In Vitro* Toxicological Evaluation of Cigarette Smoke Particulate Matter: Effect of Dimethyl Sulfoxide (DMSO) as Solvent

by

Manoj Misra¹, Robert D. Leverette¹, Jonathan T. Hamm^{1,2} and Suryanarayana V. Vulimiri¹

¹AW Spears Research Center, Lorillard Tobacco Company, 420 N. English Street, Greensboro, NC 27420.

²Current address: ICCVAM/NICEATM, Integrated Laboratory Systems, Inc., P.O. Box 13501, Research Triangle Park, NC 27709.

SUMMARY

This study examined the potential to minimize the cytotoxic or genotoxic effects that dimethylsulfoxide (DMSO), when used as solvent, has on the *in vitro* interleukin-8 (IL-8) release, mammalian cell cytotoxicity and micronuclei formation, and bacterial mutagenesis induced by cigarette smoke wet total particulate matter (WTPM). The use of DMSO as a solvent vehicle for test articles of limited water solubility is widely applied in *in vitro* assays due to its moderate toxicity to test organisms and its excellent solvent properties for both polar and non-polar compounds, such as WTPM. A significant DMSO dose-dependent depletion in the IL-8 release was observed, with or without the addition of WTPM, at concentrations spanning those typically employed in *in vitro* assays. DMSO at 3.6% reduced cell viability 40-50%. Overall, DMSO at final concentrations of 0.5% and 4.0% resulted in about 50% and 90% depletion of final IL-8 levels, respectively. DMSO-induced cytotoxicity was evident only at concentrations of 1.5% or more, a concentration higher than that typically employed in such testing. The WTPM-induced cytotoxicity was equivalent at low ranges of DMSO concentrations. DMSO concentrations of 3.6% or higher resulted in an increase of cytotoxicity by 20–25%. DMSO alone did not give rise to bacterial mutagenicity at doses from 0% to 3.9%; however, WTPM exposure with increasing levels of DMSO resulted in increased toxicity of the WTPM at doses of DMSO greater than 6.9%, as indicated by lower revertant counts. This effect suggests that for Ames assay analysis of WTPM collected in DMSO, the level of DMSO should be minimized to prevent lower revertant counts due to DMSO-induced toxicity. DMSO alone gave a dose-dependent increase in the background

micronuclei percentage, with a statistically significant increase at 4%. In the presence of WTPM, DMSO at 3% concentration resulted in a significantly higher micronucleus frequency, suggesting a possible clastogenic role of DMSO or potential cell permeability differences.

In conclusion, this study should provide guidance for the range of DMSO concentrations when used as a solvent for WTPM-mediated *in vitro* toxicological assays and therefore help in properly designing and conducting *in vitro* studies that utilize DMSO as the extraction solvent of choice. [Beitr. Tabakforsch. Int. 24(2010) 2–9]

ZUSAMMENFASSUNG

Diese Arbeit untersucht das Potential, die zytotoxischen oder genotoxischen Effekte, die durch das Lösungsmittel Dimethylsulfoxid (DMSO) verursacht wurden, zu minimieren. Die Effekte bestehen in der *in vitro* Freisetzung von Interleukin-8 (IL-8), der Säugerzellzytotoxizität, die Mikronukleusbildung und der bakteriellen Mutagenität, die durch (wasserhaltiges) Gesamtkondensat (WTPM) induziert wurden. Die Nutzung von DMSO als Lösungsmittel für Proben mit begrenzter Wasserlöslichkeit und in *in vitro*-Versuchen ist weit verbreitet. Dies liegt zum einen an der geringen Toxizität gegenüber den Testorganismen und zum anderen an den hervorragenden Löseeigenschaften von DMSO gegenüber polaren und nicht-polaren Verbindungen wie WTPM. Eine dosisabhängige Verringerung der Freisetzung von IL-8 durch DMSO konnte mit und ohne die Zugabe von WTPM beobachtet werden, und zwar bei Konzentrationen, wie sie typischerweise auch bei *in vitro*-Versuchen eingesetzt werden. Ein DMSO-Gehalt von 3,6% reduzierte die

Lebensfähigkeit der Zellen um 40–50%. Insgesamt führten DMSO-Endkonzentrationen von 0,5% und 4,0% zu einer Reduktion um 50% bzw. 90% der erreichten IL-8-Mengen. Die durch DMSO induzierte Zytotoxizität war erst bei Konzentrationen von mindestens 1,5% nachweisbar, also bei Konzentrationen, die weitaus höher sind, als die typischerweise in solchen Experimenten verwendeten Konzentrationen. Die durch WTPM induzierte Zytotoxizität war vergleichbar zu der durch niedrige DMSO-Konzentrationen erreichten. DMSO Konzentrationen von 3,6% und höher führten zu einer Erhöhung der Zytotoxizität um 20–25%. DMSO alleine führte bei Dosierungen von 0% bis 3,9% nicht zu einer höheren bakteriellen Mutagenität. Allerdings führte eine Exposition mit WTPM und ansteigenden Mengen DMSO ab einer Menge von 6,9% DMSO zu einer höheren Toxizität von WTPM, erkennbar an einer geringeren Anzahl an Revertanten. Diese Ergebnisse deuten darauf hin, dass eine Analyse von in DMSO gelöstem WTPM beim Ames Test die Menge des verwendeten DMSO so gering wie möglich gehalten werden sollte, um die durch die DMSO-Toxizität ausgelöste verringerte Revertantenzahl zu minimieren. DMSO alleine führte zu einem dosisabhängigen Anstieg des prozentualen Hintergrundanteils an Micronuklei mit einem signifikanten Anstieg bei 4% DMSO. In Anwesenheit von WTPM führten DMSO Konzentrationen ab 3% zu einer signifikant höheren Häufigkeit von Mikronuklei, was ein Hinweis auf mögliche klastogene Eigenschaften von DMSO oder auf potentielle Änderungen der Zellpermeabilität durch DMSO ist. Zusammenfassend soll diese Arbeit eine Anleitung für die Konzentrationsbereiche, in denen DMSO als Lösungsmittel für WTPM bei *in vitro* toxikologischen Experimenten eingesetzt wird, bieten und eine Hilfestellung bei der richtigen Planung und Durchführung von *in vitro* Experimenten, die DMSO als Extraktionsmittel wählen, sein. [Beitr. Tabakforsch. Int. 24 (2010) 2–9]

RESUME

Cette étude examine le potentiel pour réduire les effets cytotoxiques ou génotoxiques du diméthylsulfoxyde (DMSO), lorsqu'il est utilisé comme solvant, sur la libération d'interleukine-8 (IL-8) *in vitro*, la cytotoxicité des cellules de mammifère, la formation de micronoyaux, et la mutagenèse bactérienne induite par la matière particulaire totale humide (WTPM) de la fumée de cigarette. L'utilisation du DMSO comme solvant pour les articles de test peu soluble dans l'eau est largement appliquée aux essais *in vitro* du fait de sa toxicité modérée sur les organismes expérimentaux et son excellent pouvoir solvant pour les composés à la fois polaires et non-polaires tels que les WTPM. On a constaté une réduction dose-dépendante significative du DMSO dans la libération d'IL-8, avec ou sans addition de WTPM, à des concentrations correspondant à celles employées typiquement dans des essais *in vitro*. A 3,6 % de DMSO, la viabilité cellulaire est réduite de 40 à 50 %. Dans l'ensemble, le DMSO, à des concentrations finales de 0,5 % et 4,0 %, a entraîné une réduction d'environ 50 % et 90 % des niveaux finaux d'IL-8 respectivement. La cytotoxicité induite par le DMSO n'a

été évidente qu'à des concentrations de 1,5 % ou plus, une concentration supérieure à celle-ci est typiquement employée dans de tels tests. La cytotoxicité induite par la WTPM était équivalente à des faibles niveaux de concentrations de DMSO. Les concentrations de DMSO de 3,6 % ou plus ont entraîné une augmentation de la cytotoxicité de 20 à 25 %. Le DMSO seul n'a pas provoqué d'augmentation de la mutagenicité bactérienne à des doses de 0 % à 3,9 % ; toutefois, l'exposition de la WTPM à des niveaux croissants de DMSO a eu pour résultat une toxicité accrue de la WTPM à des doses de DMSO supérieures à 6,9 %, comme indiqué par des numérations inférieures de révertants. Cet effet suggère que, pour l'analyse du test d'Ames de la WTPM collectée dans le DMSO, le niveau de DMSO doit être minimisé pour empêcher des numérations inférieures de révertants dues à une toxicité induite par le DMSO. Le DMSO seul a induit une augmentation dose-dépendante du pourcentage de micronoyaux dans le pourcentage initial de micronoyaux, avec une augmentation statistiquement significative de 4 %. En présence de WTPM, le DMSO à 3 % de concentration a induit une fréquence de micronoyaux significativement supérieure, suggérant un éventuel rôle clastogène du DMSO ou des différences potentielles de perméabilité cellulaire.

Pour conclure, cette étude devrait servir de repère pour classer les concentrations de DMSO lorsqu'il est utilisé comme solvant pour des essais toxicologiques *in vitro* à médiation WTPM et par conséquent aider à concevoir et à réaliser correctement des études *in vitro* qui utilisent le DMSO comme solvant d'extraction. [Beitr. Tabakforsch. Int. 24 (2010) 2–9]

INTRODUCTION

The *in vitro* toxicological testing, including cytotoxicity and micronuclei induction in mammalian cells and mutagenesis in bacterial strains, has been utilized as a toxicological screening tool to assess the toxicological impact of tobacco ingredients (1) when utilizing the particulate phase of cigarette smoke aerosol labeled wet total particulate matter (WTPM) (2). WTPM contains thousands of polar and non-polar components, and constitutes the mainstream smoke particulate fraction as it is collected on a standard Cambridge glass fiber filter, as opposed to FTC "tar", which refers to WTPM minus water and nicotine. The chemical complexity of cigarette smoke WTPM renders it a uniquely challenging test article in toxicology assessments.

Dimethylsulfoxide (DMSO) has been widely applied as a vehicle for *in vitro* assays of test articles of limited water solubility due to its excellent solvent properties for both polar and non-polar compounds and its moderate toxicity to test organisms. DMSO has proven to be particularly useful as a solvent for complex combustion mixtures such as the WTPM of cigarette smoke aerosol.

Oxygen-centered radical species are believed to play a role in initiating and sustaining inflammatory processes, both as a consequence of their reactivity toward key cellular components and as intracellular triggers that stimulate the release of endogenous mediators of inflammation, such as cytokines and NF- κ B (3). Hydrogen peroxide induced

oxidative stress has been observed to promote IL-8 secretion in cultured cells (4). DMSO is a potent scavenger of free radicals, including oxygen radicals in biological systems (5). Several mechanistic studies demonstrated the inhibitory effects of DMSO on oxygen radical-mediated IL-8 induction from cells induced with LPS (6), GSNO, S-NO, spermine-NONOate (7), H₂O₂, TNF- α (8), NaCl (9), as well as aneugen and clastogen mediated micronuclei formation (10). The utility of DMSO as a solvent to achieve dissolution of WTPM for *in vitro* assays was considered in light of the reported potential of this solvent to affect several elements of the inflammatory process, including the release of the cytokine IL-8 (5).

The *in vitro* micronucleus (MN) assay, routinely used as part of a battery of genotoxic assays by regulatory agencies (11), scores the structural and chromosomal aberrations induced by both clastogens and aneugens by monitoring a mammalian cell's progression through the cell cycle (12). The *in vitro* MN assay has been used to detect the genotoxicity of single components (13) or a mixture of chemicals, such as cigarette smoke WTPM in mammalian cells (2).

The present investigation was pursued to characterize the potential of this solvent to affect *in vitro* cytotoxicity, bacterial mutagenicity, MN formation, and IL-8 release over a range of solvent concentrations spanning those commonly employed in such *in vitro* testing of WTPM. Additionally, to determine the relationship among cellular antioxidant capacity, cytotoxicity, and IL-8, we evaluated the concentration-dependent effects of DMSO on WTPM-mediated IL-8 release and cytotoxicity in A549 human lung alveolar epithelial carcinoma cells pre-treated with *d,l*-buthionine-(S,R)-sulfoximine (BSO), a cellular glutathione depleting agent and a potent inhibitor of glutathione (GSH) biosynthesis.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) unless otherwise stated.

Smoking and WTPM Preparation

Cigarettes were conditioned at 60% relative humidity at 24 °C for at least 18 h prior to machine smoking. University of Kentucky 2R4F cigarettes were smoked on a 20-port rotary smoking machine (Borgwaldt-KC, Richmond, VA) to a butt length of 3 mm from the tipping paper with the standard Federal Trade Commission (FTC) smoking regime of a 35 mL puff of 2 s duration taken once per min (14). Smoke particulate was collected on a Cambridge filter pad and extracted with DMSO to give a final stock solution of WTPM at a concentration of 40 mg/mL (w/v). The level of DMSO, present either in samples or exogenously added, is expressed as percentage volume of DMSO in total treatment dose volume (v/v).

Cell Culture and Treatment – Cytotoxicity and IL-8 Assay

The A549 cells (ATCC) were plated in 96-well plates in 200 μ L volume of complete media (Hamm F-12 media with

10% heat-inactivated fetal bovine serum (FBS)), 2 mM *L*-glutamine and 0.01 mg/mL gentamicin) at a seeding density of 50,000 cells/mL and cultured for 24 h in the cell incubator at 37 °C with 5% CO₂.

Cells were treated with different levels of DMSO and/or WTPM simultaneously for 24 h prior to Neutral Red Uptake (NRU) and IL-8 assays. For the BSO treatments, cells were pre-incubated with fresh complete media containing 500 μ M BSO overnight prior to DMSO and/or WTPM treatments. Dilutions of WTPM stock solution were made with DMSO to achieve exposure concentrations of 0–250 μ g WTPM/mL. The level of DMSO was adjusted to the desired level in the exposures with additional DMSO. For treatment, culture media was withdrawn and replaced with complete media containing the desired WTPM and DMSO concentrations followed by exposure for 24 h.

Cytotoxicity Assay

Cytotoxicity, measured by the NRU procedure was determined in A549 cells as previously described in (15, 16). Following exposure, the culture medium was replaced with 1.5% neutral red dye and incubated for 2.5 h. The plates were then washed and the cell-incorporated dye was released and quantified by measuring the absorbance at 540 nm on a Thermolabsystems (Franklin, MA) Multiskan Spectrum spectrophotometer.

IL-8 Assay

Following exposure, the cell culture media was removed and assayed for IL-8 with an ELISA detection kit (Abazyme, Inc.) by measuring the absorbance at 450 nm with a Thermolabsystems (Franklin, MA) Multiskan Spectrum spectrophotometer.

Micronucleus (MN) assay

The *in vitro* MN assay was performed in CHO-K1 cells as previously described in (10) utilizing the MN HitKit (ThermoFisher Scientific, Pittsburgh, PA).

Bacterial Mutagenesis

Ames reverse bacterial mutagenicity assays were conducted with the pre-incubation modification (17) in strains TA 98 and TA 100 with S9 activation. Aroclor induced Sprague-Dawley rat liver S9 post-mitochondrial supernatant (Moltox, Inc.), in 0.154 M KCl, was used for the S9-cocktail (0.1 M Phosphate Buffer, pH 7.4; 8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-phosphate (G-6-P), 4 mM nicotinamide adenine diphosphate (NADP), 5% (v/v) S9-fraction). *Salmonella* tester strains were exposed to increasing amounts of DMSO (% v/v) in the presence or absence of 125 μ g of WTPM. Exposures were performed as follows: 100 μ L of an overnight culture, 500 μ L of the S9-mix, plus between 5 and 150 μ L of DMSO. Exposures were shaken at 250 rpm for 30 min at 37 °C prior to the addition of 2 mL of histidine/biotin top agar and plated onto 150 mm minimal glucose agar plates. All exposures were conducted in triplicate in two independent experiments. Viability was determined by plating 10⁻⁴ dilutions of the

Figure 1. Effect of DMSO on measurement of 800 pg/mL IL-8 standard. IL-8 standard was incubated with different concentrations of DMSO followed by IL-8 measurement.

Figure 2: NRU by A549 cells exposed for 24 h to different levels of DMSO. The relative NRU is expressed as percentage of control. *Significantly different ($p < 0.05$) when compared with 0% DMSO control.

Figure 3: IL-8 release by A549 cells exposed for 24 h to different levels of DMSO. The relative IL-8 release is expressed as percentage of control. *Significantly different ($p < 0.05$) when compared with control.

exposure mixtures onto nutrient agar plates. All colonies were counted with an automated colony counter (Artek, Model 880) and compared to unexposed controls.

Statistical Analysis

Data were analyzed for any significant differences ($p < 0.05$) between control and treatment groups with one-way Analysis of Variance (ANOVA) with Tukey's post-hoc multiple comparison test with GraphPad Prism® software. Results were expressed as mean $\pm$ SD with a minimum of three replicates.

RESULTS AND DISCUSSION

DMSO has proven to be particularly useful as a solvent due to its excellent properties in the dissolution of both polar and non-polar compounds and its very moderate toxicity to test organisms. WTPM, the mainstream smoke particulate fraction collected on a standard Cambridge glass fiber filter, contains thousands of both polar and non-polar components (18). To minimize the concentration of DMSO in WTPM preparations, a concentration of 40 mg WTPM/mL DMSO was standardized for the series of *in vitro* toxicological assays utilized in this study. To avoid any solvent effect in an experimental design, where various concentrations of WTPM are used, we maintained a constant concentration of DMSO in the exposure volume. Before determining the effect of DMSO on cellular mediated inflammatory cytokine IL-8 release, we assessed the direct interaction or artificial effect, if any, of DMSO on the IL-8 assay in a cell-free system. Different levels of DMSO were added to the IL-8 standard (800 pg/mL) in a cell-free system (Figure 1) and the assay was conducted. As evident from the results, up to 4% DMSO (v/v) did not cause any significant reduction in the measurement of IL-8. This lack of interference of DMSO with IL-8 detection in the ELISA measurement system suggests that any potential effect DMSO may have on IL-8 levels would be the result of a direct effect on IL-8 release from a biological system and not an effect on IL-8 detection.

We assessed the effect of DMSO itself on the cytotoxicity, bacterial mutagenicity, MN formation, and IL-8 release *in vitro*. Figures 2 and 3 demonstrate the effect of varying the level of DMSO added to A549 cells on cytotoxicity, as measured by the neutral red uptake, and IL-8 release, respectively. DMSO significantly inhibits IL-8 release in a dose-dependent manner at concentrations spanning those typically employed in *in vitro* assays (Figure 3). A significant reduction in IL-8 release was observed at DMSO concentrations as low as 0.06% (Figure 3). However, DMSO-induced cytotoxicity was evident only at concentrations of 1.5% or more (Figure 2), a concentration range higher than that typically employed in such testing. Both bacterial mutagenicity and the MN formation assays did not reveal any mutagenic or clastogenic effects of DMSO at levels typically employed in these assays (Figure 10–13).

After demonstrating the effect of DMSO alone on the *in vitro* assays employed in this study, we determined the effects of added DMSO to WTPM samples prepared in DMSO as the solvent in order to determine the optimal DMSO level for WTPM without any solvent mediated cytotoxic or genotoxic effects.

For the NRU and IL-8 assays, a WTPM dose of 100 μ g/mL was chosen based on previous studies (unpublished data) that demonstrated quantifiable effects on cytotoxicity and IL-8 release at that dose level. The dose of WTPM for the reverse bacterial mutagenicity assay was 125 μ g WTPM per plate, a dose that has consistently been within the linear dose response for both strains TA 98 and TA 100 (data not shown). A lower WTPM dose of 25–50 μ g/mL was required for the MN assay due to WTPM-induced toxicity at higher doses within this particular assay (Figure 10).

Figure 4: NRU uptake by A549 cells exposed for 24 h simultaneously with 100 µg/mL WTPM and different levels of DMSO. The relative NRU is expressed as percentage of control. *Significantly different ($p < 0.05$) when compared with control.

Figure 5: IL-8 release by A549 cells exposed simultaneously with WTPM and different levels of DMSO. Cells were exposed for 24 h to different concentrations of DMSO and 100 µg/mL WTPM. The relative IL-8 release is expressed as percentage of control. *Significantly different ($p < 0.05$) when compared with control.

Figure 6: Effect of BSO pretreatment on NRU by A549 cells exposed with WTPM. Cells were pretreated with 500 µM BSO overnight followed by different concentrations of WTPM for additional 24 h. The relative NRU is expressed as percentage of respective control. *Significantly different ($p < 0.05$) when compared with control.

The 100 µg/mL WTPM dose in the NRU and IL-8 assays contained 0.24% DMSO, a level of DMSO that demonstrated no apparent cytotoxicity as measured by NRU (Figure 2). However, WTPM at 100 µg/mL did result in

about 50% cytotoxicity relative to control (Figure 4), suggesting WTPM-mediated toxicity. When maintaining 100 µg/mL WTPM and increasing the level of DMSO, a DMSO dose-dependent increase in cytotoxicity was observed, confirming our DMSO results.

Exposure to 100 µg/mL WTPM, containing 0.24% DMSO, resulted in about 40% increase in IL-8 release as compared to control (Figure 5), suggesting WTPM induced IL-8 release. As shown in Figure 3, IL-8 release decreases with increasing levels of DMSO. This decrease is also reflected in the presence of WTPM as the level of DMSO increases (Figure 5). The diminution of IL-8 release could be the result of DMSO scavenging of any oxygen radical species that may trigger cytokine release. MASSION *et al.* (5) reported a hydrogen peroxide (H_2O_2) dose-dependent IL-8 release in human bronchial epithelial cells and subsequently, a 1% DMSO concentration significantly decreased the H_2O_2 -mediated IL-8 release. Epithelial cells are capable of producing reactive oxygen species (ROS) after exposure to inflammatory mediators (19), such as WTPM. ROS have been demonstrated to regulate IL-8 gene expression (20), and since DMSO is a hydroxyl radical scavenger (21), it was conjectured that the oxygen radical scavenging properties of DMSO resulted in the decreased IL-8 levels mediated by H_2O_2 in airway epithelial cells (5). Such effects are manifested at sub-toxic DMSO concentrations, while cytotoxicity attributable to this solvent was only apparent at concentrations above the recommended maximum assay level of 0.5% (v/v) (22). The cytotoxicity of DMSO and WTPM appears additive, whereas the inflammatory response measurements to DMSO and WTPM appears antagonistic. The cytotoxic and inflammatory responses of cellular systems to test articles have been demonstrated to be increased in cells that are in a compromised state. Specifically, GSH, a cellular tripeptide, is an efficient endogenously synthesized antioxidant known to play an important role in the cellular antioxidant capacity. Depletion of GSH has been shown to vastly increase the toxicity of a variety of cellular insults (23). In order to examine the relationship to oxidative stress and WTPM toxicity, A549 cells were pre-treated with the GSH-depleting compound BSO. Treatment with BSO alone resulted in no significant increase in cytotoxicity (Figures 6–7), but a significant IL-8 release was observed (Figures 8–9), suggesting that BSO itself is inflammatory. As evident from Figures 7 and 9, BSO pre-treatment followed by WTPM exposures significantly increased cytotoxicity and IL-8 release, respectively; however, at lower WTPM doses (25 and 50 µg/mL), IL-8 release was significantly higher with no significant increase in cytotoxicity. The results from the NRU and IL-8 release assays with BSO pretreatment are consistent with the role of reactive oxygen species in both cytotoxicity and cytokine release (23).

The effect DMSO concentration may have on WTPM-induced genotoxic endpoints was examined with MN formation and bacterial mutagenicity assays. MN formation at WTPM doses (25, 50 and 75 µg/mL) was measured in the presence of increasing DMSO concentrations (0–4%). DMSO up to 1% final concentration did not affect the binucleated cell count (BNC) in CHO-K1 cells (Figure 10); however, as the concentration increased from 1% to 4%,

Figure 7: Effect of DMSO on NRU in BSO pretreated A549 cells treated with WTPM. Cells were pretreated with 500 μ M BSO overnight followed by additional 24 h with different concentrations of DMSO and 100 μ g/mL WTPM. The relative NRU is expressed as percentage of control. *Significantly different ($p < 0.05$) when compared with their respective control.

Figure 8: Effect of BSO pretreatment on IL-8 release by A549 cells exposed with WTPM. Cells were pretreated with 500 μ M BSO overnight followed by additional 24 h with different concentrations of WTPM for additional 24 h. *Significantly different ($p < 0.05$) when compared with control.

Figure 9: Effect of DMSO on IL-8 release in BSO pretreated A549 cells exposed with WTPM. Cells were pretreated with 500 μ M BSO overnight followed by additional 24 h with different concentrations of DMSO and 100 μ g/mL WTPM. *Significantly different ($p < 0.05$) when compared with their respective control.

Figure 10: Effect of DMSO concentration on binucleated cell count (BNC) in CHO-K1 cells. CHO-K1 cells were exposed to 0, 25, 50 and 75 μ g/mL of WTPM with added DMSO to final concentrations of 0–4% in a cytokinesis-blocked micronucleus assay. The binucleated cell (BNC) count is expressed as % control.

the BNC decreased from 96% to 49% suggesting toxicity effects. Organization for Economic Co-operation and Development (OECD) guidelines suggest an overall BNC count of $> 40\%$ to be considered a non-toxic dose (24); therefore, only MN values should be taken from those doses with BNC counts $> 40\%$. This increase in cytotoxicity, as revealed by the decrease in BNC counts with increasing levels of DMSO, is consistent with an increase in cytotoxicity in CHO-K1 cells with increasing DMSO as determined by the cytokinesis-blocked proliferation index (10). WTPM-treated CHO-K1 cells showed a dose-dependent decrease in BNC count at any given WTPM dose, suggesting an increase in toxicity with increasing WTPM concentration (Figure 10). With increasing DMSO concentrations and constant WTPM doses, the BNC counts showed a decreasing trend, suggesting greater cytotoxicity at higher DMSO concentrations. The WTPM concentration of 75 μ g/mL was observed to be toxic to the CHO-K1 cells, verified by the BNC counts of approximately 40%; therefore, the MN frequencies (MNF) from this dose were excluded from analysis. The MNF data were compared between different groups relative to the control group. It appeared that DMSO alone caused an increase in the background MNF relative to the control as the final DMSO concentration increased from 0% to 4%, the latter being statistically significant ($p < 0.05$) (Figure 11). This observation was consistent with previously reported findings (10). Despite the higher background MNF seen at 3% DMSO, a significantly higher MNF was observed for WTPM at 25 and 50 μ g/mL doses (Figure 11). Overall, DMSO was shown to contribute to MN induction at higher levels, which could potentially mask the genotoxicity of a test compound or overestimate the genotoxicity of the test compound when using excessive levels of DMSO. Caution should be exercised not to exceed a DMSO level of 2% in samples used to expose CHO-K1 cells in the *in vitro* MN assay.

Salmonella tester strains TA 98 and TA 100 pre-incubated with increasing amounts of DMSO in the presence of an S9-activation cocktail showed no increase in the number of revertants per plate when compared to background (Figures

Figure 11: Effect of DMSO concentration on micronucleus frequency (MNF) in CHO-K1 cells. CHO-K1 cells were exposed to 0, 25, and 50 µg/mL of WTPM with added DMSO to final concentrations of 0–4% in a cytokinesis-blocked micronucleus assay. The micronucleus frequency (MNF) is expressed as % control. *Significantly different ($p < 0.05$) when compared with respective 0 % added DMSO controls.

Figure 12: Effect of DMSO on TA 98 revertant counts under S9+ conditions in the presence and absence of 125 µg WTPM/plate.

Figure 13: Effect of DMSO on TA 100 revertants counts under S9+ conditions in the presence and absence of 125 µg WTPM/plate. *Significantly different ($p < 0.05$) when compared with control.

12 and 13). These results were expected since DMSO has previously been shown to be non-mutagenic in strains TA 98 and TA 100 (25).

In TA 98, the revertant counts induced by 125 µg WTPM showed a slight increase up to 3.2% added DMSO followed by a steady decrease in revertant counts to the 19.2% added DMSO (Figure 12). In TA 100, the revertant counts decreased with increasing DMSO concentrations (Figure 13). Examination of the background lawn from all the

exposures indicated hints of toxicity at the two highest levels of DMSO (13.5% and 19.2% DMSO) in the presence of WTPM. To investigate the hypothesis that the decrease in revertant counts at lower DMSO levels was the result of decreased viability, 100 µL of a 1:1000 dilution of the pre-incubation reactions were plated on nutrient agar plates. Results indicated a lower viability in the presence of WTPM at the 13.5% and 19.2% added DMSO in strain TA 98 and at the 6.9 – 19.2% added DMSO levels in TA 100 (data not shown).

It has previously been reported that DMSO does not create adverse effects in these two tester strains up to 20% in plate incorporation or 25% in pre-incubation methods (26). The results of WTPM exposure with increasing levels of DMSO indicated that the bacteria are experiencing increased toxicity at the higher doses of DMSO, resulting in lower revertant counts. This effect suggests that for the analysis of smoke condensates collected in DMSO, the amount of DMSO should not exceed 4% (v/v) final concentration in the pre-incubation exposure mixture in order to minimize toxicity not discernable through simple examination of the background lawn.

CONCLUSIONS

The practical utility of DMSO as a solvent for *in vitro* testing of WTPM must be weighed against the possibility of the inherent capacity of DMSO to affect free radicals, including oxygen radicals, or other unknown interfering mechanisms which may distort the toxicological endpoints of interest. The present demonstration of solvent effects on the cytotoxicity, bacterial mutagenicity, MN formation, and IL-8 release in response to WTPM exposure provides a practical example of the difficulties inherent in the study of this complex combustion mixture. The observations and conclusions from this study will help in properly designing and conducting *in vitro* toxicological studies that utilize DMSO as the solvent of choice.

Additionally, in light of the present and prior work documenting the practical difficulties with available WTPM solvent systems, e.g. saline, DMSO, ethanol, acetone, there emerges an additional rationale for the development, validation and standardization of solvent-free, direct cigarette smoke exposure techniques for the *in vitro* study of a freshly-generated, whole cigarette smoke aerosol.

REFERENCES

1. Roemer, E., F.J. Tewes, T.J. Meisgen, D.J. Veltel, and E.L. Carmines: Evaluation of the potential effects of ingredients added to cigarettes. Part 3. In vitro genotoxicity and cytotoxicity; Food and Chem. Tox. 40 (2002) 105–111.
2. Baker, R.R., E.D. Massey, and G. Smith: An overview of the effects of tobacco ingredients on smoke chemistry and toxicity; Food Chem. Toxicol. 42 Suppl (2004) S53–S83.
3. Kelly, A.K., M. R. Hill, K. Youkhana, F. Wanker, and J.M. Gimble: Dimethyl sulfoxide modulates NF-κB and cytokine activation in lipopolysaccharide-

- treated murine macrophages; *Infect. Immun.* 62 (1994) 3122–3128.
4. Yamamoto, K., R. Kushima, O. Kasaki, Y. Fujiyama, and H. Okabe: Combined Effect of Hydrogen Peroxide Induced Oxidative Stress and IL-1 α on IL-8 Production in CaCo-2 Cells and Normal Intestinal Epithelial Cells; *Inflammation* 27 (2003) 123–128.
 5. Massion, P.P., A. Linden, H. Inoue, M. Mathy, I.M. Grattan, and J.A. Nadel: Dimethyl sulfoxide decreases interleukin-8-mediated neutrophil recruitment in the airways; *Am. J. Physiol. Lung Cell. Mol. Physiol.* 15 (1996) L838–L843.
 6. Remick, D.G. and L. Villarete: Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates; *J. Leukocyte Biol.* 59 (1996) 471–475.
 7. Turpaev, K., D. Litvinov, and J. Justesen: Redox modulation of NO-dependent induction of interleukin 8 gene in monocyte U937 cells; *Cytokine* 23 (2003) 15–22.
 8. Dong, W., P.P. Simeonova, R. Gallucci, J. Matheson, R.P. Fannin Montuschi, L. Flood, and M.I. Luster: Cytokine expression in hepatocytes: Role of oxidant stress; *J. Interferon Cytokine Res.* 18 (1998) 629–638.
 9. Loitsch, S.M., C.V. Mallinckrodt, S. Kippengerger, D. Steinhilber, T.O.F. Wagner, and J. Bargon: Reactive oxygen intermediates are involved in IL-8 production induced by hyperosmotic stress in human bronchial epithelial cells; *Biochem. Biophys. Res. Commun.* 276 (2000) 571–578.
 10. Diaz, D., A. Scott, P. Carmichael, W. Shi, and C. Costales: Evaluation of an automated *in vitro* micronucleus assay in CHO-K1 cells; *Mutat. Res.* 630 (2007) 1–13.
 11. Marzin, D.: The position of the *in vitro* micronucleus test within the battery of screening for genotoxic potential determination and the regulatory guidelines; *Mutat. Res.* 392 (1997) 175–81.
 12. Fenech, M.: The *in vitro* micronucleus technique. *Mutat. Res.* 455 (2000) 81–95.
 13. Phelps, J.B., M.L. Garriott, and W.P. Hoffman: A protocol for the *in vitro* micronucleus test. II. Contributions to the validation of a protocol suitable for regulatory submissions from an examination of 10 chemicals with different mechanisms of action and different levels of activity; *Mutat. Res.* 521 (2002) 103–12.
 14. Ogg, C.L.: Determination of particulate matter and alkaloids (as nicotine) in cigarette smoke; *J. AOAC* 47 (1964) 356–362.
 15. Borenfreund, E. and J.A. Puerner: Toxicity determined *in vitro* by morphological alterations and neutral red absorption; *Toxicol. Lett.* 24 (1985) 119–124.
 16. Bombick, D.W. and D.J. Doolittle: The role of chemical structure and cell type in the cytotoxicity of low molecular weight aldehydes and pyridines; *In Vitro Toxicol.* 8 (1995) 349–356.
 17. Maron, D.M. and B.N. Ames: Revised methods for the *Salmonella* mutagenicity test; *Mutat. Res.* 113 (1983) 173–215.
 18. Hoffmann, D. and S.S. Hecht: Advances in tobacco carcinogenesis; in: *Chemical Carcinogenesis and Mutagenesis*, edited by C.S. Cooper and P.L. Grover, Springer-Verlag Press, New York, N.Y., 1989, pp 64–102.
 19. Kinnula, V.L., K.B. Adler, N.J. Ackley, and J.D. Crapo: Release of reactive oxygen species by guinea pig tracheal epithelial cells *in vitro*; *Am. J. Physiol. Lung Cell. Mol. Physiol.* 6 (1992) L708–L712.
 20. DeForge, L.E., A.M. Preston, E. Takeuchi, J. Kenny, L.A. Boxer, and D.G. Remick: Regulation of interleukin 8 gene expression by oxidant stress; *J. Biol. Chem.* 268 (1993) 25568–25576.
 21. Barnes, P.J.: Reactive oxygen species and airway inflammation; *Free Radical Biol. Med.* 9 (1990) 235–243.
 22. Curren, R.D., G.O. Moyer, N. Wilt, M.L. Clear, A.M. Sizemore, and G. Mun: Assessment of protocol variables in cytotoxicity assays utilizing BALB/c 3T3 cells and normal human keratinocytes; *The Toxicologist* 72 (2003) 157.
 23. Thanislak J., M. Raveendran, and H. Devaraj: Buthionine sulfoximine-induced glutathione depletion - Its effect on antioxidants, lipid peroxidation and calcium homeostasis in the lung; *Biochem. Pharmacol.* 50 (1995) 229–234.
 24. Organisation for Economic Co-operation and Development (OECD) guideline for the testing of chemicals draft proposal for a new guideline # 487: *In vitro* mammalian cell micronucleus test (MNVit); (draft version 3) (2007).
 25. National Toxicology Program: Dimethyl Sulfoxide, Study Report #229896 (1988).
 26. Maron, D., J. Katzenellenbogen, and B.N. Ames: Compatibility of organic solvents with the *Salmonella*/microsome test; *Mutat. Res.* 88 (1981) 343–350.

Corresponding author:

Manoj Misra
A.W. Spears Research Center
Lorillard Tobacco Company
420 N. English Street
Greensboro
NC 27420
E-Mail: misra@lortobco.com