

ORIGINAL CONTRIBUTION

Cytotoxicity of TNT and Its Metabolites

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The production and storage of explosives has resulted in the environmental accumulation of 2,4,6-trinitrotoluene (TNT). The biotransformation products of the nitroaromatic compound TNT and metabolites in mammalian cells in culture and their cytotoxicity are studied. We report after our analysis by reverse phase high performance liquid chromatography (HPLC) that the most prevalent biotransformation product of TNT in the NG108 neuroblastoma cells is primarily monoamino-dinitrotoluene (2Am-DNT). It causes toxic effects based on trypan blue exclusion and LDH-release colorimetric assays.

INTRODUCTION

2,4,6-Trinitrotoluene (TNT)^d and its known nitroaromatic metabolites, 2-amino-4,6-dinitrotoluene (2Am-DNT); 2,4-dinitrotoluene (DNT); 4-nitrotoluene (NT); and toluene are environmental pollutants and are of potential health concerns. TNT is used extensively in making explosives. 2,4-DNT is used in production of polyurethane foams. Significant amounts of these aromatic compounds are dumped into the soil in many areas of the United States and other parts of the world. Due to continuous leaching, these compounds also contaminate ground water. A

few studies have been conducted on the toxicity of TNT and 2,4-DNT on prokaryotic cells [1], but the knowledge about their effects on mammalian cells remain limited. Very little, if any, information is available regarding toxicity of the most prevalent metabolite of 2Am-DNT. Since we are exposed to these compounds, it was intriguing to investigate the toxicity of these compounds on mammalian cell lines such as NG108 neuroblastoma cell line. It was also of interest to analyze the principle biotransformation product of TNT in mammalian cells in order to understand whether TNT or its metabolite(s) cause toxicity.

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^d Abbreviations: TNT, 2,4,6-trinitrotoluene; 2Am-DNT, 2-Amino,4,6-dinitrotoluene; DNT, 2,4-dinitrotoluene; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; HPLC, high performance liquid chromatography; ppm, parts per million.

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MATERIALS AND METHODS

Sources of chemicals

Pure TNT and other nitroaromatic compounds such as 2Am-DNT, DNT, and NT were obtained from the Aldrich Chemical Company, St. Louis, Missouri. Analytical grade of DMSO was obtained from the Fisher Scientific Company, Pennsylvania.

Cell culture

NG108 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 3 mM glutamine, 50 µg/ml gentamycin, and 10 percent heat inactivated fetal calf serum (Life Technologies Inc., Gaithersburg, Maryland), in a 5 percent CO₂ humidified chamber at 37°C. The NG108 cells were obtained from Dr. M. Nirenberg of the National Institutes of Health, U.S.A. and were cultured as described by Dutta and Verma [2].

Preparation of TNT, 2Am-DNT, DNT, and 4-NT stock solutions

1 ppm (parts per million), 10 ppm solution and 100 ppm of these compounds in DMSO (dimethylsulfoxide) were prepared. Fresh solutions were made with each experiment. A stock solution of 10 mg/ml or 10,000 ppm was made for each compound from which the necessary dilutions were made.

Biotransformation studies of TNT in mammalian cells by HPLC analysis

Ethylacetate extraction was done on control and TNT treated neuroblastoma cells by adding equal volume of ethylacetate to the cell containing medium, vortexing and centrifuging at 3000 rpm for 10 min. The top layer was used for high performance liquid chromatography (HPLC) analysis. This analysis was done using the Hewlett-Packard series 1050 HPLC instrument equipped with LC-18, 15 cm x 4.6 mm column and an ultraviolet detector set at 254 nm. Each time, 2 µl of samples were injected in the HPLC. Isocratic elution was performed with 46 percent methanol at a constant flow rate. Identification of metabolites was done by monitoring the HPLC of pure standards. Spiking with known metabolites was done to identify specific metabolites. Specific metabolites were collected using a HPLC compatible fraction collector.

Cytotoxicity assay by trypan blue method

Cytotoxicity was assayed by trypan blue exclusion method as described by Sinensky et al. [3]. After exposing NG108 cells to 1, 10 and 100 ppm of TNT, 2Am-DNT, DNT, and 4-NT at different intervals (7, 18 and 48 hr), cells were collected and centrifuged at 4000 rpm for 15 min. Pellets were suspended in fresh 2 ml of DMEM medium, and 100 µl of this suspension was mixed with 200 µl of trypan

Table 1. Effects of TNT and its metabolites on neuroblastoma cells as tested by trypan blue and LDH release assay methods¹.

Compounds	Trypan blue method	LDH release assay method
Control ²	0	0
TNT ³	84 ± 0.94	88 ± 6.9
2Am-DNT	89 ± 0.96	90 ± 3.2
DNT	71 ± 3.2	70 ± 3.4
NT	65 ± 3.8	67 ± 3.4

¹ Percent counts ± SD; n = 3.

² In controls with DMSO but without TNT or its metabolites.

$$\text{Percent cytotoxicity} = \frac{\text{LDH released spontaneously from the medium of untreated cells}}{\text{LDH released from medium containing treated cells}} \times 100$$

blue. Dead cells were identified because they absorbed the blue as they took blue dye due to the loss of membrane polarity. Viable cells were colorless as they did not take dye due to intact cell membranes and polarity. Cells were counted on a hemocytometer under a light microscope. The cells per milliliter were counted in a hemocytometer. An average of 10 squares were multiplied by dilution factor $\times 10^4$.

Cytotoxicity assay by cytotox 96 method

The cytotox 96 assay is a colorimetric assay that quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatant is measured within 30 minutes by a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product (Promega Corporation, Madison, Wisconsin, USA). Absorbance in the visible region of the spectrum, which was proportional to the number of cells lysed, was determined in a 96-well plate reader. Two sets of NG108 cells were taken in two 96-well plates: one for spontaneous LDH release as control (i.e., for cells dying naturally) and another set for experimental purposes. A known number of cells (10^5) were grown on the 96-well plate at 37°C in a 5 percent purified CO₂ incubator overnight. Medium was replaced the next day with fresh medium containing 100 ppm of TNT, 2Am-DNT, DNT, and 4-NT in different wells. After seven hours, the plate was taken out and 50 µl of 1 M acetic acid solution was added to each well, and the absorbance was recorded at

490 nm. The LDH estimate was done using the formula above.

RESULTS

Initial exposure to 1, 10, 100, and 1000 ppm of TNT at 7, 18, and 48 hours indicated that 100 ppm of TNT exposure for 7 hours was optimum (50 percent lethal dose) for *in vitro* cytotoxicity assays. This dose and time was used for other experiments. Cytotoxicity, determined by trypan blue method indicated that TNT, 2Am-DNT, DNT, and NT at 100 ppm killed 84 percent, 89 percent, 71 percent, and 65 percent of NG108 cells respectively (Table 1). Cytotoxicity assay by cytotox 96 LDH-release assay method with TNT, 2Am-DNT, DNT and NT killed 88 percent, 90 percent, 70 percent, and 67 percent of the cells respectively (Table 1). The difference in the extent of cytotoxicity, might be due to different sensitivity of two assays.

Figure 1 shows biotransformation products of TNT in the NG108 mammalian cells by HPLC analyses indicating that 2Am-DNT is the primary visible metabolic product in the cells in culture. Other metabolic products, if any, are not visible under these experimental conditions. Authenticity of TNT and of 2Am-DNT were confirmed by spiking with the pure chemicals obtained commercially. Conversion of areas at specific retention times, as seen in the HPLC readouts, were calculated as mM concentration. Linear graphs for each chemical were then generated using these mM concentrations.

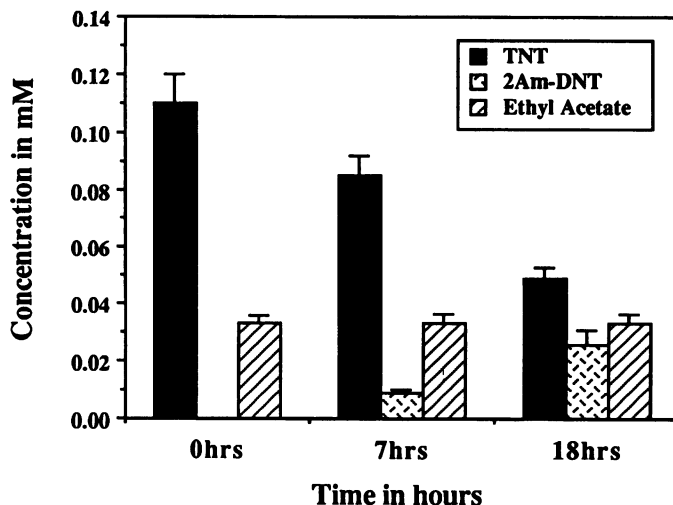


Figure 1. Summary of HPLC analyses shown as histograms expressed quantitatively as mM. HPLC analyses showing 2Am-DNT as the principle and only visible metabolite of TNT in NG108 mammalian cells. No change in ethylacetate was seen, which serves as internal control.

DISCUSSION

Understanding cytotoxicity of cells with environmental pollutants is an essential step to develop strategies for bioremediation. It was important to find out the stable metabolite of these toxic compounds. We have demonstrated that TNT, in mammalian NG 108 cells, metabolizes to 2Am-DNT, which was the most stable metabolite of TNT in cells. The other metabolites such as 2,4-DNT and 4-NT were also cytotoxic as shown by two cytotoxicity assays, but these were not produced in the neuroblastoma cells. 2Am-DNT was also found to be the most cytotoxic in comparison to other nitroaromatic compounds using both tests. Our results showed that a small amount, such as 100 ppm of these compounds would cause considerable cytotoxicity. Recently, [4], it has been reported that the urinary metabolites of TNT treated Fischer 344 rats were mutagenic in bacteria. It will be of interest and of great importance to public health to

find out the chemical nature of those TNT metabolites and their toxicity and mutagenicity in mammalian cells.

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