

Research Article

In-Vitro Investigation of the Cytotoxic and Genotoxic Effects of Benzimidazole Group Pesticides Benomyl and Carbendazim

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Abstract

Fungicides are the most effective method to control fungal microorganisms which cause plant diseases. The benzimidazole group of fungicides acts by inhibiting microtubule formation. Benomyl and its metabolite carbendazim are the most commonly used benzimidazole group systemic agricultural fungus in developing countries. Benomyl is an important teratogenic agent, have toxic effects on male reproductive system and also on the nervous system due to the mechanisms that disrupt the microtubule organization are also frequently encountered. Carbendazim is also known aneugen fungicide. In our study, the cytotoxic effects of benomyl and its metabolite carbendazim were investigated by MTT and NRU tests on human neuroblastoma cell line (SH-SY5Y) and rat kidney epithelial cell line (NRK-52E) and their genotoxic effects were tested by Comet assay. According to the results of cytotoxicity in our study, the LC50 values in SH-SY5Y and NRK52E cell lines were 108.7µM and 25.7µM for benomyl, respectively; and 201.3µM and 1619.5µM for carbendazim, respectively. As a result of our cytotoxicity study, the doses to be used in the genotoxicity assessment were determined for benomyl and carbendazim in both cell lines. According to Comet assay results it has been observed that benomyl and carbendazim have genotoxic effects on SH-SY5Y and NRK52E cell lines.

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Introduction

Fungicides have been commonly used pesticides against fungal diseases in the aim of increase crop production. However adverse effects of fungicides on other species not yet clearly identified. It is important to understand mechanisms that play role under toxic effects for effective hazard identification and risk assessment, increase efficiencies of fungicides and also protect non-target species [1]. Benzimidazole fungicides have systemic effects and selectively disrupt tubulin biosynthesis through inhibit α and β tubulin dimerisation that cause disruption in fungal spindle fibril structures. Benomyl and its metabolite carbendazim are widely used benzimidazole fungicides against to crop fungi and believed that these fungicides are nontoxic to other species except male reproductive system [2].

Benomyl is metabolized into functionally active carbendazim and this metabolite is commonly used fungicide from farmers. Benomyl and carbendazim show their toxic effects by inhibiting mitosis through binding β tubulin subunits of microtubules [3-5]. Benomyl binds mammalian neuronal tubulins with low affinity and prevent polymerization of tubulins [6]. Due to weak and slow catabolism of carbendazim, its mostly retained in the tissues [7]. In mammals, benomyl rapidly absorbed and metabolized through hydroxylation and hydrolysis in the liver and excreted into urine and feces [8]. Carbendazim; absorbed as high as 80-85% after oral exposure and then metabolizes many molecules [7]. Benomyl and carbendazim have several adverse effects as male reproductive system disruptions, teratogenicity, neurodegeneration, dermal sensitisation, tubular degeneration in kidney, liver toxicity, endocrine disruption and cancer [9-15]. There is only one study about benomyl's toxic effect on SH-SY5Y cells in the literature and no data about carbendazim cytotoxic and genotoxic effect on SH-SY5Y cells [16], and therewithal there is no data on NRK52E cell line. In this study the aim was to investigate cytotoxic and genotoxic effects of benomyl and carbendazim commercial products on SH-SY5Y and NRK52 cell lines.

Materials and Methods

Cell culture and cytotoxicity assays

Pilben 50 (benomyl) and Derosal 50 (carbendazim) commercial products were used for exposures. To evaluate cytotoxic effects of benomyl and carbendazim, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide) and NRU (Neutral Red Uptake) tests were performed on Human neuroblastoma SH-SY5Y cell (ATCC, CRL-2266) and rat kidney epithelial NRK52E cell (ATCC, CRL-1571) cells. Exposure doses were shown in table 1.

The MTT test is an in vitro cytotoxicity test on the basis of a cell culture that aims to assess cell growth and/or cell death indirectly. This method is based on the principle that mitochondrial enzyme succinate dehydrogenase break of tetrazolium ring in MTT dye and spectrophotometrically measure at 590nm. Neutral Red is an in vitro cytotoxicity test which is based on Neutral Red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride) intake by viable cell

lysosome. Accumulation of dye in lysosomes is directly proportional to the cell number.

NRK-52E MTT Exposure Doses (µM)	
Benomyl	10-100
Carbendazim	50-1100
SH-SY5Y MTT Exposure Doses (µM)	
Benomyl	6.25-250
Carbendazim	25-350
NRK-52E NRU exposure doses (µM)	
Benomyl	10-100
Carbendazim	500-1100
SH-SY5Y NRU exposure doses (µM)	
Benomyl	25-250
Carbendazim	100-350

Table 1: MTT and NRU test exposure doses.

The rat kidney proximal tubular epithelial cell line (NRK-52E) and Human Neuroblastoma Cell line (SH-SY5Y) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown at 37°C in a humidified incubator with 5% CO₂ in Dulbecco's Modified Eagles medium consisting of nutrient mixture F12 (DMEM/F12) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% antibiotic (100U/mL penicillin and 100µg/mL streptomycin).

NRK-52E and SH-SY5Y cells were seeded at 10⁴ cells into each well of 96-well plates following disaggregation of cells with trypsin/EDTA. After 24h, the cells were exposed to benomyl and carbendazim doses were shown in table 1. After 24h incubation period, cytotoxicity was assessed using MTT test. Optical densities (OD) of each well were determined at 590nm and compared, against at a reference wavelength of 670nm, using a microplate spectrophotometer system (Epoch, Erlangen, Germany). 1% DMSO were used as solvent control for all assays. All concentrations were tested in triplicates and each test was repeated triple. The absorbance values of samples were compared with those of the solvent controls (1% DMSO) after all values were corrected by subtracting the absorbance value of a blank (negative control). The cytotoxic activity was expressed as an IC₅₀, the concentration of extracts that caused a 50% inhibition of enzyme activity in the cells.

For NRU test a total of 10⁴ cells/well were plated in 96 well tissue-culture plates. After 24h incubation the cells were exposed to benomyl and carbendazim doses were shown in table 1. The cells were incubated for 24h at 37°C in 5% CO₂, then the medium was discarded. The cells were washed twice with PBS and incubated for an additional 3h in the medium supplemented with NR (50µg/ml). The cells were rinsed five times with PBS and 200µl of "fixation solution" (50% ethanol, 1% acetic acid, and 49% distilled water) was added to each well to fix the cells and bring NR into solution. The plates were shaken for 20min, and the absorbance of the solution in each well was measured in a microplate reader at 540nm using a microplate spectrophotometer system (Epoch, Erlangen, Germany). Results were expressed as the mean percentage of cell growth inhibition from three independent experiments. IC₅₀ values represent the concentrations that reduced the mean absorbance of 50% of those in the untreated cells.

Genotoxicity assays

For genotoxicity assay the alkaline comet assay was performed [17]. Human neuroblastoma SH-SY5Y cell (ATCC, CRL-2266) and rat kidney epithelial NRK52E cell (ATCC, CRL-1571) cells were seeded in 6-well plates before the treatment at 5x10⁵ cells per well. Benomyl and carbendazim exposure doses on NRK52E and SH-sy5y cells were shown in table 2. The viability of cells was checked via trypan blue dye method and cells viability was ≥80% in all concentrations.

NRK-52E Cell Line Exposure Doses					
Benomyl	10µM	5µM	2,5µM	1,25µM	Control
Carbendazim	900µM	450µM	225µM	112,5µM	Control
SH-SY5Y Cell Line Exposure Doses					
Benomyl	60µM	30µM	15µM	7,5µM	Control
Carbendazim	100µM	50µM	25µM	12,5µM	Control

Table 2: Comet test exposure doses.

Microscopic slides were covered with 0.5% Normal Melting Agarose (NMA) at about 45°C in Ca²⁺- and Mg²⁺- free PBS. Cells were mixed with 75µl of 0.5% LMA and the cell suspension was rapidly pipetted onto the first agarose layer, spread out with a coverslip and maintained on an ice-cold flat tray for 5min to solidify. After removal of the coverslip, the slides were immersed in cold lysing solution (2.5M NaCl, 100mM Na₂EDTA, 10mM Tris, 1% sodium sarcosinate, pH10) with 1% Triton X-100 and 10% DMSO added just before use, for at least 1h at 4°C. Electrophoresis was performed with 20 V/300mA at 4°C for 20min. For neutralization 0.4M tris-HCl buffer (pH7.5) buffer administered 3times for 5min. For slide examination 20mg/mL ethidium bromide was used and slides were examined under fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 400 (40x10) magnification by using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (% TDNA, tail intensity). Protocol was performed in triplicate to ensure reproducibility.

Statistics

MTT and NRU tests results calculated and evaluated with Microsoft Office Excel Programme. Data were analyzed by one-way ANOVA Dunnett t-test and expressed as mean±SE. The level of statistical significance was set at p0.05, and all analyses were performed using the statistical package SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL).

Results

Cytotoxicity results

The IC₅₀ values of benomyl and carbendazim in the NRK52E and SH-SY5Y cells were calculated via MTT. IC₅₀ values of benomyl on NRK52E and SH-SY5Y cells were 25,78µM and 108,7µM. IC₅₀ values of carbendazim on NRK52E and SH-SY5Y cells were 1619,47µM and 201,27µM (Figure 1). % inhibition values with NRU assay were shown in figure 2.

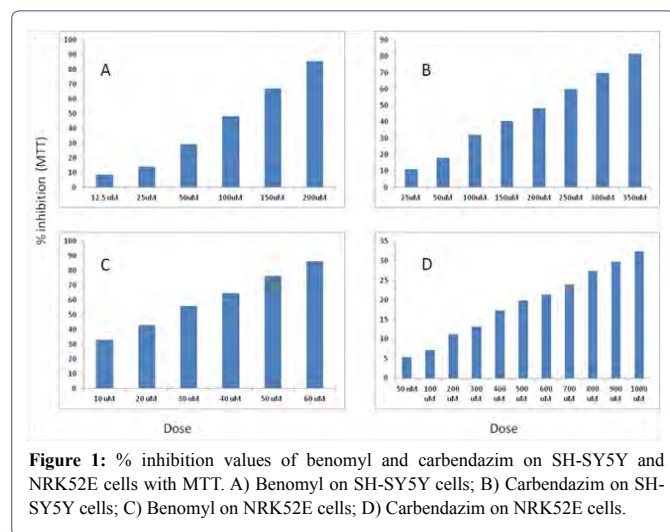


Figure 1: % inhibition values of benomyl and carbendazim on SH-SY5Y and NRK52E cells with MTT. A) Benomyl on SH-SY5Y cells; B) Carbendazim on SH-SY5Y cells; C) Benomyl on NRK52E cells; D) Carbendazim on NRK52E cells.

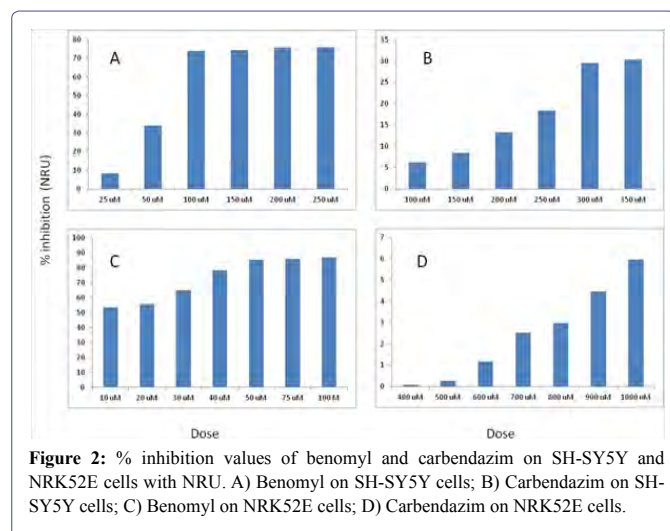


Figure 2: % inhibition values of benomyl and carbendazim on SH-SY5Y and NRK52E cells with NRU. A) Benomyl on SH-SY5Y cells; B) Carbendazim on SH-SY5Y cells; C) Benomyl on NRK52E cells; D) Carbendazim on NRK52E cells.

Genotoxicity results

The tail intensity parameters were evaluated from the comet test, after exposure of benomyl and carbendazim on SH-SY5Y and NRK52 cells. In SH-SY5Y cells, benomyl increased DNA damage in 30 and 60µM dose groups compared to control ($p < 0.05$). There were no significant difference between groups in NRK cells. There were no significant difference between groups with carbendazim exposure in SH-SY5Y cells, however in NRK cells DNA damage significantly increased with dose dependent ($p < 0.05$) (Table 3 and 4).

Discussion

The benzimidazole pesticides benomyl and its main metabolite carbendazim, are fungicides that target to microtubules and inhibit microtubule assembly and perturbing microtubule formation so this resulted with chromosomal assemble disruptions. It has been reported that carbendazim inhibit mitosis by disrupting the polymerization of mammalian tubulin into microtubules and arrest the cell cycle at the G2/M phase in turn induce apoptosis. Benomyl and carbendazim are worldwide used antifungal pesticides. It has been shown in different

studies that benomyl and carbendazim cytotoxic effects on pancreas, prostate, colon and breast tissues. And also carbendazim have role on immun system deregulation [18-20].

SH-SY5Y		P Value	NRK		P Value
Groups	Mean ±SD		Groups	Mean ±SD	
Control	3,774±0,632		Control	4,184±0,377	
7,5µM	3,191±0,607	>0,05	1,25µM	5,291±0,617	>0,05
15µM	4,712±1,372	>0,05	2,5µM	5,669±0,364	>0,05
30µM	5,2349±0,522	<0,05*	5µM	4,959±0,950	>0,05
60µM	6,306±0,344	<0,05*	10µM	3,514±0,238	>0,05

Table 3: Tail intensity values of Benomyl on SH-SY5Y and NRK cells.

Note: *significantly increased compared to control group.

SH-SY5Y		P Value	NRK		P Value
Groups	Mean±SD		Groups	Mean±SD	
Control	3,774±0,632		Control	6,467±0,336	
12,5µM	4,235±1,393	>0,05	112,5µM	8,926±1,230	<0,05*
25µM	3,869±0,772	>0,05	225µM	9,787±0,604	<0,05*
50µM	5,224±0,683	>0,05	450µM	12,45±0,932	<0,05*
100µM	5,260±1,453	>0,05	900µM	12,19±0,219	<0,05*

Table 4: Tail intensity values of Carbendazim on SH-SY5Y and NRK cells.

Note: *significantly increased compared to control group.

In our study we found that the IC50 values of benomyl on NRK52E and SH-SY5Y cells were 25,78µM and 108,7µM and carbendazim were 1619,47µM and 201,27µM. DNA damage increased dose dependent with benomyl and carbendazim in NRK cells and in SH-SY5Y cells DNA damage increased in 30 and 60µM groups compared to control. There are different studies in the literature about cytotoxic and genotoxic effects of benomyl and carbendazim on different cells. Chang et al. [20], showed cell proliferation inhibition in human endometrial cells of benomyl and carbendazim with dose dependent. Laryae et al. [18], showed that benomyl have more potent cytotoxic effect than carbendazim on T-cell leukemia, multiple myeloma, small cell lung cancer, renal adenocarcinoma, cervical adenocarcinoma, normal retinal epithelial cells and LNCaP cells. In LNCaP cells IC50 values of benomyl and carbendazim were reported as 15±5.7 and 50±9.0 mmol/l. In another study in cultured rat hepatocytes 35ug/ml benomyl decreased 49% cell viability [21]. Dierickx [22], reported benomyl and carbendazim's IC50 values in HepG2 cells are 203uM and >1750uM and in Fa32 cell 205uM and >1750uM neutral red uptake inhibition assay. In this study Dierickx classified benomyl more toxic chemical compared to carbendazim, quinalphos, carbaryl, piperonyl butoxide and 1-Aminobenzotriazole.

In another study with benomyl effects on 16HBE14o-(16HBE) human bronchial epithelial cells results indicated that IC50 values of benomyl administration for 24 or 48h are 44.2 and 7.2µM [23]. In human placental trophoblast cell line (HTR-8), compared to control group 2.5 and 5µM benomyl doses reduced cell viability by 5.79% and 6.49% and 5µM carbendazim dose decreased viability by 5.17% [24].

Benomyl and carbendazim classified as IARC group 2B possible human carcinogens. Benomyl is an aneugenic pesticide that disrupt microtubule formation. Benomyl causes micronuclei formation during cell division mechanism. It has been reported that 3.2-4.1mM benomyl concentrations associated with chromosomal abnormalities [25]. Lebaillly et al. [26], reported that, benomyl administration in human peripheral blood lymphocytes up to 500µM did not increase DNA damage with Comet Assay. 1000mg/kg benomyl induces DNA damage in Japanese quails [27]. It has been demonstrated in several different studies that carbendazim induce DNA damage in different species as *Daphnia magna*, *Eisenia foetida* earthworms, *Donax faba*, mice, rat, in human lymphocytes. In *D. magna* species it has been demonstrated with comet assay that, carbendazim induce DNA damage cumulative and were seen in all the generations with multigenerational study. In another study, carbendazim induce DNA damage with duration dependent in *Eisenia foetida* earthworms [26-32].

In conclusion, our in-vitro study results in accordance with different studies about benomyl and carbendazim's cytotoxic and genotoxic effects. While benomyl and carbendazim usage restricted in many countries, their usage still continues in many developing countries. Thus detailed studies on these fungicides about its usage currency, accumulation in the environment, detailed mechanistic studies on their toxic effects should be clarified with further studies.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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