Comparative evaluation of phenobarbital/beta-naphthoflavone versus Aroclor 1254- induced rat S9 for use in *in vitro* cytogenetic assays

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Introduction

A variety of test compounds exert genotoxic effects through indirect mechanisms requiring metabolic activation, mainly by Cytochrome P450 enzymes. While *in vivo* test systems generally possess metabolic pathways required for bioactivation, most *in vitro* test systems either lack the ability to or are inefficient at metabolizing such compounds and thus, require the use of exogenous metabolic systems, generally a rat liver S9, to mimic bioactivation during genotoxicity assessment. Environmental concerns about the use of Aroclor-1254 to induce high levels of P450 and other microsomal enzymes have led to the need for alternative methods of S9 preparation. The present study was conducted to compare phenobarbital/beta-naphthoflavone (PB/NF) induced S9 with Aroclor-1254 induced rat S9 in *in vitro* cytogenetics assays in human peripheral blood lymphocytes. Cyclophosphamide, which is a commonly used positive control, requiring metabolic activation, was evaluated at 5 and 7.5 µg/mL in the chromosome aberration (CA) and 2.5, 5, and 7.5 µg/mL in the micronucleus (MN) assays following standard assay procedures. Cytotoxicity ranged from 30 to 40% for CA and 10% to 60% for MN assays. Frequency of aberrant cells ranged from 18 to 20% (CA) for both sources of S9. Induction in %MN frequencies were 3 to 7-fold for Aroclor and 3 to 6-fold for PB/NF-induced S9. The results demonstrate comparable qualitative data between two activation/induction systems with minimal quantitative differences. Thus, this comparative human lymphocyte cytogenetic study in our new laboratory demonstrated that PB/NF-induced S9 could be used as an alternative to the Aroclor- induced rat S9 for *in vitro* genotoxicity assessment in HPBL cells.

Objective

The purpose of this study was to evaluate the potential replacement of Aroclor-induced S9 with phenobarbital/beta-naphthoflavone (PB/NF) -induced S9 in the in vitro cytogenic assay. Micronucleus and chromosome aberrations assays using human peripheral blood lymphocytes (HPBL) were conducted with above mentioned two different liver homogenate S9. Due to space limitation, results are presented for the micronucleus assay only. The most commonly used reference test compound, cyclophosphamide (CAS - 50-18-0), that requires metabolic activation to cause genotoxicity was evaluated in this project. Assay design was in the line with OECD testing guidelines for in vitro micronucleus evaluation (OECD 487). The study was conducted using the Good Laboratory Practice (GLP) regulations for nonclinical laboratory studies as a guideline.

Methods

Experimental design:

5.0 mL complete media, 0.5 mL blood, 2% v/v PHA /culture in 15 mL centrifuge tube ⟹ ~48h later, media was removed, cells were resuspended in 4mL serum free medium + 1.0 mL S9-mix and treatment performed ⟹ After 4h, treatment medium removed, fresh complete medium with cytochalasin B added. Cells were allowed to recover for additional 20h ⟹ All cultures harvested at 24h from initiation of treatment for micronucleus evaluation
CBPI was used as cytotoxicity indicator

Test system and culture condition: Whole blood cultures were initiated from healthy adult donors (18-35 years of age, non-smokers, without a recent history of radiotherapy, viral infections or the administration of drugs) in complete medium (RPMI-1640 containing 15% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 µg/mL streptomycin) by adding 0.5 mL heparinized blood per 5 mL of complete medium with 2% phytohemagglutinin (PHA) in centrifuge tubes. The cultures were incubated under standard conditions (37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air) for 44-48 hours prior to treatment initiation.

Treatment: Aroclor-induced as well as Phenobarbital/5,6 Benzoflavone induced liver homogenate S9 were obtained from MolTox; Boone, NC and used at 2% and 1.5% v/v, respectively with other cofactors mix, as per our standard operating procedure. Cyclophosphamide (CP) mixed with sterile water (vehicle) was evaluated at multiple target concentrations in 4h exposure + 20h recovery. DMSO, frequently used vehicle control in the in vitro micronucleus assay was also evaluated.

Results and Discussion

There was no qualitative difference in in the baseline cell growth based on cytokinesis blocked proliferation index (CBPI) and micronucleus frequency (MN%). Both sources of metabolic activation systems had similar potency in activation of positive control (CP) for induction of micronucleus formation (5.5-fold vs. 4.6- fold in Aroclor vs. PB/NF-induced S9). The slight differences observed were statistically insignificant.

Conclusions

Since both sources of S9 showed similar effects in the endpoints evaluated, the PB/NF-induced S9 could be used in place of Aroclor-induced S9 in the in vitro micronucleus assay.

Reference

OECD Guideline for the Testing of Chemicals, Guideline 487 (*In Vitro* Mammalian Cell Micronucleus Test). Updated and adopted 29 July 2016.

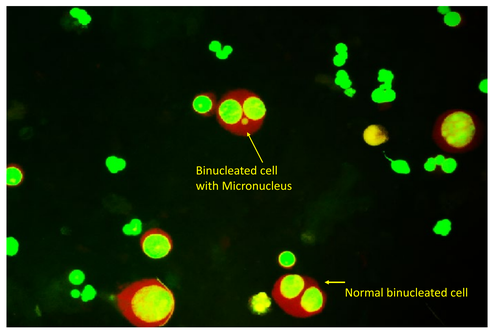

Figure 3: Microphotograph of human peripheral blood lymphocyte cells stained with acridine orange showing normal and abnormal binucleated cells.

Table 1

Impact of S9 type on CBPI and micronucleus induction

	Vehicle		T-Test	Positive Control		T-Test
	Arc	PB/NF	p-value	Arc	PB/NF	p-value
N	19	20		14	15	
CBPI	1.66±0.10	1.63±0.11	0.478	1.44±0.12	1.39±0.08	0.171
MN%	0.39±0.20	0.39±0.36	0.968	2.16±1.12	1.80±0.72	0.328

Arc=Aroclor-induced S9

PB/NF=Phenobarbital/beta-naphthoflavone-induced S9

PB/NF vs. Acr, 2-samples T-Test (Unequal)

Table 1: Showing the mean ±SD value CBPI and MN% from 5 trials with Aroclor and PB/NF- induced S9. Statistically there was no significant difference when two different sources of S9 were used on cell growth induction of MN frequency.

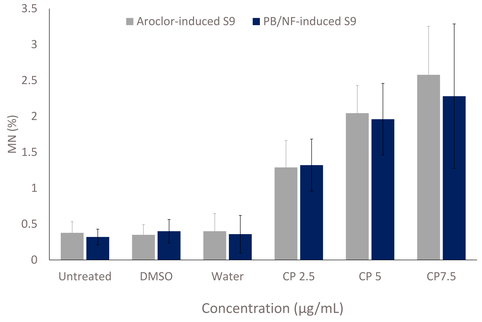

Figure 1: Showing the mean ±SD value MN% from 5 trials with Aroclor and PB/NF- induced S9. The baseline MN% of untreated and two commonly used solvent controls (DMSO and water) were similar. Statistically significant (Fisher's Exact test, p<0.01) and concentration dependent increase in the MN% was observed for positive control (CP).

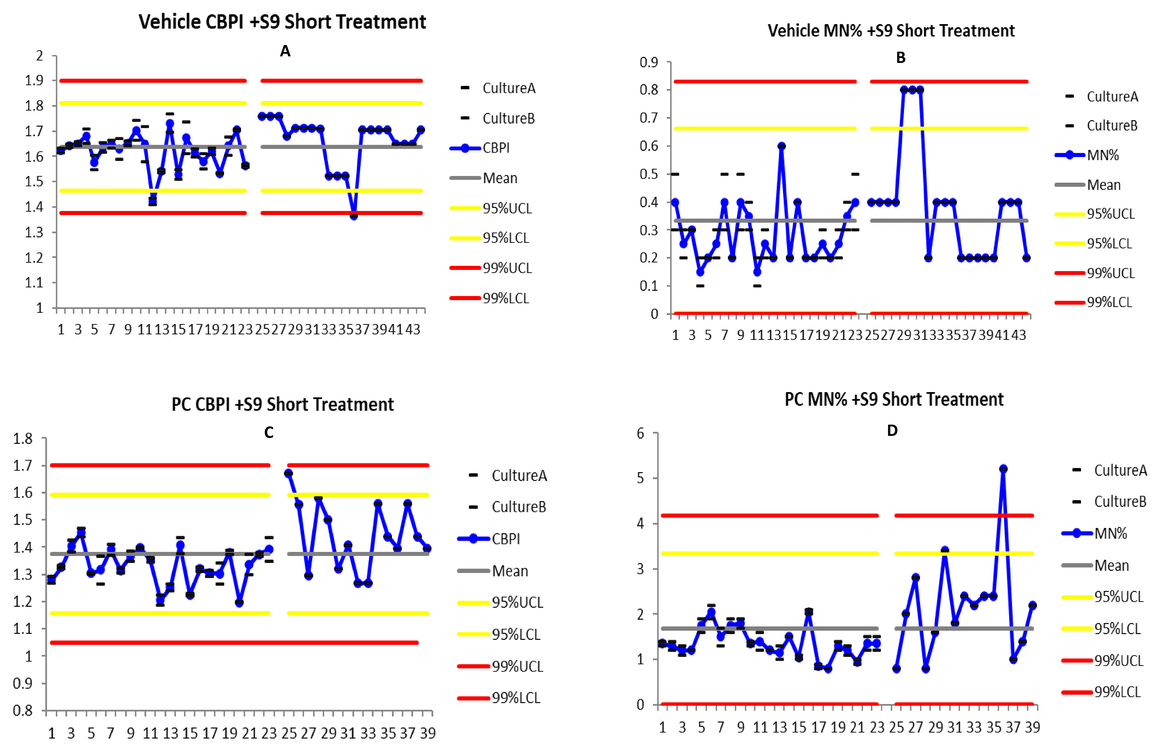

Figure 2: Graphs A to D showing the c-charts for distribution of CBPI and MN% . Data generated at newly established genetic toxicology laboratory at Inotiv, Rockville facility were compared with data of previous lab with same technical staff. Inotiv's qualification data are separated from previous data by a break. The data confirms the assay stability and sensitivity in the newly established Inotiv genetic toxicology laboratory. The use of combined previous historical control data constructed by current Inotiv staff along with the current qualification data may be used to build historical control data initially (or until enough Inotiv data points are available for compilation of a meaningful 95% range). (PC= positive control; MN = micronucleus; CBPI = cytokinesis blocked proliferation index; UCL = upper control limit; LCL = lower control limit)