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Toxicol Ind Health 2014 30: 275 originally published online 17 August 2012
DOI: 10.1177/0748233712456060

Toxicology and Industrial Health
2014, Vol. 30(3) 275–283

DOI: 10.1177/0748233712456060
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Genotoxic and antigenotoxic assessment of four newly synthesized dihydropyridine derivatives

Kadir Turhan[1], S Arda Ozturkcan[1], Zuhal Turgut[1], Mehmet Karadayi[2], Ali Aslan[3] and Medine Gulluce[2]

Abstract

The current study aims to determine the genotoxic and antigenotoxic potential of four newly synthesized dihydropyridine derivatives using *Escherichia coli* WP2 and Ames/*Salmonella* bacterial reversion assay systems. The bacterial mutant tester strains, *E. coli* WP2*uvrA* with a point mutation and *Salmonella typhimurium* TA1537 with a frameshift mutation, were used to determine genotoxic potentials of the test compounds. To determine antigenotoxic potentials of the test compounds, the same strains were also used together with positive mutagens *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) for *E. coli* WP2*uvrA* and 9-aminoacridine (9-AA) for *S. typhimurium* TA1537. According to the results, neither of the test compounds showed significant genotoxic activity on both tester strains at the tested concentrations. However, except compound 4, all the test compounds showed significant antigenotoxic activity on MNNG- or/and 9-AA-induced mutations. The inhibition rates of mutagenesis ranged from 27.0% (compound 2: 2.5 mM/plate) to 65.0% (compound 2: 0.5 mM/plate) for MNNG and from 30.6% (compound 2: 2 mM/plate) to 58.5% (compound 1: 1 mM/plate) for 9-AA genotoxicity. According to these results, it is concluded that all the test compounds do not have a mutagenic potential on the bacterial strains at the tested concentrations, and some of them have antigenotoxic potentials against MNNG- and 9-AA-induced mutagenesis.

Keywords

Dihydropyridine, genotoxicity, antigenotoxicity, bacterial reverse mutation test, MNNG, 9-aminoacridine

Introduction

Dihydropyridines (DHPs) are of importance in biological systems as a class of useful drugs, particularly as antioxidants. Some of the representative compounds of this class possess acaricidal, insecticidal, bactericidal, herbicidal, and several inhibitor activities (Abadi et al., 2009, 2010; Bertrand et al., 2010; Choi et al., 2010; Khadilkar and Borkar, 1998; Li et al., 2007; Marsh et al., 1988; Tu et al., 2004). DHP drugs, namely nifedipine, nicardipine, and amlodipine, are cardiovascular agents for the treatment of hypertension (Augstein et al., 1972; Buhler and Kiowski, 1987; Harb, 2004; Zolfigol et al., 2006). Recent studies have revealed that 1,4-DHPs exhibit several medicinal applications that include neuroprotectant and platelet antiaggregatory activity, in addition to cerebral anti-ischemic activity in the treatment of Alzheimer's disease and as chemosensitizer in tumor therapy (Boer and Gekeler, 1995; Bretzel et al., 1993; Kumar et al., 2011).

Due to their wide-range usage potential that is directly associated with human health-care, determination of genotoxic and antigenotoxic properties of 1,4-DHP derivatives is a very important strategy.

[1]Department of Chemistry, Faculty of Science and Art, Yildiz Technical University, Istanbul, Turkey
[2]Department of Biology, Faculty of Science, Ataturk University, Erzurum, Turkey
[3]Department of Biology, Faculty of Kazim Karabekir Education, Ataturk University, Erzurum, Turkey

Corresponding author:
Mehmet Karadayi, Department of Biology, Faculty of Science, Ataturk University, Erzurum 25240, Turkey.
Email: mkaradayi@atauni.edu.tr

Escherichia coli WP2 and Ames/*Salmonella* bacterial reversion assay systems are two important *in vitro* test systems commonly used for determining the genotoxic and antigenotoxic properties of natural or synthetic materials obtained from various sources (Mortelmans and Riccio, 2000; Mortelmans and Zeiger, 2000).

Thus, the present study was designed to evaluate the genotoxic and antigenotoxic potential of four newly synthesized 1,4-DHP derivatives (Figure 1) using *E. coli* WP2 and Ames/*Salmonella* bacterial reversion assay systems.

Material and methods

Synthesis of DHP derivatives

To a stirred mixture of 1,3-dione compound (1 mM), ethyl acetoacetate (1 mM) and $Yb(OTf)_3$ (5 mol%) in ethanol (5 mL), aldehyde (1 mM), and ammonium acetate (1 mM) were added at room temperature. The reaction mixture was stirred for 6 h thin layer chromatography (TLC) at room temperature, and then the resulting solid product was filtered, washed with water, and dried in a vacuum to afford the crude product. A pure product was obtained by further recrystallization using ethanol as a solvent. The filtrate containing the catalyst could be evaporated under reduced pressure to give a white solid. After completion of the reaction (monitored by TLC), the reaction mass was filtered in hot condition to separate the catalyst and poured on ice-water. The obtained solid condensation product was further purified by recrystallization in ethanol. The recovered catalyst was washed with ethyl acetate, then dried at 70°C and activated at 120°C prior to use for next run in model reaction. It was found that the recovered catalyst shows a good yield with three successive reactions (Wang et al., 2005).

Chemicals

Direct acting mutagens, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and 9-aminoacridine (9-AA), were obtained from ABCR GmbH & Co. KG (Karlsruhe, Germany) and Merck (Hohenbrunn, Germany), respectively. Other solvents and pure chemicals including magnesium sulfate ($MgSO_4$), sodium ammonium phosphate ($Na_2NH_2PO_4$), D-glucose, D-biotin, sodium chloride (NaCl), L-histidine HCl, L-tryptophane, sodium phosphate-dibasic (Na_2HPO_4), crystal violet, citric acid monohydrate, potassium phosphate-dibasic (K_2HPO_4), and sodium phosphate-monobasic (NaH_2PO_4) were obtained from Difo (New Jersey, USA), Fluka (Steinheim, Germany), Merck (Darmstadt, Germany), and Sigma (St Louis, USA).

Bacterial strains

Salmonella typhimurium TA1537 (ATCC® Number: 29630) strain was provided by The American Type Culture Collection – Bacteria Department of Georgetown University, Washington, USA, and *E. coli* WP2*uvrA* (ATCC® Number: 49979) strain was provided by LGC standards Middlesex, UK. All strains were stored at −80°C. Working cultures were prepared by inoculating nutrient broth with the frozen cultures, followed by an overnight incubation at 37°C with gentle agitation (Gulluce et al., 2010).

Viability assays and determination of test concentrations

The toxicity of chemicals toward *E. coli* WP2*uvrA* and *S. typhimurium* TA1537 was determined as described in detail elsewhere (Santana-Rios et al., 2001; Yu et al., 2001). These tests confirmed that there was normal growth of the background lawn, spontaneous colony numbers within the regular range, and no significant reduction in cell survival. Thus, for the concentrations and conditions reported here, no toxicity or other adverse effects were observed.

Bacterial reversion assay

The bacterial genotoxicity and antigenotoxicity assays were performed according to the studies described in detail elsewhere (Mortelmans and Riccio, 2000; Mortelmans and Zeiger, 2000). The known mutagens 9-AA (in methanol: 40 µg/plate) for *S. typhimurium* TA1537 and MNNG (in 10% dimethylsulfoxide (DMSO): 1 µg/plate) for *E. coli* WP2*uvrA* were used as positive controls, and 10% DMSO was used as negative control in these studies (Gulluce et al., 2011).

In the genotoxicity test performed with TA1537 strain of *S. typhimurium*, 100 µL of the overnight bacterial culture, 50 µL of the test compounds at different concentrations (0.5, 1, 1.5, 2 and 2.5 mM/plate in 10% DMSO), and 500 µL of buffer solution were added to 2 mL of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose agar plates. Histidine-independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 h.

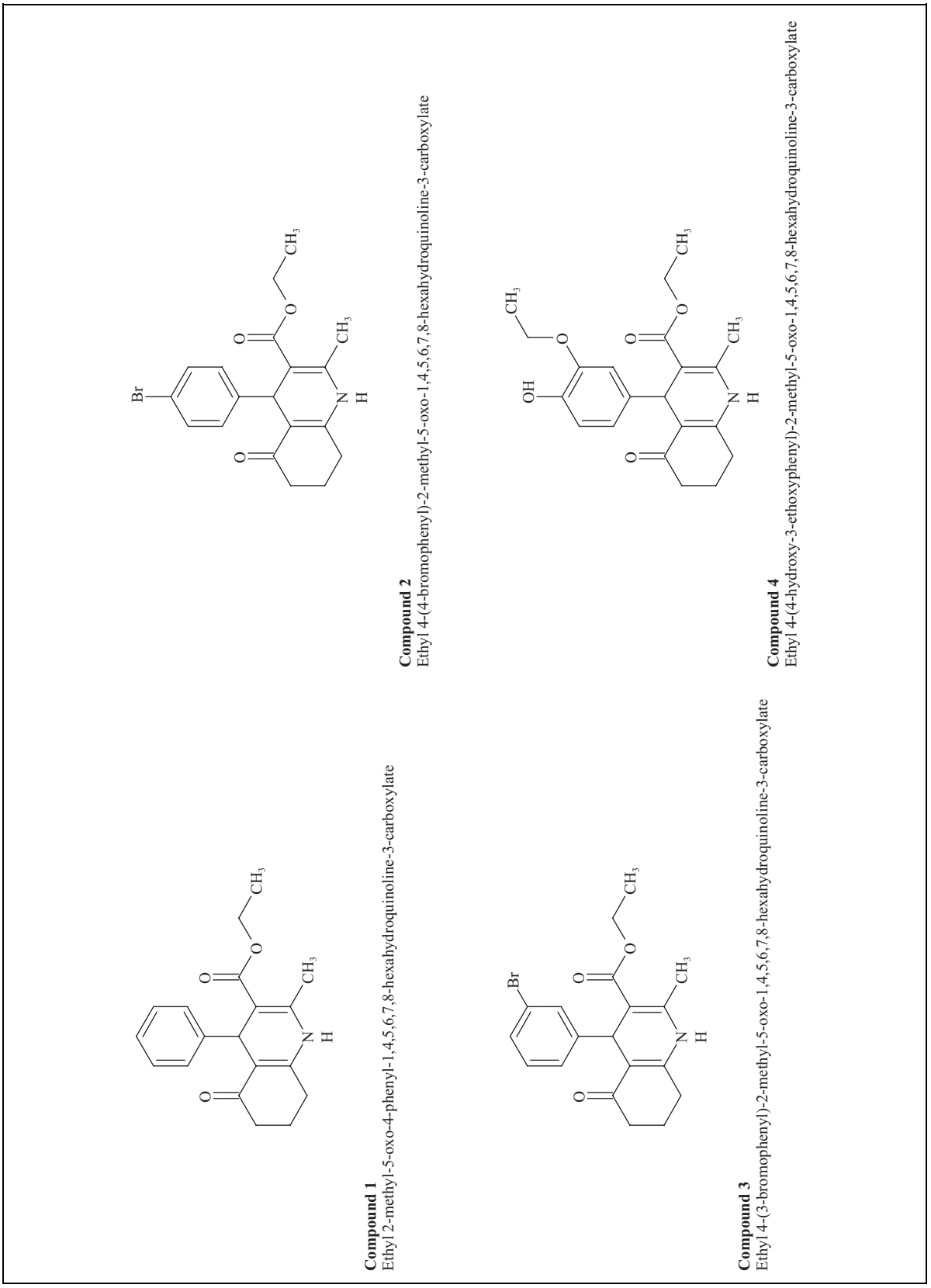

Figure 1. Four newly synthesized 1,4-dihydropyridine derivatives.

Table 1. The mutagenicity assay results of test compounds for *Escherichia coli* WP2*uvrA* and *Salmonella typhimurium* TA1537 bacterial tester strains.

Test items	Concentration (mM/plate)	Number of revertants			
		E. coli WP2*uvrA*		*S. typhimurium* TA1537	
		Mean ± SE	Mutation (%)	Mean ± SE	Mutation (%)
Compound 1	2.5	29.33 ± 0.95	–	23.33 ± 1.11	–
	2.0	30.16 ± 0.87	–	23.16 ± 1.24	–
	1.5	30.50 ± 1.38	–	21.83 ± 0.79	–
	1.0	32.00 ± 1.06	–	26.50 ± 1.33	–
	0.5	30.50 ± 0.84	–	25.16 ± 0.74	–
Compound 2	2.5	32.66 ± 0.80	–	25.33 ± 1.17	–
	2.0	30.00 ± 1.12	–	24.66 ± 1.42	–
	1.5	32.33 ± 1.22	–	24.83 ± 1.37	–
	1.0	31.66 ± 1.20	–	24.50 ± 0.92	–
	0.5	31.33 ± 1.28	–	24.83 ± 1.40	–
Compound 3	2.5	28.16 ± 0.83	–	22.83 ± 1.01	–
	2.0	33.00 ± 1.06	–	25.50 ± 1.17	–
	1.5	31.00 ± 1.23	–	23.66 ± 1.08	–
	1.0	31.50 ± 1.54	–	24.50 ± 0.84	–
	0.5	31.66 ± 0.98	–	25.16 ± 1.30	–
Compound 4	2.5	30.33 ± 1.02	–	25.33 ± 0.71	–
	2.0	33.16 ± 0.87	–	26.16 ± 0.79	–
	1.5	30.33 ± 0.98	–	25.66 ± 1.52	–
	1.0	31.16 ± 1.47	–	24.83 ± 1.19	–
	0.5	31.66 ± 1.56	–	27.33 ± 0.88	–
MNNG[a]		499.83 ± 8.26			
9-AA[a]				448.66 ± 10.95	
DMSO[a] (μl/plate)		30.66 ± 1.20		26.33 ± 0.61	

MNNG: *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; DMSO: dimethylsulfoxide; 9-AA: 9-aminoacridine.
[a]MNNG (1 μg/plate) and 9-AA (40 μg/plate) were used as positive controls for *E. coli* WP2*uvrA* and S. *typhimurium* TA1537 strains, respectively. DMSO (100 μl/plate) was used as negative control.

In the antigenotoxicity test performed with the same strain, 100 μL of the overnight bacterial culture, 50 μL of mutagen solution, 50 μL of the test compounds at different concentrations (0.5, 1, 1.5, 2 and 2.5 mM/plate in 10% DMSO), and 500 μL of buffer were added to 2 mL of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose agar plates. Histidine-independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 h.

The procedures of genotoxicity and antigenotoxicity assays described for the Ames/*Salmonella* assay are all applicable to the *E. coli* WP2 reverse mutation assay. The only procedural difference is the addition of limited tryptophan (0.01 mM) instead of histidine to the top agar (Mortelmans and Riccio, 2000).

The plate incorporation method was used to assess the results of genotoxicity and antigenotoxicity assays (Maron and Ames, 1983).

In genotoxicity assays, the mutagenic index was calculated for each concentration, which is the average number of revertants per plate divided by the average number of revertants per plate with the negative (solvent) control. A sample is considered genotoxic when a dose–response relationship was observed and a twofold increase in the number of mutants with at least one concentration was observed (Gulluce et al., 2010).

In genotoxicity assays, the inhibition of mutagenicity was calculated using the following equation (M: number of revertants/plate induced by mutagen alone, S_0: number of spontaneous revertants, S_1: number of revertants/plate induced by the test compound plus the mutagen)

$$\%\text{Inhibition} = 1 - [(M - S_1)/(M - S_0)] \times 100$$

A 25–40% inhibition was defined as moderate antigenotoxicity, 40% or more inhibition as strong

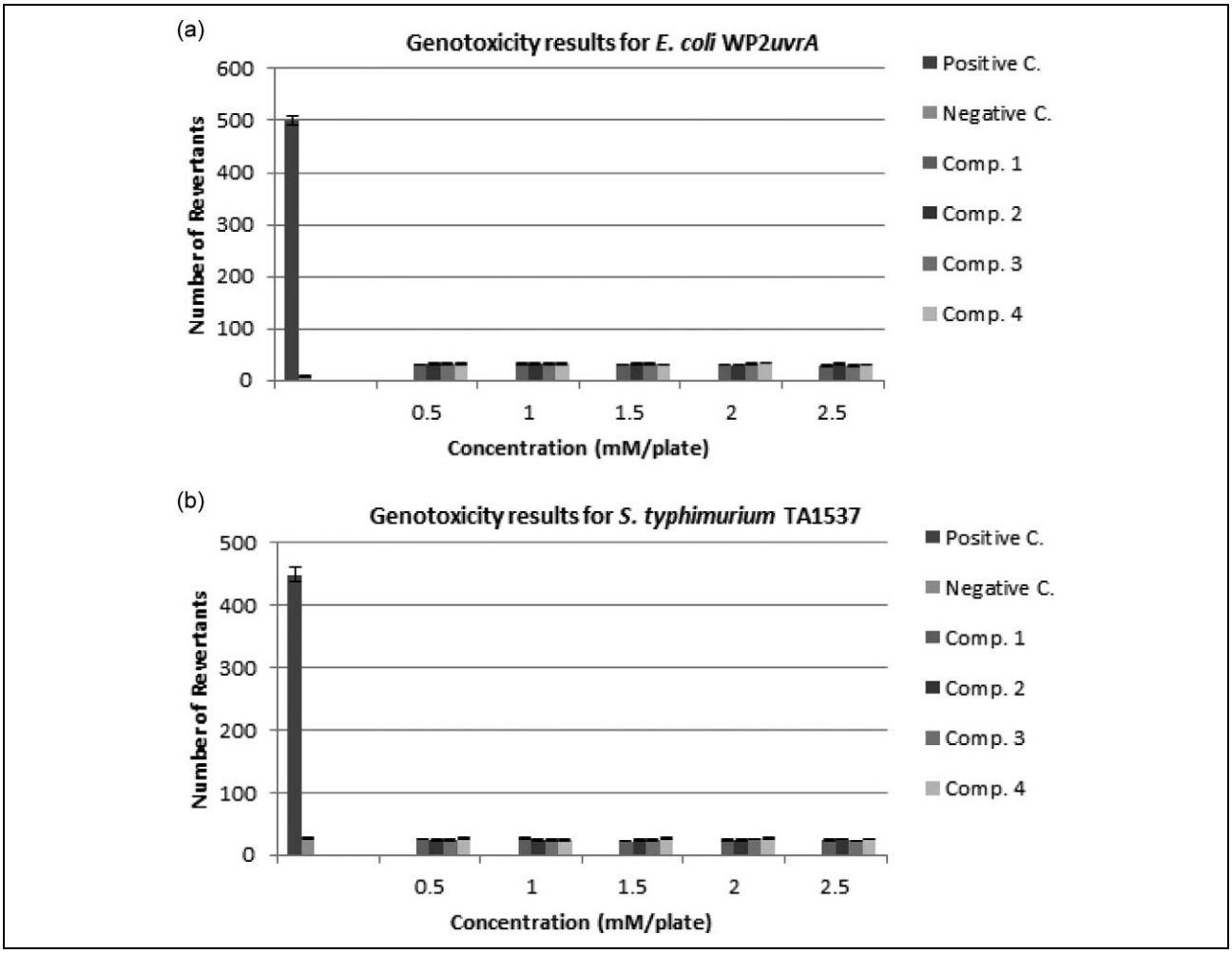

Figure 2. The genotoxicity results of the test compounds on *E. coli* WP2*uvrA* (a) and *S. typhimurium* TA1537 (b) mutant bacterial strains.

antigenotoxicity, and 25% or less inhibition as no antigenotoxicity (Gulluce et al., 2011; Turhan et al., 2012).

Statistical analysis

The results are presented as the average and standard error of three experiments with triplicate plates/dose experiment. The data were further analyzed for statistical significance using analysis of variance, and the difference among means was compared by high-range statistical domain using Tukey's test. A level of probability was taken as $p < 0.05$ indicating statistical significance (Gulluce et al., 2011; Turhan et al., 2012).

Results

The genotoxicity assay results showed that any test compound has no mutagenic activity on *E. coli* WP2*uvrA* and *S. typhimurium* TA1537 bacterial tester strains at applied concentrations (Table 1 and Figure 2(a) and (b)).

The antigenotoxic potentials of test materials were also examined against MNNG and 9-AA in *E. coli* WP2*uvrA* and *S. typhimurium* TA1537, respectively. The results were evaluated using standard plate incorporation method and summarized in Table 2 showing the antigenotoxic activities of the test materials, which were tested at five different concentrations (0.5, 1, 1.5, 2 and 2.5 mM/plate).

According to the antigenotoxicity assay results performed with MNNG and *E. coli* WP2*uvrA* strain carrying a base substation point mutation, compounds 2 and 3 have significant antimutagenic activity at various test concentrations between 0.5 and 2.5 mM/plate. The inhibition rates of these substances ranged from 27.0% (compound 2: 2.5 mM/plate) to 65.0% (compound 2: 0.5 mM/plate; Table 2 and Figure 3(a)).

Table 2. The antimutagenicity assay results of test compounds for *Escherichia coli* WP2*uvrA* and *Salmonella typhimurium* TA1537 bacterial tester strains.

		Number of revertants			
		E. coli WP2*uvrA*		*S. typhimurium* TA1537	
Test items	Concentration (mM/plate)	Mean ± SE	Inhibition (%)	Mean ± SE	Inhibition (%)
Compound 1	2.5	349.33 ± 8.37	–	356.00 ± 6.74	24.3
	2.0	353.50 ± 8.42	–	319.00 ± 7.30	**32.1**[a]
	1.5	361.33 ± 9.26	–	355.00 ± 6.49	24.5
	1.0	360.66 ± 9.60	–	195.00 ± 7.20	**58.5**[a]
	0.5	352.83 ± 7.57	–	424.33 ± 6.90	9.7
Compound 2	2.5	252.50 ± 7.89	**27.0**[a]	280.00 ± 7.82	**40.4**[a]
	2.0	171.00 ± 8.60	**50.6**[a]	326.00 ± 7.52	**30.6**[a]
	1.5	213.00 ± 6.89	**38.4**[a]	200.00 ± 6.32	**57.4**[a]
	1.0	160.50 ± 8.45	**53.6**[a]	325.00 ± 7.68	**30.9**[a]
	0.5	121.00 ± 8.74	**65.0**[a]	313.00 ± 6.31	**33.4**[a]
Compound 3	2.5	251.50 ± 7.88	**27.3**[a]	459.16 ± 7.08	2.3
	2.0	160.00 ± 7.89	**53.8**[a]	479.50 ± 7.34	–
	1.5	213.00 ± 8.81	**38.4**[a]	480.66 ± 7.88	–
	1.0	168.50 ± 8.17	**51.3**[a]	478.66 ± 6.74	–
	0.5	149.00 ± 7.57	**56.9**[a]	485.66 ± 6.60	–
Compound 4	2.5	348.16 ± 7.92	–	479.66 ± 7.05	–
	2.0	349.16 ± 9.24	–	487.66 ± 6.58	–
	1.5	349.50 ± 7.15	–	479.66 ± 7.69	–
	1.0	361.16 ± 7.36	–	485.16 ± 7.12	–
	0.5	360.33 ± 7.94	–	491.16 ± 5.99	–
MNNG[b]		346.00 ± 8.53			
9-AA[b]				470.00 ± 8.62	
DMSO[b] (μl/plate)		29.83 ± 1.37		25.16 ± 1.04	

MNNG: *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; DMSO: dimethylsulfoxide; 9-AA: 9-aminoacridine.
[a]$p < 0.05$.
[b]MNNG (1 μg/plate) and 9-AA (40 μg/plate) were used as positive controls for *E. coli* WP2*uvrA* and S. *typhimurium* TA1537 strains, respectively. DMSO (100 μl/plate) was used as negative control.

According to the other antimutagenicity assay results performed with 9-AA and *S. typhimurium* TA1537 strain carrying a frameshift mutation, compounds 1 and 2 have significant antimutagenic activity against 9-AA mutagenicity on *S. typhimurium* TA1537 strain at the tested concentrations. The inhibition rates were between 30.6% (compound 2: 2 mM/plate) and 58.5% (compound 1: 1.5 mM/plate; Table 2 and Figure 3(b)).

Discussion

Synthetic derivatives of 1,4-DHP possess important biochemical and pharmacological properties. They show modulating activity on cardiovascular and neuronal processes, on corticosteroid regulatory circuits, prevent inflammatory and diabetic processes, and some show antineoplastic, geroprotective, radioprotective, and radiosensitizing effects (Briede et al., 1999, 2002; Emanuél et al., 1985; Ivanov et al., 1990, 2004; Klegeris et al., 2002; Liutkevicius et al., 1999; Misane et al., 1998; Vartanian et al., 2004). Some of the positive effects of 1,4-DHPs are long-term; and due to their low or very low toxicity, this group of compounds appears to offer promise for medical applications (Goncharova et al., 1995; Klusa et al., 1996). Among 12 screened 1,4-DHPs that differed in chemical structure, six β-carbonyl-1,4-DHPs that are analogues of dihydronicotinamide, the hydrogen- and electron-transferring moiety of the redox coenzymes NADH and NADPH, showed antimutagenic activity and significantly reduced spontaneous and alkylation-induced point mutations and chromosome breaks in germ cells of *Drosophila*, alkylation-induced micronuclei in mouse bone-marrow cells, and radiation-induced

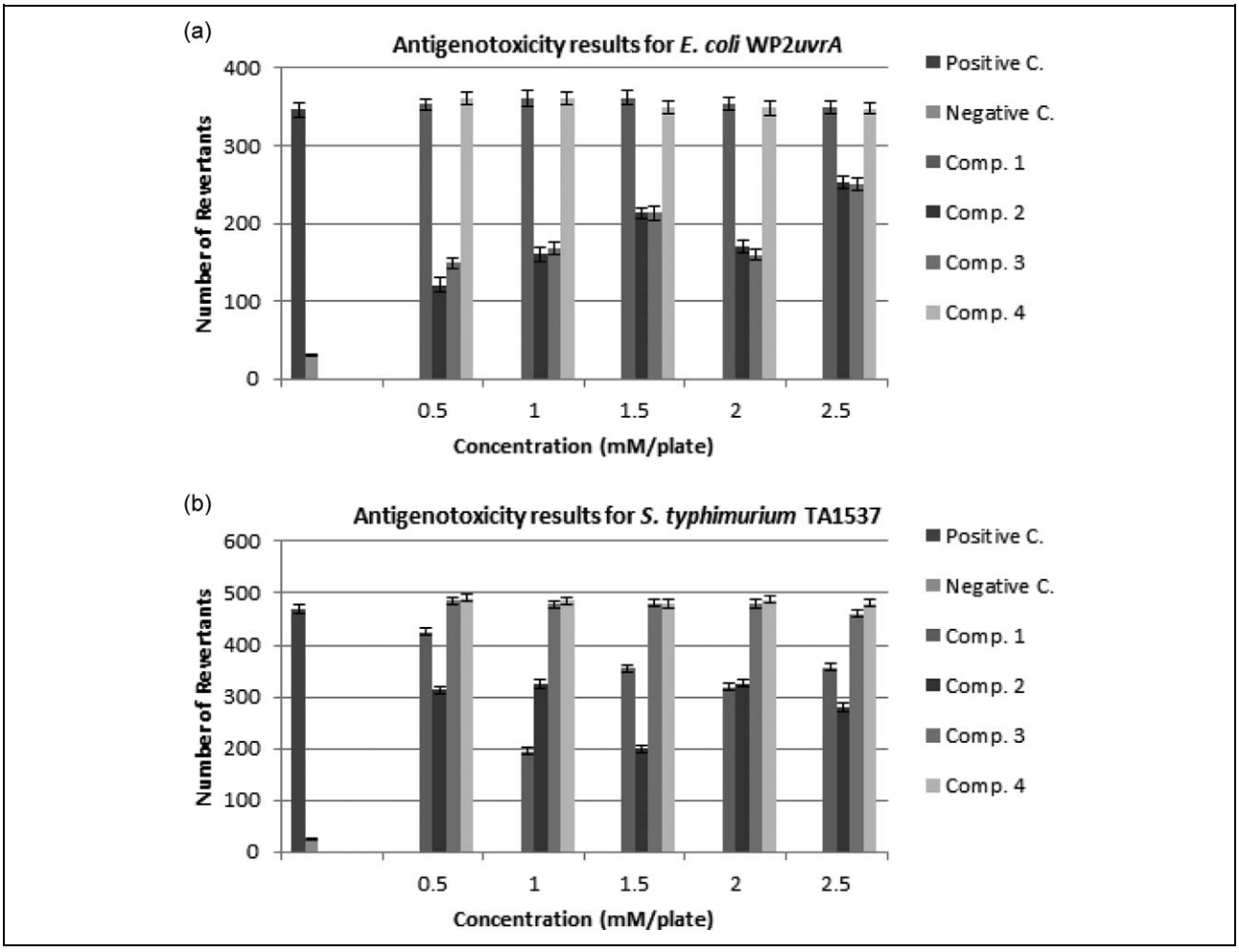

Figure 3. The antigenotoxicity results of the test compounds on MNNG- and 9-AA-induced mutations in *Escherichia coli* WP2*uvrA* (a) and *Salmonella typhimurium* TA1537 (b) mutant bacterial strains, respectively. MNNG: *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; 9-AA: 9-aminoacridine.

chromosome aberrations and other cytogenetic endpoints in fish (Goncharova, 2000; Goncharova et al., 2001; Goncharova and Kuzhir, 1989).

In the current study, the genotoxic and antigenotoxic properties of newly synthesized four DHP derivatives have been investigated using *E. coli* WP2 and Ames/*Salmonella* bacterial test systems.

According to the results, none of the test compounds showed mutagenic activity on *E. coli* WP2*uvrA* and *S. typhimurium* TA1537 tester strains. However, some of them have significant antimutagenic activity against MNNG- and 9-AA-induced mutagenesis on the same strains at the tested concentrations.

The mutagens used to determine the antimutagenic activity of the test substances in this study were MNNG for WP2*uvrA* and 9-AA for TA1537.

MNNG is a well-known carcinogen, and it is known to exert its mutagenic and lethal effects by methylation of DNA (Kumaresan et al., 1995). Previous studies showed that the formation of *O6*-methylguanine, which is one of its important products, appears to be responsible for its mutagenic action (Eadie et al., 1984; Loveless, 1969). The results of this study showed that both the test compounds have antimutagenic activity against MNNG at tested concentrations. The antimutagenicity of these substances may be explained with their inhibitor activity on the production of *O6*-methylguanine.

9-AA was used in this study as a simple intercalator mutagen. Through intercalation, 9-AA induces frameshift mutations at hotspots in which a single base, especially guanine, is repeated (Hoffman et al., 2003). The antimutagenicity assay performed with *S. typhimurium* TA1537 and 9-AA depends on the inhibition of this mechanism by test substances, which were thought as antimutagenic. In this study,

the results suggested that compounds 1 and 2 have antimutagenic activity in TA1537 strain at different concentrations (Table 2). This antimutagenic effect may be due to their inhibition capabilities by blocking 9-AA binding to DNA.

In conclusion, four newly synthesized 1,4-DHP derivatives, which were investigated in the present study, can be thought as genotoxically safe at the tested concentrations because they do not show mutagenic activity. Besides, some of them showed significant antimutagenic properties that can be valuable for the prevention and drug discovery studies against MNNG and 9-AA genotoxicity.

Funding

This study was financially supported by Yildiz Technical University (project number BAPK 2010-01-02-GEP02).

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