	50, 150, 250, 500, 1000, 1500, 2000, 4000 ppm	24 weeks	Dietary	Spleen lymphocytes	*Hprt*	Negative	Manjanatha *et al.* (2009)
Macacca mulatta	2–4 years	2.5 12.5 mg kg^{-1} twice per day	M0–M20	Oral	Peripheral blood lymphocytes	*HPRT*	Negative	Morris *et al.* (2009)
Macacca mulatta	2–4 years	2.5 12.5 mg kg^{-1} twice per day	M20	Oral	Peripheral erythrocytes	*PIG-A*	Negative	Dobrovolsky *et al.* (2009)
Rat Sprague–Dawley	29 days	3 mg kg^{-1} per day for 21 days	29 days	Oral	Peripheral erythrocytes	*Pig-A*	Negative	Dobrovolsky *et al.* (2010)
Rat Sprague–Dawley	29 days	3 mg kg^{-1} per day for 21 days	50 days	Oral	Peripheral erythrocytes	*Pig-A*	Negative	Dobrovolsky *et al.* (2010)
Rat Sprague–Dawley	29 days	3 mg kg^{-1} per day for 21 days	90 days	Oral	Peripheral erythrocytes	*Pig-A*	Negative	Dobrovolsky *et al.* (2010)

In order to further explore this issue, mutant frequencies (MF) were measured in both target (liver) and nontarget (spleen) tissue from B6C3F1 mice (Morris *et al.*, 2009; Manjanatha *et al.*, 2008, 2009; Dobrovolsky *et al.*, 2009, 2010). B6C3F1 mice were exposed to 250 to 4000 ppm of MPH in the diet for 28 days, after which no increase in the *Hprt* MF was detected in splenic lymphocytes (Manjanatha *et al.*, 2008). In a subsequent study, no increase in the *Hprt* MF was found in splenic lymphocytes of Big Blue mice after 4, 12 or 24 weeks of exposure to 4000 ppm MPH (Manjanatha *et al.*, 2009). Further, no increase in *cII* MF was detected in the liver of these mice after 4, 12 or 24 weeks of MPH exposure. Additional loci were evaluated in NHP exposed to 5 or 25 mg kg^{-1} per day MPH for a period of 20 months: *HPRT* MF and *PIG-A* MF. The *HPRT* MF was determined at monthly intervals, and both *PIG-A* and *HPRT* were determined after 20 months of continuous exposure; no increases in MPH-induced MF were detected at any time point at either locus (Morris *et al.*, 2009; Dobrovolsky *et al.*, 2009). When the *Pig-A* assay was extended to the analysis of Sprague–Dawley rats exposed to 3 mg kg^{-1} of MPH for 21 days, no increase in the MF was detected (Dobrovolsky *et al.*, 2010). Although these studies did not measure the MF in the brain, the results do not support a mutagenic role for MPH, as mediated through an 8-OH-dG pathway, in these tissues.

It has been reported that exposure to weakly mutagenic chemicals, e.g. phenobarbital, may result in a shift in the mutation spectrum without a measurable increase in the mutant frequency (Singh *et al.*, 2001). Thus, the mutation spectra were determined for the *cII* and *Hprt* genes in the mouse and the *HPRT* gene in the NHP, with no differences in the mutation spectra detected in any target gene (Manjanatha *et al.*, 2009; Morris *et al.*, 2009). These data do not support the hypothesis that MPH has mutagenic potential mediated through an 8-OH-dG or alternative pathway.

CANCER INCIDENCE IN MPH-TREATED HUMAN SUBJECTS

A major concern raised by the reported increase in chromosomal aberrations in the study of El-Zein *et al.* (2005) was that pediatric treatment with MPH would increase the risk of adult-onset cancers. The concern was heightened when the results were interpreted in light of the findings from the NTP cancer bioassay in which an increase in hepatocellular neoplasms was found in B6C3F1 mice (National Toxicology Program, 1995; Dunnick and Hailey, 1995). It is important to note, however, that the NTP concluded that the increase in the frequency of these hepatocellular neoplasms occurred through a proliferative, rather than a genotoxic, mode of action. Two lines of evidence support this conclusion. First, in a study designed to specifically determine the mode of action of MPH, no data consistent with a DNA-damaging mode of action were obtained (Manjanatha *et al.*, 2009). Second, in a study designed to assess the effects of MPH at the cellular level, MPH was found to increase cell proliferation as measured by the uptake of the thymidine analog, bromodeoxyuridine (Bartl *et al.*, 2010). With regard to the observations of increased frequencies of chromosomal aberrations reported by El-Zein *et al.* (2005), several large epidemiology studies have documented an association between increased frequencies of chromosome translocations and risk for cancer in exposed human populations (Bonassi *et al.*, 2004, 2008; Peters *et al.*, 2011). However, no increase in translocation frequencies was seen in lymphocytes of MPH-treated NHP using chromosome painting, a

Published 2012. This article is a US Government work and is in the public domain in the USA.

technique that lends itself to the efficient detection of chromosome translocations (Morris *et al*., 2009). Finally, reassuring results were reported in a large human subjects study that found no strong association between MPH treatment during childhood and increased cancer risk (Oestreicher *et al*., 2007).

CONCLUSIONS

Considerable attention has been given to the possible genetic toxicity of MPH in recent years. Because the use of MPH is widespread and increasing, involving several million children in the USA alone (Zuvekas and Vitiello, 2011), this attention is well justified. In response to the concerns about MPH, a number of studies, using diverse methods and models, and including rodents, nonhuman primates, and humans have been conducted so that a significant body of data is now available upon which to base a conclusion regarding the genotoxicity of MPH. The evidence clearly does not indicate that MPH is either mutagenic or clastogenic. Considering the extent of the in-depth research thus far conducted on this topic, it seems unlikely that further studies will change the conclusion that the therapeutic use of MPH for the treatment of ADHD does not entail significant risk of genetic toxicity. These data should be reassuring to the users of this commonly prescribed medication and their families. However, MPH continues to be investigated for other potential adverse health effects, and therefore, the lack of genetic toxicity associated with MPH does not necessarily indicate a complete absence of health hazard.

REFERENCES

Aguiar A, Eubig PA, Schantz SL. 2010. Attention deficit/hyperactivity disorder: a focused overview for children's environmental health researchers. *Environ. Health Perspect.* **118**(12): 1646–1653.

American Psychiatric Association. (2000). Diagnostic and Statistical Manual of Mental Disorders (4th edn, text rev.). APA: Washington, DC.

Andreazza AC, Frey BN, Valvassoroi SS, Zanotto C, Gomes KM, Comim CM, Cassini C, Stertz L, Riberio LC, Quevedo J, Kapczinski F, Berk M, Goncalves CA. 2007. DNA damage in rats after treatment with methylphenidate. *Prog. Neuropsychopharmacol. Biol. Psychiat.* **31**(6): 1282–1288.

Bagnyukova TV, Powell CL, Pavliv O, Tryndyak VP, Pogribny IP. 2008. Induction of oxidative stress and DNA damage in rat brain by a folate/methyl-deficient diet. *Brain Res.* **1237**: 44–51.

Bartl J, Link P, Schlosser C, Gerlach M, Schmitt A, Walitza S, Riederer P, Grunblatt E. 2010. Effects of methylphenidate: the cellular point of view. *Attent. Defic. Hyperact. Disord.* **2**(4): 225–232.

Bonassi S, Znaor A, Norppa H, Hagmar L. 2004. Chromosomal aberrations and risk of cancer in humans: an epidemiologic perspective. *Cytogenet. Genome Res.* **104**(1–4): 376–382.

Bonassi S, Norppa H, Ceppi M, Stromberg U, Vermeulen R, Znaor A, Cebulska-Wasilewska A, Fabianova E, Fucic A, Gundy S, Hansteen IL, Knudsen LE, Rossner P, Sram RJ, Boffetta P. 2008. Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. *Carcinogenesis* **29**(6): 1178–1183.

Chovanova Z, Muchova J, Sivonova M, Dvorakova M, Zitnanova I, Waczulikova I, Trebaticka J, Skodacek I, Durackova Z. 2006. Effect of polyphenolic extract, Pycnogenol, on the level of 8-oxoguanine in children suffering from attention deficit/hyperactivity disorder. *Free. Radic. Res.* **40**(9): 1003–1010.

Dobrovolsky VN, Shaddock JG, Mittelstaedt RA, Manjanatha MG, Miura D, Uchikawa M, Mattison DR, Morris SM. 2009. Evaluation of *Macaca mulatta* as a model for genotoxicity studies. *Mutat. Res.* **673**(1): 21–28.

Dobrovolsky VN, Boctor SY, Twaddle NC, Doerge DR, Bishop ME, Manjanatha MG, Kimoto T, Miura D, Heflich RH, Ferguson SA. 2010. Flow cytometric detection of Pig-A mutant red blood cells using an erythroid-specific antibody: application of the method for evaluating the *in vivo* genotoxicity of methylphenidate in adolescent rats. *Environ. Mol. Mutagen.* **51**(2): 138–145.

Dunnick JK, Hailey JR. 1995. Experimental studies on the long-term effects of methylphenidate hydrochloride. *Toxicology* **103**(2): 77–84.

El-Zein RA, Abdel-Rahman SZ, Hay MJ, Lopez MS, Bondy ML, Morris DL, Legator MS. 2005. Cytogenetic effects in children treated with methylphenidate. *Cancer Lett.* **230**(2): 284–291.

Faraone SV, Sergeant J, Gillberg C, Biederman J. 2003. The worldwide prevalence of ADHD: is it an American condition? *Wld Psychiat.* **2**(2): 104–113.

Galloway SM, Armstrong MJ, Reuben C, Colman S, Brown B, Cannon C, Bloom AD, Nakamura F, Ahmed M, Duk S *et al*. 1987. Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environ. Mol. Mutagen.* **10**(suppl. 10): 1–175.

Gomes KM, Petronilho FC, Mantovani M, Garbelotto T, Boeck CR, Dal-Pizzol F, Quevedo J. 2008. Antioxidant enzyme activities following acute or chronic methylphenidate treatment in young rats. *Neurochem. Res.* **33**(6): 1024–1027.

Hannestad J, Gallezot JD, Planeta-Wilson B, Lin SF, Williams WA, van Dyck CH, Malison RT, Carson RE, Ding YS. 2010. Clinically relevant doses of methylphenidate significantly occupy norepinephrine transporters in humans *in vivo*. *Biol. Psychiat.* **68**(9): 854–860.

Jacobson-Kram D, Mattison D, Shelby M, Slikker W, Tice R, Witt K. 2008. Methylphenidate and chromosome damage. *Cancer Lett.* **260**(1–2): 216–218.

Larsen E, Reite K, Nesse G, Gran C, Seeberg E, Klungland A. 2006. Repair and mutagenesis at oxidized DNA lesions in the developing brain of wild-type and Ogg1$^{-/-}$ mice. *Oncogene* **25**(17): 2425–2432.

Liu D, Croteau DL, Souza-Pinto N, Pitta M, Tian J, Wu C, Jiang H, Mustafa K, Keijzers G, Bohr VA, Mattson MP. 2011. Evidence that OGG1 glycosylase protects neurons against oxidative DNA damage and cell death under ischemic conditions. *J. Cereb. Blood Flow Metab.* **31**(2): 680–692.

Manjanatha MG, Shelton SD, Dobrovolsky VN, Shaddock JG, McGarrity LG, Doerge DR, Twaddle NW, Lin CJ, Chen JJ, Mattison DR, Morris SM. 2008. Pharmacokinetics, dose-range, and mutagenicity studies of methylphenidate hydrochloride in B6C3F1 mice. *Environ. Mol. Mutagen.* **49**(8): 585–593.

Manjanatha MG, Shelton SD, Dobrovolsky VN, Shaddock JG, McGarrity LG, Twaddle NW, Moore MM, Mattison DR, Slikker W Jr, Morris SM. 2009. Evaluation of mutagenic mode of action in Big Blue mice fed methylphenidate for 24 weeks. *Mutat. Res.* **680**(1–2): 43–48.

Martins MR, Reinke A, Petronilho FC, Gomes KM, Dal-Pizzol F, Quevedo J. 2006. Methylphenidate treatment induces oxidative stress in young rat brain. *Brain Res.* **1078**(1): 189–197.

Morris SM, Dobrovolsky VN, Shaddock JG, Mittelstaedt RA, Bishop ME, Manjanatha MG, Shelton SD, Doerge DR, Twaddle NC, Chen JJ, Lin CJ, Paule MG, Slikker W, Hotchkiss CE, Petibone D, Tucker JD, Mattison DR. 2009. The genetic toxicology of methylphenidate hydrochloride in non-human primates. *Mutat. Res.* **673**(1): 59–66.

Mortelmans K, Haworth S, Lawlor T, Speck W, Tainer B, Zeiger E. 1986. Salmonella mutagenicity tests: II. Results from the testing of 270 chemicals. *Environ. Mutagen.* **8**(suppl. 7): 1–119.

National Toxicology Program. 1995. NTP toxicology and carcinogenesis studies of methylphenidate hydrochloride (CAS no. 298-59-9) in F344/N rats and B6C3F1 mice (feed studies). *Natl. Toxicol. Program Tech. Rep. Ser.* **439**: 1–299.

Oestreicher N, Friedman GD, Jiang SF, Chan J, Quesenberry C Jr, Habel LA. 2007. Methylphenidate use in children and risk of cancer at 18 sites: results of surveillance analyses. *Pharmacoepidemiol. Drug Saf.* **16**(12): 1268–1272.

Peters S, Portengen L, Bonassi S, Sram R, Vermeulen R. 2011. Intra- and interindividual variability in lymphocyte chromosomal aberrations: implications for cancer risk assessment. *Am. J. Epidemiol.* **174**(4): 490–493.

Pinkel D, Thompson LH, Gray JW, Vanderlaan M. 1985. Measurement of sister chromatid exchanges at very low bromodeoxyuridine substitution levels using a monoclonal antibody in Chinese hamster ovary cells. *Cancer Res.* **45**(11 Pt 2): 5795–5798.

Ponsa I, Ramos-Quiroga JA, Ribases M, Bosch R, Bielsa A, Ordeig MT, Morell M, Miro R, de Cid R, Estivill X, Casas M, Bayes M, Cormand B, Hervas A. 2009. Absence of cytogenetic effects in children and adults with attention-deficit/hyperactivity disorder treated with methylphenidate. *Mutat. Res.* **666**(1–2): 44–49.

Published 2012. This article is a US Government work and is in the public domain in the USA.

Preston RJ, Kollins SH, Swanson JM, Greenhill LL, Wigal T, Elliott GR, Vitiello B. 2005. Comments on 'Cytogenetic effects in children treated with methylphenidate' by El-Zein *et al*. *Cancer Lett*. **230**(2): 292–294.

Schmidt AJ, Clement HW, Gebhardt S, Hemmeter UM, Schulz E, Krieg JC, Kircher T, Heiser P. 2010a. Impact of psychostimulants and atomoxetine on the expression of 8-hydroxyguanine glycosylase 1 in human cells. *J. Neural Transm*. **117**(6): 793–797.

Schmidt AJ, Krieg JC, Clement HW, Gebhardt S, Schulz E, Heiser P. 2010b. Impact of drugs approved for treating ADHD on the cell survival and energy metabolism: an *in-vitro* study in human neuronal and immune cells. *J. Psychopharmacol*. **24**(12): 1829–1833.

Singh VK, Ganesh L, Cunningham ML, Shane BS. 2001. Comparison of the mutant frequencies and mutation spectra of three non-genotoxic carcinogens, oxazepam, phenobarbital, and Wyeth 14,643, at the lambdacII locus in Big Blue transgenic mice. *Biochem. Pharmacol*. **62**(6): 685–692.

Spencer TJ, Biederman J, Ciccone PE, Madras BK, Dougherty DD, Bonab AA, Livni E, Parasrampuria DA, Fischman AJ. 2006. PET study examining pharmacokinetics, detection and likeability, and dopamine transporter receptor occupancy of short- and long-acting oral methylphenidate. *Am. J. Psychiat*. **163**(3): 387–395.

Storer RD, French JE, Haseman J, Hajian G, LeGrand EK, Long GG, Mixson LA, Ochoa R, Sagartz JE, Soper KA. 2001. $P53^{+/-}$ hemizygous knockout mouse: overview of available data. *Toxicol. Pathol*. **29**(suppl): 30–50.

Suter W, Martus HJ, Elhajouji A. 2006. Methylphenidate is not clastogenic in cultured human lymphocytes and in the mouse bone-marrow micronucleus test. *Mutat. Res*. **607**(2): 153–159.

Teo SK, San RH, Wagner VO, Gudi R, Stirling DI, Thomas SD, Khetani VD. 2003. D-Methylphenidate is non-genotoxic in *in vitro* and *in vivo* assays. *Mutat. Res*. **537**(1): 67–79.

Tripp G, Wickens JR. 2009. Neurobiology of ADHD. *Neuropharmacology* **57**(7–8): 579–589.

Tucker JD, Christensen ML, Strout CL, Carrano AV. 1986. Determination of the baseline sister chromatid exchange frequency in human and mouse peripheral lymphocytes using monoclonal antibodies and very low doses of bromodeoxyuridine. *Cytogenet. Cell Genet*. **43**(1–2): 38–42.

Tucker JD, Suter W, Petibone DM, Thomas RA, Bailey NL, Zhou Y, Zhao Y, Muniz R, Kumar V. 2009. Cytogenetic assessment of methylphenidate treatment in pediatric patients treated for attention deficit hyperactivity disorder. *Mutat. Res*. **677**(1–2): 53–58.

Walitza S, Werner B, Romanos M, Warnke A, Gerlach M, Stopper H. 2007. Does methylphenidate cause a cytogenetic effect in children with attention deficit hyperactivity disorder? *Environ. Health Perspect*. **115**(6): 936–940.

Walitza S, Kampf K, Artamonov N, Romanos M, Gnana Oli R, Wirth S, Warnke A, Gerlach M, Stopper H. 2009. No elevated genomic damage in children and adolescents with attention deficit/hyperactivity disorder after methylphenidate therapy. *Toxicol. Lett*. **184**(1): 38–43.

Walitza S, Kampf K, Oli RG, Warnke A, Gerlach M, Stopper H. 2010. Prospective follow-up studies found no chromosomal mutagenicity of methylphenidate therapy in ADHD affected children. *Toxicol. Lett*. **193**(1): 4–8.

Witt KL, Shelby MD, Itchon-Ramos N, Faircloth M, Kissling GE, Chrisman AK, Ravi H, Murli H, Mattison DR, Kollins SH. 2008. Methylphenidate and amphetamine do not induce cytogenetic damage in lymphocytes of children with ADHD. *J. Am. Acad. Child Adolesc. Psychiat*. **47**(12): 1375–1383.

Witt KL, Malarkey DE, Hobbs CA, Davis JP, Kissling GE, Caspary W, Travlos G, Recio L. 2010. No increases in biomarkers of genetic damage or pathological changes in heart and brain tissues in male rats administered methylphenidate hydrochloride (Ritalin) for 28 days. *Environ. Mol. Mutagen*. **51**(1): 80–88.

Wong AW, McCallum GP, Jeng W, Wells PG. 2008. Oxoguanine glycosylase 1 protects against methamphetamine-enhanced fetal brain oxidative DNA damage and neurodevelopmental deficits. *J. Neurosci*. **28**(36): 9047–9054.

Zuvekas SH, Vitiello B. 2011. Stimulant medication use among U.S. children: a twelve-year perspective. (E-pub ahead of print) *Am. J. Psychiat*. doi: 10.1176/appi.ajp.2011.11030387

Published 2012. This article is a US Government work and is in the public domain in the USA.