SUPPORTING INFORMATION for

Comparative *In Vitro* Toxicity of Nitrosamines and Nitramines Associated with Amine-Based Carbon Capture and Storage

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19 pages

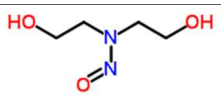
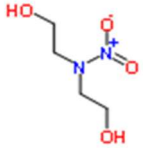
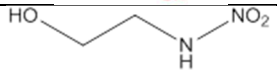
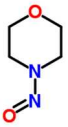
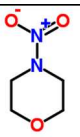
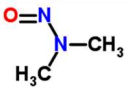
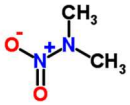
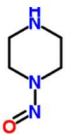
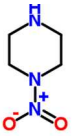
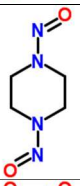
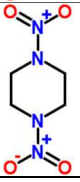
2 Tables

9 Figures

Table of Contents

The agents analyzed for their *in vitro* toxicity are listed in Table S1.

Table S1 Description of nitrosamines and nitramines evaluated in this study

Name	Formula	CASN	Source	Structure
N-Nitrosodiethanolamine	$C_4H_{10}N_2O_3$	1116-54-7	Sigma Chem. Co	
N-Nitrodiethanolamine	$C_4H_{10}N_2O_4$	4185-47-1	CanSyn Chem. Co.	
N-Nitromono-ethanolamine	$C_2H_6N_2O_3$	unavailable	Toronto Res. Chem.	
N-Nitrosomorpholine	$C_4H_8N_2O_2$	59-89-2	Sigma Chem. Co	
N-Nitromorpholine	$C_4H_8N_2O_3$	4164-32-3	Sigma Chem. Co	
N-Nitrosodimethylamine	$C_2H_6N_2O$	62-75-9	Chem. Ser-vice Co	
N-Nitrodimethylamine	$C_2H_6N_2O_2$	4164-28-7	CanSyn Chem. Co.	
1-Nitrosopiperazine	$C_4H_9N_3O$	5632-47-3	Toronto Res. Chem.	
1-Nitropiperazine	$C_4H_9N_3O_2$	42499-41-2	CanSyn Chem. Co.	
1,4-Dinitrosopiperazine	$C_4H_8N_4O_2$	140-79-4	Toronto Res. Chem.	
1,4-Dinitropiperazine	$C_4H_8N_4O_3$	4164-37-8	MP Bio-medicals	

S. typhimurium Cells

We employed *S. typhimurium* tester strain YG7108 (*hisG46*, *rfa*; △(*chl*, *uvrB, bio*) △ada_{ST}; ogt_{ST}; Cm^r; Amp^r) with and without mammalian microsomal activation. Reverse mutation is measured at *hisG46* (Figure S1).[1, 2] *S. typhimurium* strain YG7108 is highly sensitive to nitrosamines and other alkylating agents and was provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan.[3]

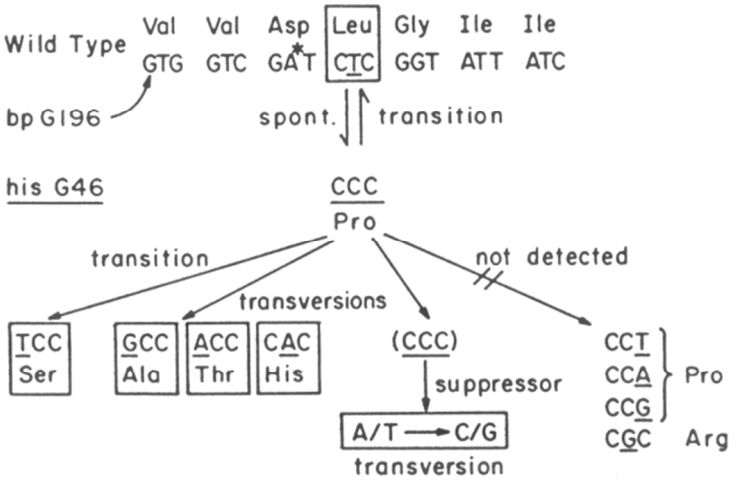


Figure S1. Reverse mutation at hisG46 in *S. typhimurium* that leads to the growth of a visible colony on selective VB medium. The mutant colonies are referred to as revertants.

YG7108 was stored as a frozen culture in Luria-Bonner (LB) plus 10% dimethylsulfoxide (DMSO) at −80°C. To construct a master plate, frozen log-phase *S. typhimurium* YG7108 cells were thawed and grown in 5 mL LB at 37°C overnight with shaking. The culture was streaked across an LB plate and incubated overnight at 37°C. The master plate was stored at 4°C. For the mutagenicity and cytotoxicity (survivorship) experiments a single colony isolate of *S. typhimuri-*

um YG7108 from the master plate was grown overnight in 100 mL LB medium with kanamycin (25 μg/mL) and chloramphenicol (10 μg/mL) at 37°C with shaking (200 rpm).[3] The following day the culture was centrifuged for 5 min at 5000 rpm (4096 ×g) using the GSA rotor in the RC5B Superspeed Centrifuge (Sorvall). The supernatant was discarded, and the bacterial pellet was suspended in 50 mL of 100 mM potassium-phosphate buffer, pH 7.4 (PPB). The suspension was centrifuged again for 5 min at 5000 rpm, and the supernatant was discarded. The pellet was suspended in 2.5-3 mL PPB. An aliquot (50 μL) of the bacteria was added to 4.95 mL PPB and the optical density was determined spectrophotometrically (660 nm). The titer of the suspension was adjusted to 6×10^9 cells/mL and the cells were kept on ice until used in the microsuspension mutagenicity assay.[4-6] Most of the nitrosamines and nitramines require metabolic activation to exhibit mutagenicity. Metabolic activation of nitrosamines and nitramines is primarily dependent on cytochrome P-450 which converts nitrosamines into DNA alkylating agents [7]. For example N-nitrodibutylamine and N-nitrodiethylamine were activated by liver microsomes and hepatocytes from rats treated with phenobarbital.[8] Nitrodimethylamine was activated with S9 microsomes into mutagenic agents.[9] Oxidative transformation is an important route of metabolism of xenobiotics. These oxidative reactions are largely catalyzed by the monooxygenase system based on cytochrome P-450. The monooxygenase system is formed by the enzyme system of cytochrome P-450 and NADPH cytochrome P-450 reductase. The haemoprotein cytochrome P-450 functions as the terminal oxidase involved in the hydroxylation of xenobiotics such as nitrosamines. A description of the cytochrome P-450-mediated monooxygenation of dimethylnitrosamine (NDMA) is presented in Figure S2.

***S. typhimurium* Microsuspension Mutagenicity Assay**

This assay was conducted in sterile round bottom 96-well microplates. Each reaction mixture (well) was composed of a known nitrosamine or nitramine concentration, 3×10^8 cells, and PPB in a total volume of 100 µL.

When including a mammalian microsomal-mediated activation system, 35 µL of an S9 mix was added for a total volume of 100 µL. S9 (post mitochondrial hepatic supernatant) from Aroclor 1254-induced male rats) was purchased from Moltox. The S9 mix consisted of 50 mM PPB pH 7.4, 10 mM $MgCl_2$, 5 mM glucose-6-phosphate, 30 mM KCl, and 4 mM NADP plus 200 µL/mL of Aroclor 1254-induced rat hepatic microsomal suspension (S9). The final S9 concentration in the treatment well was 7%. In general there were three microplate wells for each test agent concentration plus concurrent negative (buffer only) and positive controls (with S9 activation, NDMA, 250 µM; without S9 activation, ethylmethanesulfonate, 188 µM). The wells were sealed with AlumnaSeal to prevent any crossover volatilization or evaporation, and the microplate was placed in a plastic container and incubated at 37°C with shaking at 200

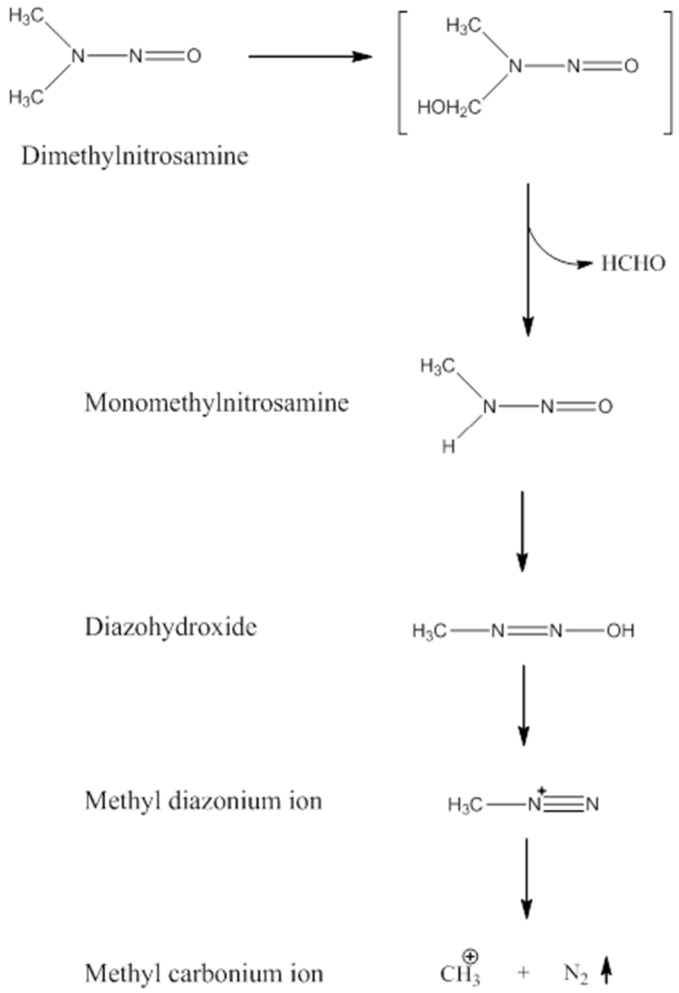


Figure S2. Metabolic activation of *N*-nitrosodimethylamine via cytochrome P-450 leading to the formation of a reactive methyl carbonium ion. This radical can alkylate DNA and induce mutation.

rpm for 60 min. After treatment, the entire contents of each well were added to 2 mL of molten histidine and biotin supplemented Vogel-Bonner (VB) top agar, mixed and poured onto selective VB plates. The plates were placed in a static 37°C incubator for 72 h. Histidine revertant colonies were counted by hand or with a New Brunswick Biotran III automatic colony counter. An example of revertant *S. typhimurium* colonies expressing mutagenicity induced by *N*-nitrodimethylamine is presented in Figure S3. The data were saved as an Excel spreadsheet. The data were analyzed from the Excel spreadsheet using the statistical and graphical functions of SigmaPlot 11 (Systat Software, Inc., San Jose, CA).

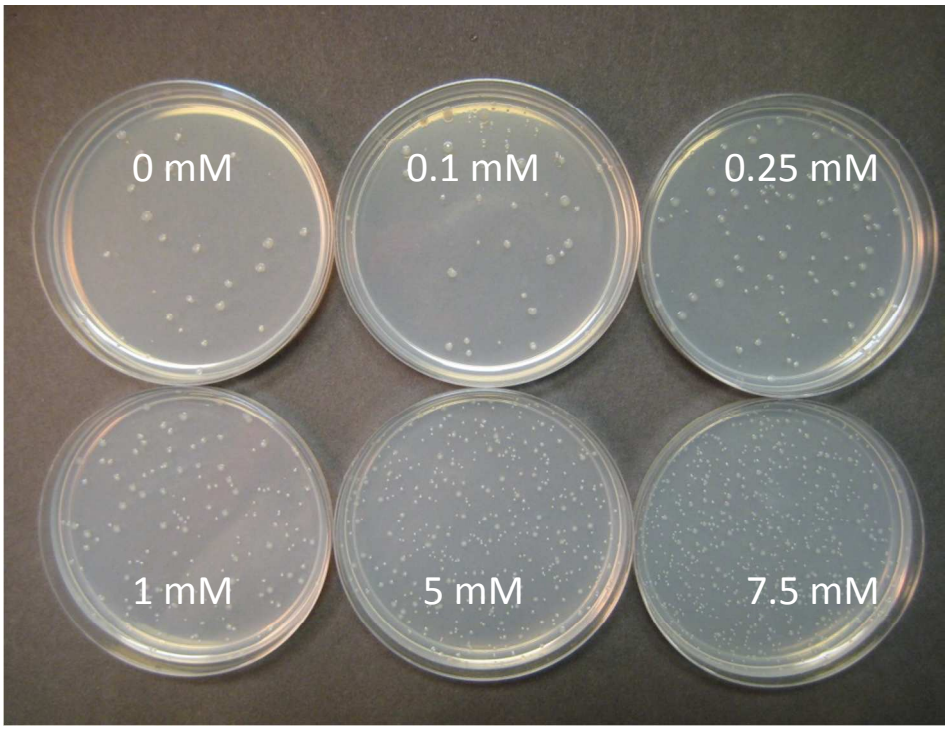


Figure S3. Mutagenicity of N-nitrodimethylamine in *S. typhimurium,* strain YG7108, after mammalian hepatic microsomal activation showing increasing numbers of histidine revertants as a function of the concentration of the nitramine.

Survivorship was measured by making serial dilutions from the treatment wells in PPB and plating on LB (complete media) plates. After incubation for 24 h the colonies were counted. The number of colonies from the negative control was set at 100% and the colonies from the treatment wells were compared and expressed as a percent of the negative control.

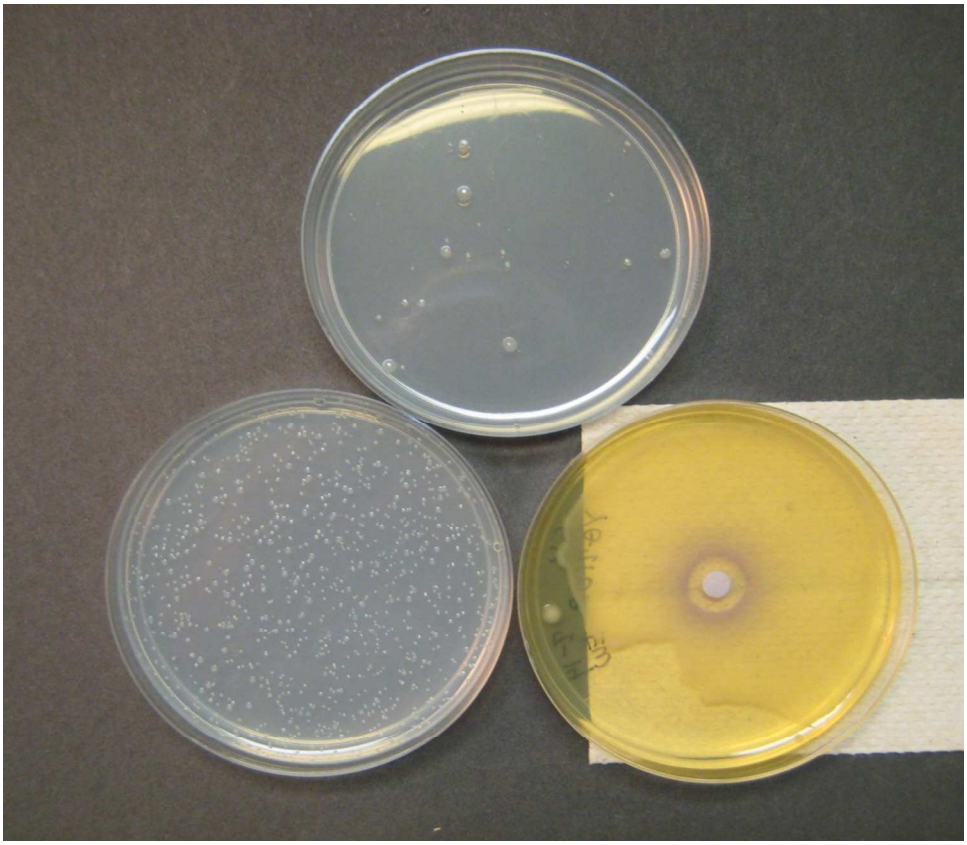


Figure S4. Diagnostic plates for the *S. typhimurium* microsuspension mutagenicity assay. A negative control is illustrated by the top plate, the left plate is the positive control *N*-nitrosodimethylamine and the right plate is the crystal violet *rfa*$^{-}$ plate showing a clear zone of inhibition.

To confirm the genotype of the YG7108 cells, 100 µL of the titered suspension was added to an LB plate, and spread with a flamed glass rod. Flamed tweezers were used to place a crystal violet disk onto the center of the plate, and the disk was tapped lightly in place. Due to

the presence of the *rfa* mutation, [1] the large molecules of crystal violet are able to enter and kill the YG7108 cells. This was indicated by a clear zone around the crystal violet disk (Figure S4).

Suspension Versus Plate Incorporation Assays

We employed a quantitative microsuspension methodology for the *S. typhimurium* mutagenicity assay instead of the more commonly used plate incorporation assay method. An overall comparison is outlined in Table S2. In the plate incorporation method the cell titer is not determined; an aliquot (usually 100 μL) of an overnight culture in complete growth medium is used. For the plate incorporation assay the test agent is added to a 2-mL volume of supplemented molten top agar along with the cells and ± S9. The top agar is poured upon a VB minimal plate. Thus the actual exposure concentration to the cells is difficult to determine. It is impossible to calculate a molar concentration and usually a gram unit per plate is used to express the concentration. The volume during the exposure time for the cells is impossible to quantify. Cells are exposed in approximately 2 mL of molten top agar which is then poured onto a VB selective plate that consists of approximately 20 mL. The cells are retained in the top agar, but the test chemical diffuses throughout the approximately 22 mL volume of agar in the petri plate.

Another difficulty is that the test agent is in contact with S9 monooxygenase enzymes and co-factors first in the 2 mL overlay agar and then this volume changes when the contents are poured upon the VB plate. Finally, in the plate incorporation and preincubation methods no quantitative cytotoxicity measurement can be made. It is important that the *S. typhimurium* cells are not killed as the concentration of the test agent is increased throughout the concentration range.

Table S2. Comparison between the plate incorporation method and the microsuspension method for the *S. typhimurium* mutagenicity assay		
Metric	Plate Incorporation Method	Microsuspension Method
Single colony isolate grown overnight in complete medium	Yes	Yes
Centrifugation and washing bacteria before treatment	No	Yes
Determination of titer before treatment (same number of cells treated for each experiment)	No	Yes. By treating a consistent number of cells, the results can be presented in revertants per 10^8 cells plated. This provides a uniform level of mutagenicity response.
Treatment times can be adjusted	No	Yes
Precise control of concentration of test agents	No — With this method the test compound and bacteria are combined in 2 mL of molten agar and poured immediately onto a plate from which diffusion occurs throughout the plate. The concentration of agent can be expressed as the concentration in the treatment tube or as the concentration in the petri plate.	Yes – With this method a known number of bacteria are exposed to the agent while shaking for defined time periods. After treatment the mixture is added to molten agar and poured onto a petri plate. The concentration of agent is expressed as the concentration during treatment.
Determination of cytotoxicity	Not possible. A background lawn that is thin or sparse is only qualitative and not a quantitative measure of toxicity.	Yes, an aliquot of treated cells may be serially diluted and plated onto complete medium, allowing for determination of toxicity.

A qualitative visual inspection of the background lawn of cells is part of the plate incorporation and preincubation procedures, but this is not a true measurement of cytotoxicity.

In the microsuspension assay that we employed for this report, *S. typhimurium* cells were washed, titered, and a specific number of cells were exposed to a test agent in a known volume of buffer with and without S9 for a specific time period. The concentrations of the test agent were expressed in molar units. An aliquot of the suspension was reserved after treatment, serial

dilutions were prepared and known volumes were plated onto complete (LB) medium. All living cells grow on LB medium and we can determine if we are conducting the assay at concentrations of test chemical that do not induce a cytotoxic response. Thus, the microsuspension procedure is a quantitative assay that provides control over cell titer, a precise measure of cytotoxicity and an exact determination of the concentration of test agent to which a specific number of cells are exposed for a specific time period. This level of precision provides high quality data on the relative mutagenic capacities and allows for a quantitative comparison amongst the test chemicals.

Chinese Hamster Ovary (CHO) Cell Assays

CHO cells are widely used for *in vitro* toxicology research. We employed CHO cell line AS52 clone 11-4-8 for the mammalian cell cytotoxicity and genotoxicity analyses of the agents generated for this project (Figure S5). This clone expresses a stable chromosome complement as well as functional p53 protein.[10 11] The CHO cells were maintained in Ham's F12 medium at 37°C in a humidified atmosphere of 5% CO_2. The cells exhibit normal morphology, express cell contact inhibition and grow as a monolayer without expression of neoplastic foci (tumor cells). CHO cells were employed for the chronic cytotoxicity assay.[12, 13] Genomic DNA damage in CHO cells was analyzed with the single cell gel electrophoresis genotoxicity assay.[14, 15]

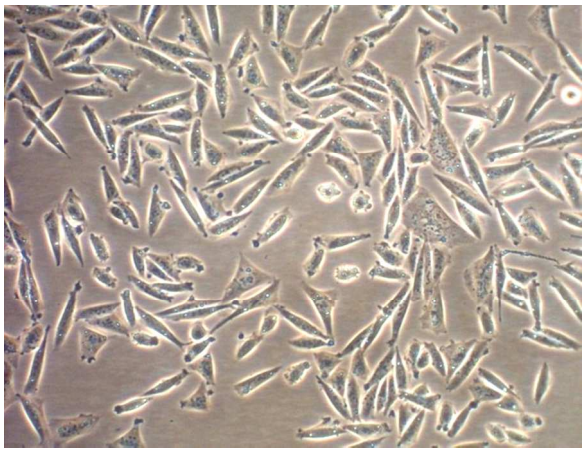


Figure S5. Photomicrograph of CHO AS52 cells, clone 11-4-8.

CHO Cell Chronic Cytotoxicity Assay

The CHO cell microplate chronic cytotoxicity assay measures the reduction in cell density as a function of the concentration of the test agent over a 72 h period.[12, 13] A 96-well flat-bottomed microplate was used to evaluate a series of concentrations of specific chemical agents. One column of eight microplate wells served as the blank control consisting of 200 μL of F12 + 5% fetal bovine serum (FBS) medium only. The concurrent negative control column consisted of eight wells with 3×10^3 CHO cells plus F12 +FBS medium. The remaining wells within the experiment contained 3×10^3 CHO cells, F12 +FBS and a known test agent concentration in a total of 200 μL. The wells were covered with a sheet of sterile Alumna Seal™. The microplate was placed on a rocking platform at 37°C for two 5 min-periods (turning the plate 90° after the first 5 min). This step is important and ensures an even distribution of cells across the bottom of the microplate wells. The cells were incubated for 72 h at 37°C at 5% CO_2. After the treatment time, the medium from each well was aspirated, the cells fixed in methanol for 10 min and stained for 10 min with a 1% crystal violet solution in 50% methanol. The microplate was washed, and 50 μL of DMSO/methanol (3:1 v/v) was added to each well, and the plate incubated at room temperature for 10 min. The microplate was analyzed at 595 nm with a BioRad microplate reader; the absorbency of each well was recorded and stored on a spreadsheet file. This assay was calibrated and there is a direct relationship between the absorbency of the crystal violet dye associated with the cell and the number of viable cells.[13] The averaged absorbency of the blank wells was subtracted from the absorbency data from each microplate well. The mean blank-corrected absorbency value of the negative control was set at 100%. The absorbency for each treatment group well was converted into a percentage of the negative control. This procedure normalizes the data, maintains the variance and allows for the combination of data from multiple micro-

plates. The data were used to generate a concentration-response curve for each test agent. Regression analysis was applied to each test agent concentration-response curve, which was used to calculate the LC_{50} value. The LC_{50} is the calculated concentration of test agent that induced a cell density that was 50% of the negative control. The original absorbency data, the blank-corrected data and the conversion to the percent of the negative control values were saved on the spreadsheet for each test agent analyzed. This CHO cell chronic cytotoxicity assay described above cannot be used with mammalian microsomal metabolic activation because the S9 mix interferes with the crystal violet staining method.

Single Cell Gel Electrophoresis Assay (SCGE)

The microplate SCGE assay was used to assess the genotoxicity and acute cytotoxicity of the test agents to CHO cells.[13, 14] SCGE is a sensitive assay that can quantitatively determine genomic DNA damage.[15-18] The SCGE assay detects genomic DNA strand breakage, DNA cross-links, alkali-labile sites and incomplete excision repair sites in individual nuclei. Upon electrophoresis in an agarose microgel, undamaged nuclei have few DNA fragments and appear as spheres when stained and viewed by fluorescence microscopy (Figure S6, top panel). Nuclei containing damaged DNA (including strand breaks and incomplete repair sites) exhibit a characteristic "comet tail" appearance after electrophoresis. Figure S6 illustrates nuclei from negative control cells (top panel), and

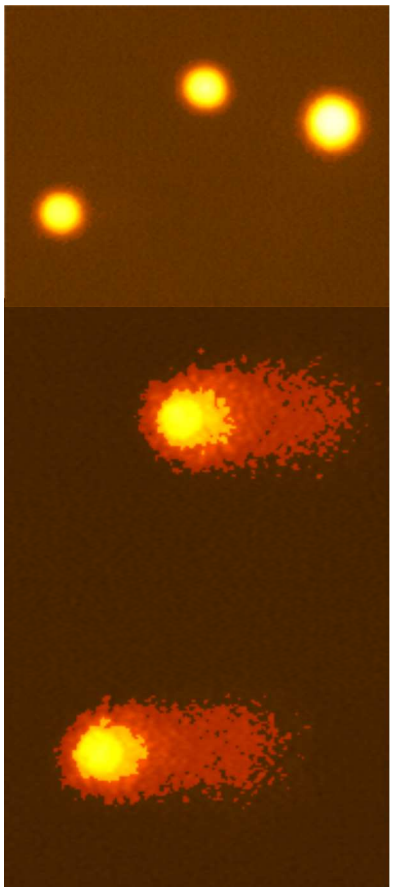


Figure S6. SCGE images of nuclei from negative control cells (top) and cells exposed to 800 µM 2AAAF (bottom).

239 nuclei from cells damaged by 2-acetoxyacetylaminofluorene (2AAAF) (bottom panel). Using a
240 digital camera, the amount of DNA that migrates away from the nucleus into the microgel
241 (%Tail DNA) is a quantifiable direct measure of DNA damage.[19]

242 The day before treatment, CHO cells from maintenance plates were washed with Hanks
243 Balanced Salt Solution and harvested with trypsin; a cell suspension was constructed with a titer
244 of 4×10^5 cells/mL. The contents of the microplate wells consisted of 100 µL of F12 medium +
245 5% FBS and 100 µL of the titered cell suspension. The microplate was placed on a rocking plat-
246 form at 37°C for two 5 min-periods (turning the plate 90° after the first 5 min). This is very im-
247 portant and ensures an even distribution of cells across the bottom of the microplate wells. After
248 rocking, the microplate was placed in the 5% CO_2 incubator. The following day the CHO cells
249 were treated with a series of concentrations of the test agent for 4 h with or without S9 microso-
250 mal activation. We employed a microplate methodology[14] with an enhanced hepatic microsomal
251 mix (S9B150) specifically designed for use with nitrosamines;[6] this consisted of 2 mM $MgCl_2$, 6
252 mM KCl, 1.5 mM glucose-6-phosphate, 1.2 mM NADP, 10 mM sodium phosphate buffer, pH
253 7.4, 2 mM $CaCl_2$ and 3.36% (v/v) S9 fraction. A typical experimental design included a negative
254 control (F12 medium without FBS), 9 concentrations of the test agent (in F12 without FBS) and
255 an appropriate positive control. We employed the direct-acting alkylating agent ethylmethanesul-
256 fonate for the –S9 positive control and NDMA as the +S9 positive control. In experiments with-
257 out S9 metabolic activation the total volume in each well was 25 µL. With S9 metabolic activa-
258 tion an increase in the total volume to 100 µL was required. After treatment, the cells were
259 washed and harvested with trypsin. A 10 µl aliquot was removed to measure acute cytotoxicity
260 using the trypan blue vital dye exclusion assay to ensure that the cells were exposed to sub-lethal
261 concentrations.[12, 20]

After embedding the remaining cells in agarose microgels, the cell membranes were lysed, and the nuclei were electrophoresed. After staining with the fluorescent DNA binding dye, ethidium bromide, the microgels were analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. A computerized image analysis system was used to measure a number of specific SCGE parameters of 25 randomly chosen nuclei per microgel. The intensity of the DNA that migrated away from the nucleus (%Tail DNA) was the primary metric of DNA damage that was used for the concentration-response curves.

Comparison of CHO Cell Cytotoxicity and Genotoxicity Among the Nitrosamines and Nitramines

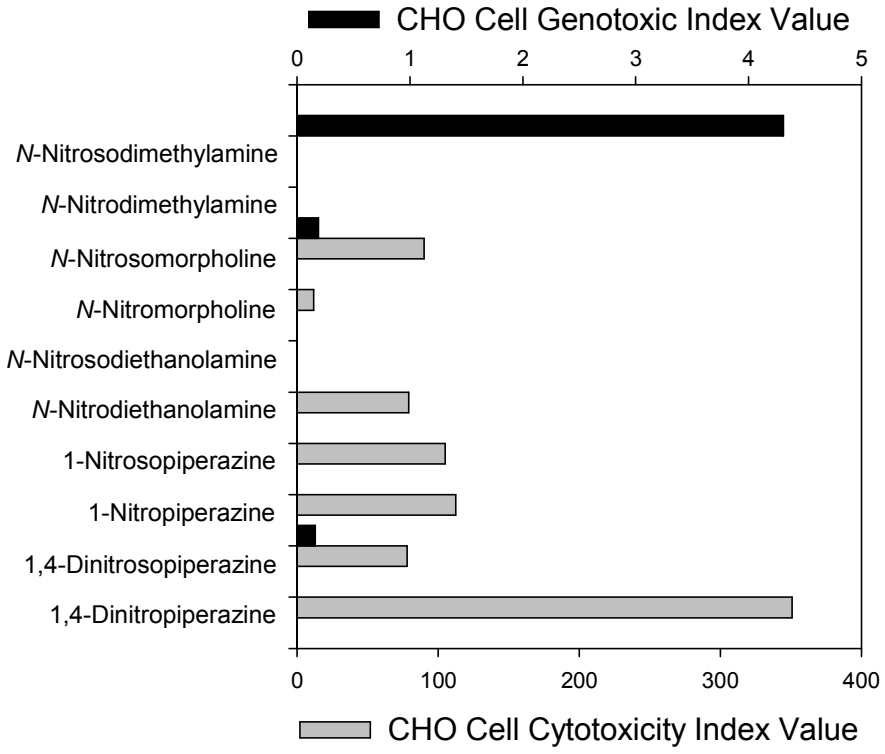


Figure S7. Comparison between the direct chronic CHO cell cytotoxicity (gray bars) and acute SCGE genotoxicity (black bars) of the nitrosamine and nitramine test chemicals analyzed in this study. The cytotoxicity index = $(LC_{50})^{-1}(10^3)$ and the genotoxic index is the reciprocal of the SCGE 50% Tail DNA value $\times 10^4$.

Comparison Between CHO Cell Genotoxicity with *S. typhimurium* Mutagenicity

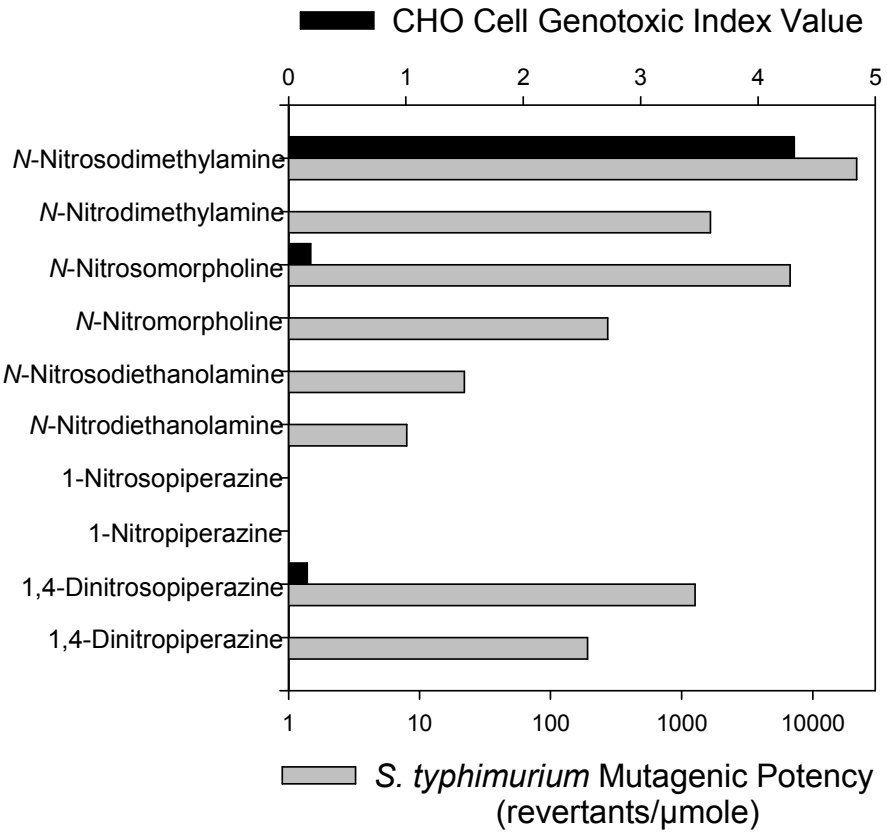


Figure S8. Comparison between mutagenicity in *S. typhimurium* YG7108 (gray bars) and genotoxicity in CHO cells (black bars). The CHO cell genotoxic index is the reciprocal of the SCGE 50% Tail DNA value $\times 10^4$.

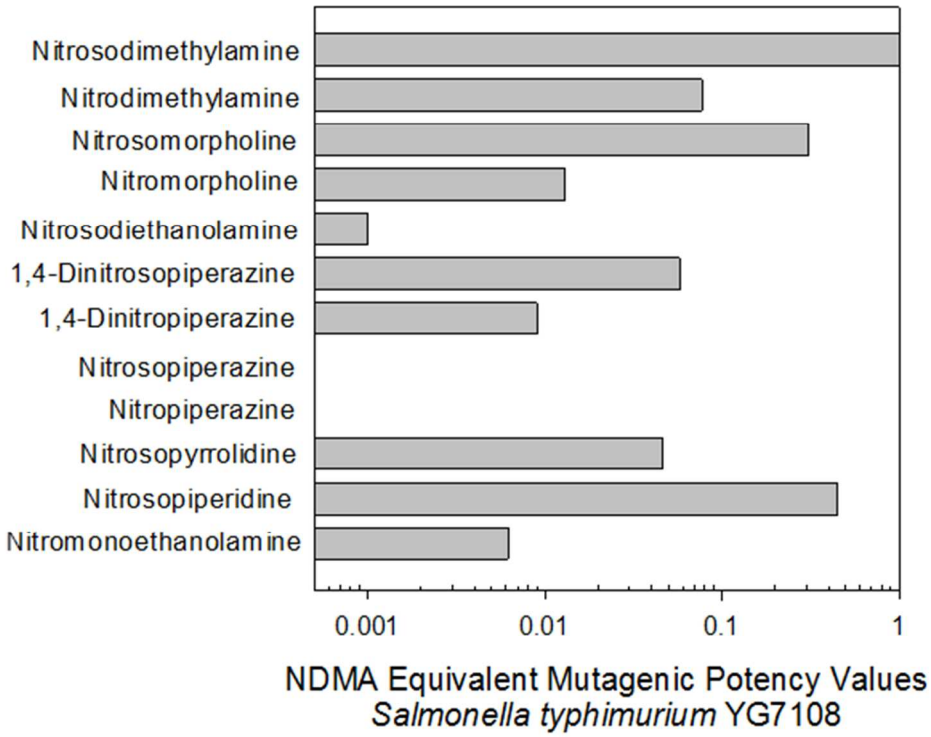


Figure S9. A comparison of the *N*-nitrosodimethylamine equivalent mutagenicity values for agents positive in *S. typhimurium* strain YG7108 +S9 metabolic activation.

References

1. Maron, D. M.; Ames, B. N., Revised methods for the Salmonella mutagenicity test. *Mutat.Res.* **1983,** *113*, (3-4), 173-215.
2. Claxton, L. D.; Umbuzeiro, G. d.; DeMarini, D. M., The Salmonella mutagenicity assay: The stethoscope of genetic toxicology for the 21st century. *Environ. Health Perspect.* **2010,** *118*, (11), 1515-1522.
3. Yamada, M.; Matsui, K.; Sofuni, T.; Nohmi, T., New tester strains of Salmonella typhimurium lacking 0^6-methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents. *Mutat.Res.* **1997,** *381*, 15-24.
4. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in *Salmonella typhimurium*. *Teratogen. Carcinogen. Mutagen.* **2002,** *22*, (2), 113-128.
5. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., A new assessment of the cytotoxicity and genotoxicity of drinking water disinfection by-products. In *Natural Organic Matter and Disinfection By-Products: Characterization and Control in Drinking Water*, Barrett, S. E.; Krasner, S. W.; Amy, G. L., Eds. American Chemical Society: Washington, D.C., 2000; pp 16-27.
6. Wagner, E. D.; Hsu, K. M.; Lagunas, A.; Mitch, W. A.; Plewa, M. J., Comparative genotoxicity of nitrosamine drinking water disinfection byproducts in *Salmonella* and mammalian cells. *Mutat.Res.* **2012,** *741*, 109–115.
7. Timbrell, J. A., *Principles of Biochemical Toxicology*. Taylor and Francis Ltd.: London, 1987; p 248.
8. Suzuki, E.; Mochizuki, M.; Sekiguchi, N.; Osabe, M.; Okada, M., In vitro metabolism of N-nitrodialkylamines. *Jpn. J. Cancer Res.* **1985,** *76*, (1), 28-36.
9. Malaveille, C.; Croisy, A.; Brun, G.; Bartsch, H., Hydroxylation and nitroreduction are required to activate dimethylnitramine into alkylating and mutagenic agents. *Carcinogenesis* **1983,** *4*, (11), 1477-1481.
10. Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J., Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* **1998,** *32*, (4), 360-368.
11. Tzang, B. S.; Lai, Y. C.; Hsu, M.; Chang, H. W.; Chang, C. C.; Huang, P. C.; Liu, Y. C., Function and sequence analyses of tumor suppressor gene p53 of CHO.K1 cells. *DNA Cell Biol.* **1999,** *18*, (4), 315-321.
12. Plewa, M. J.; Wagner, E. D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*. Water Research Foundation: Denver, CO, 2009; p 134.
13. Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2002,** *40*, (2), 134-142.
14. Wagner, E. D.; Plewa, M. J., Microplate-based comet assay. In *The Comet Assay in Toxicology*, Dhawan, A.; Anderson, D., Eds. Royal Society of Chemistry: London, 2009; pp 79-97.
15. Tice, R. R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J. C.; Sasaki, Y. F., Single cell gel/comet assay: guidelines

for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **2000,** *35*, (3), 206-221.
16. Anderson, D.; Yu, T. W.; McGregor, D. B., Comet assay responses as indicators of carcinogen exposure. *Mutagenesis* **1998,** *13*, (6), 539-555.
17. Rundell, M. S.; Wagner, E. D.; Plewa, M. J., The comet assay: genotoxic damage or nuclear fragmentation? *Environ. Mol. Mutagen.* **2003,** *42*, (2), 61-67.
18. Fairbairn, D. W.; Olive, P. L.; O'Neill, K. L., The comet assay: a comprehensive review. *Mutat.Res.* **1995,** *339*, (1), 37-59.
19. Kumaravel, T. S.; Jha, A. N., Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat.Res.* **2006,** *605*, (1-2), 7-16.
20. Phillips, H. J., Dye exclusion tests for cell viability. In *Tissue Culture: Methods and Applications*, Kruse, P. F.; Patterson, M. J., Eds. Academic Press: New York, 1973; p 406.