In Vitro Micronucleus Positive or Negative?

Background

- Drug candidate for chronic and non-life-threatening indication
- Genotoxicity Testing Summary
  - Ames (GLP and non-GLP) - Negative
  - In vivo micronucleus up to 750 mg/kg/day (GLP and non-GLP) – Negative
    - Add-on to multi dose tox study
  - In vitro micronucleus (non-GLP) – Negative
  - In vitro micronucleus (GLP) – Negative?

In Vitro Micronucleus Platform

- Exploratory and GLP assay performed in the same lab using same SOP
  - Same scorer and study director
- CHO Cells
- Manual Microscopic Scoring (Acridine Orange stain)
  - 4000 cells in vehicle control
  - 2000 cells treated
- Relative Population Doubling (%RPD) – Toxicity Assessment
- 3 Treatment Conditions
  - 3 hours without S9 with 21-hour recovery
  - 3 hours with S9 with 21-hour recovery
  - 24 hours without S9
- Historical Vehicle Control upper limit – 2.27% micronucleated cells
- Precipitate assessed in media at start of treatment and end of treatment by eye and microscopically.

In Vitro MN Results – Exploratory Assay

- Exploratory MN Study in CHO cells – Negative
  - Max dose limited to 200 µM by solubility in 3-hour treatments (with/without S9)
    - 65% Relative Population Doubling at highest dose (with S9)
  - Max dose limited to 105 µM by toxicity in 24-hour treatment
    - 46% Relative Population Doubling at highest dose
  - Assay met all criteria for a valid test compliant with OECD and ICH S2(R1) Guidelines

24-hour without S9

Concentration (uM)	%RPD	%MN
0	100	1.78
10	88	1.75
60	59	1.85
105	46	1.85
130	25	NS

3 hours without S9

Concentration (uM)	%RPD	%MN	
0	100	1.35	
10	98	1.45	
105	80	1.35	
200	73	2.20	
250	41	NS	ppt

3 hours with S9

Concentration (uM)	%RPD	%MN	
0	100	1.73	
10	103	1.25	
105	78	2.25	
200	65	1.75	ppt
CP	73	6.10*	

NS=Not Scored

CP – Cyclophosphamide 10 µM

In Vitro MN Results – GLP Assay

- Same form of compound but different batch
- Negative in 3-hour and 24-hour treatments without S9
  - Toxicity-limited dose 210 µM (3-hour) and 85 µM (24-hour)
- Single positive point in 3-hour treatment with S9
  - Tested up to 175 uM (solubility-limited dose)
  - Weak increase in %MN at 61% RPD - Statistically significant and just outside the historical control distribution

24-hour without S9

Concentration (uM)	%RPD	%MN	
0	100	1.33	
10	92	1.4	
50	66	1.45	
85	52	1.6	
90	42	NS	

3 hours without S9

Concentration (uM)	%RPD	%MN	
0	100	1.35	
50	86	1.25	
200	66	1.80	
210	57	1.25	
220	42	NS	ppt

3 hours with S9

Concentration (uM)	%RPD	%MN	
0	100	1.20	
50	102	1.43	
150	82	1.68	
175	61	2.45*	ppt
200	NS	NS	ppt
CP	67	5.95*	

NS=Not Scored

CP – Cyclophosphamide 10 µM

Repeat of 3-Hour Treatment with S9

- Positive point repeated but observed precipitate and toxicity shifted

3 hours with S9 - Repeat

Concentration (uM)	%RPD	%MN	
0	100	1.53	
10	101	2.30	
50	85	2.10	
150	76	2.65*	
175	36	NS	ppt
CP	77	5.90*	

Summary of data in CHO Cells (3 hour + S9)

- Assessment and Next Steps
  - Shifts in precipitate and toxicity suggests that the results are confounded by inconsistent solubility and resulting toxicity
  - Precipitate is particularly difficult to assess in culture media containing S9. Possible that 150 uM contained precipitate
  - Follow up study performed to determine if there is any evidence of genotoxicity and identify potential confounding effects at ≥150 uM

Exploratory

3 hours with S9

Concentration (uM)	%RPD	%MN	
0	100	1.73	
10	103	1.25	
105	78	2.25	
200	65	1.75	ppt
CP	73	6.10*	

GLP 1

3 hours with S9

Concentration (uM)	%RPD	%MN	
0	100	1.20	
50	102	1.43	
150	82	1.68	
175	61	2.45*	ppt
200	NS	NS	ppt
CP	67	5.95*	

GLP 2

3 hours with S9 - Repeat

Concentration (uM)	%RPD	%MN	
0	100	1.53	
10	101	2.30	
50	85	2.10	
150	76	2.65*	
175	36	NS	ppt
CP	77	5.90*	

Micronucleus Assessment in TK6 Cells with Samples Taken for Biomarker Assessment

- No MN induction in TK6 cells down to 71% RPD
- Steep tox between 50 and 100 uM
- Precipitate corelates with steep toxicity induction in TK6 cells
- Maximum dose limited by toxicity
- Repeat study not performed due to shifting toxicity and precipitate observed in previous studies
- Biomarker Analysis on the same culture used to supplement analysis

4 hours with S9

Concentration (uM)	%RPD	%MN	
0	100	1.28	
10	123	1.20	
50	71	1.15	
100	34	NS	ppt
CP	47	2.6*	

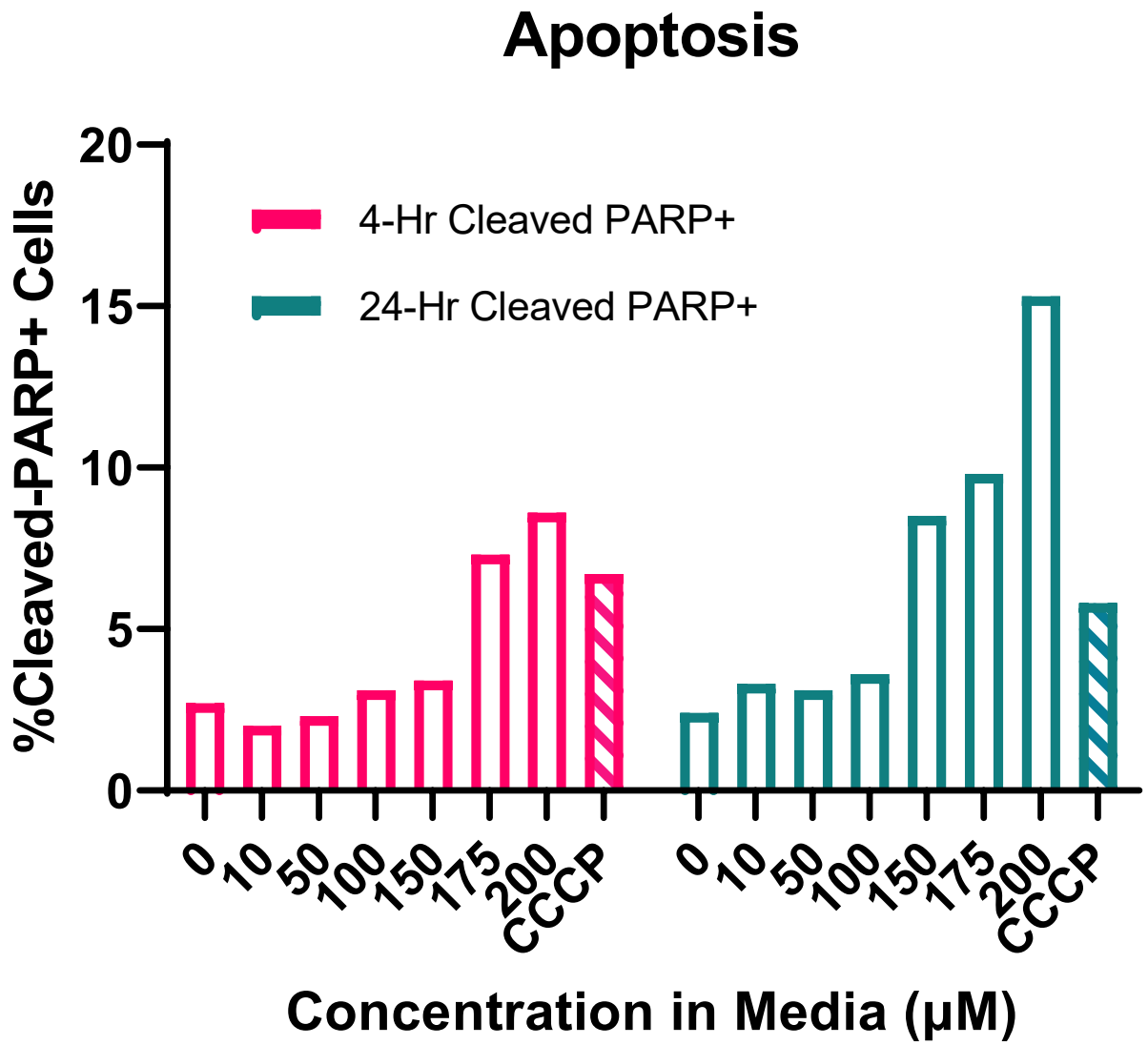
Exploratory Genotoxicity Biomarker Assay (MultiFlow™) in TK6 cells

- Antibody-based flow cytometric assay of cellular responses to genotoxicity (after 4 h & 24 h in TK6 cells
- **MultiFlow™** assay can differentiate between categories of genotoxicity:
  - ***Clastogenicity***: chromosomal breakage
    - Phosphorylated p53: when transported to the nucleus, signals DNA damage
    - gamma H2AX: when phosphorylated is an early read for double stranded DNA breaks
  - ***Aneugenicity***: abnormal number of chromosomes
    - Phospho-Histone H3: indicator of mitotic cells
    - Polyploidy: indicator of cells with greater than normal amount of DNA
  - ***Apoptosis*** – Cleaved-PARP

Positive Call Cut-off values applied (fold increases)				
	γH2AX	Nuclear p53	Phospho-H3	polyploidy
4 hr	1.33	1.16	1.99	na
24 hr	1.51	1.4	1.55	5.3

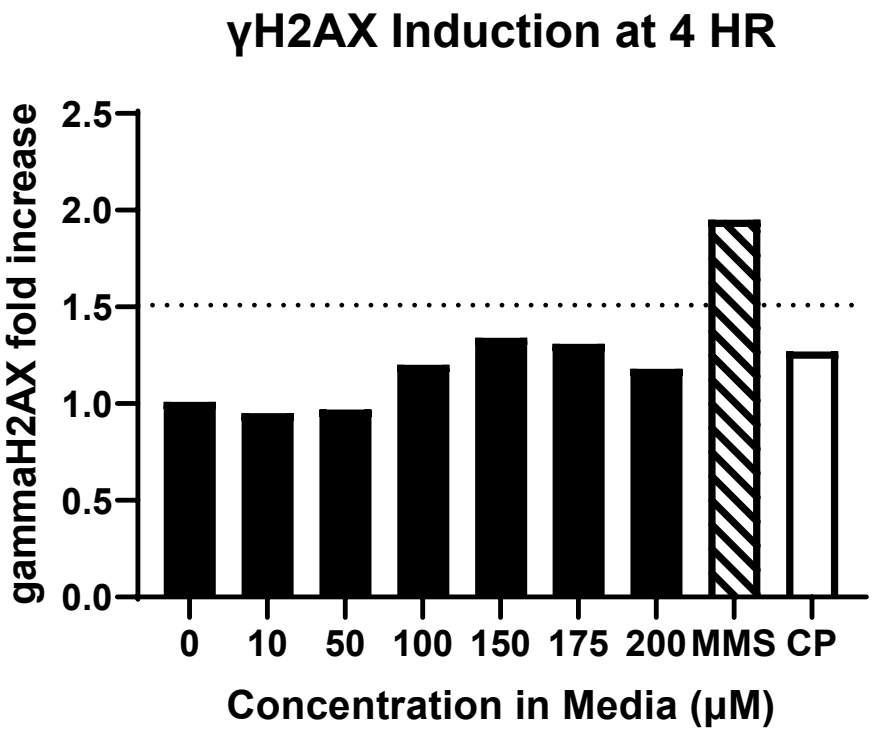
(Multiflow kit, Litron Laboratories, Rochester, NY)

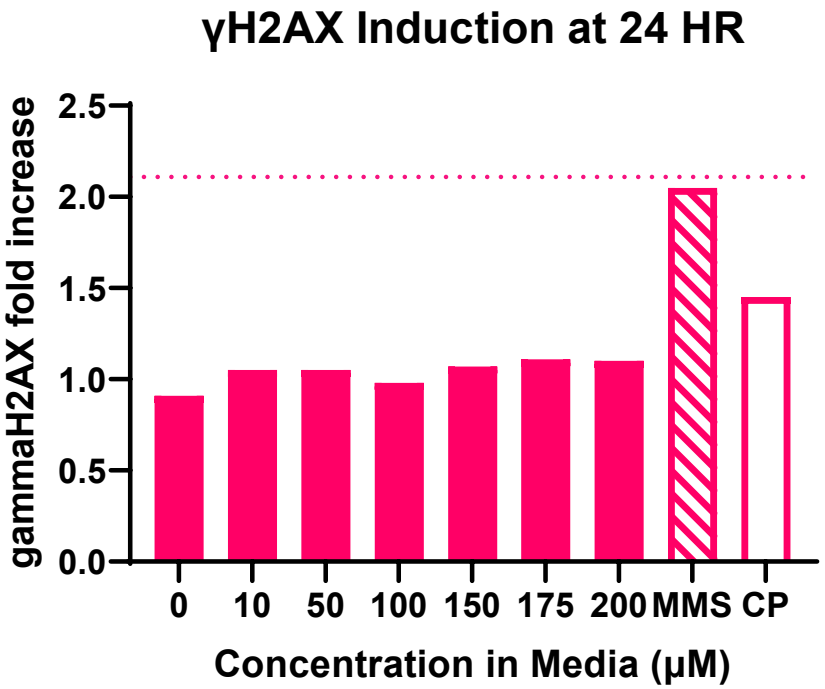
Protocol and positive controls not optimized for treatment in presence of S9 at the time studies were conducted



- Apoptosis assessed after 4 hours of treatment in the presence of S9 and after a 20 hour recovery period
- Increases in apoptosis observed at 4 and 24 hours in the concentration range where precipitate precluded MN assessment
- CCCP is positive control for apoptosis induction (not genotoxic) but cells typically treated for 24 hours continuously to induce robust response

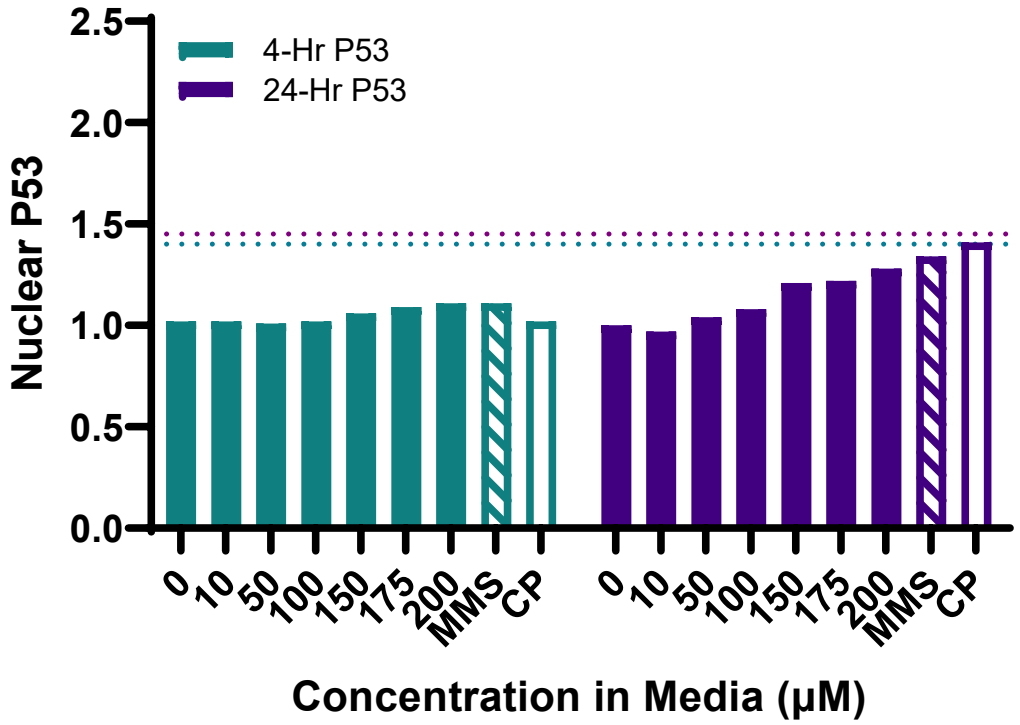
Markers of Clastogenicity: γH2AX





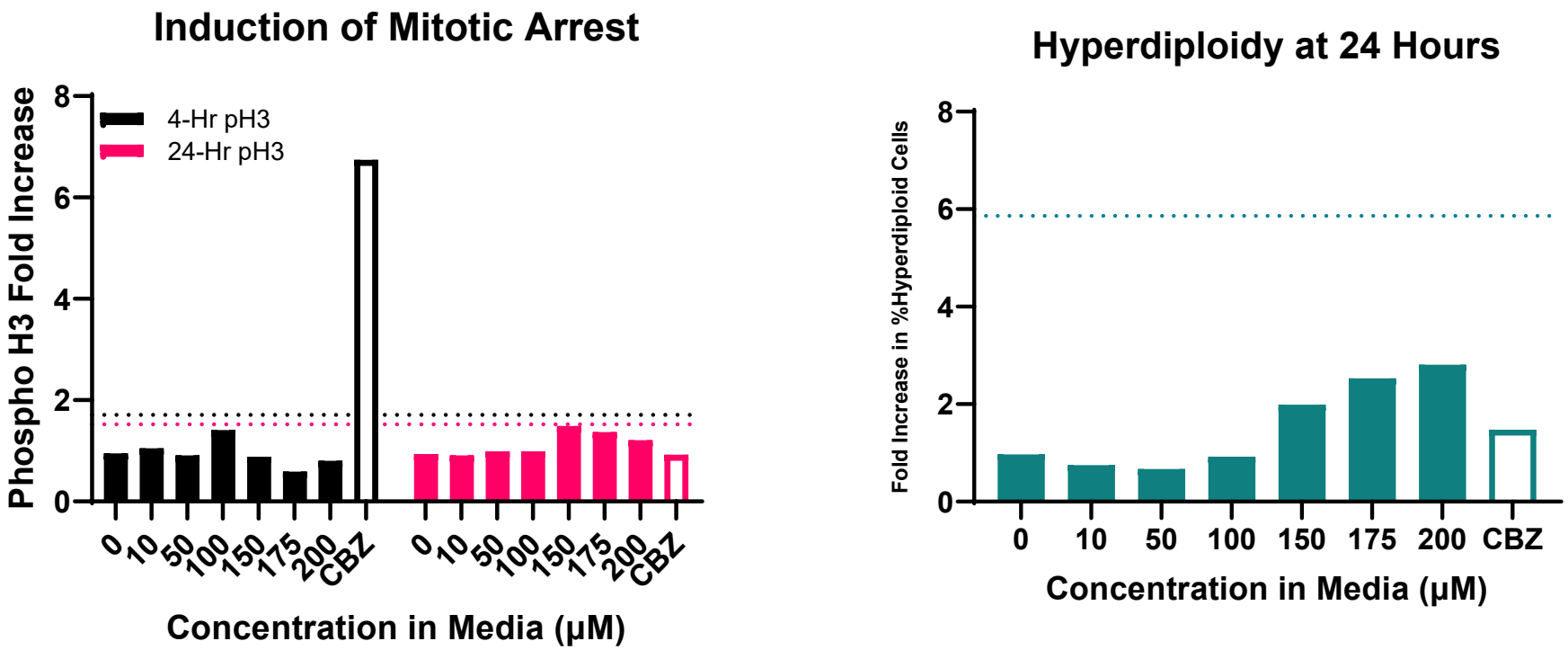
- No induction of clastogenicity in the presence of S9 for 4 hours or following 20-hour recovery
- MMS is the positive control for gammaH2AX and response observed at 4 hours but did not reach response threshold after 20-hour recovery
- Dotted line on graph represents response threshold established for the endpoint at each time point

Markers of Clastogenicity: Nuclear P53 Induction



- No induction of P53 nuclear translocation after treatment with either test article or positive controls
- Endpoint assessment not optimized for 4 hour treatment with S9 followed by 20 hour recovery period

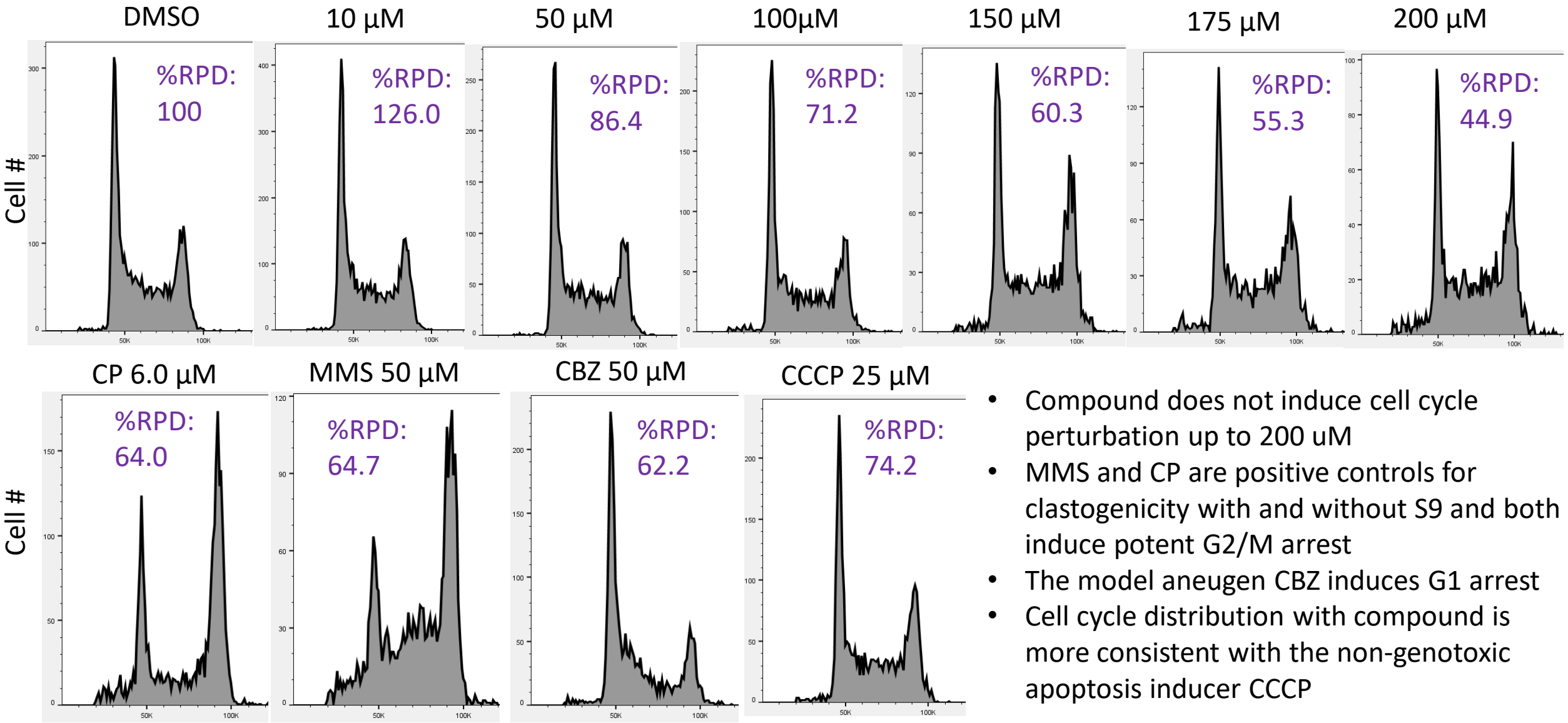
Markers of Aneugenicity



- No induction of aneugenicity in the presence of S9 for 4 hours or following 20-hour recovery
- Carbendazim (CBZ) is the positive control for aneugenicity and response observed at 4 hours but did not reach response threshold after 20-hour recovery
- Dotted line on graph represents response threshold established for the endpoint at each time point

TK6 Cell Cycle Analysis 24 hrs

Note: y-axis is not the same in all graphs



- Compound does not induce cell cycle perturbation up to 200 uM
- MMS and CP are positive controls for clastogenicity with and without S9 and both induce potent G2/M arrest
- The model aneugen CBZ induces G1 arrest
- Cell cycle distribution with compound is more consistent with the non-genotoxic apoptosis inducer CCCP

Summary of the GLP In Vitro Micronucleus Assay

- Compound did not induce micronuclei in both treatments without metabolic activation (3-hour and 24-hour) when tested up to toxicity-limiting concentrations
- Compound led to a statistically significant increase in micronuclei in the 3-hour treatment condition with metabolic activation
  - The response was just outside the historical control range
  - The response reproduced and corelated with precipitate, which was inconsistent between studies and difficult to assess in the presence of S9
  - Follow-up studies in TK6 cells showed no induction in micronuclei or any biomarker of genotoxicity up to 200 uM
  - The observed increase in micronuclei in the presence of S9 was confounded by precipitate and is therefore considered not biologically relevant and uninterpretable.
- Compound is negative for induction of micronuclei in the in vitro micronucleus assay.

Questions

- Do you agree that the compound is negative for induction of micronuclei in vitro and the results in the presence of S9 could not be interpreted?
  - In the context of total WoE
- What additional studies would you have done (or would like to see) to conclude that the compound does not induce chromosome damage
  - Chromosome aberration analysis likely also confounded by ppt and apoptosis

Back UP

Genotoxicity Biomarker Analysis in TK6 cells (4-hour treatment +S9 with 21- hour recovery)

- No evidence of genotoxicity up to the maximum concentration tested (200 uM) at either 4 hours or 24 hours
- Precipitate and steep induction of apoptosis starting at 150 uM

												Aneugenicity Biomarkers		
						Clastogenicity Biomarkers								
				Fold Increase										
Metabolic Activation	Compound	Concentration (µM)	24 hr % RNC	4 hr γH2AX	24 hr γH2AX	4 hr p53	24 hr p53	4 hr % Phospho-Histone H3	24 hr % Phospho-Histone H3	24 hr % Polyploidy	% Apoptotic Cells at 24 Hr[1]	4hr Cleaved PARP+	24hr Cleaved PARP+	Excluded Due to: P, T, A[2]
	Threshold Values:			**1.51**	**2.11**	**1.4**	**1.45**	**1.71**	**1.52**	**5.86**	**30**			
	DMSO	1%	104.9	0.99	1.09	0.98	1	1.05	1.06	1.03	3.4	2.7	4	
	DMSO	1%	95.1	1.01	0.91	1.02	1	0.95	0.94	0.97	2.8	2.7	2.4	
		10	126	0.95	1.05	1.02	0.97	1.05	0.91	0.75	2.4	2	3.3	
		50	86.4	0.97	1.05	1.01	1.04	0.91	0.99	0.67	2.6	2.3	3.1	
		100	71.2	1.2	0.98	1.02	1.08	1.41	0.99	0.92	4.7	3.1	3.6	
		150	60.3	1.34	1.07	1.06	1.21	0.88	1.49	1.99	7.5	3.4	8.5	ppt
		175	55.3	1.31	1.11	1.09	1.22	0.59	1.37	2.53	10.2	7.3	9.8	ppt
		200	44.9	1.18	1.1	1.11	1.28	0.8	1.21	2.81	15.4	8.6	15.3	ppt
	Cyclophosphamide	6	64	1.27	1.45	1.02	1.41	0.55	0.9	2	6	2	6.6	
	MMS	50	64.7	**1.95**	2.05	1.11	1.34	1.42	0.79	2.16	6.2	4.3	4.7	
	CBZ	50	62.2	1.27	0.77	1.18	1.01	**6.74**	0.92	1.48	7.2	5.2	7	
	CCCP	25	74.2	1.37	1.03	1.15	1.12	**3.44**	1.18	1.44	6	6.7	5.8	