Genotoxicity of a low-dose nitrosamine mixture as drinking water disinfection byproducts in NIH3T3 cells

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Table S1. The physical traits, classifications and risks of nine nitrosamine compounds which as disinfection byproducts

Compounds	Abbr.	No. CAS	MW (g/mol)	Density (g/mL)	Grading	Drinking Water Conc. at E6 Risk Level [a] (ng/L)	Drinking Water Quality Standard, Guideline or Notification Level
N-Nitrosodimethylamine [d]	NDMA	62-75-9	74.08	1.01	B2 [b]	0.7	9 ng/L [e]; 10 ng/L [f]; 100 ng/L [g]
N-nitrosomethylethylamine [d]	NMEA	10595-95-6	88.11	0.96	B2 [b]	2	no guideline
Nnitrosodiethylamine [d]	NDEA	55-18-5	102.14	0.95	B2 [b]	0.2	10 ng/L [f]
N-nitrosopyrrolidine [d]	NPYR	930-55-2	100.12	1.09	B2 [b]	20	no guideline
N-nitrosopiperidine	NPIP	100-75-4	114.15	1.06	Human carcinogen [c]	n/a	no guideline
N-nitrosomorpholine	NMOR	59-89-2	116.12	1.32	Human carcinogen [c]	n/a	no guideline
N-nitrosodi-n-propylamine [d]	NDPA	621-64-7	130.19	0.92	B2 [b]	5	10 ng/L [f]
N-nitrosodi-n-butylamine [d]	NDBA	924-16-3	158.24	0.91	B2 [b]	6	no guideline
N-nitrosodiphenylamine	NDPhA	86-30-6	198.22	1.23	n/a	n/a	n/a

[a] Quantitative estimate of lifetime carcinogenic risk from oral exposure at 1 in 10^6 risk level, (US EPA IRIS: www.epa.gov/iris); [b] US EPA IRIS;

[c] International Agency for Research on Cancer (IARC); [d] included in the Unregulated Contaminant Monitoring Rule 2(UCMR-2);

[e] Ontario Drinking Water Quality Standard; [f] Notification level in California ;

[g] World Health Organization guideline and Australian Drinking Water Guideline;

B2: Probable human carcinogen;

n/a: information not available.

Table S2. The doses of nitrosamines and their mixtures for Ames CBMN, Comet and 8-OHdG assays

Groups	Exposure Conc. for Ames test (ng/L)	Ames (ng/plate)	Exposure Conc. for CBMN, Comet and 8-OHdG assays (ng/L)
NDMA		Actual Conc. = 10	
1×	7	7×10^{-3}	10
10×	7 0	7×10^{-2}	100
100×	7×10^{2}	0.7	10×10^{2}
1000×	7×10^{3}	7	10×10^{3}
NDEA		Actual Conc. = 5	
1×	3.5	3.5×10^{-3}	5
10×	35	3.5×10^{-2}	5 0
100×	3.5×10^{2}	3.5×10^{-1}	5×10^{2}
1000×	$3.\ 5\times 10^{3}$	3.5	5×10^{3}
NEMA		Actual Conc. = 3	
1×	2.1	2.1×10^{-3}	3
10×	21	2.1×10^{-2}	30
100×	2.1×10^{2}	2.1×10^{-1}	3×10^{2}
1000×	2.1×10^{3}	2.1	3×10^{3}
Mixture		Actual Conc. = 10 + 5 + 3	
1×	7 + 3.5 + 2.1	$(7 + 3.5 + 2.1) \times 10^{-3}$	10 + 5 + 3
10×	$(7 + 3.5 + 2.1) \times 10^{1}$	$(7 + 3.5 + 2.1) \times 10^{-2}$	$(10 + 5 + 3) \times 10^{1}$
100×	$(7 + 3.5 + 2.1) \times 10^{2}$	$(7 + 3.5 + 2.1) \times 10^{-1}$	$(10 + 5 + 3) \times 10^{2}$
1000×	$(7 + 3.5 + 2.1) \times 10^{3}$	7 + 3.5 + 2.1	$(10 + 5 + 3) \times 10^{3}$

Table S3. Comparative mutagenicity of nitrosamines in S. *typhimurium* TA 98 and TA100, without (−S9) and with (+S9) metabolic activation

Chemical	S9[a]	Conc. range (fold)	2 × Mutat Conc. (fold)[b]	MI[c]	r^2
		Strain TA 98			
Control	-S9/+S9	0	Negative	1.0	NA
NDMA	-S9	1-1000	Negative	1.2	NA
NDMA	+S9	1-1000	1000	2.1*	0.98
NDEA	-S9	1-1000	Negative	1.3	NA
NDEA	+S9	1-1000	Negative	1.4	NA
NMEA	-S9	1-1000	Negative	1.3	NA
NMEA	+S9	1-1000	Negative	1.9	NA
Mixture	-S9	1-1000	Negative	1.2	NA
Mixture	+S9	1-1000	100	2.4*	0.97
Mixture	+S9	1-1000	1000	3.3*	0.97
		Strain TA100			
Control	-S9/+S9	0	Negative	1.0	NA
NDMA	-S9	1-1000	Negative	1.2	NA
NDMA	+S9	1-1000	Negative	1.7	0.99
NDEA	-S9	1-1000	Negative	1.1	NA
NDEA	+S9	1-1000	Negative	1.9	NA
NMEA	-S9	1-1000	Negative	1.2	NA
NMEA	+S9	1-1000	Negative	1.7	NA
Mixture	-S9	1-1000	Negative	1.3	NA
Mixture	+S9	1-1000	1000	3.1*	0.98

[a] Plychlorinated biphenyl-induced hepatic microsomal mixture (MolTox Inc.).

[b] The concentration of the sample induced 2 × increase over the number of the negative control.

[c] Mutagenic index: (Number of His^+ induced in the sample)/(Number of spontaneous His^+ in the negative control). MI > 2 was considered the sample was mutagenic and was indicated by asterisk.

r^2: Coefficient of determination of the goodness of fit for the regression analyses.

Positive controls without S9 mix were: 2,7-AF (100μg/plate) for TA98, 1624 ± 193 colonies; 2-AF (10μg/plate) for TA100, 3004 ± 212 revertants.

Positive controls with S9 mix were: 2-AF (10μg/plate) for TA98, 3602 ± 268 colonies; NaN_3 (1μg/plate) for TA100, 1856 ± 156 revertants.

Blank culture medium were used for confirming that there was no other strain pollution.

NA: not applicable.

Table S4. Con A Agglutination assay

Group	Conc. of Con A (μg/ml)				
	0	12.5	25	50	100
Control	-	-	-	-	+
NAms-treated	-	-	+	+	+

Control represents the normal, untransformed cells; NAms-treated represents the transformed cells that developed after exposure to NAms mix of 1000 folds for 72 h. "−" indicates negative result with cells were dispersive. "+" indicates positive with cells agglutinated together.

Table S5. Soft Agar assay

Group	Cloning efficiency (%) ($\bar{x}$±SD)
Control	0
NAms-treated	15.62±1.89*

Control represents the normal, untransformed cells; NAms-treated represents transformed cells that developed after exposure to NAms mix of 1000 folds for 72 h. * p<0.05 indicate the cloning efficiency significant difference from control group.

Table. S6. The Sequence of Base designed for this study

Name of Primer	Sequence of Base (5' to 3')		TM ℃
	Forward primer	Reverse primer	
P21	AGCAAAGTGTGCCGTTGTCT	TCAAAGTTCCACCGTTCTCG	60
P53	AAGTCACAGCACATGACGGA	TACAAATTTCCTTCCACCCG	60
CDC25A	TGACTGCCGATACCCATATG	GAACACGACAATGACACGCT	60
CDC2/CDK1	ATTGTGTTTTGCCACTCCCG	ATTCCAAACGCTCTGGCAAG	60
CyclinB1	ATCCTTGCAGTGAGTGACGT	TCCTCCAGTTGTCGGAGATA	60
GADD45A	ATAACGTGGTACTGTGCCTG	CAGGATGTTGATGTCGTTCT	60
chk1	TACTGCAATGTTGGCTGGAG	AAGCCAGAGGAGCAGAATCA	60
chk2	GAACAAGCGCCTGAAAGAAG	ATTCTCCGGCTTTAAGTCCC	60
GAPDH	GGCAAATTCAACGGCACAGT	ACGACATACTCAGCACCGGC	60

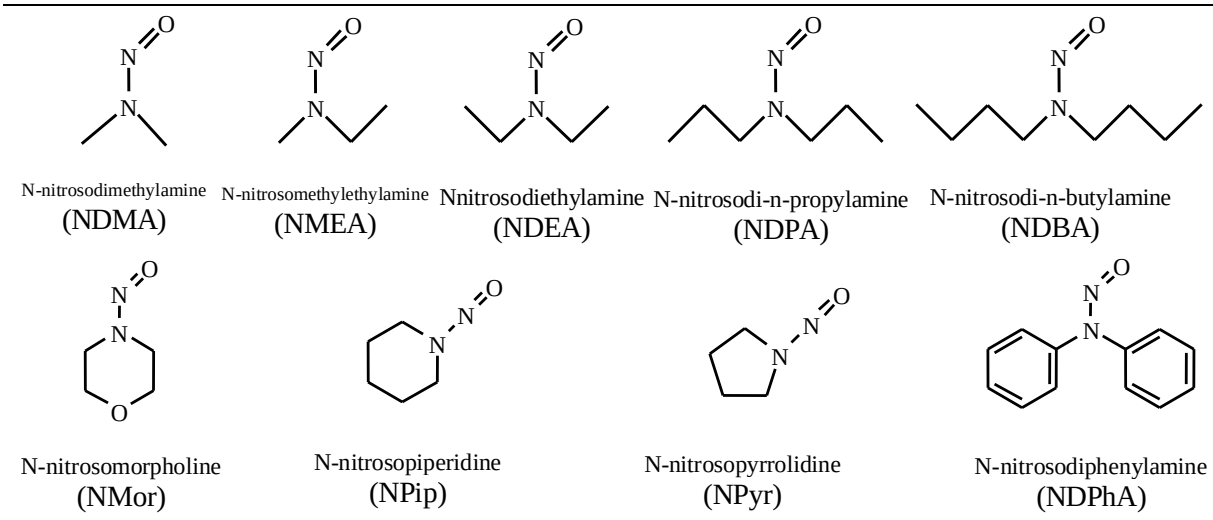

Figure S1. The structures of nine nitrosamine compounds

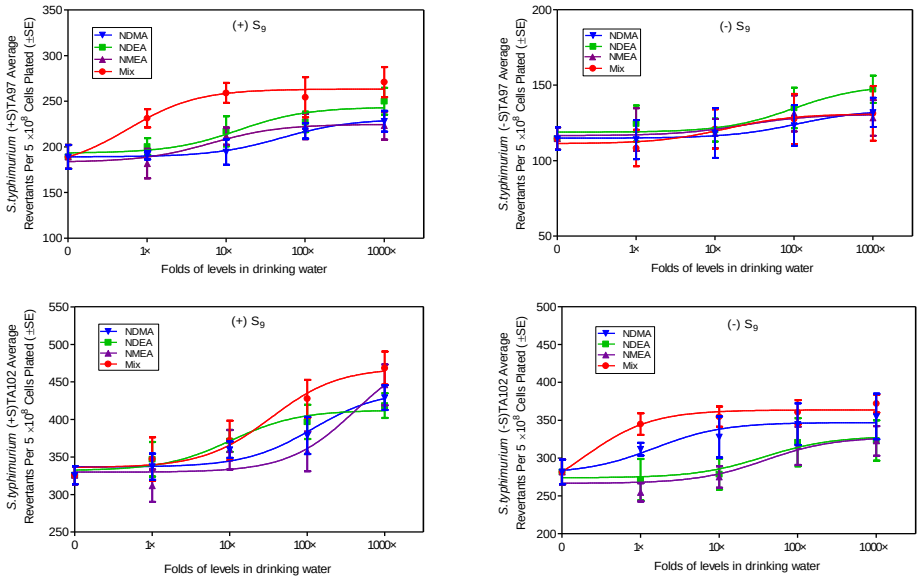

Figure S2. Concentration–response curves of mutagenicity for NDMA, NDEA, NMEA and their mixture in S. *typhimurium* TA97 and TA102, without (−S9) and with (+S9) metabolic activation.

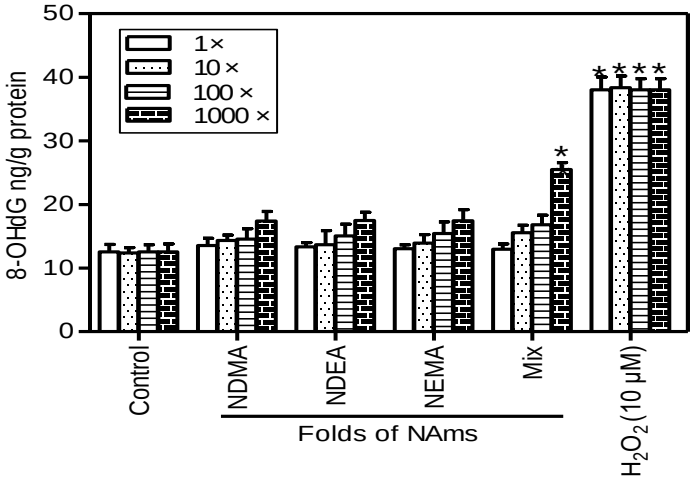

Figure S3. Enzyme-linked immunosorbent assay for 8-OHdG detection in NIH/3T3 cells. The effect of single nitrosamines and their mixture on the formation of 8-OHdG evaluated by the ELISA. NIH/3T3 cells were exposed to single Nitrosamine and their mixture with 0, 1-, 10-, 100-, 1000-fold and 10μM H_2O_2 (positive control) for 6 h. Data represents the Mean ± SD obtained in three independent experiments (n = 3). * p <0.05 indicate significant difference from control group.

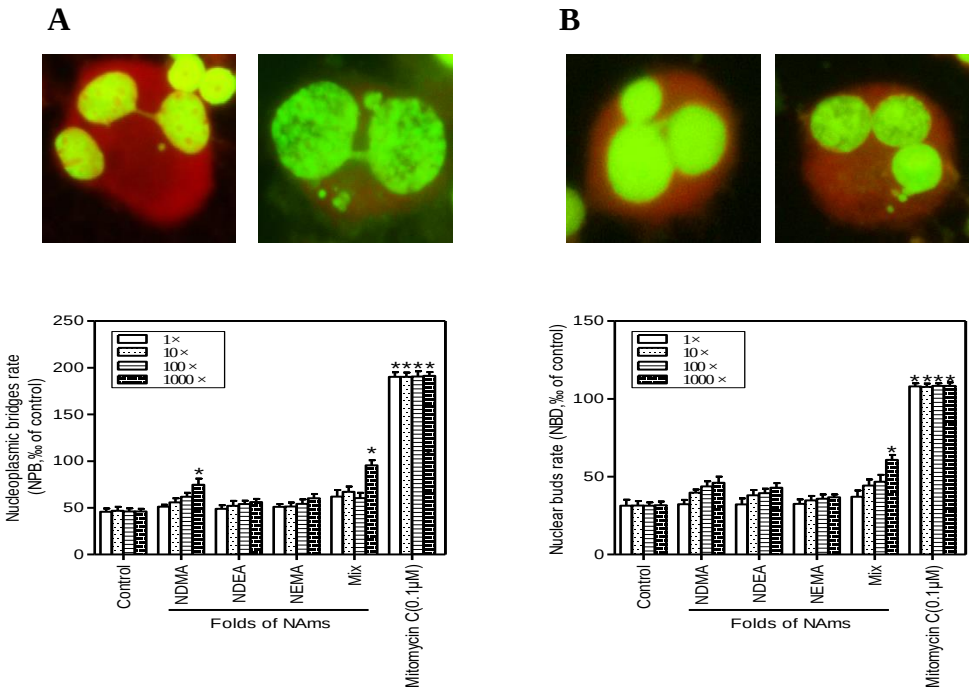

Figure S4. Results of Cytokinesis-Block Micronucleus (CBMN) assay. Upper A and B indicate the images of NUBDs and NPBs. Lower A and B indicate the rate of NUBDs and NPBs respectively. * p<0.05 indicate significant difference from control group.

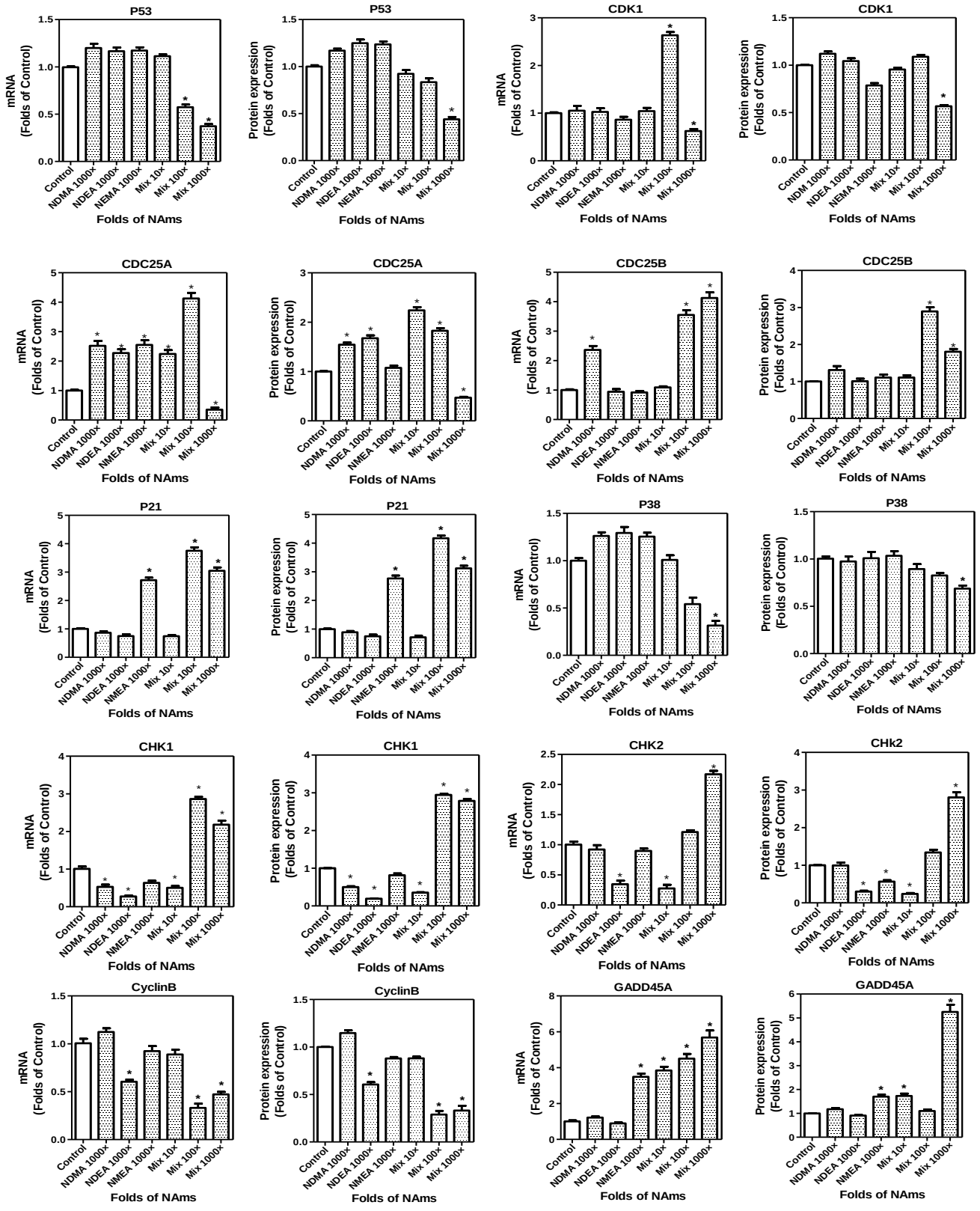

Figure S5. Results of gene and protein expression. * $p < 0.05$ indicate significant difference from control group.

This supporting information (**Table S1.** and **Figure S1.**) provides structures and physical property of nine nitrosamines compounds which as DBPs with their associated classifications, risks and guideline notification level. **Table S2.** showed dose design of Nitrosamines and their mixtures for Ames assay, CBMN assay, Comet assay and 8-OHdG assay. **Table S3.** presented the mutagenicity of Nitrosamines in S. *typhimurium* TA 98 and TA100, without (−S9) and with (+S9) metabolic activation. (**Table S4.** and **Table S5.)** indicated the Con A Agglutination assay and Soft Agar assay. **Table S6.** indicated the sequence of base designed for this study. **Figure S2.** provided the concentration–response curves of mutagenicity for NDMA, NDEA, NMEA and their mixture in S. *typhimurium* TA97 and TA102, without (−S9) and with (+S9) metabolic activation. **Figure S3.** presented the result of enzyme-linked immunosorbent assay for 8-OHdG evaluated by the ELISA in NIH/3T3 cells. **Figure S4.** indicated the results of CBMN assay. **Figure S5.** are the results of gene and protein expression. * $p < 0.05$ indicate significant difference from control group.