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Quarterly ISSN: 0148-0545
INFORMA HEALTHCARE, TELEPHONE HOUSE, 69-77 PAUL STREET, LONDON, ENGLAND, EC2A 4LQ

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Drug and Chemical Toxicology

http://informahealthcare.com/dct
ISSN: 0148-0545 (print), 1525-6014 (electronic)

Drug Chem Toxicol, Early Online: 1–5
 DOI: 10.3109/01480545.2013.866136

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ORIGINAL ARTICLE

Inhibition of the mutagenic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 9-Aminoacridine by indenopyridines in the *Salmonella typhimurium* tester strain 1537 and *E. coli*

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Abstract

The goal of the present research was to determine the protective potential of five newly synthesized indenopyridine derivatives against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 9-aminoacridine (9-AA) induced mutagenesis. MNNG sensitive *Escherichia coli* WP2*uvrA* and 9-AA sensitive *Salmonella typhimurium* TA1537 were chosen as the bacterial tester strains. All of the test compounds showed significant antimutagenic activity at various tested concentrations. The inhibition rates ranged from 25.6% (Compound **2** - 1 mM/plate) to 68.2% (Compound **1** - 2.5 mM/plate) for MNNG and from 25.7% (Compound **4** - 1 mM/plate) to 76.1% (Compound **3** - 2.5 mM/plate) for 9-AA genotoxicity. Moreover, the mutagenicity of the test compounds was investigated by using the same strains. None of the test compounds has mutagenic properties on the bacterial strains at the highest concentration of 2.5 mM. Thus, the findings of the present study give valuable clues to develop new strategies for chemical prevention from MNNG and 9-AA genotoxicity by using synthetic indenopyridine derivatives.

Keywords

Ames/Salmonella test, antimutagenicity, *E. coli* WP2 test, indenopyridine derivatives, indenopyridines, mutagenicity

History

Received 29 April 2013
Revised 24 September 2013
Accepted 10 October 2013
Published online 16 December 2013

Introduction

Recently, mutation-based research studies have growing interest due to rising knowledge about the relationship between mutagenesis and its detrimental effects. In this regard, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 9-Aminoacridine (9-AA) are known as two model mutagens, widely effect on many living organisms causing gene mutations. MNNG is a well-known carcinogen and used as a chemical methylating agent causing DNA damages that allow for the exploring pathways of DNA repair, DNA damage response, and mutagenesis (Wyatt & Pittman, 2006). On the other hand, 9-AA is an intercalating agent that belongs to the Acridine family, known as model frame-shift mutagens and frequently used in bacterial assay systems. In the mutagenesis mechanism, it binds to DNA non-covalently by intercalation that frequently results in frame-shift mutagenesis (Hoffmann et al., 2003). So, 9-AA has been used as a model mutagen to investigate potential hazards of the intercalating agents, and formations of related disorders in living organisms.

To date, there have been a lot of studies focused on MNNG and 9-AA genotoxicity in order to determine their effect mechanisms and prevention perspectives because of their wide-range hazardous potential (Hoffmann et al., 2003; Wyatt & Pittman, 2006). In this manner, natural or synthetic heterocyclic compounds have a great importance to develop new drug formulations with a significant protective potential against mutagens and their negative effects (Gulluce et al., 2010, 2012).

Recently, idenopyridine derivatives have attracted attentions with their broad range use potentials (Debache et al., 2009; Hild et al., 2004; Wang et al., 2008). Especially, significant anticarcinogenic effects of some idenopyridine derivatives give promising data for development of preventive strategies against mutation related disorders (Ghorab & Al-Said, 2012).

The present study was designed to evaluate the mutagenic and antimutagenic potentials of five newly synthesized indenopyridines by using Ames/*Salmonella* and *E. coli* WP2 bacterial reverse mutation assay systems. Thus, 9-AA-sensitive *S. typhimurium* TA1537 and MNNG-sensitive *E. coli* WP2*uvrA* were chosen as the bacterial tester strains.

Materials and methods

Chemicals

Direct acting mutagens 9-Aminoacridine (9-AA) and *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were obtained from Sigma-Aldrich (St. Louis, MO) and ABCR GmbH & Co. KG (Karlsruhe, Germany) respectively. Other solvents and pure chemicals including magnesium sulfate

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($MgSO_4$), sodium ammonium phosphate ($Na_2NH_2PO_4$), D-glucose, D-biotin, sodium chloride (NaCl), L-histidine HCl, L-tryptophan, sodium phosphate-dibasic (Na_2HPO_4), crystal violet, citric acid monohydrate, potassium phosphate-dibasic (K_2HPO_4), sodium phosphate-monobasic (NaH_2PO_4) were also obtained from Difco (New Jersey), Fluka (Steinheim, Germany), Merck (Darmstadt, Germany) and Sigma (St. Louis, MO).

Synthesis method for indenopyridine derivatives

To a stirred mixture of 1,3-indandione compound (1 mmol), ethyl acetoacetate (1 mmol) and $Yb(OTf)_3$ (5 mol%) in ethanol (5 mL), aldehyde (1 mmol) and ammonium acetate (1 mmol) were added at room temperature. The reaction mixture was stirred for 6 h (TLC) at room temperature then the resulting solid product was filtered, washed with water, and dried in 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 re-crystallization 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. And it was found that the recovered catalyst shows good yield with three successive reactions (Wang et al., 2005).

Five pure indenopyridine derivatives were obtained after this process and these were named as ethyl 4-(5-bromofuran-2-yl)-2-methyl-5-oxo-4,5-dihydro-1*H*-indeno[1,2-*b*]pyridine-3-carboxylate (MW: 414.3 g/mol) for compound **1**, ethyl 2-methyl-5-oxo-4-phenyl-4,5-dihydro-1*H*-indeno[1,2-*b*]pyridine-3-carboxylate (MW: 345.5 g/mol) for compound **2**, ethyl 4-(4-bromophenyl)-2-methyl-5-oxo-4,5-dihydro-1*H*-indeno [1,2-*b*]pyridine-3-carboxylate (MW: 424.3 g/mol) for compound **3**, ethyl 4-(3-phenoxyphenyl)-2-methyl-5-oxo-4,5-dihydro-1*H*-indeno[1,2-*b*]pyridine-3-carboxylate (MW: 437.5 g/mol) for compound **4** and ethyl 4-(3,4-dimethoxyphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (MW: 371.4 g/mol) for compound **5**.

Bacterial tester strains

E. coli WP2*uvrA* (ATCC® Number: 49 979) strain was provided by LGC standards Middlesex, UK, and *S. typhimurium* TA1537 (ATCC® Number: 29 630) strain was provided by The American Type Culture Collection – Bacteria Department of Georgetown University, Washington, DC. 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 (Oh et al., 2008).

Viability assay and determination of test concentrations

The toxicity of chemicals toward *E. coli* WP2*uvrA* and *S. typhimurium* TA1537 strains 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 reverse mutation assay

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

In the mutagenicity 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, 1.0, 1.5, 2.0 and 2.5 mM 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.

In the antimutagenicity 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, 1.0, 1.5, 2.0 and 2.5 mM 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 mutagenicity and antimutagenicity assays described for the Ames/*Salmonella* test 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 & Riccio, 2000).

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

In mutagenicity 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 mutagenic when a dose-response relationship was observed and a two-fold increase in the number of mutants with at least one concentration was observed (Gulluce et al., 2010; Santos et al., 2008).

In antimutagenicity assays, the inhibition of mutagenicity was calculated by 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): $\%\text{Inhibition} = 1-[(M-S_1)/(M-S_0)] \times 100$.

25–40% inhibition was defined as moderate antimutagenicity; 40% or more inhibition as strong antimutagenicity;

and 25% or less inhibition as no antimutagenicity (Evandri et al., 2005).

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 (ANOVA), 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., 2010).

Results

The mutagenicity assay results of this study clearly showed that none of the test compounds have mutagenic activity on *S. typhimurium* TA1537 and *E. coli* WP2*uvrA* bacterial strains at tested concentrations (Table 1).

The possible antimutagenic potential of test materials was examined against 9-AA and MNNG induced mutagenesis in *S. typhimurium* TA1537 and *E. coli* WP2*uvrA* bacterial strains, respectively. The results were evaluated by using standard plate incorporation method and summarized in Table 2 showing the antimutagenic activities of the test materials at five different concentrations (0.5, 1, 1.5, 2 and 2.5 mM/plate).

Table 1. The mutagenicity assay results of the test materials without MNNG for *E. coli* WP2*uvrA* and 9-AA for *S. typhimurium* TA1537 tester strains.

		Number of revertants			
		E. coli WP2*uvrA*		*S. typhimurium* TA1537	
Test items	Concentration (mM/plate)	Mean ± S.E.	Mut. %	Mean ± S.E.	Mut. %
Compound **1**	0.5	33.33 ± 1.35	–	27.83 ± 1.44	–
	1.0	32.16 ± 1.07	–	27.83 ± 0.60	–
	1.5	32.33 ± 1.45	–	25.83 ± 1.30	–
	2.0	34.00 ± 1.15	–	27.16 ± 0.79	–
	2.5	32.66 ± 1.22	–	26.66 ± 0.71	–
Compound **2**	0.5	33.83 ± 1.53	–	27.33 ± 0.66	–
	1.0	32.66 ± 1.22	–	27.00 ± 0.89	–
	1.5	32.00 ± 1.46	–	28.50 ± 0.61	–
	2.0	34.83 ± 1.74	–	26.16 ± 0.70	–
	2.5	33.00 ± 1.54	–	27.00 ± 1.34	–
Compound **3**	0.5	32.83 ± 1.30	–	26.66 ± 0.66	–
	1.0	34.83 ± 0.65	–	28.33 ± 0.80	–
	1.5	36.00 ± 1.34	–	28.16 ± 1.40	–
	2.0	32.16 ± 1.60	–	25.66 ± 0.82	–
	2.5	34.00 ± 1.63	–	26.66 ± 0.98	–
Compound **4**	0.5	32.50 ± 1.05	–	26.66 ± 0.76	–
	1.0	32.50 ± 1.91	–	26.50 ± 1.36	–
	1.5	35.33 ± 1.97	–	26.00 ± 0.85	–
	2.0	32.66 ± 1.30	–	25.83 ± 0.87	–
	2.5	34.83 ± 1.32	–	27.00 ± 0.77	–
Compound **5**	0.5	32.83 ± 1.62	–	26.50 ± 1.05	–
	1.0	35.00 ± 1.21	–	27.16 ± 1.24	–
	1.5	33.66 ± 0.95	–	26.16 ± 1.01	–
	2.0	32.83 ± 1.19	–	28.33 ± 0.66	–
	2.5	34.50 ± 0.99	–	28.00 ± 1.57	–
MNNG*		452.00 ± 13.26			
9-AA*				563.33 ± 10.60	
DMSO* (µl/plate)		34.00 ± 1.23		27.50 ± 0.56	

*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 (dimethylsulfoxide; 100 µl/plate) was used as negative control.

In the antimutagenicity assay performed with MNNG and *E. coli* WP2*uvrA* strain carrying a base substation point mutation, all the test compounds showed significant antimutagenic activity at various test concentrations between 0.5–2.5 mM/plate except Compound **4**. Moreover, Compound **1** and **5** showed dose-dependent antimutagenic activity against MNNG mutagenicity on *E. coli* WP2*uvrA* strain. The inhibition rates of all antimutagenic test materials were between 25.6% (Compound **2** - 1 mM/plate) and 68.2% (Compound **1** - 2.5 mM/plate) (Table 2).

For the other antimutagenicity assay performed with 9-AA and *S. typhimurium* TA1537 strain carrying a frame-shift mutation, only Compound **3** and **4** showed significant antimutagenic activity against 9-AA mutagenicity on *S. typhimurium* TA1537 strain at tested concentrations. The antimutagenic activities of these compounds were not dose-dependent and the inhibition rates were between 25.7% (Compound **4** - 1 mM/plate) and 76.1% (Compound **3** - 2.5 mM/plate) (Table 2).

Discussion

Researching mutagenic and antimutagenic properties of various synthetic substances is a very important strategy owing to their large scale usefulness in medicine (Agar et al. 2010; Avunduk et al., 2008; Gulluce et al., 2010). In this manner, synthetic dihydropyridine derivatives have great importance due to their versatile and broad range effectiveness. Dihydropyridine derivatives possess a variety of biological activities and drugs such as nifedipine, nicardipine and amlodipine are effective cardiovascular agents for the treatment of hypertension (Debache et al., 2009; Mager et al., 1992; Mannhold et al., 1992; Wang et al., 2008). In particular, indenopyridines (azafluorenes) are one of the most important privileged medicinal scaffolds, which were developed initially as antihistamines (Augstein et al., 1972) but shown inadvertently to cause antispermatogenic effects (Hild et al., 2004) in various species and are useful inhibitors of spermatogenesis in animals, and showed a fungicidal activity (Hild et al., 2001). Compounds with this motif show a wide range of pharmacological activities. Hydrogenated indenopyridines have valuable therapeutic uses (Meyer et al., 1994). They lower serum lipids, in particular the triglycerides, and are used for the therapy of primary hyperlipidemias and certain other hyperlipidemias. They also have potential antidepressant activity (Kunstmann & Fischer, 1984).

On the other hand there is a balance between the therapeutic and toxicological effects of a compound, which determine the limits of the usefulness of a pharmacological drug. Therefore, the potential mutagenic effect of any drug has to be determined during its development process (Resende et al., 2012). Besides, the antimutagenic activities of drugs also determine their preventive and therapeutic potentials by causing significant reductions in the occurrence of *in-vivo* or *in-vitro* mutational events (Fahmi et al., 2013).

Table 2. The mutagenicity assay results of the test materials with MNNG (1 μg/plate) for *E. coli* WP2*uvrA* and 9-AA (40 μg/plate) for *S. typhimurium* TA1537 tester strains.

		Number of revertants			
		E. coli WP2*uvrA*		*S. typhimurium* TA1537	
Test items	Concentration (mM/plate)	Mean ± S.E.	Inhib. %	Mean ± S.E.	Inhib. %
Compound 1	0.5	706.33 ± 10.31	–	628.00 ± 10.56	–
	1.0	408.00 ± 12.08	42.0*	649.16 ± 11.93	–
	1.5	325.00 ± 11.09	53.8*	618.33 ± 08.79	–
	2.0	310.00 ± 09.33	56.0*	635.50 ± 11.52	–
	2.5	224.00 ± 08.09	68.2*	632.00 ± 12.41	–
Compound **2**	0.5	436.00 ± 09.88	38.1*	633.66 ± 11.39	–
	1.0	523.50 ± 11.72	25.6*	641.50 ± 13.47	–
	1.5	622.00 ± 11.78	11.6	618.66 ± 11.47	–
	2.0	711.50 ± 10.69	–	632.00 ± 13.61	–
	2.5	533.50 ± 13.30	24.2	623.66 ± 10.98	–
Compound **3**	0.5	468.00 ± 11.94	33.5*	346.00 ± 10.68	43.6*
	1.0	602.00 ± 12.36	14.5	314.00 ± 09.33	48.9*
	1.5	451.00 ± 11.02	35.9*	418.00 ± 08.26	31.9*
	2.0	511.00 ± 09.28	27.4*	233.50 ± 09.38	62.0*
	2.5	502.50 ± 11.64	28.6*	147.00 ± 08.13	76.1*
Compound **4**	0.5	722.83 ± 08.20	–	337.00 ± 10.06	45.11*
	1.0	712.83 ± 10.05	–	456.00 ± 07.48	25.7*
	1.5	714.33 ± 10.89	–	475.00 ± 10.02	22.6
	2.0	706.66 ± 10.10	–	426.00 ± 08.42	30.6*
	2.5	723.33 ± 11.79	–	526.00 ± 11.07	14.33
Compound **5**	0.5	605.00 ± 13.68	14.1	640.33 ± 11.36	–
	1.0	425.50 ± 07.98	39.6*	615.16 ± 10.12	–
	1.5	418.00 ± 11.27	40.6*	614.66 ± 10.34	–
	2.0	320.00 ± 12.30	54.6*	616.83 ± 11.39	–
	2.5	306.00 ± 08.48	56.5*	618.16 ± 08.61	–
MNNG**		704.00 ± 10.39			
9-AA**				614.00 ± 8.48	
DMSO** (μl/plate)		36.66 ± 1.83		25.83 ± 1.60	

*A statistical significant value for antimutagenic activity ($p < 0.05$).
**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 (dimethylsulfoxide; 100 μl/plate) was used as negative control.

The mutagenicity assay results clearly show that all the test compounds do not have mutagenic activity in *S. typhimurium* TA1537 and *E. coli* WP2*uvrA* strains at all tested concentrations.

Moreover, the antimutagenicity assay results show that test compounds have antimutagenic activity in *E. coli* WP2*uvrA* and *S. typhimurium* TA1537 strains at various tested concentrations including 0.5, 1, 1.5, 2 and 2.5 mM/plate.

In this study, 9-AA for *S. typhimurium* TA1537 and MNNG for *E. coli* WP2*uvrA* were chosen as mutagenic agents to determine antimutagenic activity of the test substances.

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 (Hoffmann 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. According to the results, Compound **3** and **4** have antimutagenic activity in *S. typhimurium* TA1537 strain at different concentrations (Table 2). The antimutagenic effects of these compounds may be due to their inhibition capabilities by blocking 9-AA binding to DNA.

MNNG is a well-known carcinogen, and it is known as prototypical methylating agent with S_N1-type effect mechanism. Previous studies showed that the formation of 7-methylguanine (*N*7-MeG), 3-methyladenine (*N*3-MeA), O^6-methylguanine (O^6-MeG) appears to be mainly responsible for its mutagenic action (Wyatt & Pittman, 2006). The results of this study showed that Compound **1**, **2**, **3** and **5** have antimutagenic activity against MNNG at various tested concentrations. The antimutagenicity of these substances may be explained with their inhibitor activity on the formation of related adducts.

Conclusion

In conclusion, all test substances investigated in the present study can be considered genotoxically safe at the tested concentrations, and all of them provided important antimutagenic properties. These activities are valuable towards an extension of the employ of these compounds as new therapeutic or preservative ingredients, and the data can be also supported by performing complicate test systems resulting in more reliable results for human health applications.

Declarartion of interest

This study was supported by YILDIZ TECHNICAL UNIVERSITY with the project number of BAPK 2012-01-02-KAP05.

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