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Mutagenic activities of ten imidazole derivatives in *Salmonella typhimurium*

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Abstract: Ten imidazole derivatives were tested for mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 both in the absence and presence of metabolic activation by the microsomal fraction S9 mix. In a general manner, derivatives tested exhibited a greater mutagenic activity in the TA100 strain comparing to the responses in TA 98. In the standard plate incorporation assay, 8 of these substances (80%) were found to be mutagenic for at least one of the two strains in the presence or absence of metabolic activation. Two compounds showed positive results in TA98 and 6 compounds were also mutagenic in TA100 without S9. In the presence of S9 mix, all of the 10 substances were non-mutagenic in TA98, whereas 4 compounds were positive in TA100. The results suggested the mutagenic potentials of the imidazole derivatives particularly inducing the reversion of base-pair substitutions. According to the structure-activity relationships phenyl groups in position 2 with different substituents can confer the mutagenic activity of the tested compounds. Methyl groups in different positions of these phenyl substituents can cause different types of mutations. This mutagenic effect is observed more clearly when the phenyl group is inhibited with a nitro group.

Key words: imidazole derivatives; mutagenicity; *Salmonella typhimurium*.

Abbreviations: AZS, sodium azide; β-NADP, β-nicotinamide adenine dinucleotide phosphate; DMSO, dimethylsulfoxide; NPD, 4–nitro-*o*-phenylenediamine.

Introduction

Heterocyclic compounds containing nitrogen are important in many biological systems. Imidazole nucleus constitutes the basic structure of some endogenous substances. Imidazole derivatives have been introduced into the structure of many drug molecules used in the medicine and veterinary, particularly due to their antifungal, antiinflammatory, analgesic, and antiallergic activities (Hrelia et al. 1998; Meriç & Işikdağ 2000).

Research and development of imidazole derivatives has increasingly expanded in the last decades. Although imidazole and its principal metabolites like hydantoin, hydantoic acid, *N*-acetyl-imidazole and histamine were reported as non-mutagenic biological agents, relatively few of the synthetic imidazole derivatives with different substituents are available with acceptable low toxicity (Forster et al. 1992; Hrelia et al. 1998). This concerns especially nitroimidazoles, a group that suffers from the property of being mutagenic and carcinogenic in a variety of experimental models (Voogd et al. 1979).

The aim of the present study was to investigate the mutagenic activities of some substituted imidazole compounds synthesized by Işikdağ & Meriç (1998). There are no reports available on the mutagenicity of the ten imidazole derivatives tested in the present study. For this reason, the *Salmonella* mutagenicity assay according to Maron & Ames (1983) was applied. The continuing popularity of the *Salmonella* mutagenicity assay depends on the ability of prescreening a large number of chemicals for the identification of genotoxic hazards and the elucidation of the biochemical mechanisms of mutagenesis (Mamber et al. 1993; Josephy et al. 1997). In addition, the test is used for its high sensitivity and recognized validity (Kaplan et al. 2004). In the present study, the compounds were tested on *Salmonella typhimurium* strains TA98 and TA100 using the standard plate incorporation procedure in the presence and absence of metabolic activation.

Material and methods

Chemicals

The chemicals were purchased from the following manufacturers: nutrient broth, bacto agar (Oxoid), ampicillin trihydrate, β-nicotinamide adenine dinucleotide phosphate (β-NADP), 3–methylcholantren, crystal violet, sodium chloride (Sigma), dimethylsulfoxide (DMSO), 4–nitro-*o*-phenylenediamine (NPD) (Aldrich), sodium azide (AZS), magnesium chloride, potassium chloride, potassium phosphate, citric acid monohydrate, sodium ammonium phosphate,

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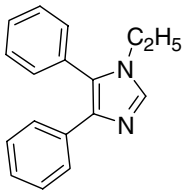
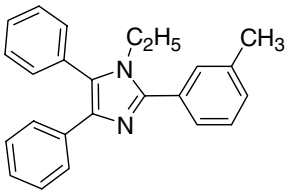
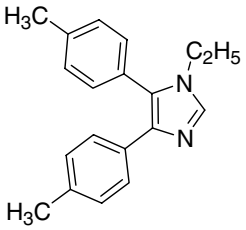


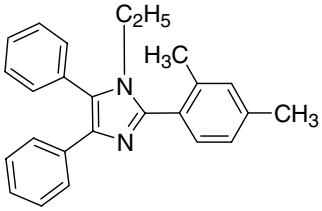
(1) 1-Ethyl-4,5-diphenyl-1H-imidazole



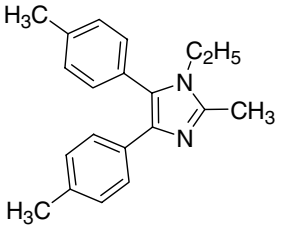
(6) 1-Ethyl-2-(m-methylphenyl)-4,5-diphenyl-1H-imidazole



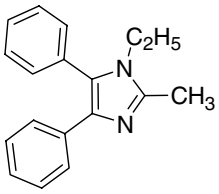
(2) 1-Ethyl-4,5-di-(p-tolyl)-1H-imidazole



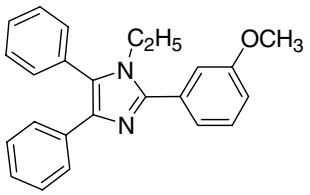
(7) 1-Ethyl-2-(o-p-dimethylphenyl)-4,5-diphenyl-1H-imidazole



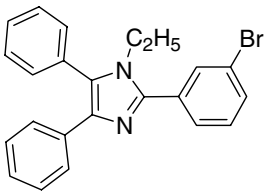
(3) 1-Ethyl-2-methyl-4,5-di-(p-tolyl)-1H-imidazole



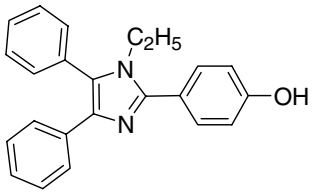
(8) 1-Ethyl-2-methyl-4,5-diphenyl-1H-imidazole



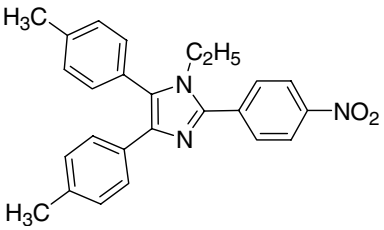
(4) 1-Ethyl-2-(m-methoxyphenyl)-4,5-diphenyl-1H-imidazole



(9) 1-Ethyl-2-methyl-4,5-diphenyl-1H-imidazole



(5) 1-Ethyl-2-(p-hydroxyphenyl)-4,5-diphenyl-1H-imidazole



(10) 1-Ethyl-2-(p-nitrophenyl)-4,5-diphenyl-1H-imidazole

Fig. 1. Structural formula of 10 imidazole derivatives.

sodium dihydrogen phosphate, disodium hydrogen phosphate (Merck).

Test materials and sample preparation

Ten imidazole derivatives were kindly provided by Prof. Dr İ. Işıkdağ (Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey).

These compounds were synthesized and structure elucidations of the compounds were achieved using spectral data and elemental analyses results at the Faculty of Pharmacy, Anadolu University (Işikdağ & Meriç 1999). Chemical structures of the compounds are shown in Figure 1.

Imidazole derivatives were dissolved in DMSO and tested at five increasing doses of concentrations (0.0001, 0.001, 0.01, 0.1, 1 mg/plate). The doses of test materials used in the mutation assays were selected in the cytotoxicity assay. One hundred μL of a suitable dilution of an overnight bacterial culture and the test compounds at different concentrations were added to 2 mL top agar. The top agar was then poured onto nutrient agar plates. After 24 h incubation at 37 °C cytotoxicity assessment was made (Dean et al. 1984).

Table 1. The results of the solvent controls and positive controls in *Salmonella* mutagenicity test with or without metabolic activation.[a]

Chemicals (μg/plate)	Revertants/plate	
	−S9	+S9
DMSO	23 ± 3.9*	30 ± 3.1*
	123 ± 22.4**	136 ± 7.0**
4–NPD (20)	1223 ± 74.2*	
AZS (1.5)	586 ± 51.4**	
2–AF (10)		714 ± 16.4*
		692 ± 41.8**

[a] −S9: without metabolic activation; +S9: with metabolic activation; DMSO: dimethylsulfoxide (solvent control); 4-NPD: 4-nitro-*o*-phenylene diamine; AZS: sodium azide; 2-AF: 2 aminoflourene (positive controls); *: use of TA 98; **: use of TA 100.

Tester strains

Salmonella typhimurium TA98 and TA100 strains were kindly provided by Dr. Bruce Ames (Biochemistry Department, University of California, Berkeley, CA, USA).

Preparation of the liver homogenate S9 fraction

Sprague-Dawley male rats were injected intraperitionally with 3-methylcholanthrene, diluted in corn oil (80 mg/kg body weight) five days before they were sacrificed. The preparation of S9 fraction is based on the procedure of Garner et al. (1972). All steps of the procedure were carried out at 0–4 °C using cold, sterile solutions and glassware. The S9 mix consisted of 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM β-NADP, 100 mM sodium phosphate (pH 7.4) and S9 in a concentration of 0.04 mL per mL of the mix. In all experiments with metabolic activation, 0.5 mL of this mix was added to the plates.

Mutagenicity tests

The *Salmonella* mutagenicity assays were carried out according to the standard plate incorporation procedure described by Maron & Ames (1983). The *S. typhimurium* strains used were TA98 for detecting the frameshift mutagens and TA100 for detecting base-pair substitution mutagens. The strains were checked routinely for histidine requirement, crystal-violet sensitivity, ampicillin resistance, ultraviolet-light sensitivity and spontaneous reversion rate.

Treatments without S9 were conducted by adding 0.1 mL of a fresh overnight culture of the tested strain (approximately 1×10^9 bacteria/mL) and the sample to be tested to 2 mL of molten top agar. These reagents were mixed and poured on the surface of a plate containing 30 mL of minimal glucose agar. Treatments with metabolic activation were conducted by adding 0.5 mL of the S9 mix. Plates were incubated at 37 °C for 48 h in the dark and revertants to histidine independence were evaluated.

Solvent control (DMSO) and positive controls (1.5 μg/plate AZS for TA100 and 20 μg/plate NPD for TA98 in the absence of metabolic activation, 10 μg/plate 2–AF for both of the two strains in the presence of metabolic activation) were included in each assay and the results are given in Table 1. All plates were set up in triplicate; all experiments were reproduced at least twice.

Statistical analysis

A test sample was classified as mutagenic if it produced a dose-response curve and if it gave a significant response in at least one concentration more than the control incidence (Sarrif et al. 1997; Hrelia et al. 1998; Suter et al. 2002). Student-t test was used for the comparisons with solvent control. The results were considered to be statistically significant at $P \leq 0.05$. Significantly decreased number of revertants compared to solvent control was considered as the proof of toxicity (Maron & Ames 1983).

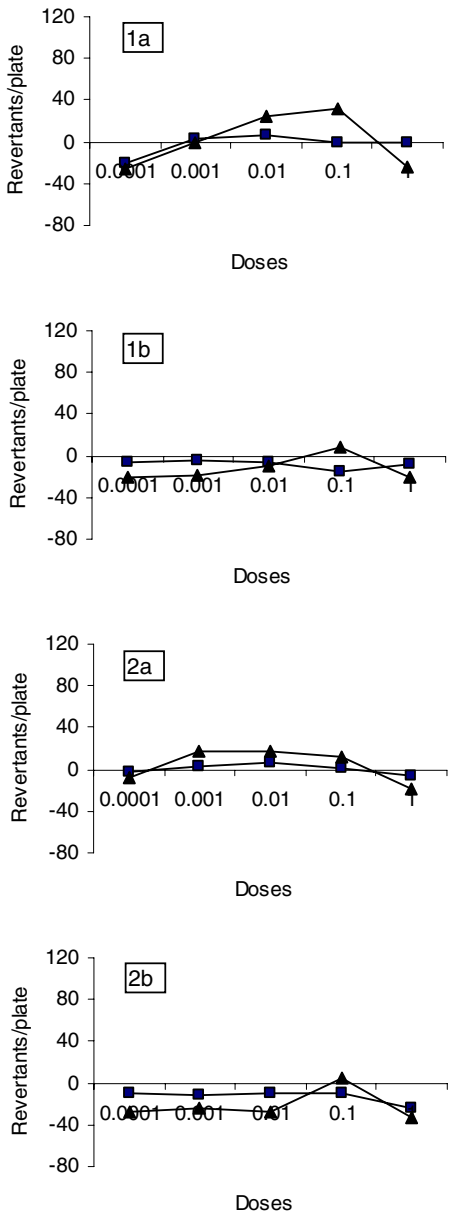


Fig. 2. Dose-response (0.0001, 0.001, 0.01, 0.1, 1 mg/plate) curves of the revertants induced by 1-ethyl-4,5-diphenyl-1H-imidazole [1] and 1-ethyl-4,5-di-(p-tolyl)-1H-imidazole [2] in the absence (a) and in the presence (b) of metabolic activation. The results are the mean of six plates from two separate experiments. Solvent control values have been subtracted. Result of each concentration was compared with the solvent control by Student-t test. * $P \leq 0.05$. ■ TA 98, ▲ TA100.

Results and discussion

The mutagenicity of the ten imidazole derivatives determined from the individual dose-response curves (Figs 2–

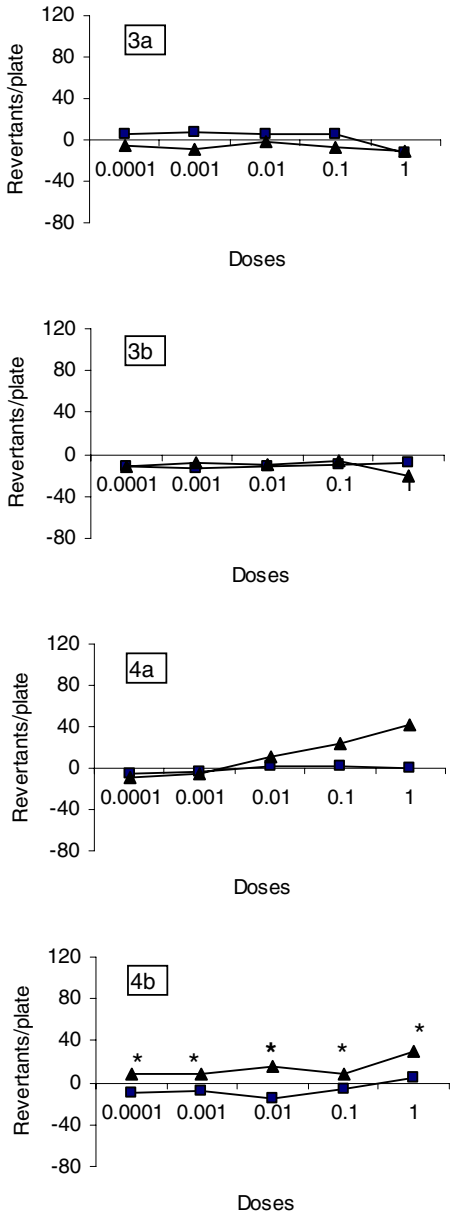


Fig. 3. Dose-response (0.0001, 0.001, 0.01, 0.1, 1 mg/plate) curves of the revertants induced by 1-ethyl-2-methyl-4,5-di-(p-tolyl)-1H-imidazole [3] and 1-ethyl-2-(m-methoxyphenyl)-4,5-diphenyl-1H-imidazole [4] in the absence (a) and in the presence (b) of metabolic activation. For the rest of the details, see the legend to Figure 2.

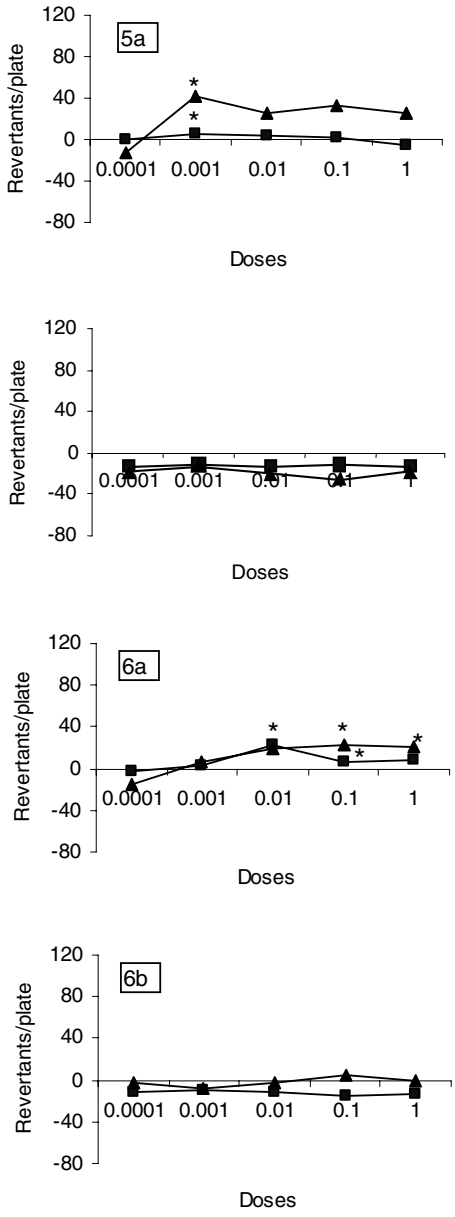


Fig. 4. Dose-response (0.0001, 0.001, 0.01, 0.1, 1 mg/plate) curves of the revertants induced by 1-ethyl-2-(p-hydroxyphenyl)-4,5-diphenyl-1H-imidazole [5] and 1-ethyl-2-(m-methylphenyl)-4,5-diphenyl-1H-imidazole [6] in the absence (a) and in the presence (b) of metabolic activation. For the rest of the details, see the legend to Figure 2.

6) is presented in Table 2. In a general manner, the ten imidazole derivatives and their metabolic byproducts tested were found weakly mutagenic and strongly toxic. We also obtained the non-linear dose-response curves. The presence of different substituents resulted in variations in the mutagenicity of the imidazole derivatives.

In their study which constitutes the basic principles of the *Salmonella* mutagenicity assay, Maron & Ames (1983) occasionally obtained non-linear dose-response curves. When a test compound is weakly mutagenic and strongly toxic, it may be difficult to find a range of concentrations in which the mutagenic potential is not masked by toxicity. In the present study, a dose-related increase in the number of revertants when compared to the solvent control and/or statistically significant response in at least one concentration more than the control incidence was considered as the proof of mutagenicity (Maron & Ames 1983; Sarrif et al. 1997; Hrelia et al. 1998; Mortelmans & Zeiger 2000; Suter et al. 2002).

TA98 and TA100 strains displayed different mutagenic sensitivities to compounds in this study. In a general manner, derivatives tested exhibited a greater mutagenic action on the TA100 strain as compared to the responses in TA98 and were regarded as mutagens inducing the reversion of base-pair substitutions. The

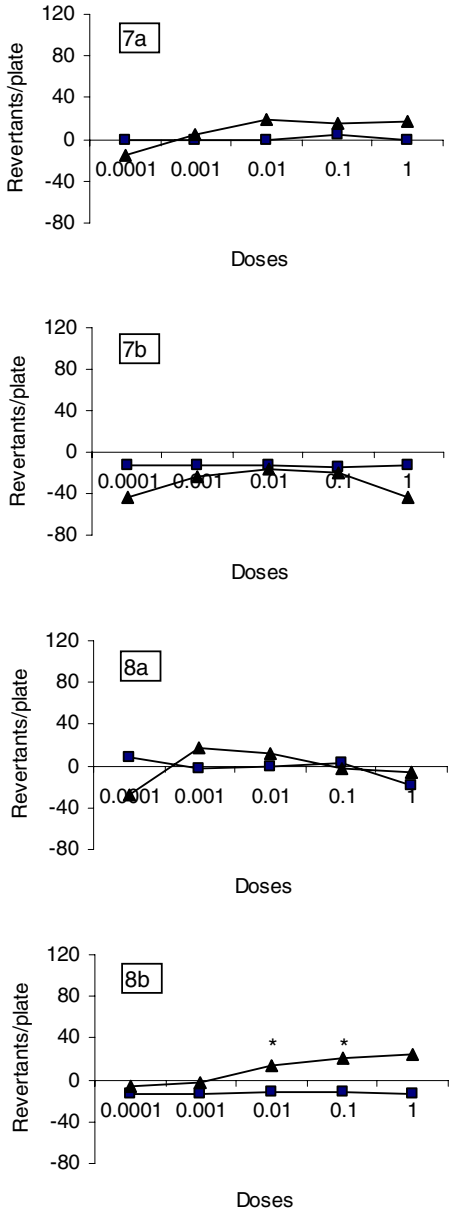


Fig. 5. Dose-response (0.0001, 0.001, 0.01, 0.1, 1 mg/plate) curves of the revertants induced by 1-ethyl-2-(o,p-dimethylphenyl)-4,5-diphenyl-1H-imidazole [7] and 1-ethyl-2-methyl-4,5-diphenyl-1H-imidazole [8] in the absence (a) and in the presence (b) of metabolic activation. For the rest of the details, see the legend to Figure 2.

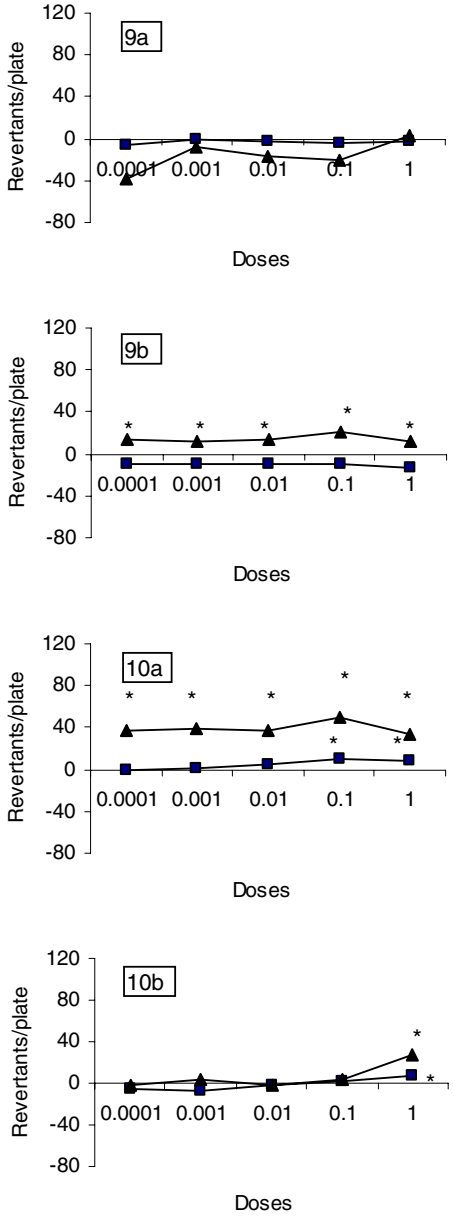


Fig. 6. Dose-response (0.0001, 0.001, 0.01, 0.1, 1 mg/plate) curves of the revertants induced by 1-ethyl-2-(m-bromophenyl)-4,5-diphenyl-1H-imidazole (9) and 1-ethyl-2-(p-nitrophenyl)-4,5-di-(p-tolyl)-1H-imidazole [10] in the absence (a) and in the presence (b) of metabolic activation. For the rest of the details, see the legend to Figure 2.

general prevalence of induction of the base-pair substitution mutation for some imidazole derivatives was also reported by other authors (Voogd et al. 1979; Hrelia et al. 1998).

Eight of the 10 substances tested were found to be mutagenic for at least one of the two strains in the presence or in the absence of metabolic activation. Two compounds showed positive results in TA98 and 6 compounds were also mutagenic in TA100 without S9. In the presence of S9 mix, all of the 10 substances were non-mutagenic in TA98, whereas 4 compounds were positive in TA100.

With regard to structure-activity relationships, 1-ethyl-4,5-diphenyl-1H-imidazole [1] (which does not have any substituents in position 2), 1-ethyl-4,5-di-(p-tolyl)-1H-imidazole [2] and 1-ethyl-2-methyl-4,5-di-(p-tolyl)-1H-imidazole [3] were found non-mutagenic for both of the two strains. 1-ethyl-2-(m-methoxyphenyl)-4,5-diphenyl-1H-imidazole [4] showed a weak direct-acting mutagenicity in TA100 and this mutagenic effect strengthened by the addition of the S9 microsomal fraction.

In the absence of exogenous metabolism the increases of revertant colonies were significant in both tester strains treated with 1-ethyl-2-(p-hydroxyphenyl)-4,5-diphenyl-1H-imidazole [5] and 1-ethyl-2-(m-methyl-

Table 2. Mutagenic activities of the ten imidazole derivatives.[a]

Test chemical	TA 98		TA 100	
	−S9	+S9	−S9	+S9
[1] 1-Ethyl-4,5-diphenyl-1H-imidazole	−	−	$+^{TD}$	−
[2] 1-Ethyl-4,5-di-(p-tolyl)-1H-imidazole	−	$-^{TD}$	$-^{TD}$	−
[3] 1-Ethyl-2-methyl-4,5-di-(p-tolyl)-1H-imidazole	−	$-^{T}$	$-^{T}$	$-^{T}$
[4] 1-Ethyl-2-(m-methoxyphenyl)-4,5-diphenyl-1H-imidazole	−	−	+	+
[5] 1-Ethyl-2-(p-hydroxyphenyl)-4,5-diphenyl-1H-imidazole	$+^{TD}$	$-^{T}$	$+^{TD}$	$-^{T}$
[6] 1-Ethyl-2-(m-methylphenyl)-4,5-diphenyl-1H-imidazole	−	$-^{T}$	+	−
[7] 1-Ethyl-2-(o,p-dimethylphenyl)-4,5-diphenyl-1H-imidazole	−	$-^{T}$	+	$-^{T}$
[8] 1-Ethyl-2-methyl-4,5-diphenyl-1H-imidazole	−	$-^{T}$	$-^{T}$	$+^{T}$
[9] 1-Ethyl-2-(m-bromophenyl)-4,5-diphenyl-1H-imidazole	−	$-^{T}$	$-^{T}$	+
[10] 1-Ethyl-2-(p-nitrophenyl)-4,5-di-(p-tolyl)-1H-imidazole	+	+	+	+

[a] −S9: without metabolic activation; +S9: with metabolic activation; +: mutagenic; −: non-mutagenic; T: toxic; TD: dose-related toxicity; $+^{TD}$: highest doses are toxic after mutagenic effect.

phenyl)-4,5-diphenyl-1H-imidazole [6] for some doses. 1-ethyl-2-(o,p-dimethylphenyl)-4,5-diphenyl-1H-imidazole [7] was weakly mutagenic in TA98 without metabolic activation. Differences in the mutation types of the compounds having methyl groups in *meta*, *ortho* and *para* positions of the phenyl substituents in position 2 were observed. This may give the opinion that the same substituents in different positions can cause different interactions with DNA. The addition of S9 microsomal fraction conferred the mutagenic activity of 1-ethyl-2-methyl-4,5-diphenyl-1H-imidazole [8] and 1-ethyl-2-(m-bromophenyl)-4,5-diphenyl-1H-imidazole [9] in TA100. Therefore metabolites of these two substances should be considered for their genotoxic potentials.

Forster et al. (1992) performed the *Salmonella* mutagenicity assays with imidazole and its principal metabolites like hydantoin, hydantoic acid, *N*-acetylimidazole and histamine, a structurally related compound which is widely distributed in mammalian tissues. The results of that study indicated that imidazole and its metabolites are unlikely to present a mutagenic or carcinogenic hazard. However, our results were in parallel with some reports available on the mutagenicity of imidazole derivatives containing methyl and nitro groups in particular. The genotoxic properties of a number of 1-methyl-1*H*-imidazole derivatives were investigated and it was reported that 5-nitroimidazoles were genotoxic (Kato et al. 1990; Zani et al. 1995). Compounds containing methyl groups in position 2 were found mutagenic or toxic. However compounds which have tolyl groups in positions 4 and 5 were not mutagenic, except for 1-ethyl-2-(p-nitrophenyl)-4,5-di-(p-tolyl)-1H-imidazole [10] with a nitrophenyl group in position 2.

The results of the present study indicate that phenyl groups in position 2 with different substituents can confer the mutagenic activity of the tested compounds. Methyl groups in different positions of these phenyl substituents can cause different types of mutations. This mutagenic effect is observed more clearly when the phenyl group is inhibited with a nitro group. Mutagenicity of nitro groups have been reported in many studies (Voogd et al. 1979; Zani et al. 1995; Ludolph et al. 2001). But it is known that mutagenicity of nitro groups can be affected by the other substituents. Ludolph et al. (2001) reported that the mutagenicity of nitrostilbenes decreased with the size of the alkyl substituents. In their study about the mutagenic action of nitroimidazoles Voogd et al. (1979) found a relationship between the chemical structure and mutagenic action of methyl-nitroimidazoles.

Eighty % of the compounds tested were observed to have mutagenic activities. Although the Ames test is a well-established method with high sensitivity, mutagenicity tests may occasionally exhibit false-positive results and it is not possible to draw a conclusive statement based solely on a single study (Maslat et al. 2002; Kaplan et al. 2004). In this connection, the imidazole derivatives should be more investigated in subsequent studies in order to compare the results from different test systems before they are considered as pro-drug molecules.

Acknowledgements

We thank Prof. Dr. İ. Işıkdağ and Dr. A. Meriç (Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey) who synthesized and gave us the test substances as gifts. This research was supported by Anadolu University by the project number 031055.

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