GENOTOXICITY OF 2-NITROFLUORANTHENE IN HUMAN LYMPHOBLASTOID CELLS *IN VITRO*

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By

TANYA BURGAR-SULIGOJ, B.SC.

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AUTHOR: Tanya Burgar-Suligoj

SUPERVISOR: Dr. Darrell J. Tomkins

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ABSTRACT

A micronucleus assay was developed using HSC-3TO, a human continuous lymphoblastoid cell line (LCL) and cytochalasin B (Cyt-B) to block cytokinesis. Mitomycin C (MMC), a chemotherapeutic agent and an inducer of micronuclei in various human cell types, was used as a positive control. MMC induced micronuclei in HSC-3TO at levels over six times that of control cells when cells were treated with the D_{50} dose (i.e., the dose of MMC which was toxic to 50% of treated cells). It was found that harvest time was a very important variable and that a harvest time of 108 hours was optimal.

2-Nitrofluoranthene (2-NFA), a nitrated polycyclic aromatic hydrocarbon, the most abundant nitroarene found in the particulate organic matter fraction of ambient air and a potent direct-acting mutagen in the Ames Salmonella assay, was tested using the established CBMN assay protocol. Doses (20, 200 and 2000 ng/mL 2-NFA) were chosen based on the estimated yearly total body exposure to 2-NFA in Hamilton. 2-NFA was not toxic to HSC-3TO at a dose of 1000 times the estimated yearly total body exposure and did not produce elevated levels of micronuclei at a dose of 100 times the estimated yearly total body exposure.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CBMN assay	Cytokinesis block micronucleus assay
Cyt-B	Cytochalasin B
D_{10}	Dose at which 10% of cells survive
D_{50}	Dose at which 50% of cells survive
EBV	Epstein-Barr virus
GLM	General linear model
LCL	Lymphoblastoid cell line
MMC	Mitomycin C
2-NFA	2-Nitrofluoranthene
Nitro-PAH	Nitrated polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PDT	Population doubling time
PMBC	Percent micronucleated binucleate cells
POM	Particulate organic matter
%RS	Percent relative survival
sd	Standard deviation
TM	Total micronuclei
TVCN	Total viable cell number

1. INTRODUCTION

> The level of genetic integrity of human populations is increasingly under threat due to industrial activities that result in exposure to chemical and physical genotoxins.
>
> Michael Fenech, 1993a

Epidemiological studies have consistently shown an association between particulate air pollution and not only exacerbations of illness in people with respiratory disease but also excess mortality in polluted cities (Dockery et al., 1993; Seaton et al., 1995). Particulate matter is a term used to describe particles which are suspended in air. They may be produced by natural processes (pollen, salt spray, soil erosion) or by human activity (flyash, soot, cars driving over dirt roads, vehicular emissions, wind erosion, tobacco smoke) and range in size from 0.005 to 500 μm (Godish, 1991; Leutwyler, 1993). The organic constituent of atmospheric particulate matter is termed particulate organic matter, or POM. A variety of organic compounds including polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs have been found to be adsorbed to the surfaces of particles such as soot and are thus found in POM (Ramdahl et al., 1986; Godish, 1991).

Seaton et al. (1995) calculated that during a pollution episode, each lung acinus could receive an average of

30 million particles and each alveolus about 1500 particles every 24 hours with about 50% being deposited. What is not known is whether the particles themselves cause damage or whether blame belongs to the chemicals they carry deep into the lungs (Leutwyler, 1993). Many such chemicals have been found to be bacterial mutagens and warrant further testing in mammalian assays in order to assess the risk to human health.

The evaluation of the risk of an environmental agent to human health is a multidisciplinary, multi-tiered process. The first step is hazard identification, the qualitative determination of whether a chemical is causally linked to a toxic effect (NRC, 1983). The Ames Salmonella assay is widely used as a preliminary indicator of mutagenic potential because of its high sensitivity to many types of chemicals. Mammalian assays are employed to complement microbial assays because of the differences in genome organization and genetic complexity (Li and Loretz, 1991).

A widely used mammalian assay is the micronucleus test, applied *in vivo* using mice and *in vitro* using various cell types including human lymphocytes. The micronucleus test is advantageous for use in human health risk assessment because it detects chemicals which cause both chromosomal breakage and aneuploidy. Since cytogenetic damage in humans is generally associated with severe clinical disorders, it is imperative to determine if chemicals to which man may be

exposed are capable of inducing this type of genetic damage (Heddle et al., 1983). A wide range of chemicals have been found to be positive in the micronucleus test including known aneugens such as colchicine (Jenssen and Ramel, 1980; Elhajouji et al., 1995), colcemid (Heddle et al., 1983; Rudd et al., 1991), vincristine sulfate (Eastmond and Tucker, 1989; Mavournin et al., 1990; Gudi et al., 1992) and carbendazim (Jenssen and Ramel, 1980; Elhajouji et al., 1995) and known clastogens such as methyl methanesulfonate (Jenssen and Ramel, 1980; Heddle et al., 1983; Elhajouji et al., 1995) and MMC (see below). Various environmentally occurring chemicals have been tested and found to be positive including the polycyclic aromatic hydrocarbon benzo[a]pyrene (Mavournin, et al., 1990; Vian et al., 1993; Warshawsky et al., 1995), and the N-heterocyclic aromatic hydrocarbon 7H-dibenzo(c,g)carbazole (Warshawsky et al., 1995).

The *in vivo* mouse bone marrow micronucleus test may be considered relatively insensitive because the dose delivered to the target cells is dependent on the uptake, metabolism and transport of the chemicals (Miller et al., 1994). Furthermore, the dose is limited by the toxicity of the test chemical to the whole animal (*ibid*). The *in vivo* micronucleus test is also limited by its being a murine test. Although exposure of mice to pollutants can provide information on toxicity, mice are not humans and

extrapolation of data to humans cannot be done with certainty (Godish, 1991).

No assay is capable of detecting all genotoxic chemicals. All of the data pertaining to mutagenic activity are considered in providing a risk estimate for whether an agent is a potential human mutagen (Valcovic, 1991). With this framework in mind, it was the objective of this study to develop a human *in vitro* micronucleus test to screen environmental chemicals for chromosomal effects. This assay may be used in conjunction with other assays in order to predict a chemical's potential detrimental effect on human health.

1.1 Objectives

The first objective of this study was to determine a protocol for the cytokinesis block micronucleus (CBMN) assay using HSC-3TO, a human lymphoblastoid cell line and mitomycin C (MMC), a chemical known to be positive in both the *in vivo* and *in vitro* micronucleus test, and to use this assay as a screening tool for chemicals suspected of causing chromosomal damage. The second objective was to test 2-nitrofluoranthene (2-NFA), an environmental contaminant of concern, in the CBMN assay using the newly established protocol.

1.2 Human Lymphoblastoid Cells

HSC-3TO, a continuous human lymphoblastoid cell line, was chosen as the experimental cell line for this study. Infection of human B cells with Epstein-Barr virus (EBV) *in vitro* results in their immortalization and the creation of a lymphoblastoid cell line (LCL; Pope et al., 1968; Ring, 1994). Epstein-Barr virus (EBV) is a human B lymphotropic herpesvirus which is endemic in all human populations (Neitzel, 1986; Ring, 1994). Immortalization in cultured cells is defined as a set of events that leads to the continuous division of cells but not necessarily associated with mechanisms essential for malignant transformation or tumorigenicity (Khoobyarian and Marczynska, 1993). LCLs are said to be "latently" infected and the cells express only a limited number of viral genes (Ring, 1994).

The establishment and use of human LCLs is of great practical importance in human genetic toxicology for several reasons: the original material is easily obtained from any patient, EBV-transformed cell lines exhibit chromosomal stability up to high passages, LCLs have minimal media requirements for growth in suspension and can thus be cultivated up to high density with minimal work and cost. LCLs are also always available because of their continuous proliferation (Neitzel, 1986). Lymphocytes have a limited lifespan and new samples may be difficult or impossible to obtain if patients are no longer available (*ibid*).

1.3 The Cytokinesis Block Micronucleus (CBMN) Assay

Historically, the basis of the micronucleus assay was the identification of Howell-Jolly bodies in erythrocytes in hematological practice. Although mature erythrocytes do not contain DNA, chromosomal fragments from broken chromosomes or chromosomes which lag behind due to abnormal segregation during cell division may be included in daughter nuclei. Main nuclei are expelled from the mature erythrocytes but secondary nuclei from the fragments or lagging chromosomes are retained as Howell-Jolly bodies, or "micronuclei" (Schmid, 1975; Heddle et al., 1983).

The micronucleus test was developed as a simpler and faster alternative to metaphase chromosome analysis. This test detects not only chromosome breakage (clastogenesis) but loss of entire chromosomes (aneuploidy; Schmid, 1975).

Clastogenesis is defined as chromosome or chromatid breakage. Many chemicals are known to break chromosomes and are termed *clastogens*. A series of mechanisms, many of them still unknown, play a role in chromosome breakage (Natarajan, 1984; Savage, 1990; Therman and Susman, 1993). The first event is some sort of DNA damage termed a *lesion*. The damage may lead directly to chromosome breakage or one of many aberrations may occur: base alkylation, inter- and intrastrand cross-linkage, or intercalation, an insertion of the mutagen in between base pairs in the double helix. The lesions may go through DNA replication where they may

undergo repair or misrepair by various repair systems. Misrepair can lead to chromosome breakage.

Aneuploidy is defined as a change in the number of chromosomes other than an exact multiple of the haploid number (Therman and Susman, 1993; Parry et al., 1995). Aneuploidy may be induced by chemical action upon a wide variety of cellular targets involved in cell division as well as the chromosomes themselves (Parry et al., 1995). Consequently its induction may not always be correlated with gene mutation or chromosomal aberration induction, where the predominant site of action is on the DNA (*ibid*).

Micronucleus frequency, as a measure of cytogenetic damage, was first used in plant cells (Evans et al., 1959). The micronucleus test was further developed in the early 1970's and extended to animal cells (Heddle et al., 1983; Garewal et al., 1993). The work of von Ledebur and Schmid (1973) was particularly important from a historical perspective because it led directly to the development of the simple *in vivo* test based on the identification of micronuclei in the polychromatic erythrocytes (PCE's) of mouse bone marrow (Schmid, 1975).

Practical and ethical reasons limit the human tissues in which micronuclei can be assessed to exfoliated epithelial cells and peripheral blood cells (Fenech, 1991). In addition to human peripheral blood lymphocytes which have been used extensively in the micronucleus assay, human

lymphoblastoid cells have recently been used successfully in the *in vitro* micronucleus assay (Zhang et al., 1995).

Expression of micronuclei is dependent on one cell division subsequent to the chemical insult. Furthermore micronuclei are lost in further cell divisions. As a result, the conventional micronucleus technique is imprecise because the cells which have undergone one and only one division and the micronuclei in them cannot be identified separately from the total population of lymphocytes (Fenech and Morley, 1985). In order to ensure only those cells which have completed one division are scored for micronuclei, Fenech and Morley (1985) developed the cytokinesis block micronucleus (CBMN) assay in which cells are blocked in cytokinesis by cytochalasin B (Cyt-B). The resulting easily recognizable binucleate cells, which have undergone only one division, are the only cells scored for micronuclei. Fenech (1991) compared estimates of the micronucleus frequency obtained by treating human lymphocytes with mitomycin C using the cytokinesis block method and the conventional method. The results clearly demonstrated that the conventional assay grossly underestimates micronucleus induction.

Cyt-B is one of a group of fungal metabolites which paralyze many different types of cell movements including cell locomotion, phagocytosis and cytokinesis (Carter, 1967). Cyt-B, which inhibits the polymerization of actin

(MacLean-Fletcher and Pollard, 1980; Cooper, 1987; Ohmori et al., 1992), blocks cytokinesis (cytoplasmic division) by preventing the formation of the contractile ring. The contractile ring, a cytoskeletal structure of actin filaments, pulls the plasma membrane inward, dividing the cell in two during cytokinesis (Alberts et al., 1989). Cyt-B does not inhibit the microtubule-dependent separation of chromosomes during nuclear division (Alberts et al., 1989), nor does it cause chromosome abnormalities or cell toxicity (Carter, 1967; Ridler and Smith, 1968; Prosser et al., 1988). Cyt-B has not been found to increase the frequency of micronucleated binucleate cells in human lymphocytes (Fenech and Morley 1985; Lindholm et al., 1991), although reports of Cyt-B's role in micronuclei induction in whole blood cultures are conflicting (Prosser et al., 1988; Surralles et al., 1992; Surralles et al., 1994), perhaps because the influence of erythrocytes in the CBMN assay has not been well evaluated (Surralles et al., 1994).

Parry et al. (1995) outlined several recommendations made by a UK Environmental Mutagen Society working group for the testing of chemicals in the micronucleus assay:

1. Preliminary assessment of the cytotoxicity of the test material should be made before selecting concentrations to be tested for the induction of micronuclei.

2. Normally, at least duplicate cultures for each concentration tested are established and the assay should be repeated in time; a second experiment need not be an exact replicate but may examine slightly different concentrations, treatment times or sampling times.
3. The criteria for scoring micronuclei of Countryman and Heddle (1976) should be used (outlined in Methods and Materials) and scoring should only be done in cells with intact cytoplasm.

A practical use of the CBMN assay is for biological dosimetry of exposure to genotoxic agents. Genotoxic agents present in the environment can be ingested, inhaled, or absorbed through the skin and mucous membranes (Workman and Livingston, 1993). It is important to develop a simple and reliable technique for biological dosimetry of exposure to genotoxic agents because such exposures may lead to carcinogenic events (Fenech, 1993b).

There is a connection between the carcinogenicity of various agents and their ability to break chromosomes (Therman and Susman, 1993). This inference is based on the widespread occurrence of chromosomal abnormalities in cancer cells (Mitelman, 1988). The best-known example of a chromosome aberration associated with malignancy is the Philadelphia chromosome (a G chromosome with about half of its long arm missing), first described in chronic

myelogenous leukemia (CML) by Nowell and Hungerford (1960). Individuals having genetic syndromes in which chromosomal breakage rates are elevated are also characterized by an increased risk of developing cancer (German, 1983). A role for chromosome breakage, translocation or loss is implicated in the sequence of events leading to the development of neoplasia, as such changes can activate oncogenes or result in the loss of tumor anti-oncogenes or suppressor genes (Cooper, 1984; Flier et al., 1988).

Epidemiological studies of cancer in humans are frequently limited by low prevalence rates. Multidisciplinary studies that evaluate the results of different endpoints related to the carcinogenic process may provide a useful surrogate to epidemiology to identify the risk associated with genotoxicants (Anwar, 1991). Proper and relevant methods for genotoxicity assessment need to be used both at the experimental and at the human exposure assessment levels (*ibid*). These levels of testing (*ibid*) using 2-NFA as a relevant example are:

1. **External exposure:** environmental monitoring of 2-NFA in air
2. **Internal absorption:** biological monitoring of 2-NFA or 2-NFA metabolites in blood
3. **Biological effect:** genetic monitoring such as *in vivo* measurements of micronuclei induction by 2-NFA
4. **Clinical Response:** epidemiological effects of 2-NFA

The CBMN assay for the detection of micronuclei in human lymphoblastoid cells is a practical, inexpensive technique which may serve as an experimental method at the "biological effect" level for the assessment of genotoxicity of environmental chemicals of concern.

1.4 Mitomycin C (MMC): A Positive Control

Mitomycin C (MMC) is an antibiotic produced by several Streptomyces species (Szybalski and Iyer, 1964) which is a direct-acting mutagen in the Ames Salmonella assay (McCann et al., 1975; Levin et al., 1982; Quillardet et al., 1985). MMC mutagenesis is unique in that it reverts only strains which have a functional excision repair system (Levin et al., 1982).

Dose-dependent cytotoxicity of MMC has been demonstrated in numerous cell types including cultured porcine pulmonary artery endothelium (Hoorn et al., 1995), human fibroblasts (Rudd et al., 1991) and human peripheral lymphocytes (Mark et al., 1994).

MMC causes various types of chromosome aberrations in human lymphocytes including chromosome breakage (Morad et al., 1973; Shiraishi et al., 1979; Woods et al., 1995) and sister chromatid exchange (SCEs; Shiraishi et al., 1979; Mark et al., 1994; Woods et al., 1995). MMC-induced lesions and SCE in human lymphocytes have also been shown to

persist for at least two and up to three successive cell generations (Daza et al., 1992).

MMC has been shown to induce micronuclei in a variety of human cell types including human T4-, T8-, and B-cells (Holmen et al., 1994), human lymphocytes (Elhajouji et al., 1995; Migliore et al., 1990), human keratinocytes (van Pelt et al., 1991) and human fibroblasts (Rudd et al., 1991).

MMC is known to produce a reversible arrest and/or delay in S phase and in G2 (Mauro et al., 1986). As a consequence of this MMC-induced mitotic delay, harvest time has been shown to be very important when assaying for MMC-induced damage (Morad et al., 1973; Ceccherini et al., 1988; Armstrong et al., 1992). Hoorn et al. (1995) demonstrated that cultured porcine pulmonary artery endothelium cells experienced significant cross-linking and inhibition of proliferation when exposed to concentrations of MMC which caused little or no cytotoxicity, suggesting that DNA cross-linking may be implicated in the antimitotic effects of MMC.

The formation of DNA cross-links by MMC was first demonstrated by Iyer and Szybalski (1963). In studies using *B. subtilis*, they found that the degree of cross-linking increased with increasing concentration of MMC, or increasing periods of exposure and that the continuous presence of MMC in the cultures was not a requirement for persistence of the observed effects on the DNA.

MMC is proposed to be metabolized by the enzyme DT-diaphorase, a two-electron reductase, to a metabolite capable of alkylating DNA (Siegel et al., 1992; Prakash et al., 1993). The major covalent adducts formed by MMC both *in vitro* and *in vivo* have been isolated and characterized. Two monoadducts and two bisadducts have been discerned with one of the bisadducts representing interstrand cross-links and the other originating from intrastrand cross-links in DNA (Basu et al., 1993). Monadducts are generally formed in comparable proportions to cross-links (*ibid*). MMC monoadducts are strong blocks of DNA replication *in vitro* and are thus likely to be toxic lesions *in vivo* (*ibid*).

MMC is an inducer of micronuclei in various human cell types. MMC has been used consistently in numerous laboratories as a positive control in both the *in vivo* and *in vitro* micronucleus assay. On the basis of its widely accepted association with micronucleus induction, it was chosen as a positive control for the detection of clastogenesis and/or aneuploidy in the development of an *in vitro* CBMN assay using HSC-3TO.

1.5 2-NFA: Environmental Contaminant of Concern

A major class of nitro-PAHs identified in anthropogenic emissions (Paputa-Peck et al., 1983) and urban POM (Ramdahl et al., 1986) are the nitropyrenes and nitrofluoranthenes. 1-nitropyrene and 3-, 7- and 8-nitrofluoranthene have been

identified in diesel and gasoline exhaust (Paputa-Peck et al., 1983; Ciccioli et al., 1989). 2-Nitrofluoranthene (2-NFA) has, to date, never been identified as having come from combustion sources (Ramdahl et al., 1986; Ciccioli et al., 1989; Tokiwa et al., 1993). However, 2-NFA is formed in the atmosphere by the gas-phase reaction of fluoranthene with N_2O_5 (Sweetman et al., 1986; Atkinson et al., 1990) and by the gas-phase reaction of OH radicals with fluoranthene in the presence of NO_x (Arey et al., 1986; Atkinson et al., 1990).

2-NFA is the most abundant nitro-PAH in ambient POM in cities throughout the world, including cities in southern California (Zielinska et al., 1989; Arey et al., 1987), Aurskog, St. Louis, Washington, D.C. (Ramdahl et al., 1986) and Hamilton (Legzdins et al., 1994b). The concentration of 2-NFA in air in Hamilton, Ontario (0.006 ng/m^3; Legzdins et al., 1994b) is considerably lower than levels found in Torrance, California (0.3 ng/m^3; Arey et al., 1987) and in Milan, Italy (0.4 ng/m^3; Ciccioli et al., 1993).

2-NFA is considered a potential human health hazard for several reasons, but primarily because of its association with inhalable air particulate. Inhalable air particulate refers to particles (and consequently their adsorbed 2-NFA) which have a diameter of less than 10 μm (Godish, 1991). Fine particulate air pollution includes particles less than 2.5 μm (Dockery et al., 1993). Fine particles are thought

to pose a particularly great risk to health because they are more likely to be toxic than larger particles and can be breathed more deeply into the lungs (Dockery et al., 1993).

2-NFA is a potent direct-acting mutagen in the Ames Salmonella assay. The mutagenicity and metabolism of 2-NFA in bacteria has been studied using various strains of *Salmonella typhimurium*. TA98 is the standard tester strain TA1535 (Ames et al., 1975) with a resistance transfer factor (R factor) contained in the plasmid pKM101 (McCann et al., 1975). TA98 contains the classical bacterial nitroreductase and O-acetyltransferase enzymes. TA98 contains the *hisD3052* mutation in the *hisD* gene coding for histidinol dehydrogenase (Ames et al., 1975; Maron and Ames, 1983). The *hisD3052* mutation has the sequence -C-G-C-G-C-G-C-G- near the site of a -1 frameshift mutation in the hisD gene (Gletten et al., 1975). TA98 detects various frameshift mutations (Ames et al., 1975; Maron and Ames, 1983). TA98 also contains the mutation *rfa* which causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases the permeability to large molecules such as benzo[a]pyrene that do not permeate the normal cell wall efficiently (Ames et al., 1973). TA98 also contains the mutation *uvrB*, a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens (Ames et al., 1973). Strains TA98NR and TA98/1,8-DNP are TA98 strains which are

deficient in nitroreductase (McCoy et al., 1981) and O-acetyltransferase enzymes (McCoy et al., 1983) respectively. Strain YG1021 was developed by Watanabe et al. (1989) by cloning the nitroreductase gene of *Salmonella typhimurium* into pBR322 and introduced the plasmids carrying the gene into TA98. The resulting strain, YG1021, overproduced nitroreductase and was extremely sensitive to the mutagenic action of many nitroarenes. Strain YG1024 was also developed by Watanabe et al. (1990) by cloning the acetyltransferase gene of *Salmonella typhimurium* into pBR322 and introduced the plasmids carrying the gene into TA98. The resulting strain, YG1024, overproduced acetyltransferase and was extremely sensitive to the mutagenic action of many nitroarenes and aromatic amines.

The direct-acting (i.e., without S9) mutagenic response of 2-NFA has been found to be greater in strain TA98 than in TA98NR and TA98/1,8-DNP (Zielinska et al., 1987; Shane et al., 1991; Ball et al., 1994), indicating an important role for the nitroreductase and O-acetyltransferase enzymes in the mutagenicity of 2-NFA in bacteria. The importance of these two enzymes is further supported by the heightened mutagenic response of 2-NFA in strains YG1021 and YG1024 over that in TA98 (Ball et al., 1994, Legzdins et al., 1994a, 1994b).

2-NFA has been identified as a major source of mutagenic potency in Hamilton air particulate (Legzdins et

al., 1994a). Air particulate samples were collected on filters at an air quality monitoring station in downtown Hamilton. A subfraction of the non-polar extract (containing high molecular weight PAH and nitro-PAH) was prepared from the filters and analyzed for mutagenic activity. Using strain YG1021, 2-NFA was identified as the major peak of mutagenicity and accounted for approximately half of the mutagenic activity detected in the subfraction.

Data on the mutagenic response of 2-NFA in TA98, TA98NR and TA98/1,8-DNP with S9 are conflicting (Zielinska et al., 1987; Shane et al., 1991). Zielinska et al. (1987) identified two 2-NFA metabolites formed from the incubation of 2-NFA with S9 and tested these metabolites in the Ames assay using strains TA98, TA98NR and TA98/1,8-DNP with and without S9. 9-hydroxy-2-nitrofluoranthene expressed mutagenicity in strain TA98 which was elevated over that of 2-NFA indicating that the 9-hydroxy isomer cannot be viewed as a detoxification product. 8-hydroxy-2-nitrofluoranthene expressed mutagenicity in strain TA98 which was lower than that of 2-NFA. Both 8- and 9-hydroxy-2-nitrofluoranthene expressed a five-fold lower direct-acting mutagenicity in strains TA98NR and TA98/1,8-DNP. These results indicate that the mutagenicity of the metabolites were dependent upon nitroreduction and further O-acetylation of the resulting N-hydroxyarylamine intermediates. The mutagenicities of 8- and 9-hydroxy-2-nitrofluoranthene were substantially

suppressed by the addition of S9 in all three test strains suggesting that further oxidation of 8- and 9-hydroxy-2-nitrofluoranthene by S9 leads to less mutagenic or non-mutagenic products.

Chemicals which induce frameshift mutations due to the formation of covalent DNA adducts either as a result of base-displacement mechanism or because of reaction in the DNA groove are active primarily in strain TA98 (Rosenkranz and Mermelstein, 1983). 2-NFA expresses direct-acting mutagenicity in strain TA98, and DNA adducts have been found in both bacterial and mammalian DNA after treatment with 2-NFA. Using ^{32}P-postlabelling analysis, Herreno-Saenz et al. (1994) identified N-(deoxyguanosin-8-yl)-2-aminofluoranthene, a DNA adduct formed in both *Salmonella typhimurium* TA98 and the DNA isolated from the liver of neonatal mice treated with 2-NFA.

Several mono- and di-nitrofluoranthenes have been tested in the mouse micronucleus assay. Differences in micronucleus induction between chemically treated and untreated mice were observed for 3,7-diNFA and 3,9-diNFA (Tokiwa et al., 1988). Micronucleus induction by 3-NFA was not observed (*ibid*).

There is clearly a need to test for the genotoxicity of 2-NFA in humans at the "biological effect" level. 2-NFA has thus far not been tested for its ability to induce micronuclei. Its status as an environmental contaminant of

concern makes it a good candidate for genotoxic testing in the established CBMN assay protocol. If 2-NFA induces either clastogenesis or aneuploidy, the resulting chromosome fragments or chromosomes should be captured as micronuclei.

The detailed objectives of this study were as follows. In order to establish the present CBMN assay, several variables, the growth of the HSC-3TO, the optimal concentration of Cyt-B, the optimal duration of Cyt-B treatment and the optimal experimental seeding density, had to be determined. The growth of MMC-treated cells was investigated in order to establish if MMC induced mitotic delay. MMC-induced mitotic delay was accounted for when assaying for micronuclei-induction. Having established a CBMN assay using MMC as a valid positive control, it was necessary to optimize the assay using 2-NFA. This was done by examining 2-NFA-induced toxicity and mitotic delay in HSC-3TO and accounting for these variables in the CBMN assay protocol.

2. MATERIALS AND METHODS

2.1 Cell Lines

HSC-3 is a human lymphoblast cell line which was derived by Ishida and Buchwald (1982) from the peripheral blood lymphocytes of a healthy adult male donor. HSC-3 was subsequently transformed by Epstein-Barr virus (EBV) into a lymphoblastoid cell line and made 6-thioguanine-resistant (HSC-3T) and ouabain-resistant (HSC-3TO; Ishida and Buchwald, 1982; Buchwald, personal communication).

TK6 (H2BT), a human lymphoblastoid cell line heterozygous at the TK locus ($TK^{+/-}$; Skopek et al., 1978), was isolated from WI-L2, a human lymphoblastoid cell line which was established from the spleen of a 5-year-old patient having hereditary spherocytic anemia (Levy et al., 1968). Why WI-L2 emerged as a continuous cell line is unknown. There is no direct evidence that these cells are indeed tumor cells (*ibid*). TK6 cells have a deficiency in O^6-alkylguanine-DNA alkyltransferase (AGT) activity (Goldmacher et al., 1986; Bronstein et al., 1991). This deficiency does not allow for the removal of O^6-alkylguanine DNA adducts. TK6 is competent in nucleotide excision repair (NER; Bronstein et al., 1991).

The HSC-3TO subpopulation of verified HSC-3TO used in this thesis was obtained by selecting HSC-3TO cells for 6-thioguanine- and ouabain-resistance. This was done as an extra precaution to ensure only HSC-3TO cells were present in culture. The cell population was selected for by growing HSC-3TO in selection media containing concentrations of ouabain (1×10^{-6} mol/L) and 6-thioguanine (1×10^{-5} mol/L) which were not toxic to HSC-3TO but toxic to TK6. Cells were seeded at 1×10^5 cells/mL in 30 mL of selection media and allowed to proliferate for one week after which they were maintained in regular media.

2.2 Culturing of Lymphoblastoid Cells

HSC-3TO and TK6 cells were maintained in RPMI-1640 medium (Department of Pathology, McMaster University) supplemented with 1% L-glutamine (Department of Pathology, McMaster University), 1% Hepes buffer (Department of Pathology, McMaster University) and 10% fetal bovine serum (Hyclone, catalogue #A-1115-L), henceforth referred to as media. Cells were maintained at concentrations of 2.5×10^5 and 1×10^5 cells/mL respectively in 30 mL in T-75 culture flasks (Corning catalogue #25110-75) and kept in a 37°C, humidified, 5% CO_2 incubator. All tissue culture work was performed in a containment level 2 laminar flow hood (Medical Research Council of Canada, 1989).

Cells were seeded for routine maintenance by transferring cells to a 50 mL centrifuge tube (Falcon catalogue #4-2070-1) and centrifuging at 1000 RPM (180 x g) for 10 minutes (International Equipment Co. IEC Centra-7 Centrifuge), henceforth referred to as centrifuging. The supernatant was aspirated and the cells were resuspended in 5 mL of fresh prewarmed medium by vortexing. An aliquot was transferred from the tube into a counting tube (Sarstedt catalogue #55.476.005). A 1:10 dilution was prepared by adding 20 μL of cells to 180 μL of phosphate buffered saline (Gibco catalogue #14190-029). Twenty μL of this 1:10 dilution was added to 20 μL of trypan blue (Gibco catalogue #630-5250PE) to give a 1:20 dilution of the original aliquot of cells. Aliquots of this final cell suspension were placed in both sides of a hemacytometer (SPotlite Hemacytometer; American Scientific Products catalogue #B3175) and eight large squares were counted at 400X magnification using a Leitz Laborlux 12 light microscope. The number of viable cells per millilitre in the original culture was calculated as follows:

$$TVCN = (TLC \div 8 \text{ Squares}) \times 10^{4*} \times 20^{**}$$

Where:

TVCN = Total Viable Cell Number/mL
TLC = Total number of Live Cells
* = dilution factor per large square of hemacytometer
** = dilution factor per millilitre of cell suspension

Cells were prepared for freezing in liquid nitrogen or in a -70°C freezer using the same procedure and were frozen at a density of 1 x 10^7 cells in 1 mL in a 2 mL plastic cryovial (Nalgene catalogue #5000-0020) in media supplemented with 10% dimethyl sulfoxide (DMSO; BDH). Cells were immediately placed in a -70°C freezer in a plastic container to allow freezing at approximately -1°C per minute. Cells were either kept in the -70°C freezer or transferred after 1-7 days to liquid nitrogen for long-term storage at -180°C.

Cells were recovered from frozen vials by rapidly thawing vials in a 37°C water bath and adding the 1 mL aliquot to 10 mL of prewarmed media in a 15 mL centrifuge tube (Falcon catalogue #4-2095-5). The tube was centrifuged, medium removed and cells resuspended in 1 mL of medium. Cells were counted using a hemacytometer and seeded at the desired density in a T-25 (Falcon Catalogue #3014) or T-75 culture flask.

It should be noted that all experiments utilized HSC-3TO. Cells were seeded at 2.5 x 10^5 cells/mL 48 hours prior to experimental set-up, unless otherwise stated.

2.3 Growth Determination Experiments

Growth curve experiments were performed in order to determine the long-term (10-11 days) and short-term (48 hours) growth characteristics of HSC-3TO. Three 15 mL

centrifuge tubes were prepared for each counting time. Cells were seeded at 2.5 x 10^5 cells/mL in a sufficient volume and 5 mL of this mass culture was aliquoted to each 15 mL centrifuge tube. Tubes, with caps loosened to allow for gas exchange, were placed in the incubator. Cells in short-term growth determination experiments were counted every 4 hours for 28-48 hours. Cells in long-term growth determination experiments were counted at 4 hours after seeding and every 24 hours for 240-264 hours (10-11 days). Counting involved centrifuging cells in the 15 mL centrifuge tubes, removing media, adding 1 mL of fresh media, preparing a 1:2, 1:4 or 1:10 dilution and counting cells in a hemacytometer.

2.4 Optimization of the CBMN Assay

Three experiments were performed in order to determine the optimal conditions for the CBMN assay. Cyt-B (Sigma Catalogue #C 6762) was prepared by adding 2.5 mL of filtered DMSO to the original ampule containing 5 mg of Cyt-B to make a stock solution at a concentration of 2 mg/mL. The entire solution was then filtered and dispensed in 75 μL aliquots to 2 mL plastic vials and stored at -20°C.

The purpose of the first experiment was to determine the dose of Cyt-B which would provide the highest percent binucleate cells. Five doses of Cyt-B were tested: 0, 1.5, 3, 6, 12 μg/mL. Triplicate cultures were prepared for each

dose of Cyt-B. Cells were seeded at 5 x 10^5 cells/mL (twice the final density) in a sufficient volume and 2.5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. Cyt-B was added to the cultures upon seeding and the final volume of the cultures brought up to 5 mL with media. Cultures were harvested after 32 hours and slides were made and scored for percent binucleate cells as described below (see Section 2.5.3).

The purpose of the second experiment was to determine the duration of Cyt-B exposure which produced the highest percent binucleate cells. Triplicate cultures were prepared for each duration of Cyt-B exposure. Cells were seeded at 2.5 x 10^5 cells/mL in a sufficient volume and 5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. Cells were treated with 3 μg/mL Cyt-B upon seeding as described below (see Section 2.5.2). Cells were harvested after 24, 28, 32 and 36 hours and scored for percent binucleate cells as described below (see Section 2.5.3).

The third experiment was performed to determine the experimental seeding density which produced the highest percent binucleate cells. Triplicate cultures were prepared for each seeding density. Cells were seeded at 1 x 10^5, 2.5 x 10^5 and 5 x 10^5 cells/mL in sufficient volumes and 5 mL of each cell suspension aliquoted to 15 mL centrifuge tubes. Cells were treated with 3 μg/mL Cyt-B upon seeding as described below (see Section 2.5.2). Cells were

harvested after 24 hours and scored for percent binucleate cells as described below (see Section 2.5.3).

2.5 MMC Experiments

2.5.1 Dose-Response Experiments

The purpose of the dose-response experiments was to determine the toxicity of MMC under various experimental conditions. For a summary of the protocols used in the dose-response experiments, see Table M1. Toxicity was measured by determining the D_{10} and D_{50} doses, the doses of MMC which caused 10% and 50% relative survival respectively.

MMC (Sigma catalogue #M 0503) was prepared by adding 2 mL of 70% ethanol (Commercial Alcohols Inc.) to the original ampule containing 2 mg MMC to make a solution at 1 mg/mL. This solution was pipetted up and down approximately 50 times to dissolve the MMC in the ethanol. This solution was filtered into a 50 mL centrifuge tube containing 38 mL alpha-MEM medium (Gibco Catalogue #12571-030) to make a solution at 50 μg/mL. This solution was inverted several times, vortexed and dispensed in 1 mL aliquots into 2 mL plastic vials and stored at -20°C.

Group A Experiments:

Cells were seeded at 2 x 10^5 cells/mL (twice the experimental seeding density) in a sufficient volume and dispensed in 0.5 mL aliquots to each well of a 24-well

Table M1: Summary of Dose-Response Protocols Used to Evaluate the Growth of HSC-3TO in MMC

Group	Seeding Density (per mL)	Seeding Volume	Seeding Vessel	#cells/ng MMC at 1000 ng/mL	Length of Exposure	Duration of Allowed Growth
A	1×10^5	1 mL	24-well plate	100	Continuous	5-6 days
B	1×10^5	Treated in 10 mL; Seeded in 1 mL	Treated in 15-ml centrifuge tube for 24 hours. Cells reseeded at 1×10^5 viable cells/mL in 24-well plate.	100	24-hours	6 days
C	1×10^5	1 mL	24-well plate	100	Continuous	48 hours
D	1×10^5	5 mL	15-mL tube	100	Continuous	48 hours
E	2.5×10^5	5 mL	15-mL tube	250	Continuous	48 hours
F	2.5×10^5	5 mL	T-25 flask	250	Continuous	48 hours
G	2.5×10^5	5 mL	15-mL tube	250	Continuous	108 hours

tissue culture plate. Four replicates of each of six doses of MMC were tested: 0, 10, 30, 100, 300, and 1000 ng/mL. Doses were prepared at twice the experimental concentration by serial dilution of the MMC stock in media in volumes sufficient for four replicates and 0.5 mL of each dose was aliquoted to each of the appropriate four wells. The final experimental volume was 1 mL. The plates were placed in the incubator and the colour of the media in each well recorded daily. Cells were counted when the media for the first three doses (0, 10 and 30 ng/mL MMC) turned yellow (4-6 days).

Counting involved pipetting the cells in each well up and down approximately 20 times with a p1000 pipette and transferring the cells to counting tubes. A different pipette tip was used for each well. Cells were counted by preparing a 1:2, 1:4 or 1:10 dilution and counting the diluted cells in a hemacytometer.

Group B Experiment:

Cells were seeded at 2.0 x 10^5 cells/mL (twice the experimental seeding density) in a sufficient volume and dispensed in 5 mL aliquots to 15 mL centrifuge tubes. Cells were treated with MMC in mass cultures which were later reseeded into a 24-well plate. MMC doses (0, 100, 500, 1000, 1500 and 2000 ng/mL) were prepared by serial dilution of the MMC stock in media at twice the experimental concentration in sufficient volumes; 5 mL of each dose was

dispensed to the appropriate 15 mL centrifuge tubes. The final experimental volume was 10 mL. Tubes were vortexed, caps loosened and placed in the incubator. After 24 hours cells treated with each dose of MMC were reseeded at 1.0×10^5 (viable) cells/mL in 5 mL and 1 mL of cell suspension was aliquoted to each of four replicate wells. The rest of the protocol followed exactly that of Group A.

Group C Experiment:

Group C experiment was prepared according to the protocol of the Group A experiments except rather than waiting for the media of the first three doses to turn yellow, cells were counted after 48 hours.

Group D Experiment:

Cells were seeded at 2×10^5 cells/mL (twice the experimental seeding density) in a sufficient volume and dispensed in 2.5 mL aliquots to 15 mL centrifuge tubes. Two replicates of each of five doses of MMC were tested: 0, 100, 500, 1000 and 2000 ng/mL. Doses were prepared by serial dilution of the MMC stock in media at twice the experimental concentration in volumes sufficient for two replicates and 2.5 mL of each dose was dispensed to the appropriate 15 mL centrifuge tubes. The final experimental volume was 5 mL. The tubes were then placed in the incubator. After 48 hours of allowed growth, cells were counted. Cells were counted by centrifuging the tubes, removing media, adding 1 mL of fresh media, resuspending and

performing a 1:2 dilution and counting the diluted cells in a hemacytometer.

Group E Experiment:

Group E was performed using the protocol of Group D except that the experimental seeding density was 2.5×10^5 cells/mL and doses of MMC used were 0, 40, 400, 1000 and 2000 ng/mL.

Group F Experiment:

Group F was performed using the protocol of Group E except that the seeding vessel used was a T-25 flask rather than a 15 mL centrifuge tube.

Group G Experiment:

Group G was performed using the protocol of Group E except that cells were counted after 108 hours rather than 48 hours and doses of MMC used were 0, 10, 100 and 1000 ng/mL.

2.5.2 Modified Growth Determination Experiments

Modified growth determination experiments were performed in order to determine if MMC causes mitotic delay at the doses tested and to determine if Cyt-B effectively blocks cytokinesis in cells treated with various doses of MMC for different durations of time. Two modified growth determination experiments were performed, each containing slightly different variables.

Modified Growth Experiment I:

Replicate cultures were prepared for two counting times, 24 and 48 hours, for each of three doses of MMC (0, 100 and 1000 ng/mL) without Cyt-B and with 3 μg/mL Cyt-B. Cells were seeded at 2.0 x 10^5 cells/mL (twice the experimental seeding density) in a sufficient volume and 2.5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. MMC doses were prepared by serial dilution of the stock MMC in media at two times the final dose and 2.5 mL was added to the appropriate 15 mL centrifuge tubes. Three μg/mL Cyt-B was added to the appropriate tubes upon seeding. In order to treat cells with Cyt-B at a concentration of 3 μg/mL, each 75 μL aliquot of stock Cyt-B was first diluted 10-fold to a 200 μg/mL solution by adding 675 μL phosphate buffered saline (PBS; McMaster University, Department of Pathology). This solution was further diluted to a final concentration of 3 μg/mL by adding 75 μL to each 5 mL culture. After each specified growth time, tubes were centrifuged, the old media was aspirated and cells were resuspended in fresh media. A small volume (approximately 20 μL) was removed from each tube and placed in a counting tube. Cells were counted by preparing a 1:2 dilution and counting the diluted cells in a hemacytometer. Total viable cell numbers and standard deviations were calculated.

Modified Growth Experiment II:

Modified growth experiment II was performed using the protocol of modified growth experiment I with the following modifications. The final total viable cell number was 2.5×10^5 cells/mL and the counting times were 72 and 108 hours.

2.5.3 CBMN Assay

Doses of MMC used for all CBMN experiments were 0, 100 and 1000 ng/mL. Three μg/mL Cyt-B was used for all CBMN experiments and added to cultures as outlined above (see Section 2.5.2).

Experiments #1 and #2:

Cells were seeded at 5×10^5 cells/mL (twice the final density) in a sufficient volume and 2.5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. Three replicates were set up for each dose of MMC. MMC doses were prepared at twice the final concentration by serial dilution of the MMC stock in media and 2.5 mL of each dose was added to the appropriate tubes, making the final experimental volume 5 mL. Cells were treated with Cyt-B upon seeding as described above (see Section 2.5.2). Tubes were vortexed, their caps loosened and placed in the incubator. After 24 hours (Experiment #1) or 48 hours (Experiment #2) cells were harvested and slides were made as described below.

Experiment #3:

Cells were seeded at 5 x 10^5 cells/mL (twice the final density) in a sufficient volume and 2.5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. Three replicates were set up for each dose of MMC. MMC doses were prepared at twice the final concentration by serial dilution of the MMC stock and 2.5 mL of each dose was added to the appropriate tubes, making the final experimental volume 5.0 mL. Tubes were vortexed, their caps loosened and placed in the incubator. After 72 hours, cells were treated with Cyt-B as described above (see Section 2.5.2) and placed back in the incubator. After 108 hours, cells were harvested and slides were made and scored as described below.

Harvesting Cultures:

Cells were first washed by centrifuging the tubes, removing old media, adding 5 mL of PBS to each tube and vortexing. Tubes were centrifuged again, the PBS was removed, 5 mL of hypotonic (0.075 mol/L KCl) was added to each tube and the tubes were vortexed. The tubes were then placed in the 37°C incubator for 10 minutes. Tubes were centrifuged again, the hypotonic was removed and 5 mL of fix (5:1 methanol:glacial acetic acid) was added to each tube after which they were placed in the refrigerator (4°C) for 30 minutes. Tubes were centrifuged again, the old fix was removed, 5 mL of fresh fix was added and the tubes were

placed back in the refrigerator for the night. Slides were made 1-7 days after refrigeration.

Slide making:

During the slide-making procedure, all tubes and slides (Clay Adams catalogue #3017) were kept on ice. Prior to use, slides were wiped clean with a Kimwipe and placed in 100% methanol or 95% ethanol in the -20°C freezer. On the day of use, slides were transferred to 0°C double distilled H_2O. Experimental tubes which had been stored in the refrigerator were centrifuged, the old fix was removed and 5 mL of fresh fix was added. The pelleted cells were broken up by first tapping on the tube and then running the tubes gently along a tube rack and pipetting up and down 100 times with a siliconized pasteur pipette. With the same pipette, a drop of cells was dropped from a height of approximately 2 cm onto a slide held at a 45° angle. Slides were tapped on either side to remove excess fix and placed on a slide warmer (Fisher Scientific) set at medium heat. The dried slides were stained with 5% Giemsa (BDH) in 10% Gurr buffer (BDH) in a Coplin jar for 10 minutes. Slides were rinsed with double distilled H_20 and placed on the slide warmer to dry.

Slide scoring:

The percent yield of binucleate cells was determined by counting 100 cells and recording them as being either binucleate or mononucleate cells. Cells with two distinct

nuclei and intact cytoplasm were scored as binucleate while cells with one distinct nucleus and intact cytoplasm were scored as mononucleate. Three slides from each replicate were scored.

One thousand binucleate cells were scored from each replicate of each dose for micronuclei. Criteria for scoring micronuclei were adapted from Countryman and Heddle (1976) and were as follows:

1. Diameter less than 1/3 the main nucleus
2. Non-refractibility (to exclude small stain particles)
3. Colour the same as or lighter than the main nuclei (to exclude large stain particles)
4. Location within the cytoplasm of the binucleate cell

An example of a micronucleated binucleate cell which fits these criteria is presented in Figure M1.

2.6 2-Nitrofluoranthene Experiments

2.6.1 Dose-Response Experiments

The purpose of the dose-response experiments was to determine the doses at which 2-NFA was toxic to HSC-3TO. A wide range of doses of 2-NFA was tested in preliminary dose-response experiments. Cells were seeded at 1×10^5 cells/mL in a sufficient volume and 1 mL of this cell suspension was aliquoted to each well of a 24-well tissue culture plate. Two replicates were prepared for each of 12 doses of 2-NFA: 0, 25, 50, 100, 250, 500, 1000, 2000,

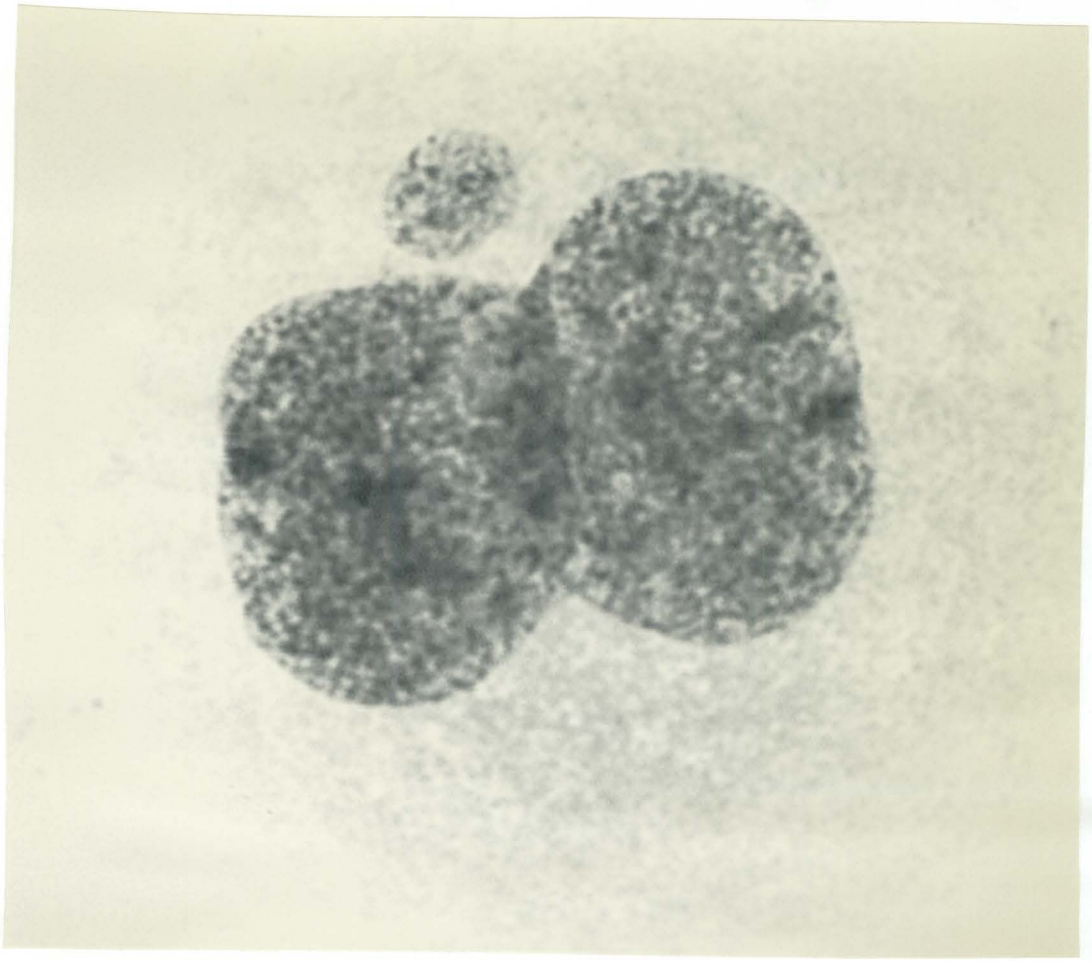

Figure M1: A Micronucleated Binucleate Cell

5000, 10000, 15000 and 20000 ng/mL. These doses were chosen to include the doses found to be positive in the Ames test (Zielinska et al., 1988; Shane et al., 1991). The maximum final concentration of DMSO in each well was 1%. 2-NFA doses were prepared as follows. A small unknown mass of 2-NFA was dissolved in dichloromethane (DCM). The molarity of this solution was determined using a spectrophotometer (see below). An appropriate amount of this solution of 2-NFA in DCM was placed in a glass vial and placed in a hot water bath to evaporate the DCM. To the remaining 2-NFA was added an appropriate amount of DMSO to make a stock solution of 2-NFA in DMSO at 2×10^3 ng/mL. A 1:40 and 1:20 dilution of the stock was made in DMSO in order to make solutions at 50 ng/mL and 100 ng/mL respectively. 2-NFA was added to the appropriate wells.

Final 2-NFA dose-response experiments utilized the same protocol as the MMC Group A dose-response experiments with the following modifications. One experiment tested only HSC-3TO while two others tested both HSC-3TO and TK6. Doses were based on the estimated yearly average concentration of 2-NFA in Hamilton air (0.006 ng/m^3; Legzdins et al., 1994b). Every 24 hours, we inhale approximately 10,000 L or 10 m^3 of air (Weinberger et al., 1986). Therefore, every year, our potential total exposure to 2-NFA is 20 ng. Doses of 2-NFA tested were 2×10^3, 5×10^3, 1×10^4 and 2×10^4 ng/mL (each with 0.5% DMSO), a media control

(0 2-NFA and 0 DMSO) and a DMSO control (0 2-NFA and 0.5% DMSO).

The doses tested in the final dose-response experiments began at 2 x 10^3 ng/mL because 2 x 10^3 ng/mL was the highest dose tested in the preliminary dose-response experiments in which no toxicity was observed. Doses in the final dose-response experiments were therefore higher in order to try to elicit a toxic response.

Doses in the final dose-response experiments were administered in higher volumes in order to try and avoid the replicate variability in the preliminary dose-response experiments. The stock 2-NFA was prepared by dissolving 2 mg of 2-NFA (Chemsyn, CSL-85-034-04-33) in 2 mL of DCM in a glass vial to make a stock solution of 2-NFA of 1 mg/mL or 1 x 10^3 ng/μL. This stock was stored at -20°C. Before each experiment, the concentration of the stock 2-NFA was verified. To a glass vial was added 1000 μL ethanol. Two μL of the ethanol was removed using a 10 μL syringe (Hamilton Co.) and 2 μL of the stock 2-NFA added to the glass vial. The vial was vortexed and the absorbance of the 1:500 2-NFA dilution at λ_{260} was determined using a spectrophotometer (Pharmacia LKB Ultraspec III). The molarity of the dilution was determined using Beer's law:

The molarity of the 1:500 dilution = A at λ_{260} ÷ EC at λ_{260}

Where:

A at λ_{260} = Absorbance of 2-NFA in ethanol at 260 nm
EC at λ_{260} = The extinction coefficient of 2-NFA in ethanol at 260 nm

The molarity of the stock 2-NFA was then easily determined and compared to the expected stock concentration of 1×10^3 ng/μL.

In preparing 2-NFA doses for dose-response experiments, the required amount of 2-NFA was taken from the stock and placed in a glass vial. This vial was placed in a hot water bath in the fume hood where the DCM readily evaporated. The residual 2-NFA was redissolved in the appropriate amount of DMSO and vortexed. 2-NFA in DMSO and DMSO were then added to aliquots of media (one aliquot for each dose) in order to obtain final solutions at two times the final dose of 2-NFA with a maximum concentration of DMSO of 1% in every dose except the media control (where neither 2-NFA in DMSO nor DMSO alone were added). The 2-NFA doses were aliquoted in 0.5 mL amounts to the appropriate wells. The wells were topped up to 1 mL with cells in media, making the final concentration of DMSO 0.5% in every well except those of the media control. Cells were counted as described above (see Section 2.5.1, Group A) after 4-7 days.

Total viable cell number for each replicate, a mean TVCN for each dose and a percent survival of the cells using mean TVCN relative to the DMSO control were calculated. Regression analyses were not performed.

2.6.2 Modified Growth Determination Experiments

One modified growth determination experiment was performed utilizing 2-NFA and HSC-3TO. The purpose of this

experiment was to determine if 2-NFA causes mitotic delay at the doses tested and if Cyt-B is effective in blocking 2-NFA treated cells from proliferating.

Replicate cultures were prepared for two counting times, 24 and 48 hours, for each of three doses of 2-NFA (20, 200 and 2000 ng/mL), a media control (0 2-NFA and 0 DMSO) and a DMSO control (0 2-NFA and 0.5% DMSO), without Cyt-B and with 3 μg/mL Cyt-B. Experimental cells were seeded at 2.0×10^5 cells/mL (twice the seeding density) in a sufficient volume and 2.5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. 2-NFA doses were prepared in media in sufficient volumes at two times the final dose following the same protocol as the dose-response experiments (see Section 2.6.1) and 2.5 mL of each dose was added to the appropriate tubes. The final experimental volume was 5 mL. Three μg/mL Cyt-B was also added to the appropriate tubes upon seeding as described above (see Section 2.5.2). Cells were counted as in the modified growth determination experiments with MMC (see Section 2.5.2, Modified Growth Experiment I).

2.6.3 CBMN Assay

Three replicates for each dose of 2-NFA were used in both CBMN experiments: 20, 200 and 2000 ng/mL, a media control (0 2-NFA) and a DMSO control (0 2-NFA, 0.5% DMSO). Dose preparation was similar to that of the dose-response experiments (see Section 2.6.1). Three μg/mL Cyt-B was used

for all CBMN experiments and added to cultures as described above (see Section 2.5.2).

Experiment I:

Cells were seeded at 2 x 10^5 cells/mL (twice the final density) in a sufficient volume and 2.5 mL of this cell suspension was aliquoted to each 15 mL centrifuge tube. 2-NFA doses were added to the appropriate tubes in 2.5 mL volumes, making the final experimental volume 5 mL. Tubes were vortexed, their caps loosened and placed in the incubator. After 24 hours, cells were treated with 3 μg/mL Cyt-B as described above (see Section 2.5.2) and placed back in the incubator. After 48 hours, cells were harvested and slides were made and scored as described above (see Section 2.5.3).

Experiment II:

Experiment II was performed according to the protocol of MMC CBMN assay Experiment II with the exception that cells were seeded at a final density of 1 x 10^5 cells/mL.

2.7 Statistical Methods

All statistical analyses were performed using Minitab Release 8 statistical software (Minitab Inc., 1991). All analyses of variance (ANOVA) were one-way. For all statistical analyses, a level of significance (α) of 0.05 was chosen.

Analysis of Variance (ANOVA) was used to assess inter- and intraexperimental variability, optimization of the CBMN assay data, the effect of harvest time on the percent micronucleated binucleate cells (PMBC) and on the total number of micronuclei (TM), the effect of MMC dose on PMBC and TM, 2-NFA micronuclei data and modified growth experiment data.

General Linear Modelling (GLM) was used to assess short- and long-term growth of HSC-3TO cells, MMC micronuclei data and 2-NFA dose-response data. Models are specified in Appendices AI-AIV.

MMC dose-response data were analyzed as follows. After cells were counted, total viable cell number was calculated for each replicate of each dose. For each replicate, a percent survival relative to the 0 dose was calculated (%RS). Regression analyses were performed on %RS data in order to determine the D_{10} and D_{50} values. Because the log transformation of the %RS and the square root transformation of the dose provided the best fit to a straight line, the prediction of the D_{10} and D_{50} values were determined using these transformations. For Group A experiments, a %RS of cells at each dose compared to the 0 dose (100% survival) was determined using mean total viable cell numbers. For each experiment in Group A an equation was fit to the data. The D_{10} and D_{50} values were predicted from this equation. For Groups B-G an equation was fit to the data for each

replicate. The D_{10} and D_{50} values were predicted from these equations. Student t-tests were performed in order to compare the mean D_{10} doses of Groups A-G.

3. RESULTS

3.1 Growth of HSC-3TO Cells

3.1.1 Long-term Growth of HSC-3TO Cells

Total viable cell numbers (TVCN) for two long-term growth experiments are shown in Table R1. TVCN at 4 hours of allowed growth is less than seeding TVCN for all replicates. This decrease in cell number upon seeding is part of the *lag phase*.

The data in Table R1 were fit to a General Linear Model (Appendix Table AI-1). Both Experiment and Hours (duration of allowed growth) had a significant effect ($p<0.001$ for both) on the total number of viable cells. The adjusted mean TVCN derived from GLM (Table R1) were plotted against hours of allowed growth (see Figure R1). Figure R1 demonstrates two phases of growth. A *growth phase* was seen from approximately 4-50 hours after which cell proliferation slowed until a *plateau phase* was reached at around 120 hours. The adjusted TVCN from 4-50 hours were fit to a straight line using linear regression (R-sq=87.3%, p=0.065). Growth from 4-50 hours was linear and could be described by equation (1):

$$TVCN = 7.09 + 0.308 \times H \quad (1)$$

Where:
TVCN = Total Viable Cell Number
H = Number of Hours of Allowed Growth

Table R1: Total Viable Cell Number (TVCN) of Three Replicates for Two Long-term Growth Experiments, Mean TVCN and Standard Deviation (sd) Based on Six Replicates and Adjusted Mean TVCN Derived from GLM
Note: TVCN at Seeding was 12.5 x 10^5.

Duration of Allowed Growth (hours)	TVCN x 10^5 Experiment #1			TVCN x 10^5 Experiment #2			Mean TVCN (sd) x 10^5	Adj Mean TVCN x 10^5
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3		
4	9.6	8.1	8.1	8.8	8.0	8.3	8.5 (0.6)	8.4
24	13.5	13.7	16.5	12.9	16.2	15.9	14.8 (1.6)	14.8
48	22.7	20.0	25.8	-	-	-	22.8 (2.9)	18.8
50	-*	-	-	23.0	21.1	20.5	21.5 (1.3)	25.6
72	-	-	-	19.4	19.3	19.8	19.5 (0.3)	23.6
85	26.8	25.9	33.3	-	-	-	28.7 (4.0)	24.6
96	35.4	34.5	34.9	25.8	21.0	20.5	28.7 (7.1)	28.7
120	28.8	49.3	33.4	23.3	28.1	22.9	31.0 (9.8)	31.0
144	42.4	34.6	33.9	22.9	29.9	-	32.7 (7.1)	31.9
168	39.8	40.5	30.5	24.8	21.3	35.4	32.1 (7.9)	32.1
192	36.5	32.5	32.4	24.5	23.1	38.3	31.2 (6.2)	31.2
216	47.8	41.8	31.0	32.9	28.5	26.4	34.7 (8.3)	34.7
240	44.6	34.5	35.9	28.3	23.3	24.8	31.9 (8.0)	31.9
264	-	-	-	29.0	32.8	25.5	29.1 (3.7)	33.2

-* = No data

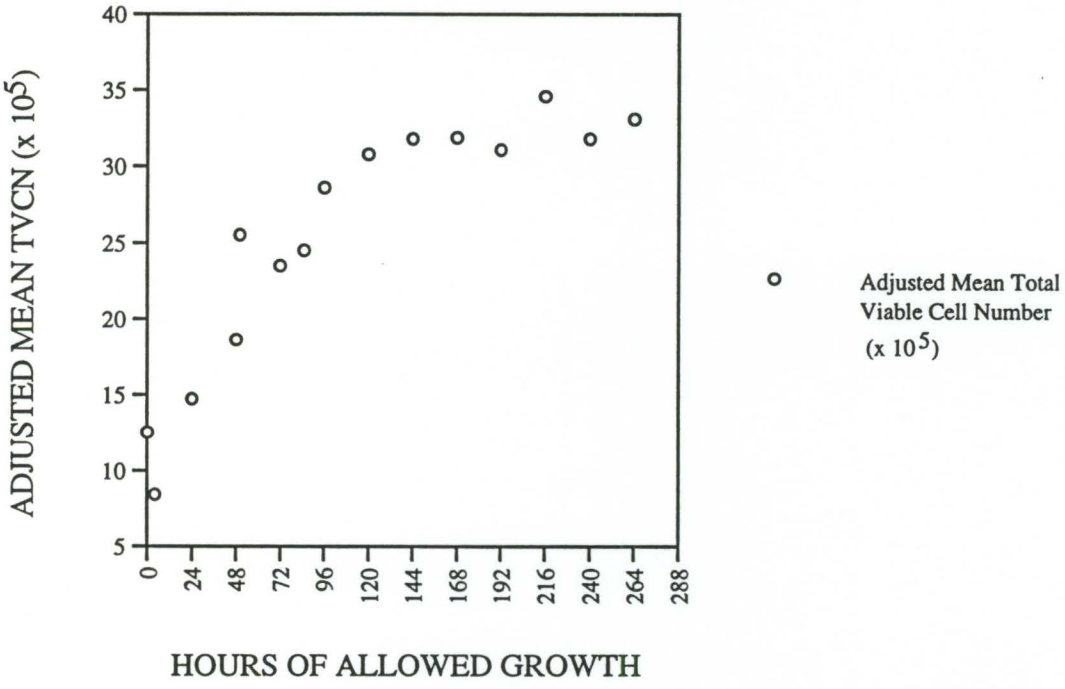

Figure R1: Long-term Growth of HSC-3TO Cells

From this equation, a population doubling time (PDT) of 33 hours was determined:

TVCN at 10 hours = 10.17 (from equation 1)
10.17 x 2 = 20.34 (plug into equation 1)
Hours = 43.02 hours
43.02 hours - 10.17 hours = 33 hours

A lag time of 18 hours was also determined from this equation by solving for hours when TVCN reached the original TVCN of 12.5×10^5.

Cells reached plateau at approximately 120 hours (see Figure R1), after which no significant growth was apparent. The mean TVCN and standard deviation at plateau was $32.3\ (1.3) \times 10^5$, derived from the adjusted TVCN from 120-264 hours.

3.1.2 Short-term Growth of HSC-3TO Cells

The data in Table R2 were fit to a General Linear Model (Appendix Table AI-2). Both Experiment and Hours (duration of allowed growth) had a significant effect on TVCN ($p<0.001$ for both). The adjusted mean TVCN (Table R3) were plotted against hours of allowed growth (Figure R2) and fit to a straight line using linear regression (R-sq=92.8%, $p<0.001$). The growth of HSC-3TO from 4-48 hours was linear and could be described by equation (2):

$$TVCN = 5.78 + 0.291 \times H \quad (2)$$

Where:
TVCN = Total Viable Cell Number
H = Number of Hours of Allowed Growth

Table R2: Total Viable Cell Number (TVCN) of Three Replicates for Three Short-term Growth Experiments
Note: TVCN at Seeding was 12.5×10^5.

Duration of Allowed Growth (hours)	TVCN x 10^5 Experiment #1			TVCN x 10^5 Experiment #2			TVCN x 10^5 Experiment #3		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
4	7.2	9.4	8.0	6.8	6.6	5.5	7.2	6.7	8.7
16	8.9	9.6	8.7	11.5	10.7	9.0	10.0	9.5	11.5
20	13.4	8.1	10.5	9.8	9.9	10.6	10.6	15.6	13.3
24	13.1	10.3	13.1	12.0	11.0	10.6	15.3	19.8	13.8
28	14.4	13.3	13.0	10.0	11.5	10.7	22.0	17.2	24.9
32	11.5	9.7	10.3	14.2	10.7	13.9	16.7	16.1	20.2
36	14.9	11.8	12.1	10.8	10.9	15.6	21.0	16.9	16.2
40	16.0	28.5	26.0	13.3	15.3	-	19.8	18.3	17.2
44	16.3	22.2	25.7	16.3	15.9	16.4	20.0	17.2	17.3
48	13.9	20.8	18.8	16.4	13.2	17.3	34.3	14.7	29.1

Table R3: Mean Total Viable Cell Number (TVCN; Based on Nine Replicates; see Table R2) and Standard Deviation (sd) and Adjusted Mean TVCN Derived from GLM of Short-term Growth Data

Duration of Allowed Growth (hours)	Mean TVCN x 10^5 (sd)	Adj Mean TVCN x 10^5
4	7.4 (1.2)	7.4
16	10.0 (1.1)	10.0
20	11.3 (2.3)	11.3
24	13.2 (3.0)	13.2
28	15.2 (5.2)	15.2
32	13.7 (3.5)	13.7
36	14.5 (3.4)	14.5
40	19.3 (5.3)	18.7
44	18.6 (3.4)	18.6
48	19.8 (7.3)	19.8

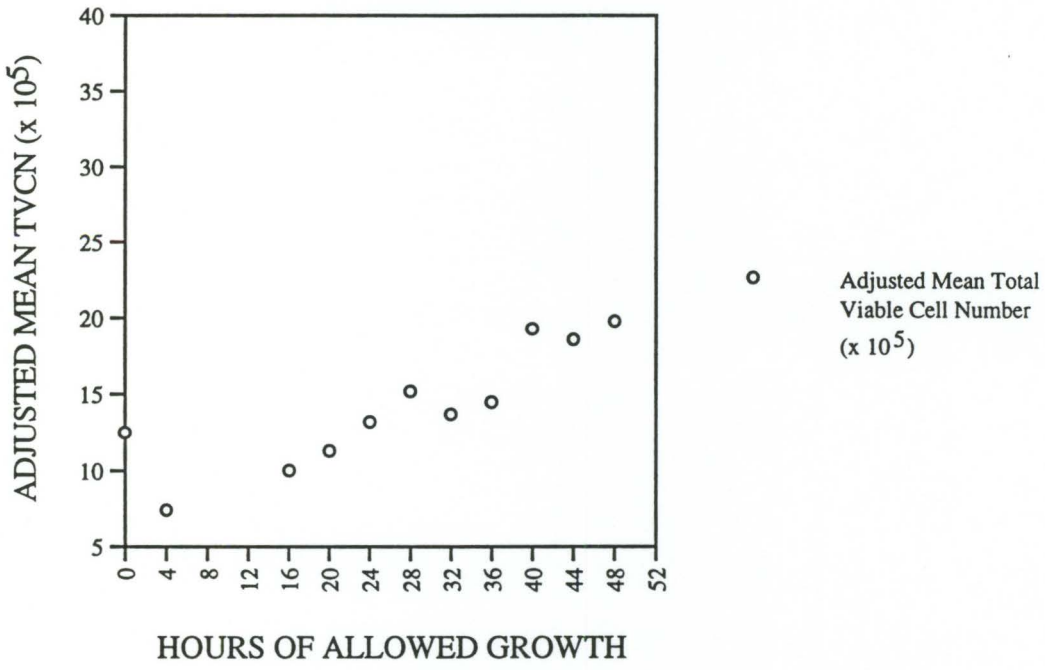

Figure R2: Short-term Growth of HSC-3TO Cells

From this equation, a PDT of 30 hours was determined:

TVCN at 10 hours = 8.69 (from equation 1)
8.69 x 2 = 17.38 (plug into equation 1)
Hours = 39.86 hours
39.86 hours - 10 hours = 30 hours

A lag time of 23 hours was also determined from this equation by solving for hours when TVCN reached the original TVCN of 12.5×10^5. The population doubling time and lag time for short-term and long-term growth were comparable.

3.2 The Cytokinesis Block Micronucleus (CBMN) Assay

3.2.1 Optimal Concentration of Cytochalasin B (Cyt-B)

Analysis of variance was performed on the replicate data shown in Table R4 (Appendix Table AII-1). Replicate variation was observed only at 3 μg/mL Cyt-B. Replicate data were used to calculate a mean percent binucleate cells for each dose of Cyt-B (Table R5).

ANOVA of the data in Table R4 (Appendix Table AII-2) showed that Cyt-B dose had a significant effect on the percent binucleate cells ($p=0.009$). T-tests were performed to compare the mean percent binucleate cells obtained for each dose of Cyt-B. There were statistically significant differences between the percent binucleate cells of control cultures (0 μg/mL Cyt-B) and Cyt-B-treated cultures at all doses ($p<0.001$). There was no statistically significant difference between the percent binucleate cells obtained after treatment with 1.5 or 3 μg/mL Cyt-B, and with 3 or 6 μg/mL Cyt-B.

Table R4: Percent Binucleate Cells Obtained after Treatment with Various Doses of Cyt-B for 32 Hours
Note: Based on 100 Cells Scored

Replicate-Slide	Binucleate Cells (%) Obtained after Treatment with Cyt-B (µg/mL)				
	0	1.5	3.0	6.0	12.0
1-1	5	39	48	37	31
1-2	2	21	53	36	36
1-3	4	43	47	37	45
2-1	8	-*	16	39	23
2-2	8	-	17	57	14
2-3	4	-	10	48	35
3-1	7	41	48	53	38
3-2	7	36	45	28	19
3-3	4	41	33	56	40

-* No Data

Table R5: Mean Percent Binucleate Cells after Treatment with Various Doses of Cyt-B

Cyt-B (µg/mL)	Mean % Binucleate Cells (sd)
0	5.4 (2.1)
1.5	36.8 (8.1)
3	35.2 (16.7)
6	43.4 (10.3)
12	31.2 (10.4)

There was, however, a statistically significant reduction in the percent binucleate cells obtained after treatment with 12 μg/mL as compared with 6 μg/mL Cyt-B (p=0.025). Three μg/mL Cyt-B was chosen for the CBMN assay.

3.2.2 Optimal Duration of Cyt-B Treatment

Analysis of variance was performed on the replicate data shown in Table R6 (Appendix Table AII-3). There was no statistically significant variance due to replicates. Replicate data were used to calculate a mean percent binucleate cells for each duration of Cyt-B treatment (Table R7). Analysis of variance of the data in Table R6 (Appendix Table AII-4) showed that duration of exposure of cells to Cyt-B did not have a significant effect on the percent binucleate cells.

3.2.3 Optimal Initial Seeding Density

Analysis of variance was performed on the replicate data shown in Table R8 (Appendix Table AII-5). Replicate variation was seen only at a seeding density of 2.5×10^5 cells/mL. Replicate data were used to calculate a mean percent binucleate cells for each seeding density (Table R9).

ANOVA of the data in Table R8 (Appendix Table AII-6). showed that seeding density had a statistically significant effect on the percent binucleate cells (p=0.020). T-tests were performed to compare the mean percent binucleate cells for each seeding density. There was a statistically

Table R6: Percent Binucleate Cells Obtained after Treatment with 3 µg/mL Cyt-B for Various Lengths of Time
Note: Based on 100 cells scored

Replicate-Slide	Binucleate Cells (%) Obtained after Various Durations of Treatment (hours) with 3 µg/mL Cyt-B			
	24	28	32	36
1-1	41	29	19	20
1-2	39	8	23	36
1-3	15	24	24	26
2-1	28	40	20	17
2-2	19	21	15	27
2-3	20	24	30	22
3-1	31	20	21	20
3-2	18	19	21	23
3-3	24	16	16	22

Table R7: Mean Percent Binucleate Cells and Standard Deviation (sd) after Various Durations of Treatment with 3 µg/mL Cyt-B

Cyt-B (hours)	Mean % Binucleate Cells (sd)
24	26.1 (9.3)
28	22.3 (8.9)
32	21.0 (4.5)
36	23.7 (5.6)

Table R8: Percent Binucleate Cells Obtained after Treatment of HSC-3TO Cells Seeded at Various Seeding Densities with 3 μg/mL Cyt-B for 24 hours Note: Based on 100 Cells Scored

Replicate-Slide	Binucleate Cells (%) Obtained after Seeding at Various Densities (x 10^5)		
	1.0	2.5	5.0
1-1	16	-*	22
1-2	7	-	14
1-3	19	-	28
2-1	11	22	26
2-2	11	18	29
2-3	12	18	30
3-1	26	25	18
3-2	4	26	25
3-3	7	21	26

-* No data

Table R9: Mean Percent Binucleate Cells and Standard Deviation (sd) after Treatment of HSC-3TO Cells Seeded at Various Seeding Densities with 3 μg/mL Cyt-B for 24 hours

Seeding Density (x 10^5)	Mean % Binucleate Cells (sd)
1.0	12.5 (1.4)
2.5	25.1 (6.4)
5.0	24.2 (3.7)

significant increase in the percent binucleate cells obtained using a seeding density of 2.5 x 10^5 cells/mL as opposed to 1.0 x 10^5 cells/mL ($p=0.001$). No statistically significant difference was found in percent binucleate cells between using 2.5 x 10^5 cells/mL and 5.0 x 10^5 cells/mL.

3.3 Mitomycin C (MMC)

3.3.1 Dose-Response Experiments

Table M1 (see Methods and Materials section) summarizes the various protocols used in determining the response of HSC-3TO to MMC. The effect of MMC on HSC-3TO was variable depending upon which experimental condition was employed. Several variables were examined: the length of exposure of HSC-3TO to MMC, the duration of allowed growth (i.e., the amount of time the cells were allowed to grow after treatment before being counted), the seeding vessel, and the seeding density and culture volume (which combined to give an estimate of the number of cells exposed to each nanogram of MMC). The effect of MMC on HSC-3TO can be gauged by examining the D_{10} and D_{50} (the dose of MMC required to kill 90% and 50% of cells respectively). D_{10} and D_{50} doses for all groups are shown in Table R10.

The effect of the length of MMC exposure was determined by comparing Group A to Group B. A t-test showed there was no statistically significant difference between the mean D_{10} of Group A (196.3; sd 12.1) and that of Group B

Table R10: D_{10} and D_{50} Values Obtained by Regression Analysis of the Log Transformation of the Relative Survival Versus the Square Root Transformation of the Dose of MMC, Mean Values and Standard Deviations (sd)

Dose	Group						
	A	B	C	D	E	F	G
D_{10}	211 184 201 189	228 187 207 269	1430 1110 1200 1300	3870 2280	3400 3900	4590 3600	1000 1170 1510
Mean D_{10} (sd)	**196.3 (12.1)**	**222.8 (35.1)**	**1260 (137)**	**3075 (1124)**	**3650 (354)**	**4095 (700)**	**1230 (260)**
D_{50}	20 25 23 26	31 10 16 31	161 64 153 119	269 142	411 376	549 301	84 98 147
Mean D_{50} (sd)	**23.5 (2.6)**	**22.0 (10.7)**	**124.3 (44.1)**	**205.5 (89.8)**	**393.5 (24.7)**	**425.0 (175.4)**	**109.7 (33.1)**

(222.8; sd 35.1). MMC exerted the same effect whether it was present for 24 hours or for 5-6 days.

The importance of the duration of allowed growth in cells seeded at the lower cell density (1×10^5 cells/mL) was determined by comparing Group A and B to Group C. D_{10} values from Group A and B were pooled. A t-test showed a statistically significant difference ($p<0.001$) between the mean D_{10} of Group A and B (209.5; sd 28.1) and that of Group C (1260; sd 137). A significantly lower dose of MMC was needed to produce the same amount of killing if cells were allowed to grow for 5-6 days as opposed to 48 hours.

The effect of seeding and treating cells in a 15-mL tube versus a 24-well plate was examined by comparing Group C and Group D. A t-test demonstrated no statistically significant difference between the mean D_{10} of Group C (1260; sd 137) and that of Group D (3075; sd 1124).

The effect of seeding and treating cells in a 15-mL tube versus a T-25 flask was examined by comparing Group E and Group F. A t-test demonstrated no statistically significant difference between the mean D_{10} of Group E (3650; sd 354) and that of Group F (4095; sd 700).

The D_{10} values for Group E and F were pooled and the mean compared to the mean D_{10} of Group D in order to determine the effect of different seeding density and seeding volume (i.e., the number of cells exposed to each nanogram of MMC). A t-test demonstrated no statistically

significant difference between the mean D_{10} of Group E and F (3873; sd 521) and that of Group D (3075; sd 1124).

The effect of duration of allowed growth of cells seeded at the higher cell density (2.5 x 10^5 cells/mL) was examined by comparing the mean D_{10} of Group E and F and the mean D_{10} of Group G. The mean D_{10} of Group E and F (3873; sd 521) was over three times as large as the mean D_{10} of Group G (1230; sd 260) and a t-test showed this difference to be statistically significant ($p<0.001$). As with cells seeded at the lower density, cells seeded at the higher density required a significantly lower dose of MMC to produce the same amount of killing if cells were allowed to grow for 108 hours (4.5 days) as opposed to 48 hours.

3.3.2 Modified Growth Experiments

Cell numbers for Experiments I and II cannot be directly compared because of different initial seeding density. Since it was found that cell density did not affect the cytotoxicity of MMC (see section 3.3.1), both experiments were used to provide information about the growth of MMC-treated cells. This information was useful in the interpretation of CBMN assay results.

TVCN for Experiment I are shown in Table R11. Examination of TVCN of Cyt-B-untreated cells showed that by 24 hours control cells had recovered from lag and had once again reached seeding density while cells treated with 100 and 1000 ng/mL MMC had not. By 48 hours control cells had

Table R11: **Modified Growth Experiment I: Mean Total Viable Cell Number (TVCN) and Standard Deviation (sd) of Cells Treated with Various Doses of MMC for 24 and 48 Hours and Treated with and without 3 μg/mL Cyt-B upon Seeding for 24 or 48 Hours**

MMC (ng/mL)	TVCN ($\times 10^5$) at Seeding	TVCN ($\times 10^5$) After 24 Hours of Allowed Growth (sd) 0 μg/mL Cyt-B	TVCN ($\times 10^5$) After 24 Hours of Allowed Growth (sd) 3 μg/mL Cyt-B	TVCN ($\times 10^5$) After 48 Hours of Allowed Growth (sd) 0 μg/mL Cyt-B	TVCN ($\times 10^5$) After 48 Hours of Allowed Growth (sd) 3 μg/mL Cyt-B
0	5.0	5.9 (0.7)	3.3 (0.0)	9.8 (1.3)	2.4 (0.4)
100	5.0	3.8 (0.9)	2.8 (0.1)	4.8 (0.2)	2.5 (0.2)
1000	5.0	3.2 (0.9)	2.8 (0.0)	2.5 (0.1)	2.5 (0.1)

demonstrated proliferation to approximately 1.7 times their number at 24 hours, cells treated with 100 ng/mL MMC had recovered from lag and had reached seeding density, and cells treated with 1000 ng/mL had not recovered from lag. TVCN of Cyt-B-treated cells remained at lag phase levels(i.e., below that of initial seeding TVCN) after 24 and 48 hours and a t-test demonstrated a statistically significant reduction in number of Cyt-B-treated cells between 24 and 48 hours ($p=0.006$) indicating an effective block of proliferation.

TVCN for Experiment II are shown in Table R12. After 72 hours growth without Cyt-B, control cells and cells treated with 100 ng/mL MMC had recovered from lag and demonstrated proliferation of 1.7 and 1.5 times their original TVCN respectively. Cells treated with 1000 ng/mL MMC had not yet recovered from lag and had not yet reached initial seeding TVCN. After 108 hours control cells demonstrated increased TVCN from that at 72 hours, while TVCN for cells treated with 100 and 1000 ng/mL MMC decreased. Although TVCN of cells treated with 100 and 1000 ng/mL MMC indicated a decrease in TVCN between 72 and 108 hours, a small proportion of MMC-treated cells still divided and were captured by Cyt-B (Table R12). T-tests demonstrated no statistically significant difference between TVCN of control cells at 72 hours and 108 hours with or without Cyt-B. This indicates that control cells did not

Table R12: **Modified Growth Experiment II: Mean Total Viable Cell Number (TVCN) and Standard Deviation (sd) for Cells Treated with Various Doses of MMC for 108 Hours and Treated with and without 3 μg/mL Cytochalasin B after 72 Hours until 108 Hours**

MMC (ng/mL)	Mean TVCN ($\times 10^5$) at Seeding	Mean TVCN ($\times 10^5$) After 72 Hours of Allowed Growth (sd) 0 μg/mL Cyt-B	Mean TVCN ($\times 10^5$) After 108 Hours of Allowed Growth (sd) 0 μg/mL Cyt-B	Mean TVCN ($\times 10^5$) After 108 Hours of Allowed Growth (sd) 3 μg/mL Cyt-B	Percent Binucleate Cells After 108 Hours of Allowed Growth 3 μg/mL Cyt-B
0	12.5	20.7 (1.6)	25.2 (1.7)	24.1 (3.8)	25
100	12.5	18.1 (4.1)	13.1 (2.4)	14.0 (0.6)	16
1000	12.5	8.9 (1.2)	3.2 (0.8)	2.5 (0.5)	8

proliferate significantly between 72 and 108 hours, suggesting that cell density was reaching saturation for this experiment. However, it should be noted that, with only two replicates, the power to detect a difference would be low. Percent binucleate data (Table R12), however, indicated that a proportion of control cells did divide between 72 and 108 hours and were captured by Cyt-B (25%).

3.3.3 Percent Micronucleated Binucleate Cells and Total Micronuclei

When the data in Tables R13 and R14 were each fit to a General Linear Model (Appendix Tables AIII-1 and AIII-2 respectively) it was shown that harvest time (i.e., the length of time after treatment before harvesting), dose of MMC and the interaction of harvest time and dose had a significant effect on the percentage of micronucleated binucleate cells (PMBC; $p<0.001$) and on the total number of micronuclei (TM; $p<0.001$).

Analysis of variance was used to compare PMBC (Appendix Table AIII-3) and TM (Appendix Table AIII-4) of Experiment #1 and Experiment #2 at each dose. As there was no statistically significant variance due to experiment, data for the two experiments at each dose were pooled.

The effect of harvest time on PMBC and TM at each dose was examined by analysis of variance (Appendix Tables AIII-5 and AIII-6 respectively). At 0 ng/mL MMC, harvest time had no statistically significant effect on PMBC or on TM.

Table R13: **MMC CBMN Assay Results: Percent of Binucleate Cells Containing at Least one Micronucleus (PMBC) and Mean Percent Binucleate Cells (BC) Note: Micronucleus Data Based on 1000 Binucleate Cells Scored and Binucleate Data Based on 100 Binucleate Cells Scored (Mean of Three Replicates).**

MMC (ng/mL)	Percent of Binucleate Cells Containing at Least one Micronucleus (PMBC) and Percent Binucleate Cells (BC)									
	24-hour MMC Treatment 24-hour Cyt-B treatment			48-hour MMC Treatment 48-hour Cyt-B Treatment			24+48 pooled data Mean %PMBC (sd)	108-hour MMC Treatment 36-hour Cyt-B Treatment (From 72-108 hours)		
	% PMBC	Mean %PMBC (sd)	Mean % BC (sd)	% PMBC	Mean %PMBC (sd)	Mean % BC (sd)		% PMBC	Mean %PMBC (sd)	Mean % BC (sd)
0	0.7 1.0 1.0	0.9 (0.2)	27.3 (8.7)	1.4 0.9 1.3	1.2 (0.3)	42.9 (11.2)	1.1 (0.3)	0.6 2.2 1.9	1.6 (0.9)	25.1 (2.4)
100	0.8 0.3 1.2	0.8 (0.5)	28.9 (7.0)	0.4 1.3 0.7	0.8 (0.5)	52.4 (11.7)	0.8 (0.4)	10.7 12.8 8.3	10.6 (2.3)	15.8 (2.2)
1000	1.9 1.5 2.1	1.8 (0.3)	24.9 (6.3)	2.4 1.9 1.7	2.0 (0.4)	31.0 (2.7)	1.9 (0.3)	12.8 10.5 7.5*	10.3 (2.7)	7.7 (1.5)

*Based on 24 binucleate cells with micronuclei out of 320 binucleate cell scored.

Table R14: MMC CBMN Assay Results: Total Number of Micronuclei in 1000 Binucleate Cells (TM)

MMC (ng/mL)	Total Number of Micronuclei in 1000 Binucleate Cells Scored (TM)						
	24-hour MMC Treatment 24-hour Cyt-B treatment		48-hour MMC Treatment 48-hour Cyt-B Treatment		24+48-hour MMC Treatment (Exp's #1 and #2 pooled) Mean TM (sd)	108-hour MMC Treatment 36-hour Cyt-B Treatment (From 72-108 hours)	
	TM	Mean TM (sd)	TM	Mean TM (sd)		TM	Mean TM (sd)
0	8 12 11	10.3 (2.1)	15 10 13	12.7 (2.5)	11.5 (2.4)	6 24 21	17.0 (9.6)
100	8 4 13	8.3 (4.5)	4 14 7	8.3 (5.1)	8.3 (4.3)	127 157 98	127.3 (29.5)
1000	21 19 18	19.3 (1.5)	30 21 17	22.7 (6.7)	21.0 (4.7)	145 119 94*	119.3 (25.5)

*Based on 30 micronuclei counted in 320 binucleate cells

A statistically significant effect of harvest time on PMBC and TM was demonstrated at 100 ng/mL MMC and at 1000 ng/mL ($p<0.001$ for both).

ANOVA (Appendix Tables AIII-7 and AIII-8) and t-tests were used to examine the effect of dose of MMC on PMBC and TM. At a 24-48 hour harvest time, a significant effect of dose on PMBC ($p<0.001$) and TM ($p=0.003$) was seen only at 1000 ng/mL. At a 108 hour harvest time, a significant effect of dose on PMBC and TM was seen at both 100 and 1000 ng/mL MMC (PMBC: $p=0.023$ and $p=0.033$ respectively; TM: $p=0.025$ and $p=0.023$ respectively).

The mean PMBC found in cultures treated with 100 ng/mL MMC and harvested after 108 hours (10.6; sd 2.3) was over 13 times that found after harvesting after 24-48 hours (0.8; sd 0.4). The mean PMBC found in cultures treated with 1000 ng/mL MMC and harvested after 108 hours (10.3; sd (2.7) was over 5 times that found after harvesting after 24-48 hours (1.9; sd 0.3).

Similar results are seen by examining TM. The mean TM found in cultures treated with 100 ng/mL MMC and harvested after 108 hours [127.3; sd 29.5) was over 15 times that found after harvesting after 24-48 hours (8.3; sd 4.3). The mean TM found in cultures treated with 1000 ng/mL MMC and harvested after 108 hours (119.3; sd 25.5) was over 5 times that found after harvesting after 24-48 hours (21.0; sd 4.7).

3.4 2-Nitrofluoranthene (2-NFA)

3.4.1 Dose Response Experiments

Percent survival and TVCN from preliminary experiments with cells treated with various doses of 2-NFA are shown in Table R15. No effect on relative survival was seen in doses of 2-NFA of up to 5000 ng/mL with the exception of one dose (250 ng/mL) in one replicate. This was likely due to sporadic fluctuation in culture conditions (seeding, gas exchange). Consequently, subsequent experiments tested doses of 2-NFA higher than 2×10^3 ng/mL.

The data in Tables R16 and R17 were fit to a General Linear Model (Appendix IV Table IV-1). Cell line ($p<0.001$), days of allowed growth ($p<0.001$), dose of 2-NFA ($p<0.001$) and the interaction of days of allowed growth and dose ($p=0.005$) all had a statistically significant effect on TVCN.

3.4.2 Modified Growth Experiments

Table R18 indicates the TVCN of 2-NFA-treated cells. By 24 hours of growth Cyt-B-untreated cells at all doses of 2-NFA had recovered from lag phase and had once again reached seeding density. Analysis of variance of the replicate data for all doses treated without Cyt-B indicated no variance due to dose at either 24 or 48 hours. (Appendix IV Table AIV-2). Cyt-B-untreated cells had doubled their population between 24 and 48 hours, and a t-test showed a

Table R15: **Preliminary Dose-Response Experiments: Total Viable Cell Number (TVCN) of Two Replicates; Mean TVCN and Standard Deviation (sd; Based on Two Replicates); Percent Survival of HSC-3TO in 2-NFA Relative to the 0 Dose (%RS)**

2-NFA (ng/mL) 1% DMSO	Experiment #1			Experiment #2		
	TVCN x 10^5	Mean TVCN (sd) x 10^5	% RS	TVCN x 10^5	Mean TVCN (sd) x 10^5	% RS
0	-	11.6	100	15.5	14.0	100
	11.6	(-)		12.6	(2.1)	
25	3.9	13.8	118	11.8	17.5	125
	23.8	(14.1)		23.1	(8.0)	
50	5.7	13.0	112	16.9	19.0	136
	20.3	(10.4)		21.1	(3.0)	
100	4.1	13.0	112	10.4	15.5	111
	22.0	(12.7)		20.5	(7.1)	
250	4.6	12.0	103	3.1	7.0	50
	19.3	(10.4)		10.9	(5.5)	
500	-	22.8	197	15.3	1.51	108
	22.8	(-)		14.8	(0.4)	
1000	13.9	13.9	120	20.7	18.7	134
	-	(-)		16.6	(2.9)	
2000	23.3	12.1	104	20.2	17.1	122
	0.9	(15.9)		14.0	(4.4)	
5000	22.3	11.6	100	7.1	8.3	59
	0.9	(15.2)		9.4	(1.6)	
10000	5.4	3.0	26	13.8	8.8	63
	0.7	(3.3)		3.7	(7.1)	
15000	6.1	3.2	27	13.4	10.4	74
	0.3	(4.1)		7.4	(4.2)	
20000	1.7	1.7	14	9.9	8.5	60
	-	(-)		7.0	(2.1)	

Table R16: Total Viable Cell Number (TVCN; x 10^5) of Four Replicates of HSC-3TO Treated with Various Doses of 2-NFA; Mean TVCN and Standard Deviation (sd; x 10^5) and Percent Survival Relative to the DMSO Control (% RS)

2-NFA (ng/mL) 0.5% DMSO unless otherwise indicated	Experiment #1 (4 days)			Experiment #2 (5 days)			Experiment #3 (7 days)		
	TVCN x 10^5	Mean TVCN (sd)	% RS	TVCN x 10^5	Mean TVCN (sd) x 10^5	% RS	TVCN x 10^5	Mean TVCN (sd) x 10^5	% RS
Media Control 0 2-NFA 0 DMSO	23.2 21.8 28.3 21.8	23.8 (3.1)	96	15.2 - 14.7 18.9	16.3 (2.3)	91	23.1 22.1 30.5 30.0	26.4 (4.4)	66
DMSO Control 0 2-NFA	28.2 25.9 - 19.9	24.7 (4.3)	100	19.3 16.8 18.5 17.3	18.0 (1.1)	100	38.7 44.9 47.6 29.7	40.2 (7.9)	100
2 x 10^3	29.8 25.0 22.8 21.1	24.7 (3.8)	100	22.2 22.7 20.4 20.6	21.5 (1.2)	119	35.4 25.8 35.6 27.3	31.0 (5.2)	77
5 x 10^3	20.2 24.3 29.4 27.5	25.4 (4.0)	103	20.8 19.2 14.5 15.2	17.4 (3.1)	97	31.0 36.3 26.6 26.5	30.1 (4.6)	75
1 x 10^4	28.4 26.3 25.7 24.1	26.1 (1.8)	106	18.3 16.0 15.0 18.2	16.9 (1.6)	94	23.7 23.4 25.9 23.8	24.2 (1.2)	60
2 x 10^4	24.5 20.7 23.7 20.6	22.4 (2.0)	91	15.8 12.1 15.7 13.5	14.3 (1.8)	79	25.0 27.1 27.1 24.0	25.8 (1.6)	64

Table R17: **Total Viable Cell Number (TVCN; x 10^5) of Four Replicates of TK6 Treated with Various Doses of 2-NFA; Mean TVCN and Standard Deviation (sd; x 10^5) and Percent Survival Relative to the DMSO Control (% RS)**

2-NFA (ng/mL) 0.5% DMSO unless otherwise indicated	Experiment #1 (4 days)			Experiment #2 (5 days)		
	TVCN x 10^5	Mean TVCN (sd)	% RS	TVCN x 10^5	Mean TVCN (sd) x 10^5	% RS
Media Control 0 2-NFA 0 DMSO	38.5 42.5 31.8 44.0	39.2 (5.5)	116	39.3 34.8 41.5 39.8	38.9 (2.9)	95
DMSO Control 0 2-NFA	28.3 40.5 35.5 30.8	33.8 (5.4)	100	44.0 41.0 37.8 41.0	41.0 (2.5)	100
2 x 10^3	33.0 33.3 30.8 37.8	33.7 (2.9)	100	33.8 34.3 32.8 34.3	33.8 (0.7)	82
5 x 10^3	41.5 37.8 30.5 31.5	35.3 (5.2)	104	36.5 31.3 32.3 26.5	31.7 (4.1)	77
1 x 10^4	36.3 30.0 31.0 32.8	32.5 (2.8)	96	27.3 29.3 37.3 41.5	33.9 (6.7)	83
2 x 10^4	32.8 31.5 34.0 30.5	32.2 (1.5)	95	31.5 34.8 33.0 32.5	33.0 (1.4)	80

Table R18: **Mean Total Viable Cell Number (TVCN) and Standard Deviation (sd) of Cells Treated with Various Doses of 2-NFA for 24 and 48 Hours and Treated with and without 3 μg/mL Cyt-B upon Seeding for 24 or 48 Hours of Allowed Growth**

2-NFA (ng/mL) 0.5% DMSO unless otherwise indicated	Mean TVCN ($\times 10^5$) at Seeding	Mean TVCN ($\times 10^5$) After 24 Hours of Allowed Growth (sd) 0 μg/mL Cyt-B	Mean TVCN ($\times 10^5$) After 24 Hours of Allowed Growth (sd) 3 μg/mL Cyt-B	Mean TVCN ($\times 10^5$) After 48 Hours of Allowed Growth (sd) 0 μg/mL Cyt-B	Mean TVCN ($\times 10^5$) After 48 Hours of Allowed Growth (sd) 3 μg/mL Cyt-B
Media Control 0 2-NFA 0 DMSO	5.0	5.5 (5.1)	3.3 (3.2)	9.5 (10.2)	3.6 (2.5)
DMSO Control 0 2-NFA	5.0	5.1 (5.1)	3.3 (3.7)	13.2 (8.8)	3.5 (2.0)
20	5.0	4.9 (6.2)	3.0 (2.9)	10.5 (10.6)	3.5 (2.5)
200	5.0	6.4 (5.4)	2.5 (3.2)	11.4 (11.3)	3.3 (3.6)
2000	5.0	5.6 (6.3)	- (3.8)	12.1 (11.9)	2.8 (2.6)

statistically significant increase in mean TVCN between 24and 48 hours ($p<0.001$).

Cyt-B-treated cells remained at lag phase levels after 24 and 48 hours. Analysis of variance of replicate data of cells treated with Cyt-B showed no statistically significant variance due to dose at 24 or at 48 hours (Appendix IV Table AIV-3). T-tests showed statistically significant differences between the TVCN of Cyt-B-treated and untreated cells at 24 hours ($p<0.001$) and at 48 hours ($p<0.001$) indicating proliferation of Cyt-untreated cells. A t-test found no statistically significant difference in TVCN of Cyt-B-treated cells between 24 and 48 hours indicating a block in cell proliferation for the entire culture period.

3.4.3 Percent Micronucleated Binucleate Cells

Analysis of variance of the data in Table R19 (Appendix Table AIV-4) showed that harvest time (i.e., the length of time after treatment before harvesting), dose of 2-NFA and the interaction of harvest time and dose did not have a statistically significant effect on percent micronucleated binucleate cells.

Table R19: 2-NFA CBMN Assay Results: Percent of Binucleate Cells Containing at Least One Micronucleus

2-NFA (ng/mL) 0.5% DMSO Unless Otherwise Indicated	Percentage of Binucleate Cells Containing at Least one Micronucleus Based on Scoring of 1000 Binucleate Cells					
	Experiment #1 48-hour 2-NFA Treatment 24-hour Cyt-B Treatment (From 24-48 hours)			Experiment #2 108-hour 2-NFA Treatment 36-hour Cyt-B Treatment (From 72-108 hours)		
	Replicate 1	Replicate 2	Mean (sd)	Replicate 1	Replicate 2	Mean (sd)
Media Control 0 2-NFA 0 DMSO	0.5	0.5	0.5 (0.0)	0.6	0.7	0.7 (0.1)
DMSO Control 0-2-NFA	0.8	0.7	0.8 (0.1)	0.7	0.7	0.7 (0.0)
20	0.7	0.5	0.6 (0.1)	0.9	0.3	0.6 (0.3)
200	0.3	0.4	0.4 (0.1)	0.6	0.7	0.7 (0.1)
2000	0.3	0.4	0.4 (0.1)	0.7	0.3	0.5 (0.3)

4. DISCUSSION

Prior to the initiation of the present investigation the testing of chemicals in the micronucleus test was performed primarily *in vivo* using mice and *in vitro* using human lymphocytes and fibroblasts. Human lymphoblastoid cells were used successfully by Zhang et al. (1995), however their test was limited by its exclusion of Cyt-B. Zhang et al. (1995) did not feel that Cyt-B was necessary because established cell lines are continuously dividing and are always a target for the micronucleus assay. Exclusion of Cyt-B may cause underestimation of micronuclei because cells which have not divided after the genotoxic insult will not show micronuclei. In addition, cells which have divided more than once after the genotoxic insult may no longer contain micronuclei. The presently established micronucleus assay combines the practicality of a lymphoblastoid cell line and the accuracy of Cyt-B for the testing of chemicals for chromosomal damage in human cells.

4.1 The Human *in vitro* CBMN Assay

The establishment of a protocol for the CBMN assay using HSC-3TO required first the examination of the growth of cells in culture in order to establish when cells were

best used for experimentation, and second the optimization of the primary variables of the assay (dose of Cyt-B, duration of Cyt-B exposure and initial seeding density).

From the long- and short-term growth curves for HSC-3TO cells, it was established that there was a significant lag phase of 18-23 hours before which cells attained a linear growth phase with a population doubling time of 30 to 33 hours. Cells reached a plateau of approximately 32.3×10^5 cells by 120 hours. These parameters (Freshney, 1994) were used to optimize experimental conditions for the CBMN assay.

Three μg/mL Cyt-B was chosen for use in the CBMN Assay because it provided a sufficiently high percent binucleate cells, it was the original dose used in the CBMN assay (Fenech and Morley, 1985), and is the most prevalent dose cited in the literature (Surralles et al., 1994).

Cyt-B produced comparable percent binucleate cells when HSC-3TO was treated for 24-36 hours. The duration of treatment of Cyt-B anywhere from 24-36 hours was chosen for the CBMN assay because it was shown to capture a sufficient amount of proliferating cells as binucleate cells.

The experimental seeding density of 2.5×10^5 cells/mL provided a significantly higher percent binucleate cells after a 24-hour Cyt-B treatment than 1.0×10^5 cells/mL. The higher density of 5.0×10^5 cells/mL did not produce a significantly higher percent binucleate cells than

2.5 x 10^5 cells/mL. A seeding density of 2.5 x 10^5 cells/mL was chosen for the CBMN assay.

4.2 Mitomycin C

Having optimized the time in culture during which cells are best used for experimentation and the primary CBMN assay variables, it was next necessary to optimize the use of MMC in the CBMN assay in order to elicit a positive response.

4.2.1 Dose-Response Experiments

The cytotoxicity of MMC has been attributed to the formation of monoadducts which have been shown to be strong blocks of DNA replication *in vivo* (Basu et al., 1993) and to the formation of intra- and interstrand DNA cross-links (Szybalski and Iyer, 1964). Interstrand DNA cross-links represent an important class of chemical damage since they prevent DNA strand separation and hence can constitute complete blocks to DNA replication and transcription (Friedberg, 1985).

In order to have relevant doses of MMC for the CBMN assay, the toxicity of MMC in various experimental conditions was examined. In agreement with results from other laboratories using various human cell types (Rudd et al., 1991; Mark et al., 1994), MMC caused a dose-dependent increase in cytotoxicity. The duration of allowed growth of the culture had a great effect on the degree of cytotoxicity, with increased periods of allowed growth

having had increased cytotoxicity. This was evidenced by lower D_{10} and D_{50} values after 4-6 days as opposed to 48 hours. The continuous presence of MMC in the cultures did not produce greater cytotoxic effects than a 24-hour treatment, suggesting that MMC is rapidly degraded under conditions of tissue culture. Dose-response experiment Group G provided a D_{10} dose of approximately 1000 ng/mL MMC and a D_{50} dose of approximately 100 ng/mL MMC. Under the same experimental conditions (CBMN assay experiment #3), these doses provided highly elevated micronucleus frequencies (see below, Section 4.3.3).

4.2.2 Modified Growth Experiments

As it has been established that MMC causes mitotic delay (Mauro et al., 1986), it was important to determine the growth of HSC-3TO cultures treated with the chosen doses, 100 and 1000 ng/mL MMC.

By 24 hours in culture, untreated cells had reached seeding density and were thus no longer in lag phase. Cells treated with 100 and 1000 ng/mL MMC had not yet reached seeding density and were still in lag phase. By 48 hours in culture, untreated cells had proliferated to 1.7 times their TVCN at 24 hours and were thus in their growth phase. This was in agreement with short and long-term growth experiments. Cells treated with 100 ng/mL MMC had reached seeding density and were thus no longer in lag phase.

Cells treated with 1000 ng/mL MMC had not yet reached seeding density and were still in lag phase.

By 72 hours, untreated cells had demonstrated proliferation of 1.7 times their TVCN at seeding, a growth comparable to that seen in long-term growth experiments, and had thus reached growth phase. Cells treated with 100 ng/mL MMC had also reached growth phase by 72 hours since TVCN of these cells was comparable to that of untreated cells at 72 hours. Cells treated with 1000 ng/mL had not yet reached seeding density by 72 hours and were thus still in lag phase.

There was no statistically significant difference found between TVCN of untreated cells at 72 and 108 hours, indicating cells had reached saturation density by 72 hours. Percent binucleate cells at 108 hours in untreated cultures was 25%, however, indicating proliferation of a population of cells between 72 and 108 hours. As plateau is an equilibrium between cell proliferation and cell loss, the cells which did divide between 72 and 108 hours were captured by Cyt-B. TVCN of cells treated with both 100 and 1000 ng/mL MMC decreased from 72 to 108 hours, indicating greater cell loss. MMC-treated cells were still being captured by Cyt-B between 72 and 108 hours, however, indicating a small amount of proliferation offset by a larger amount of cell loss.

The effectiveness of Cyt-B to block proliferation for up to 48 hours was seen by the inability of all Cyt-B-treated cultures to escape lag phase after 48 hours in culture. The effectiveness of Cyt-B in blocking proliferation between 72 and 108 hours is difficult to interpret using TVCN data because, for untreated cells, TVCN did not change, and for MMC-treated cells, TVCN decreased. Percent binucleate data indicated that for MMC-treated and untreated cells, cells were blocked by Cyt-B between 72 and 108 hours. As there was no evidence of enhanced proliferation between 72 and 108 hours, both MMC-treated and untreated cells were likely not escaping a Cyt-B block during this time. Cyt-B effectively blocked proliferation of MMC-treated and untreated HSC-3TO in both treatment protocols.

4.2.3 CBMN Assay

Depending upon the harvest time, MMC induced micronuclei in HSC-3TO at levels which were 1.7-6.6 times that of control cells. The effect of harvest time on micronuclei induction can be explained by MMC-induced mitotic delay. When DNA synthesis is artificially blocked, as it has been shown to be by MMC monoadducts (Basu et al., 1993), mitosis is delayed until after the block has been removed and DNA synthesis has been completed (Alberts et al., 1989). This is known as mitotic delay. Because only those cells which have completed mitosis can express

micronuclei, a cell culture which is in mitotic delay will not have dividing cells which can show micronuclei. MMC-treated cultures which remained in lag phase when untreated cultures had demonstrated proliferation were experiencing mitotic delay.

After 24 hours, cells treated with 100 and 1000 ng/mL MMC were still in mitotic delay. After 48 hours, cells treated with 100 ng/mL showed only limited proliferation. This could explain why after 24-48 hours, cultures treated with 100 ng/mL did not show PMBC or TM levels that were elevated over those of controls. The small proportion of cells treated with 100 ng/mL MMC which divided and were captured as binucleate cells at 24-48 hours (Table R13) were those least damaged by MMC. These cells did not demonstrate micronuclei. Highly damaged cells were either still in the process of repair and experiencing mitotic delay, or had already died.

Although cells treated with 1000 ng/mL were still in mitotic delay after 48 hours, significantly elevated PMBC or TM levels (1.7 and 1.8 times that of the control respectively) were seen after 24-48 hours. Percent binucleate cells indicated that a small proportion of MMC-treated cells did divide before 48 hours. This small proportion of cells which were able to divide and were captured as binucleate cells were more damaged than those at 100 ng/mL MMC. This rise in the number of damaged cells at

1000 ng/mL MMC was manifested as a slight rise in PMBC and TM.

By 72 hours cells which had been treated with 100 ng/mL MMC had proliferated while cells treated with 1000 ng/mL MMC were still in mitotic delay. At a harvest time of 108 hours, although cells treated with 100 and 1000 ng/mL MMC had decreased in number since 72 hours, a small proportion of cells were still dividing and were captured by Cyt-B. These highly damaged cells demonstrated highly elevated PMBC and TM.

After 108 hours 50% of the cells treated with 100 ng/mL MMC (D_{50}) were dead and 90% of the cells treated with 1000 ng/mL MMC (D_{10}) were dead. As a consequence of this enhanced cytotoxicity at 1000 ng/mL, fewer cells progressed through the cell cycle being captured as binucleate cells when cultures were treated with 1000 ng/mL versus 100 ng/mL MMC. Those cells most damaged may not have survived or been able to undergo cell division. This could explain why PMBC and TM levels at 1000 ng/mL were not elevated over those seen at 100 ng/mL MMC.

The MMC doses shown to be effective in inducing micronuclei in human lymphoblastoid cells were relevant in light of the serum concentrations of MMC found in humans after bolus injection of MMC in chemotherapy treatment. After injection of 30 mg, 20 mg, or 10 mg i.v., the maximum serum concentrations are 2400 ng/mL, 1700 ng/mL and

520 ng/mL MMC respectively (Canadian Pharmaceutical Association, 1996). While doses used for chemotherapy are many times higher than environmentally relevant exposures, these data demonstrate that the doses used here were biologically relevant.

The six-fold increase in percent micronucleated binucleate cells obtained in this study is comparable to that found in the literature for various human cell types. Elhajouji et al. (1995) recorded 12% micronucleated binucleate cells at 334 ng/mL MMC versus 2.16% at the control in human lymphocytes, an almost six-fold increase. Rudd et al. (1991) recorded 10% micronucleated binucleate cells at 1000 ng/mL MMC versus 1.4% at the control in human fibroblasts, a seven-fold difference. van Pelt et al. (1991) found a five- to twenty-fold increase in the percent micronucleated binucleate cells at 1000 ng/mL MMC over controls in human keratinocytes. Zhang et al. (1995) recorded 9.4% micronucleated binucleate cells in human lymphoblastoid cell cultures treated with MMC versus 1.2% in culture cultures, nearly an eight-fold increase.

The mechanism by which MMC induces micronuclei has not been elucidated, although many inferences can be drawn from the action of MMC on DNA. MMC breaks human chromosomes (Morad et al., 1973; Shiraishi et al., 1979; Woods et al., 1995). These chromosome fragments become contained in micronuclei (Fenech, 1993a). Exactly how MMC causes

chromosome breakage, however, can only be hypothesized. MMC is known to cause several DNA lesions, base alkylation (Siegel et al., 1992; Prakash et al., 1993) and inter- and intrastrand cross-linkage (Basu et al., 1993), all of which may lead to chromosome breakage via DNA misrepair (Natarajan, 1984; Savage, 1990; Therman and Susman, 1993). Cells have an excision-repair system which normally removes these adducted chemicals by cleaving the phosphodiester backbone of the DNA on either side of the adduct, excising the entire lesion and replicating the excised DNA using the other strand as a template (Strauss et al., 1983; Alberts et al., 1989). Although, ideally for the cell, DNA damage is repaired before the cell cycle is allowed to continue, DNA replication may begin before all adducts are removed from the DNA (Strauss et al., 1983). Damage which remains, either because the excision-repair systems are unable to deal with the number of adducts present or because the cells are genetically incompetent for excision, can lead to mutation or cell death (*ibid*).

It has been observed that MMC induces a high frequency of O^6-methylguanine (O^6meG) lesions that in man can be removed by a methyltransferase reaction (Hittelman, 1985). It has been shown that a number of exogenous alkylated purines, including O^6meG, induce micronuclei in Chinese hamster cells as well as aneuploidy and polyploidy in human lymphocytes (Bonatti et al., 1986; Abbondandolo et al.,

1989; Bonatti et al., 1995). The conversion of O^6meG to O^6me-5′GMP is necessary for the induction of aneuploidy (Bonatti et al., 1995). Various substituted nucleotides have been reported to interfere with components of the mitotic apparatus (for a review, see *ibib*). These results suggest that the nucleotide pool may be one possible target for the aneugenic action of alkylating agents (*ibid*). MMC, an alkylating agent, has been shown to increase the kinetochore positive fraction of micronuclei in MMC-treated human fibroblasts (Rudd et al., 1991) and human lymphocytes (Elhajouji et al., 1995) suggesting that there is an increase in the fraction with whole chromosomes.

4.3 2-Nitrofluoranthene

4.3.1 Dose-Response Experiments

In order to test 2-NFA in the established CBMN assay protocol appropriate doses had to first be established. Statistical analysis of 2-NFA dose-response data indicated an effect of duration of allowed growth and dose on cell survival in 2-NFA. Survival of HSC-3TO was lower in all doses of 2-NFA (with one exception) as duration of allowed growth increased from four to five to seven days. A similar effect was seen in TK6 although only two sets of data were available (four and five days of allowed growth). If there is a delayed effect of 2-NFA on cell survival, it may take several days before it is discernable.

It must be kept in mind, however, that the doses chosen far exceed the actual dose encountered by cells within the body. Also, even when exposed for 7 days to the highest dose of 2-NFA (2×10^4 ng/mL), HSC-3TO cultures still demonstrated over 60% survival.

4.3.2 Modified Growth Experiments

It was important to establish the growth of HSC-3TO in the appropriate doses of 2-NFA before testing these doses in the established CBMN assay protocol. If 2-NFA caused mitotic delay, harvest time would be an important variable for eliciting a positive response in the CBMN assay.

By 24 hours all cells had experienced comparable growth having once again reached seeding density and were thus no longer in lag phase. By 48 hours all cells had experienced comparable growth having doubled their population from 24 hours. By 48 hours cells were in growth phase. 2-NFA did not cause mitotic delay in HSC-3TO after treatment for 24 or 48 hours.

TVCN of Cyt-B-treated cultures remained below that at seeding after 24 and 48 hours of 2-NFA exposure and there was no increase in TVCN between 24 and 48 hours. As with MMC-treated and untreated cells, Cyt-B effectively blocked the proliferation of 2-NFA-treated and untreated cells for the entire culture period of 48 hours.

4.3.3 CBMN Assay

2-NFA did not cause micronuclei in HSC-3TO at doses of up to 2000 ng/mL after harvest times of 48 and 108 hours. There are several possible explanations for the lack of induction of micronuclei in 2-NFA-treated human cells. First of all, if 2-NFA was able to bind to serum proteins present in the culture media, delivery to cells may have been minimal.

2-NFA is known to be a potent direct-acting bacterial mutagen. CASE, a structure-activity relational method, found that the typical nitroarene moieties which were found to be major activating fragments for mutagenicity in Salmonella are not discriminatory for the induction of micronuclei (Yang et al., 1992).

Nitro-containing chemicals require the reduction of their nitro-function to the corresponding hydroxylamines for exerting their mutagenicity (Prough et al., 1988; Watanabe et al., 1989). Bacterial nitroreductase and acetyltransferase enzymes have been found to be important in the mutagenicity of 2-NFA in bacteria (Zielinska et al., 1987; Shane et al., 1991). There are conflicting results, however, as to the importance of S9 enzymes when they are added to bacteria as an exogenous metabolizing system. What is not known is if the same metabolizing enzymes which act on 2-NFA in bacterial cells act on 2-NFA in human cells. Evidence exists that 2-NFA can be metabolized to a non-toxic

substance in bacteria. Zielinska et al. (1987) tested the metabolites of 2-NFA formed from incubation of 2-NFA with S9, 8- and 9-hydroxy-2-nitrofluoranthene, in TA98, TA98NR and TA98/1,8-DNP and found the mutagenicity to be substantially suppressed, indicating further reduction of 2-NFA metabolites to non-toxic products. It is possible that in human cells, 2-NFA is metabolized to a non-toxic substance.

2-NFA was considered a potential human mutagen because it is known to cause DNA adducts in both bacterial and mammalian cells. It has been suggested that, for steric reasons, all nitroarenes having more than two rings will form DNA adducts with the C-8 position of guanine (Rosenkranz et al., 1985). Herreno-Saenz et al. (1994) found the adduct N-(deoxyguanosin-8-yl)-2-aminofluoranthene in the DNA of *Salmonella typhimurium* TA98 and neonatal mice with 2-NFA. When Salmonella was treated with 10 and 20 μM 2-NFA, the levels of adduct were 0.11 and 0.20 adducts per 10^6 nucleotides respectively. The levels of adduct in neonatal mice sacrificed 24 hr, 48 hr and 7 days after 2-NFA was administered were found to be 6.9, 5.2, and 4.0 adducts per 10^9 nucleotides respectively. 2-NFA-induced DNA adducts were clearly much less extensive in mice than in bacteria.

The C^8-guanine adduct induced by 2-NFA may not have the same biological consequences as adducts formed at other positions of guanine. There are several atoms in guanine to

which a chemical may become covalently linked (Friedberg, 1985). Normally, the C^8 position of guanine in duplex DNA in the B conformation is relatively hindered sterically and is rather inaccessible to a bulky adduct (*ibid*). In contrast, MMC forms adducts in DNA by interaction with the N^2 or N^7 positions of guanine (Basu et al., 1993; Prakash et al., 1993; Sastry et al., 1995) and methyl lesions at the O^6 position of guanine (Hittleman, 1985). The O^6 and N^7 atoms of guanine are more accessible to adducts than the C^8 atom of guanine (Friedberg, 1985). Unlike the C^8 atom, the O^6 and N^2 atoms of guanine are involved in hydrogen bonding to cytosine in the opposite DNA strand (*ibid*). MMC monoadducts at the N^2 position of guanine have been shown to be strong blocks of DNA replication (Basu et al., 1993). In contrast, the C^8-guanine adduct of 2-aminofluorene was found not to be a significant block to DNA replication (Basu et al., 1993). 2-NFA-induced C^8-guanine adducts may be similarly ineffective at blocking DNA replication. Benzo[a]pyrene, which induces an adduct at the N^2 position of guanine (Friedberg, 1985), was found to only moderately inhibit lymphocyte proliferation (Warshawsky et al., 1995). It has been hypothesized that the unusually severe replication block caused by MMC monoadducts in comparison with other DNA lesions is inherent in the unique noncovalent, hydrogen-bonding capacities of the MMC residue, which may induce additional interaction in DNA with other

nucleotides (Basu et al., 1993). This increased thermodynamic stability of a distorted structure at the replication fork results in the polymerase block (*ibid*). Covalent adducts which are capable of bypass are largely hydrophobic in nature and lack the capacity for similar, polar interactions (*ibid*). Therefore, adduct formation is not discriminatory for the induction of micronuclei.

It has been found that the micronucleus assay is relatively insensitive to detect chemicals that predominantly induce excision-repairable lesions such as DNA adducts (Fenech and Neville, 1992; Surralles et al., 1995). 2-NFA is highly mutagenic in bacterial strains derived from strain TA98 (Zielinska et al., 1987; Shane et al., 1991; Ball et al., 1994). These strains do not have a functional excision repair system. Fenech and Neville (1992) devised a method of using cytosine arabinoside (ARA) to convert excision-repairable DNA lesions to micronuclei within one cell cycle. ARA exerts its effect by inhibiting the DNA polymerization step (gap filling) that normally occurs after excision of damaged bases during nucleotide or base excision repair (Friedberg, 1985). As a result excision repair sites are converted to single strand breaks (Fenech and Neville, 1992). Using this method, Surralles et al. (1995) found that MMC, a micronuclei-inducing chemical, did not greatly induce excision repair. If 2-NFA induces excision-repairable lesions, the CBMN assay would not detect this

damage in excision repair competent cells.

The testing of 2-NFA in the ARA CBMN method or with excision repair-deficient human cells should be considered in the further genotoxic testing of 2-NFA. In addition, 3,7- and 3,9-diNFA, chemical relatives of 2-NFA which have been shown to induce micronuclei in the mouse *in vitro* micronucleus test (Tokiwa et al., 1988), should be tested in the established CBMN assay to provide a valid comparison to the mouse *in vitro* assay.

5. CONCLUSIONS

The primary objective of this study was to develop a human *in vitro* micronucleus assay which could be used as a screening tool in conjunction with other mammalian assays to estimate the human mutagenic potential of environmental chemicals. A CBMN assay using HSC-3TO as the test cell line and MMC as a valid positive control was established. Treatment of HSC-3TO cells with 100 ng/mL MMC for 108 hours with a 3 μg/mL Cyt-B block administered after 72 hours until harvest provided levels of micronucleated binucleate cells and total number of micronuclei which were over six times those of controls. A harvest time of 108 hours was necessary in order to see elevated micronuclei at 100 ng/mL MMC because of MMC-induced mitotic delay. At 100 ng/mL MMC, cells required a minimum of 48 hours to proliferate beyond lag phase. The established assay is therefore able to detect micronuclei after a genotoxic insult, although multiple harvest times should be considered when testing a novel chemical.

2-NFA did not cause micronuclei in HSC-3TO after either of two harvest times. Therefore, the response of 2-NFA in the established CBMN assay was negative.

The human *in vitro* CBMN assay was established as a preliminary screening assay for environmental chemicals of concern and, as with other assay systems, it is not without its limitations. Humans do not experience acute high-dose exposures to 2-NFA, but chronic low-dose exposures. The established assay does not address chronic low-dose exposures. As it was the objective to create a fast and effective screening test, long-term treatment was not considered. Results obtained using the established assay could be used to screen chemicals for further *in vivo* testing. The established protocol could also be extended to include low-dose chronic exposure. An additional limitation is that, although a human cell line was used, the assay is an *in vitro* system which does not mimic real life (*in vivo*) conditions. It is not known how much 2-NFA is actually retained in humans after exposure (i.e., the difference between what is inhaled and what is exhaled), what the target organs are, the degree to which 2-NFA is absorbed by the blood and the effect of metabolism and/or detoxification.

The finding that 2-NFA did not cause micronuclei in the present CBMN assay does not negate its status as an environmental contaminant of concern. No protocol, no matter how extensive, can detect all genotoxic chemicals (Kirkland et al., 1994). For a given agent, each kind of damage (toxicity, mutagenesis, chromosomal effects) has its

own distinct biological importance (Cleaver, 1989). Micronuclei induction is one of a battery of genotoxic endpoints that could be studied in human cell lines in *vitro*. Several others such as sister chromatid exchange, unscheduled DNA synthesis (to detect gap-filling after excision-repair), or ^{32}P-post-labelling (for the detection of 2-NFA-induced adducts) could be used in conjunction with the mouse *in vivo* micronucleus test before 2-NFA is considered a substance which is not a threat to human health.

The established CBMN protocol using HSC-3TO has advantages in that it is an easy and inexpensive assay which requires little prior knowledge, training or materials and can be employed on a large scale. It has the potential to identify chemicals capable of inducing aneuploidy which is particularly relevant to human health and not readily assessed by other in vitro tests. The human *in vitro* CBMN assay should be used in conjunction with other assays to assess the human health risk of environmental chemicals.

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APPENDIX I:
Data from Growth Experiments

Table AI-1: **Analysis of Variance Table for Long-term Growth Data Fit to a General Linear Model**
Model: $y_{ijk} = \mu + a_i + b_j + e_{k(ij)}$

Source	df	Seq SS	Adj SS	Adj MS	F	p
Hours	1	880.2	880.2	880.2	35.33	<0.001
Expt	13	4309.8	4302.7	331.0	13.28	<0.001
Error	53	1320.6	1320.6	24.9		
Total	67	6510.6				

Expt = Experiment
Hours = Duration of allowed growth

Table AI-2: **Analysis of Variance Table for Short-term Growth Data Fit to a General Linear Model**
Model: $y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{k(ij)}$

Source	df	Seq SS	Adj SS	Adj MS	F	p
Expt	2	338.2	325.7	162.9	17.99	<0.001
Hours	9	1325.5	1298.7	144.3	15.94	<0.001
Expt*Hours	18	399.2	399.2	22.2	2.45	0.005
Error	59	534.1	534.1	9.1		
Total	88	2597.0				

Expt = Experiment
Hours = Duration of allowed growth

APPENDIX II:
Data from the Cytokinesis Block Micronucleus Assay

Table AII-1: Analysis of Variance Table for Optimal Dose of Cyt-B Experiment Replicate Data

Cyt-B (μg/mL)	Source	df	SS	MS	F	p
0	Rep Error Total	2 6 8	14.9 21.3 36.2	7.4 3.6	2.09	0.204
1.5	Rep Error Total	1 4 5	37.5 291.3 328.8	37.5 72.8	0.51	0.513
3.0	Rep Error Total	2 6 8	2044.2 175.3 2219.6	1022.1 29.2	34.98	<0.001
6.0	Rep Error Total	2 6 8	215.0 635.0 850.0	107.0 106.0	1.01	0.417
12.0	Rep Error Total	2 6 8	272.2 591.3 863.6	136.1 98.6	1.38	0.321

Rep = Replicate

Table AII-2: Analysis of Variance Table for Mean Optimal Dose of Cyt-B Data

Source	df	SS	MS	F	p
[Cyt-B] Error Total	4 9 13	2519.6 860.9 3380.5	629.9 95.7	6.59	0.009

[Cyt-B] = Dose of Cyt-B

Table AII-3: Analysis of Variance Table for Optimal Duration of Cyt-B Treatment Experiment Replicate Data

Cyt-B (hours)	Source	df	SS	MS	F	p
24	Rep	2	144.9	72.4	0.79	0.497
	Source	6	552.0	92.0		
	Error	8	696.9			
28	Rep	2	168.0	84.0	1.10	0.392
	Source	6	458.0	76.3		
	Error	8	626.0			
32	Rep	2	12.7	6.3	0.26	0.781
	Source	6	147.3	24.6		
	Error	8	160.0			
36	Rep	2	60.7	30.3	0.98	0.428
	Source	6	185.3	30.9		
	Error	8	246.0			

Rep = Replicate

Table AII-4: Analysis of Variance Table for Mean Cyt-B Duration Data

Source	df	SS	MS	F	p
Cyt-B Duration	3	42.8	14.3	0.88	0.490
Error	8	129.3	16.2		
Total	11	172.1			

Table AII-5: Analysis of Variance Table for Optimal Seeding Density Experiment Replicate Data

Seeding Density ($\times 10^5$)	Source	df	SS	MS	F	p
1.0	Rep	2	10.9	5.4	0.09	0.915
	Error	6	363.3	60.6		
	Total	8	374.2			
2.5	Rep	2	246.2	123.1	9.39	0.014
	Error	6	78.7	13.1		
	Total	8	324.9			
5.0	Rep	2	80.2	40.1	1.66	0.268
	Error	6	145.3	24.2		
	Total	8	225.6			

Rep = Replicate

Table AII-6: Analysis of Variance Table for Optimal Seeding Density Experiment Data

Source	df	SS	MS	F	p
Seeding Density	2	294.8	147.4	7.84	0.021
Error	6	112.8	18.8		
Total	8	407.7			

APPENDIX III:
Mitomycin C

Table AIII-1: Analysis of Variance Table for MMC Percent Micronuclei Data Fit to a General Linear Model: $y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{k(ij)}$

Source	df	Seq SS	Adj SS	Adj MS	F	p
HT	2	232.8	232.8	116.4	77.08	<0.001
MMC Dose	2	61.6	61.6	30.8	20.40	<0.001
HT*Dose	4	100.1	100.1	25.0	16.56	<0.001
Error	18	27.2	27.2	1.5		
Total	26	421.7				

HT = Harvest time

Table AIII-2: Analysis of Variance Table for MMC Total Number of Micronuclei Data Fit to a General Linear Model
Model: $y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{k(ij)}$

Source	df	Seq SS	Adj SS	Adj MS	F	p
HT	2	33119.2	33119.2	16559.6	86.77	<0.001
MMC Dose	2	8612.7	8612.7	4306.4	22.56	<0.001
HT*Dose	4	14627.0	14627.0	3656.8	19.16	<0.001
Error	18	3435.3	3435.3	190.9		
Total	26	59794.3				

HT = Harvest time

Table AIII-3: Analysis of Variance Table for Comparison of Percent Micronuclei at each Dose in Experiment #1 (24 hour harvest time) and Experiment #2 (48 hour harvest time).

MMC (ng/mL)	Source	df	SS	MS	F	p
0	Experiment	1	0.14	0.14	2.70	0.176
	Error	4	0.20	0.05		
	Total	5	0.34			
100	Experiment	1	0.002	0.002	0.01	0.933
	Error	4	0.83	0.21		
	Total	5	0.83			
1000	Experiment	1	0.04	0.04	0.37	0.574
	Error	4	0.45	0.11		
	Total	5	0.49			

Table AIII-4: Analysis of Variance Table for Comparison of Total Number of Micronuclei at each Dose in Experiment #1 (24 hour harvest time) and Experiment #2 (48 hour harvest time).

MMC (ng/mL)	Source	df	SS	MS	F	p
0	Experiment	1	8.2	8.2	1.53	0.284
	Error	4	21.3	5.3		
	Total	5	29.5			
100	Experiment	1	0.0	0.0	0.00	1.000
	Error	4	93.3	23.3		
	Total	5	93.3			
1000	Experiment	1	16.7	16.7	0.71	0.446
	Error	4	93.3	23.3		
	Total	5	110.0			

Table AIII-5: Analysis of Variance Table for MMC Percent Micronuclei Data: Effect of Harvest Time (24+48 hours and 108 hours) at each Dose.

MMC (ng/mL)	Source	df	SS	MS	F	p
0	HT Error Total	1 7 8	0.5 1.8 2.3	0.5 0.3	2.10	0.191
100	HT Error Total	1 7 8	192.7 11.0 203.7	192.7 1.6	123.00	<0.001
1000	HT Error Total	1 7 8	139.5 14.6 154.1	139.5 2.1	66.79	<0.001

Note: Data for 24 and 48 hour harvest time were pooled.
HT = Harvest time

Table AIII-6: Analysis of Variance Table for MMC Total Number of Micronuclei Data: Effect of Harvest Time (24+48 hours and 108 hours) at Each Dose.

MMC (ng/mL)	Source	df	SS	MS	F	p
0	HT Error Total	1 7 8	60.5 215.5 276.0	60.5 30.8	1.97	0.204
100	HT Error Total	1 7 8	28322 1834 30156	28322 262	108.10	<0.001
1000	HT Error Total	1 7 8	19339 1411 20750	19339 202	95.96	<0.001

Note: Data for 24 and 48 hour harvest time were pooled.
HT = Harvest time

Table AIII-7: Analysis of Variance Table for MMC Percent Micronuclei Data: Effect of Dose at each Harvest Time.

Harvest Time (Hours)	Source	df	SS	MS	F	p
24+48	Dose Error Total	2 15 17	4.2 1.7 5.9	2.1 0.1	19.13	<0.001
108	Dose Error Total	2 6 8	157.4 25.7 183.1	78.7 4.3	18.36	0.003

Note: Data for 24 and 48 hour harvest time were pooled.
Dose = Dose of MMC

Table AIII-8: Analysis of Variance Table for MMC Total Number of Micronuclei Data: Effect of Dose at each Harvest Time.

Harvest Time (Hours)	Source	df	SS	MS	F	p
24+48	Dose Error Total	2 15 17	521.4 232.8 754.3	260.7 15.5	16.80	<0.001
108	Dose Error Total	2 6 8	22710 3227 25937	11355 538	21.11	0.002

Note: Data for 24 and 48 hour harvest time were pooled.
Dose = Dose of MMC

APPENDIX IV:
2-Nitrofluoranthene

Table AIV-1: Analysis of Covariance Table for 2-NFA Dose Response Data: TVCN Data Fit to a General Linear Model
Model: $y_{ijk} = \mu + bX_{ij} + A_i + B_j + AB_{ij} + e_{ijk}$

Source	df	Seq SS	Adj SS	Adj MS	F	p
Cell Line	1	2342.2	3336.4	3336.4	188.71	<0.001
DG	2	1294.3	1294.3	647.1	36.60	<0.001
Dose	4	417.0	586.4	146.6	8.29	<0.001
DG*Dose	8	430.8	430.8	53.9	3.05	0.005
Error	83	1467.5	1467.5	17.7		
Total	98	5951.8				

DG = Days of Allowed Growth

Table AIV-2: 2-NFA Modified Growth: Analysis of Variance Table for 2-NFA Dose Response TVCN Data (0 Cyt-B) at 24 and 48 Hours

Time (Hours)	Source	df	SS	MS	F	p
24	Dose	3	0.94	0.31	0.79	0.558
	Error	4	1.58	0.39		
	Total	7	2.52			
48	Dose	3	2.25	0.75	0.31	0.820
	Error	4	9.80	2.45		
	Total	7	12.05			

Dose = Dose of 2-NFA

Table AIV-3: 2-NFA Modified Growth: Analysis of Variance Table for 2-NFA TVCN Data (3 Cyt-B) at 24 and 48 Hours

Time (Hours)	Source	df	SS	MS	F	p
24	Dose	3	1.02	0.34	3.13	0.19
	Error	3	0.32	0.11		
	Total	6	1.34			
48	Dose	3	0.70	0.23	0.53	0.69
	Error	4	1.76	0.44		
	Total	7	2.46			

Dose = Dose of 2-NFA

Table AIV-4: Analysis of Variance Table for 2-NFA Percent Micronuclei Data

Source	df	SS	MS	F	p
Harvest Time	1	0.06	0.06	1.98	0.189
2-NFA Dose	4	0.20	0.05	1.66	0.234
Harvest Time*Dose	4	0.08	0.02	0.63	0.651
Error	10	0.31	0.03		
Total	19	0.65			