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ARAŞTIRMA MAKALESİ/**RESEARCH ARTICLE**

DETERMINATION OF MUTAGENIC ACTIVITIES OF FOUR DERIVATIVES OF TRI (SUBSTITUTED) PHENYL IMIDAZOLE İN *Salmonella typhimurium* USING THE AMES ASSAY

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ABSTRACT

In this study, we investigated mutagenic activities of four tri (substituted) phenyl imidazole derivatives, such as 2-(2-hydroxyphenyl)-4,5-bis-(4-methylphenyl) imidazole, 2-(4-nitrophenyl)-4,5-bis-(4-methylphenyl) imidazole, 2-(2-hydroxyphenyl)-4,5-bis-(4-methoxy-phenyl) imidazole , 2-(1,3-benzoxazole-5-yl)-4,5-bis-(4-methylphenyl) imidazole, in Salmonella typhimurium TA98 and TA100, with and without metabolic activation (S9 mix) using plate-incorporation and pre-incubation methods of the Ames assay. The chemical compounds used for the study were applied in doses of 10μg/pl, 100μg/pl, 1000μg/pl, 2500μg/pl. While 2-(2-hydroxyphenyl)-4,5-bis-(4-methylphenyl) imidazole, 2-(4-nitrophenyl)-4,5-bis-(4-methylphenyl) imidazole and 2-(1,3-benzoxazole-5-yl)-4,5-bis-(4-methylphenyl) imidazole were found to have a strong mutagenic effect in dose of 2500μg/pl in strains of TA100 depending upon the dose. Therefore, it could be said that these compounds have a higher potential of causing frame-shift mutations in comparison with point mutation. As for 2-(2-hydroxyphenyl)-4,5-bis-(4-methoxyphenyl) imidazole, a weak mutagenic activity was determined in strains of both TA98 and TA100.

Key Words: Tri (substituted) phenyl imidazole derivatives, Mutagenicity, Ames assay.

DÖRT FARKLI TRİ (SÜBSTİTÜE) FENİL İMİDAZOL TÜREVİNİN MUTAJENİK AKTİVİTESİNİN *Salmonella typhimurium*'DA AMES TEST SİSTEMİ İLE ARAŞTIRILMASI

ÖZ

Bu çalışmada, 2-(2-hidroksifenil)-4,5-bis-(4-metilfenil) imidazol, 2-(4-nitrofenil)-4,5-bis-(4-metilfenil) imidazol, 2-(2-hidroksifenil)-4,5-bis-(4-metoksifenil) imidazol, 2-(1,3-benzoksazol-5-il)-4,5-bis-(4-metil fenil) imidazol olmak üzere dört farklı tri (substitüe) fenil imidazol türevlerinin mutajenik aktiviteleri, Ames testi plak inkorporasyon ve ön inkübasyon yöntemi ile, Salmonella typhimurium TA98 ve TA100 suşları kullanılarak, S9 karışımı varlığ ında ve yokluğ unda araştırıldı. Deneylerde 10μg/pl, 100μg/pl, 1000μg/pl, 2500μg/pl olmak üzere dört farklı doz kullanıldı. 2-(2-hidroksifenil)-4,5-bis-(4-metilfenil) imidazol, 2-(4-nitrofenil)-4,5-bis-(4-metilfenil) imidazol ve 2-(1,3-benzoksazol-5-il)-4,5-bis-(4-metilfenil) imidazol adlı maddelerin 2500μg/pl dozda TA98 suşunda kuvvetli mutajenik etki göstericiken, TA100 suşunda doza bağ lı olarak zayıf mutajenik etki gösterdikleri belirlendi. Dolayısıyla bu maddelerin çerçeve kayması mutasyonu oluşturma potansiyelinin nokta mutasyonu oluşturma potansiyelinden daha fazla olduğ u söylenebilir. 2-(2-hidroksifenil)-4,5-bis-(4-metoksifenil) imidazol adlı maddenin ise hem TA98 hem de TA100 suşunda zayıf mutajenik aktivitesi olduğ u saptandı.

Anahtar Kelimeler: : Tri (substitüte) fenil imidazol türevleri, Mutajenite, Ames test sistemi.

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1. INTRODUCTION

There is an interesting relationship between biological effects, structures and mutagenic activities of chemical compounds. It has recently captured much interest from a practical point of view, such as the synthesis of compounds devoid of mutagenicity, as well as from a theoretical point of view such as the mechanisms of action (Kalopsis, 1991). Studies into structure-activity relationship of various compounds have been carried out from 1980s onwards (Kurt et al., 1983; Chung et al., 2000). Imidazole and its derivatives from an important class of heterocyclic aromatic amines. The most widely prescribed drug for the treatment of gastric ulcers has a generic name, which is cimetidine, and this is one of the synthetic imidazole derivatives (Corey, 1996). Imidazole have various pharmacological effects such as analgesic, antiinflammatory, antiprotozoal, nematosid, tumor inhibitor, sedative, hypolipidemic, and hypocolesterolemic, gastric secretion inhibitor, gastric acid neutralizer and antiallergic activities (Meriç and Işıkdağ , 2000). 1-benzyl-3-cetyl-2-methylimidazolium iodide (NH125), which is a newly-synthesized imidazole derivative, has been reported to suppress various histidine protein kinases and therefore has been regarded as an effective antibacterial agent (Yamamoto et al., 2000). One study has emphasized that the compound of clotrimazol is an antifungal imidazole derivative, and that it affects cellular Ca^{+2} homeostasis, and that has a cytotoxic affect upon acute lymphoblastic leukaemia cells (Ito, 2002).

It is essential that chemical compounds synthesised for various purposes should be tested thoroughly in terms of their mutagenic activities before they are made commercially available. The Ames test has been used extensively for testing the potential of a chemical compound to interact with DNA and thus cause mutation. It is known to be the most commonly used assay for the assessment of mutagenic potential of compounds that human beings are exposed to in their daily lives (Rexroat et al., 1995). In this study, the mutagenic activities of four different tri (substituted) phenyl imidazoles that are particularity synthesized as pre-drugs have been investigated using two different methods of the Ames test, which are plate-incorporation and pre- incubation methods.

2. MATERIAL and METHODS

2.1. Bacterial Stains

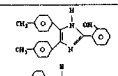
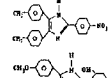
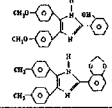
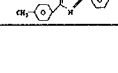
Salmonella thyphimurium his- TA98 and TA 100 were provided by Dr. Bruce Ames (University of California, Berkeley, USA), whose genetical inspection and preservation were conducted as was suggested in the literature (Maron and Ames, 1983).

2.2. Chemicals

The derivatives tested in our study tri (substituted) phenyl imidazole were all provided by Prof. Dr. İlhan IŞIKDAĞ, Prof. Dr. Ümit UÇUCU and Yrd. Doç. Dr. Asiye MERİÇ (University of Anadolu, Faculty of Pharmacy, Eskişehir, TURKEY). Chemical structures and some physicochemical features of tri (substituted) phenyl imidazole are presented in Table 1.

As a positive mutagen sodium azide and 2-aminofluorene, 3-methyl cholanthrene, D-glucose-6-phosphate, β-NADP were purchased from Fluka and glucose and dimethylsulfoxide (DMSO) from Merck and nutrient broth and nutrient agar from Oxoid.

Table 1. Structures and Physicochemicall Properties of Tri (substituted) Phenyl Imidazole Derivatives.

No	Compounds	Structure	Molecular Formula	Molecular Weight (g/mol)	Melting Point (°C)
1	2-(2-hydroxyphenyl)-4,5-bis-(4-methylphenyl) imidazole		$C_{23}H_{20}N_2O$	324,41	170
2	2-(4-nitrophenyl)-4,5-bis-(4-methylphenyl) imidazole		$C_{23}H_{19}N_3O_2$	325,21	161
3	2-(2-hydroxyphenyl)-4,5-bis-(4-methoxyphenyl) imidazole		$C_{23}H_{21}N_2O_3$	323,41	159
4	2-(1,3-benzoxazole-5-yl)-4,5-bis-(4-methylphenyl) imidazole		$C_{28}H_{23}N_3O_2$	321,41	172

2.3. Determination of the Cytotoxic Dose

Tri (substituted) phenyl imidazole derivatives were prepared in different concentrations through being dissolved in DMSO. An overnight bacteria culture of 0,1 ml as well as the above mentioned compound prepared in different concentrations study tri (substituted) phenyl imidazole of 0,1 ml were both added into the top agar of 2 ml. Afterwards, they were poured onto nutrient agar plates and then were incubated at 37 °C for 24 hours. The doses in which no growth of bacteria could be observed were accepted as cytotoxic doses. Therefore, doses below cytotoxic doses (2500μg/pl, 1000μg/pl, 100μg/pl, 10μg/pl) were the ones used for the study (Dean et al., 1985).

2.4. Preparation of S9 Mix

S9 mix was prepared for the purpose of being used in the phase requiring metabolic activation of plate incorporation and pre-incubation methods. Male Spraque-Dawley albinos were induced with a single intraperitonal dose of 3-methylcholanthrene (125 mg/kg) dissolved in corn oil and sacrificed on the 5th day following the administration. Their livers were immediately excised and postmitochondrial fraction (S9 mix) was

prepared (Maron and Ames, 1983). The components of the standart S9 mix are 8mM $MgCl_2$, 33mM KCl, 5mM glucose—6-phosphate, 4mM NADP, 100mM sodiumphosphate (pH 7,4). S9 was added into the mix in such a way that 0,004 ml of S9 could be present in per 1 ml of the S9 mix. The mix was prepared fresh for each mutagenicity assay and were kept on ice for a while in order for it not to lose its activity, as was suggested in the literature (Maron and Ames, 1983).

2.5 Plate-incorporation Test

Histidine-biotine of 0.2 ml, a fresh overnight culture of the tester strain of 0.1 ml, the test chemical of 0.1 ml and S9 mix of 0.5 ml were all added into the top agar. The mix obtained was then poured out onto a minimal glucose agar plate. The compound was tested with and without S9 mix and positive control plates were included in each assay. Revertant colonies were counted after each one of them had been incubated at 37 °C for 48 hours, as suggested in the literature (Maron and Ames, 1983).

2.6. Pre-incubation Test

The most widely used test modification of the plate-incorporation test is the pre-incubation assay. First, the test chemical of 0.1 ml and a fresh overnight culture of the tester strain were incubated at 37 °C for 30 minutes. Then, these were added into the top agar. Next, histidine-biotine of 0.2 ml was added into the mix. Finally, it was poured out onto a minimal glucose agar. The revertant colonies were counted following the incubation provided at 37 °C for 48 hours (Maron and Ames, 1983).

2.7. Statistical Analysis

All statistic analyses were performed using One-way ANOVA, Dunnett-test. p value less than 0.005 was considered statistically significant. Each experiment was carried out twice, with three plates being used for each case.

3. RESULTS and DISCUSSION

Considering the structures of the test chemicals, the mutagenic activities of which were aimed to be determined, it should be noted that three phenyl chains are attached to imidazol, and that there are also methyl ($-CH_3$), methoxy ($-OCH_3$), nitro (NO_2), hydroxyl (-OH) and benzoxazole groups that are annexed to these three phenyl chains. One study in the literature that investigated some chemical compounds that bear a remarkable resemblance to the chemical investigated in our study has reported that methyl and methoxy activate the aromatic chain in orto and para positions. They have also reported that methyl and methoxy interact with DNA, depending on the hydrophobic characteristic of methyl and due to the negative charge induced by unpaired electron pairs on oxygen as far as methoxy is concerned. In addition, phenyl has been reported to have an impact on macromolecules by means of electrophylic aromatic substitution reactions (Mercangöz and Tüylü, 2000).

The number of revertant colonies of Salmonella typhimurium TA98 and TA100 strains obtained as a result of the test chemicals are presented in Table 2. When the plate- incorporation method was applied, the first and the third compounds did not show mutagenic impacts of any doses on the strains of either TA98 or TA100 in the absence of S9 mix. However, the first compound showed mutagenic impact of doses of 1000 µg/pl and 2500µg/pl on the strains of TA98 in the presence of S9 mix, while the third compound showed doses of 100µg/pl, 1000µg/pl and 2500µg/pl on the strains of TA98 in the presence of S9 mix. The first compound showed mutagenic impact of doses of 100µg/pl, 1000 µg/pl and 2500µg/pl on the strains of TA100 in the presence of S9 mix, while the third compound showed doses of only 2500µg/pl on the strains of TA100 in the presence of S9 mix.

As for the pre-incubation method, in which S9 mix was never excluded, various doses of mutagenic impact were observed for both the first and the third compounds. The first compound showed mutagenic impacts of 100µg/pl, 1000µg/pl and 2500µg/pl on the strains of TA98, while the third compound showed mutagenic impacts of only 2500µg/pl the strains of TA98. The first compound showed mutagenic impacts of 100µg/pl, 1000µg/pl and 2500µg/pl on the strains of TA100, while the third compound showed mutagenic impacts of only 1000µg/pl and 2500µg/pl the strains of TA100. A similar study pointed out that the mutagenic activity of 2-amino-1-methyl-phenylimidazo 4,5-8 pyridine (PHIP) increased following the addition of S9 mix (Bounaratti and Felton, 1990). Therefore, we suggest that mutagenic effects of some chemicals might increase in the case of a metabolic activation. Another study employing the Ames test reported that —OH group present in benzo[c]-phenanthrene diol epoxite plays a role in the emergence of its mutagenecity depending on configuration (Ponten et al., 2000). Therefore, we conclude that —OH group, annexed to the third phenyl chain at the second carbon, could have an impact upon mutagenicity in the first and the third compounds.

Considering the numbers of revertant colonies of the second compound, when the plate incorporation method was employed, there was a mutagenic impact of 1000μg/pl and 2500μg/pl on the strains of TA98 both in the absence and presence of S9 mix. When the plate incorporation method was employed for the strains of TA100, there was a mutagenic impact on all the doses in the absence of S9 mix, but there was a mutagenic impact only in doses of 1000μg/pl and 2500μg/pl in the presence of S9 mix. As for the pre-incubation method, in which S9 mix was never excluded, there was a mutagenic impact only in the doses of 1000μg/pl and 2500μg/pl. As for the strains of TA100, there was a mutagenic impact only in the dose 2500μg/pl. A study into nitrophenanthrenes emphasized that nitro groups have an effect on mutagenic activities depending on the number of groups as well as on the positions of attachment (Sera et al., 1996). Therefore, we can suggest that $-NO_2$ group, annexed to the third phenyl chain at the fourth carbon, could have an impact upon mutagenicity.

Considering the numbers of revertant colonies of the fourth compound, when the plate incorporation method was employed, there was a mutagenic impact of 1000μg/pl and 2500μg/pl in the strains of TA98 and TA100 both in the absence and presence of S9 mix. When the pre-incubation method was employed, S9 mix was never excluded. There was a mutagenic impact only in doses of 2500μg/pl in TA98. As for the strains of TA100, there was a mutagenic impact in the doses of 100μg/pl, 1000μg/pl and 2500μg/pl. It can be seen that benzoxazole is annexed to the third phenyl chain in the fourth compound. One of the components including the benzoxazol group, 3-[2-(benzoxazol-2-yl) ethyl]-5-ethyl-6-methyl-pyridine-2(1H)-one has been reported to have an anti-viral activity in cell culture and has also been reported to be a specific inhibitor of human immunodeficiency virus type I (HIV-I) reverse transcriptase (RI) (Prueksaritanont et al., 1994). While there are studies into biological activities of benzoxazol, we could not encounter any study into the potential of mutagenic impact in the literature. Therefore, we hope our study will shed light on this obscure aspect.

We did not determine any mutagenic activities of DMSO, which is used as the dissolver of the chemicals tested.

Table 2. Results of Plate-incorporation and Pre-incubation Tests.

No	Compounds	Dose μg/pl	Plate-incorporation method				Pre-incubation method	
			TA 98		TA100		TA 98	TA100
			(-S9 mix)	(+S9 mix)	(-S9 mix)	(+S9 mix)	(+S9 mix)	
1	2-(2-hydroxyphenyl) 4,5- bis- (4-methylphenyl) imidazole	10	27±4	46±7	84±8	120±11	48±3	120±6
		100	28±3	48±8	84±10	161±10*	59±5*	161±7*
		1000	31±6	62±7*	90±8	154±9*	74±9*	154±8*
		2500	34±8	79±8*	100±7	157±9*	84±7*	157±9*
2	2-(4-nitrophenyl) 4,5-bis- (4-methylphenyl) imidazole	10	30±6	40±1	117±6*	110±1	44±8	117±10
		100	42±4	41±6	138±7*	121±3	46±5	123±8
		1000	53±3*	69±8*	147±4*	147±7*	64±6*	124±7
		2500	60±5*	86±9*	148±7*	158±10*	91±8*	173±10*
3	2-(2-hydroxyphenyl) 4,5- bis- (4-methoxyphenyl) imidazole	10	30±8	50±5	100±2	106±9	37±8	106±8
		100	32±6	52±3*	102±5	103±9	46±7	121±8
		1000	33±9	51±6*	101±7	120±8	46±8	174±8*
		2500	38±11	66±11*	103±9	164±9*	63±9*	186±9*
4	2- (1,3-bezoxazole-5-yl) 4,5- bis- (4-methylphenyl) imidazole	10	40±5	45±6	105±5	107±6	35±9	109±6
		100	41±4	50±8	108±6	121±9	35±8	154±5*
		1000	46±8*	72±8*	120±8*	175±9*	42±9	176±9*
		2500	50±8*	83±9*	173±10*	190±9*	69±9*	194±9*
	Sodium azide (NA)	1,5	-	-	988±20	-	-	-
	2-aminofluorene (2AF)	10	-	965±11	-	978±12	970±20	987±11
	DMSO		30±9	45±7	91±8	102±5	45±2	105±6
	Spontaneous revertant colony numbers		30±4	38±6	95±8	110±8	38±6	110±8

Results are presented as mean ± S.D.

* $p < 0,005$

Revertant colony numbers were evaluated with one way ANOVA, Dunnett-t test and then the values found statistically significant were marked in table 1. However, according to the procedure of the Ames test, values reaching a level twice as high as or higher than spontaneous revertant colony numbers are accepted to be mutagenic (Maron and Ames, 1983). For this reason, although they were found to be statistically significant, chemical compounds whose colony numbers were not twice as big as spontaneous revertant colony numbers were accepted as weak mutagenic. In this respect, values reaching a level twice as high as or higher than spontaneous revertant colony numbers were obtained for the strains of TA98. Therefore, we can suggest that the tested compounds were more effective with the strains of TA98 in comparison with TA100. In other words, when compared to point mutations, these compounds were determined to have a higher potentiality of causing frame-shift mutations.

Our study results seem to be in agreement with those of one study which reports that various derivatives of the compounds discussed in our study have a more effective mutagenic activity with the strains of TA98 in comparison with the strains of TA100 (Mercangöz and Tüylü, 2000). On the other hand, we determined some differences between the doses in which mutagenic impacts were present depending on the presence and absence of S9 mix. This study has reported that the difference between the groups annexed to diphenyl compounds attached to imidazol chain at the second carbon may cause different biological activities (Mercangöz and Tüylü, 2000). We believe that the differences between the study of Mercangöz and Tüylü and our study could have come from the fact that the groups attached to phenyl chain were different.

In conclusion, we suggest that groups constituting chemical compounds could have a vital role in the mutagenic impacts of chemical compounds. Our studies will continue in order to bring more light to the relationship between the newly-synthesized chemicals and mutagenic activities.

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