Mutagenic and cytotoxic activities of benfuracarb insecticide

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Received: 13 July 2014 / Accepted: 27 October 2014 / Published online: 8 November 2014
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Abstract Benfuracarb is a carbamate insecticide used to control insect pests in vegetables and it has anti-acetylcholinesterase activity lower than other carbamates. Cytotoxic effects of benfuracarb were evaluated by using root growth inhibition (EC_{50}), mitotic index (MI), and mitotic phase determinations on the root meristem cells of *Allium cepa* and mutagenic effects were determined in *Salmonella typhimurium* Ames test by TA98 and TA100 strains with and without metabolic activation. In Allium test, 1 % DMSO was used as negative control group and 10 ppm MMS was used as positive control group. 75 ppm concentration of benfuracarb was found as EC_{50} . In MI and mitotic phases determination study, 37.5, 75 and 150 ppm doses of benfuracarb were used. Dose-dependent cytotoxic activity was found by root growth inhibition and MI studies. It was identified that mitotic inhibition activity of benfuracarb was higher than 10 ppm MMS. In Ames test, mutagenic activity

was not observed and over 200 $\mu\text{g}/\text{plate}$ of benfuracarb was determined as cytotoxic to *S. typhimurium* strains. Benfuracarb can be called as “mitotic inhibitor” but not called as mutagen.

Keywords Benfuracarb · Carbamates · Ames test · Allium test · Cytotoxicity · Mutagenic activity

Introduction

Pesticides are known as toxic chemicals for our nature and they are used excessively in the agricultural lands. Their residues can be present in foods and these residues can be hazardous for human and other living organisms. Carbamates are one of the main classes of insecticides. Nowadays carbamate pesticides have become valuable, because of their broad spectrum in activity and low toxicity.

Carbamates represent one of the main category of synthetic organic pesticides and are used on a large scale worldwide (Paiga et al. 2009). Carbamate compounds are generally called anticholinesterases. In the presence of inhibitors, acetylcholinesterase becomes progressively inhibited and is not further capable of hydrolyzing acetylcholine to choline and acetic acid (Jokanovic 2009; Jokanovic and Maksimovic 1997). The acute toxicity of the different carbamates ranges from highly toxic to only slightly toxic or non-toxic (IPCS 1986). Due to the potential

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Fig. 1 Chemical structure of benfuracarb

risk of carbamates on human health, the evaluation of their toxicity is routinely required for their safe use (Weyermann et al. 2005).

The main clinical symptoms of the carbamate intoxication are muscarinic signs (miosis, salivation, sweating, lacrimation, rhinorrhea, abdominal cramping, vomiting, diarrhea, urinary incontinence, bronchospasm, dyspnea, hypoxemia, bradycardia, bronchial secretions, pulmonary edema and respiratory failure), nicotinic signs (less frequent; muscular twitching, fasciculations, muscle weakens including the respiratory muscles, paralysis, tachycardia, hypertension) and rarely, central nervous system signs (Rosman et al. 2009).

Benfuracarb (Fig. 1) is a carbamate insecticide used to control insect pests in citrus, maize, sugar beet and vegetables. In rats, benfuracarb is metabolized rapidly to carbofuran, one of the main metabolites (Tomlin 1997). Rare fatal cases due to pesticide have been recently reported (Kenneth et al. 1992; Picotte and Perreault 1991; West et al. 1997; Kintz et al. 1997). Because benfuracarb is widely used and has a high toxicity, there is the possibility of fatal poisoning due to accidental ingestion or for suicidal or homicidal purposes (Lee et al. 1999). For the toxicological evaluation of benfuracarb poisoning, benfuracarb and carbofuran must be quantified simultaneously in biological materials because the toxicity of carbofuran (oral LD50 value in rats; 8 mg/kg) is 17 times higher than benfuracarb (oral LD50 value in rats; 138 mg/kg) (Tomlin 1997).

Test systems to determine the genotoxicity or mutagenicity can be divided into groups based on the biological system employed and their genetic endpoint detected. Bioassays with prokaryotes enable the detection of agents that induce gene mutation and primary DNA damages. On the other hand, analyses with eukaryotes enable the detection of a greater damage extent, varying from gene mutations to

chromosome damages and aneuploidies (Houk 1992; Leme and Marin-Morales 2009). Prokaryotic tests supported by eukaryotic test systems provided more reliable results to understand whether chemicals have any damaging effect on genes or not.

The *Allium* test is a short-term test with many advantages: low cost, easy to handle, good chromosome conditions for the study of chromosome damage or disturbance of cell division including the evaluation of risks of aneuploidy. The *Allium* material is well known and has been used for the study of basic mechanisms as well as for scoring the effects of chemicals. Root tip cells of *Allium cepa* are suitable for such cytological tests. The *Allium* test is a sensitive test, indicating excellent correlation to other test systems. The sensitivity of the *Allium* test is on the same level as, for instance, test systems using algae or human lymphocytes (Fiskesjö 1985).

The *Salmonella typhimurium*/microsome assay (Salmonella test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses a number of *Salmonella* strains with preexisting mutations that leave the bacteria unable to synthesize the required amino acid, histidine, and therefore unable to grow and form colonies in its absence (Mortelmans and Zeiger 2000). Ames test have been widely used by some researchers in order to determine the mutagenicity of the pesticides and plant extracts (Aiub et al. 2002; Cortes-Eslava et al. 2013; Eren and Özata 2014).

Materials and methods

Pesticide

Benfuracarb ($C_{20}H_{30}N_2O_5S$) was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Cas no: 82560-54-1).

Allium test

Onions (*A. cepa*, $2n = 16$) were used in the *Allium* test system. The *Allium* test was performed according to Fiskesjö (1985). Different concentrations of the pesticides (6.25, 12.5, 25, 50, 75 and 100 ppm) were used for the root growth inhibition test. $EC_{50}/2$, EC_{50} and

$EC_{50} \times 2$ concentrations of these extracts were used for mitotic index (MI) studies.

Root growth inhibition test (EC_{50} determination)

Onions were grown in freshly made distilled water for 24 h and then exposed for 4 day to the control group and other concentrations of benfuracarb. In order to determine the efficient concentration (EC_{50}) values, ten roots from each onion were cut off at the end of the treatment period and length of each root was measured. The concentration that decreased the root growth about 50 % when compared to the negative control group (1 % DMSO), was accepted as EC_{50} value. To determine the possible toxic effects on roots, $EC_{50}/2$, EC_{50} and $EC_{50} \times 2$ concentrations of benfuracarb were assessed in the *Allium* MI test.

Mitotic index (MI) determination

Onions (*A. cepa*, $2n = 16$) were used in the *Allium* test system. Five onion bulbs were treated with distilled water and different concentrations of benfuracarb for 72 h. At the end of 24, 48 and 72 h, root tips were cut and fixed in ethanol:glacial acetic acid (3:1) then were hydrolyzed in 1 N HCl at 60 °C for 7 min. Root tips from each concentration were stained with Feulgen dye for 1 h. Five slides were prepared for each concentration and 1,000 cells/per slide were counted. Totally about 5,000 cells were evaluated for each concentration. In the MI study, about 5,000 cells were counted, and MI % was determined with the following formulation.

$$MI \% = \frac{\text{divided cell number}}{\text{total cell number}} \times 100$$

Ames mutagenicity test

Salmonella typhimurium test strains and chemicals

Salmonella typhimurium tester strains TA98 and TA100 were obtained from the Hacettepe University, Turkey. While TA98 was used for the determination of frame shifts, TA100 was used for the determination of base pair exchanges.

S9 from rat liver (Sprague–Dawley), Bacto agar, nutrient broth No. 2 (Oxoid), 2-aminoanthracene (2AA), β -nicotinamide-adenine dinucleotide phosphate (β -NADP), glucose-6-phosphate (G6P), mitomycin-C (MMC), ampicillin, histidine and basic

fuchsin were obtained from Sigma-Aldrich. Sodium azide (SA), citric acid monohydrate, NaOH, KCl, and NaCl were purchased from Riedel (Buchs, Switzerland). 4-Nitro-*o*-phenylenediamine (NPD), 2AA and 2-aminofluorene (2AF) were purchased from Fluka (Buchs, Switzerland).

Ames test

Cytotoxic doses of benfuracarb were determined from the tested concentrations (6.25, 12.5, 25, 50 and 100 $\mu\text{g}/\text{plate}$) by the method of Dean et al. (1985). The Ames test was performed as a standard plate incorporation assay with *S. typhimurium* strains TA98 and TA100 with or without metabolic activation (Maron and Ames 1983). Selection of the strains was based on the testing and strain selection strategies of Mortelmans and Zeiger (2000). These strains were tested on the basis of associated genetic markers. For each tester strain, a specific positive control was always used to test the experimental flaws, if any. While NPD was used for TA98, and sodium azide (SA) was used for TA100 as positive controls without metabolic activation. On the other hand, 2AF and 2AA were used as positive controls with metabolic activation for TA98 and TA100 strains, respectively.

Statistics

The SPSS computer program was used to analyze the variance of the data. The one-way analysis of variance (ANOVA) and Dunnett's *t* test (2-sided) were used for the statistical analyses.

Results

In *Allium* root growth test results are shown in Table 1. The effective concentration (EC_{50}) was found approximately as 75 ppm. Dose-dependent cytotoxic effect of benfuracarb was observed in *Allium* EC_{50} test. Table 2 summarizes the effect of benfuracarb on MI and mitotic phases in root meristematic cells treated for 24–72 h. At all used concentrations of benfuracarb, MI values decreased compared to the control at each exposure time. The highest inhibition was obtained at 150 ppm at 72 h exposure time, and the lowest inhibition was at 37.5 ppm at 24 h. The variation of MI showed

Table 1 Root growth inhibition test results of benfuracarb

Test substance	Concentration (ppm)	Mean root length $\pm$ SD (cm)	% Inhibition
Control	–	5.52 $\pm$ 0.23	–
1 % DMSO	–	5.37 $\pm$ 0.24	–
Benfuracarb	6.25	4.25 $\pm$ 0.20*	20.86
	12.5	4.07 $\pm$ 0.13*	24.21
	25	3.66 $\pm$ 0.19*	31.84
	50	3.27 $\pm$ 0.27*	39.11
	75	2.70 $\pm$ 0.17*	49.72
	100	2.38 $\pm$ 0.25*	55.68

SD standard deviation, DMSO dimethyl sulfoxide

* Means difference is significant at the level of 0.05 (Dunnett's *t* test, 2-sided)

statistically significant results at all concentrations and treatment periods of benfuracarb. Most interesting result obtained from MI study was the more cytotoxic effects of used concentrations of benfuracarb compared to the positive control (10 ppm MMS).

All concentrations of benfuracarb caused changes in the percentage of mitotic phases compared to the control group (Table 2). Percentages of mitotic phases of control in the 24 h experiment were 82.20 ± 6.16 for prophase, 1.07 ± 0.41 for metaphase, 1.05 ± 0.32

for anaphase and 0.93 ± 0.32 for telophase. For 48 h treatment, the values were 78.98 ± 6.24 , 1.00 ± 0.15 , 1.01 ± 0.13 and 0.82 ± 0.29 and finally for 72 h treatment mitotic phases (%) values were 77.24 ± 6.11 , 0.87 ± 0.18 , 0.98 ± 0.12 and 0.79 ± 0.36 , respectively. But percentages of mitotic phases of benfuracarb were dose-dependently decreased and values were below the percentages of the negative and positive control group. The characteristic effect caused by the tested preparations was an increase of prophase index and simultaneous decrease of metaphase, anaphase and telophase index compared to the control.

Table 3 indicated the results of the Ames test. The evaluation of the Ames test results were performed according to United States Environmental Protection Agency (USEPA 1996) methods. If the revertant frequency is 2.0 or higher over the negative control or dose-related increase in the number of revertant colonies in one or more strains, tested materials were accepted as mutagenic (Mortelmans and Zeiger 2000).

Firstly, cytotoxic doses of benfuracarb were tried to be determined. According to the results, over 200 μ g/plate of benfuracarb was determined as cytotoxic. Therefore, the doses below this value were employed in the tests.

Table 2 Effects of benfuracarb pesticide on mitotic index and mitotic phases of *Allium cepa* root meristematic cells

Concentration (ppm)	Time (h)	Counted cell number	Mitotic index (MI) $\pm$ SD*	Mitotic phases (%) $\pm$ SD			
				Prophase	Metaphase	Anaphase	Telophase
Negative control (1 % DMSO)	24	5,039	85.26 $\pm$ 5.29	82.20 $\pm$ 6.16	1.07 $\pm$ 0.41	1.05 $\pm$ 0.32	0.93 $\pm$ 0.32
Positive control (10 ppm MMS)		4,839	42.35 $\pm$ 7.36*	39.76 $\pm$ 4.18	1.05 $\pm$ 0.21	0.79 $\pm$ 0.19	0.75 $\pm$ 0.24
37.5		4,921	34.72 $\pm$ 8.89*	32.43 $\pm$ 3.16	0.90 $\pm$ 0.12	0.70 $\pm$ 0.26	0.69 $\pm$ 0.13
75		5,131	28.93 $\pm$ 3.91*	26.82 $\pm$ 2.98	0.86 $\pm$ 0.10	0.69 $\pm$ 0.42	0.66 $\pm$ 0.35
150		5,053	24.79 $\pm$ 5.19*	22.89 $\pm$ 3.01	0.67 $\pm$ 0.23	0.59 $\pm$ 0.21	0.65 $\pm$ 0.26
Negative control (1 % DMSO)	48	5,092	81.81 $\pm$ 4.89	78.98 $\pm$ 6.24	1.00 $\pm$ 0.15	1.01 $\pm$ 0.13	0.82 $\pm$ 0.29
Positive control (10 ppm MMS)		5,073	36.16 $\pm$ 5.16*	33.05 $\pm$ 5.12	0.96 $\pm$ 0.21	0.77 $\pm$ 0.22	0.77 $\pm$ 0.36
37.5		5,229	24.94 $\pm$ 3.78*	22.88 $\pm$ 4.56	0.81 $\pm$ 0.08	0.69 $\pm$ 0.28	0.56 $\pm$ 0.06
75		5,112	21.52 $\pm$ 5.12*	19.92 $\pm$ 2.54	0.72 $\pm$ 0.24	0.64 $\pm$ 0.31	0.24 $\pm$ 0.08
150		5,202	20.24 $\pm$ 4.02*	18.91 $\pm$ 2.21	0.56 $\pm$ 0.22	0.54 $\pm$ 0.25	0.23 $\pm$ 0.11
Negative control (1 % DMSO)	72	5,219	79.88 $\pm$ 6.84	77.24 $\pm$ 6.11	0.87 $\pm$ 0.18	0.98 $\pm$ 0.12	0.79 $\pm$ 0.36
Positive control (10 ppm MMS)		5,333	32.12 $\pm$ 3.45*	29.86 $\pm$ 3.23	0.85 $\pm$ 0.22	0.70 $\pm$ 0.41	0.71 $\pm$ 0.30
37.5		5,224	19.12 $\pm$ 2.56*	17.33 $\pm$ 3.64	0.76 $\pm$ 0.07	0.52 $\pm$ 0.20	0.47 $\pm$ 0.0
75		5,045	18.51 $\pm$ 2.88*	17.22 $\pm$ 2.10	0.56 $\pm$ 0.09	0.51 $\pm$ 0.24	0.22 $\pm$ 0.09
150		5,091	17.11 $\pm$ 1.95*	16.00 $\pm$ 1.77	0.43 $\pm$ 0.14	0.49 $\pm$ 0.16	0.18 $\pm$ 0.08

SD standard deviation, DMSO dimethyl sulfoxide, MMS methyl methanesulfonate

* Means difference is significant at the level of 0.05 (Dunnett's *t* test, 2-sided)

Table 3 Mutagenicity of benfuracarb towards *S. typhimurium* TA98 and TA100 strain with or without metabolic activation

Agent	Amount (µg/plate)	Number of his ⁺ revertants/plate mean ± SD*			
		TA98		TA100	
		−S9	+S9	−S9	+S9
Benfuracarb	100	34.00 ± 3.24	46.80 ± 4.38	117.40 ± 8.65	175.40 ± 13.07
	50	31.80 ± 2.77	45.60 ± 3.78	110.80 ± 7.56	159.20 ± 5.97
	25	31.40 ± 5.41	45.40 ± 2.30	107.20 ± 8.47	134.00 ± 7.21
	12.5	30.80 ± 2.17	43.00 ± 2.45	99.40 ± 2.88	134.40 ± 18.53
	6.25	29.00 ± 2.24	40.00 ± 7.84	97.80 ± 11.76	132.80 ± 2.77
Negative Control (DMSO)	100	29.00 ± 2.83	42.00 ± 3.67	92.00 ± 3.74	128.80 ± 2.79
SA	10			2,722.40 ± 80.46*	
2AA	5				2,189.00 ± 99.72*
2AF	200		963.00 ± 23.28*		
NPD	200	1,287.00 ± 46.73*			

SD standard deviation, SA sodium azide, 2AA 2-aminoanthracene, 2AF 2-aminofluorene, NPD 4-nitro-*o*-phenylenediamine

* Means statistically significant at the level of 0.05 (Dunnett's *t* test, 2-sided)

Spontaneous revertants were within the normal values for the two strains examined. The positive control mutagens (SA, 2AF, NPD, 2AA) showed significant increases relative to the spontaneous mutation rate in the two tested strains. While the highest revertant colony number was 175.40 ± 13.07 µg/plate in the TA100 with S9, and the lowest was 29.00 ± 2.24 µg/plate in the TA98 without S9. The results were not found to be statistically significant by the Dunnett's *t* test (2-sided). These results showed that benfuracarb had no mutagenic activity in the tested concentrations.

Discussion

Although carbamates present low bioaccumulation potentials and short-term toxicity (relatively short biological half lives and are fairly rapidly metabolized and excreted), they are considered hazardous to the environment and human health being included in the priority list released by the United States Environmental Protection Agency (USEPA 1992). Aldicarb, benfuracarb, pirimicarb, propoxur and thiobencarb are five of the most widely used carbamate pesticides (Ruiz et al. 2006). Benfuracarb, pirimicarb, propoxur and thiobencarb are not very toxic to mammals (Ündeğer and Başaran 2005) and are used for weed control in rice and other crops (Fernandez et al. 2000).

Benfuracarb itself was investigated in a number of genotoxicity tests in vivo and in vitro toxicity studies with hens, mallard ducks, quail, carp, *Daphnia magna*, and bees. As a result of these studies benfuracarb was found toxic to birds, bees and highly toxic to fish and aquatic organisms. Oral LD₅₀ values for hens, mallard ducks and bees were 92, > 3,950 mg/kg, and 0.16 µg/bee, respectively. LC₅₀ values for quails and carps were >5,300 mg/kg and 0.65 mg/l, EC₅₀ value of *Daphnia magna* was found 9.9 µg/l. Studies evaluating the mutagenic potential of benfuracarb have shown the compound to be non-mutagenic and not carcinogenic (MSDS Benfuracarb 2012).

Benfuracarb, carbosulfan and carbofuran were tested to evaluate acute and chronic toxicity to aquatic organisms. Acute toxicity tests were performed on *Brachionus calyciflorus*, *Daphnia magna*, and *Thamnocephalus platyurus*, whereas chronic tests were conducted on *Pseudokirchneriella subcapitata*, and *Ceriodaphnia dubia*. The results indicated acute and higher chronic toxicity from all three pesticides to aquatic organisms (Iesce et al. 2006).

Benfuracarb at the dose of 15 mg/kg led to an increase of the frequency of micronucleus containing reticulocytes in the mouse bone marrow only after an expression time of 24 h (Stehrer-Schmid and Wolf 1995). On the other hand, no significant differences over control were detected when CHO-K1 cells were

exposed to benfuracarb at any of the concentrations tested in NR or MTT assays (Ruiz et al. 2006).

Investigation on the hydrolysis and photolysis of benfuracarb and carbosulfan under natural conditions provides evidence concerning the selective decay to carbofuran and/or phenol. Carbofuran is found to be more persistent and toxic (Iesce et al. 2006). It was estimated that death was induced by the combined toxicity of benfuracarb itself and its metabolite carbofuran (Lee et al. 1999).

Benfuracarb is belonging to the *N*-methylcarbamate insecticides that are derived from carbofuran. It was reported that positive results were obtained from carbofuran in the Ames test with the *S. typhimurium* strains TA1535 and TA98 and in the SCE assay with human lymphocytes and in a gene mutation assay with V79 cells. But benfuracarb was found inactive in the Ames test according to unpublished data by Stehrer-Schmid. Benfuracarb insecticide had no influence on the frequencies of gene conversion and reverse mutation in the yeast *S. cerevisiae* D7 when tested with and without metabolic activation (Stehrer-Schmid and Wolf 1995). Ames test results of this research were similar to Stehrer-Schmid's *S. cerevisiae* mutagenicity data and unpublished Ames data. So mutagenic activity was not observed in Ames test with TA98 and TA100 *S. typhimurium* strains.

Since a number of carbamate compounds are known to induce aneuploidy by interference with the formation of the mitotic spindle while showing uncertain results in mutagenicity tests, benfuracarb and several other structural related compounds were investigated in the in vitro tubulin assembly assay. Benfuracarb showed a marked activity in the form of an inhibition of the tubulin polymerization (Stehrer-Schmid and Wolf 1995). Allium root growth inhibition length results showed that benfuracarb had mitotic inhibition effect on meristematic cells. A concentration of 75 ppm of benfuracarb reduced the root length by about 49.72 % (2.70 ± 0.17 cm) and in generally dose-dependent inhibition was observed in this study. Therefore, this concentration was accepted as EC₅₀. In addition to the root growth inhibition test, Allium MI study was supported these results. Dose-dependent mitotic inhibition and significant decreases in mitotic phases were determined in the MI study. Especially, both MI % and phases % of benfuracarb were lower than the positive control (10 ppm MMS) results. All mitotic phases were lower than the control

group (1 % DMSO) but percentages of prophase in benfuracarb treatments were clearly lower than the control group results. Allium test results showed that benfuracarb arrested the cell cycle at G2-M stage or inhibited the tubulin polymerization as mentioned by Stehrer-Schmid.

This study indicated that benfuracarb has cytotoxic activity, like other carbamates but it has no mutagenic effects, not similar to other carbamates especially carbofuran, a derivative form. This study indicated that MI activity of benfuracarb was higher than that of the positive control (10 ppm MMS) and a concentration of 75 ppm was found an effective concentration (EC₅₀) of benfuracarb. As a result, it can be said that benfuracarb has similar toxic activity like the other carbamates.

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