Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2014; 39(3):328–335
doi: 10.5505/tjb.2014.25238

Research Article [Araştırma Makalesi]

Yayın tarihi 12 Kasım 2014 © TurkJBiochem.com
[Published online November 12, 2014]

Genotoxicity of potent antiviral 1-[(2-aminophenyl)thio]-1-phenyl-2-nitrobutane derivatives designed as drug agents

[İlaç etken maddesi olarak tasarlanmış kuvvetli antiviral 1-[(2-aminofenil)tiyo]-1-fenil-2-nitrobütan türevlerinin genotoksisitesi]

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Registered: 30 May 2013; Accepted: 15 January 2014
[Kayıt Tarihi: 30 Mayıs 2013; Kabul Tarihi: 15 Ocak 2014]

ABSTRACT

Objective: Genotoxic potentials of six selected nitrobutane (I) derivatives designed as drug agents were tested here for the first time using umu-microplate test system. An important principle in drug development is to perform safety tests of previously determined significant drug activity in in vitro assays. This may be even more crucial than its efficiency in terms of experimental conditions, since it is important in chemotherapy to treat without risk for the patient.

Methods: Umu-microplate test system is especially designed for detecting the mutagenicity of nitro compounds. 1-[(2-aminophenyl)thio]-1-phenyl-2-nitrobutane (I) derivatives involve nitro groups. Therefore umu-microplate test system has been chosen for our analysis. Evaluation of the SOS inducing activity of the tested compounds was examined with the umu- microplate test system using *Salmonella typhimurium* NM1011 (overexpressed NR (nitroreductase)) and *S.typhimurium* NM2009 (overexpressed O-At (O-acethyltransferase))strains which are sensitive to nitro compounds. Chlorophenol red-β-D-galactopyranoside (CPRG) and O-nitrophenyl-β-D- galactopyranoside (ONPG) were used as substrate in the enzyme assays and also the well-known genotoxic nitro compound, 4-nitroquinoline 1-oxide (4NQO), was the positive control in the test.

Results: Although the β-galactosidase activities with using CPRG were three fold higher than ONPG, parallel results were obtained for both substrates and strains with all compounds tested. For all compounds, the induction of *umu*C gene expression was found to be almost the same for the strains that overexpress NR and O-At. The derivatives tested didn't caused an evident induction in both strains overexpressed NR and O-At enzymes which have a role in metabolic activation mechanism of nitro compunds.

Conclusion: Our study showed that, 1-[(2-aminophenyl)thio]-1-phenyl-2-nitrobutane derivatives have no genotoxic effects in this test system. This result is a very important data making them a potential drug candidate.

Key Words: Nitro compounds, umu-microplate, genotoxicity, drug

Conflict of Interest: Authors have no conflict of interest.

ÖZET

Amaç: Bu çalışmada, ilaç etken maddesi olarak tasarlanmış altı adet nitrobütan türevinin genotoksik potansiyelleri, umu-mikroplak test sistemi ile değerlendirilmiştir. İn vitro testlerde anlamlı bir ilaç aktivitesinin saptanması durumunda, güvenlik testlerinin uygulanması, ilaç geliştirmede çok önemli bir ilkedir. Kemoterapide hastayı risk oluşturmadan tedavi etmek esas olduğundan, güvenlik testleri ilacın deneysel koşullardaki etkinliğinden bile daha fazla önem taşıyabilir.

Metod: Umu-mikroplak test sistemi özel olarak nitrolu bileşiklerin mutajenitesini saptamak için tasarlanmıştır. 1-[(2-aminofenil)tiyo]-1-fenil-2-nitrobütan (I) türevleri de nitro grubu içermektedir, bu nedenle çalışmamızda bu test sistemi seçilmiştir. SOS indükleme aktivitelerinin değerlendirildiği umu-mikroplak test sisteminde, nitrolu bileşiklere hassas olarak geliştirilmiş, NR (nitroredüktaz) enzimini normalden fazla ifade eden *Salmonella typhimurium* NM1011 ve O-At(O-asetiltransferaz) enzimini normalden fazla ifade eden *S. typhimurium* NM2009 suşları kullanılmıştır. Enzim assay ortamında klorofenol-red-β-D-galaktopiranosid (CPRG) ve o-nitrofenil-β-D-galaktopiranosid (ONPG) substrat olarak ve genotoksik ajan olduğu bilinen 4-nitroquinoline 1-oxide (4NQO) de pozitif kontrol olarak kullanılmıştır.

Bulgular: CPRG ile elde edilen β-galaktosidaz (β-gal) aktiviteleri ONPG ile elde edilenlerden 3 kat daha fazla olmakla birlikte, her iki substrat ve her iki suş için test edilen tüm bileşiklerle paralel sonuçlar elde edilmiştir. Tüm bileşikler için, NR ve O-At enzimlerini normalden fazla ifade eden iki ayrı suşta, umuC gen ifadesinin indüksiyonu hemen hemen aynı bulunmuştur. Test edilen bileşikler, nitrolu bileşiklerin metabolik aktivasyon mekanizmasında görevli NR ve O-At enzimlerini normalden fazla ifade eden bu iki suşta belirgin bir indüksiyona neden olmamıştır.

Sonuç: 1-[(2-aminofenil)tiyo]-1-fenil-2-nitrobütan türevlerinin hiçbiri umu-mikroplak test sisteminde genotoksik etki göstermemiştir. Bu sonuç, ilaç adayı olarak değerlendirilmelerinde çok önemli bir veridir.

Anahtar Kelimeler: Nitrolu bileşikler, umu-mikroplak, genotoksisite, ilaç

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

An important principle in drug development is to perform safety tests of previously determined significant drug activity in in vitro assays [1]. In therapeutics research, the nitro compounds are part of an important group of drugs with multiple pharmacological activities. However, in drug design, the inclusion of a nitro group in a molecule changes the physico-chemical and electronic properties and is associated with increased mutagenicity and carcinogenicity [2-5].

1-[(2-aminophenyl)thio]-1-phenyl-2 nitrobutane (I) derivatives (Figure 1) involve nitro groups [6], for this reason, we evaluated mutagenicity of this drug agents by employing umu-microplate test system which provides sensitive test for detecting mutagenicity of nitro compounds [7,8]. Syntheses and detailed structural analyses of 1-[(2-aminophenyl) thio]-1-phenyl-2-nitrobutane (I) derivatives were reported in our previous studies. Compound (I) derivatives are the Michael type addition products and they have been synthesized by the addition of 2-aminothiophenol to the active double bond of β-Ethyl-β-nitrostyrenes. Synthesized nitrobutane derivatives (I) exhibited potent antiviral activity. According to antiviral activity tests results, the R groups have major contribution to antiviral activity [6]. The highest antiviral activity was found in derivative Ib and Ic which contain bromine and chlorine at C4 of the aromatic ring (para position), respectively. The bromine atom attracts electrons from the ring. Similarly the antiviral activity of the derivative Ic was in second order. The results have shown that electron withdrawing groups from the ring are more active than the nonsubstituted derivative (Ia). Contrary to the Ib and the Ic derivatives, the Id and Ie derivatives carrying bulky groups at C4 of the aromatic ring were 10 times less active. Although no electron withdrawal effect was present, the activity of If was much higher than the nonsubstituted ring derivative Ia, since the methyl group does not exert a negative steric effects such as the Id and Ie derivatives and in addition has a positive protective effect on the aromatic ring. Antiviral activities of all nitrobutane (I) derivatives were found to be higher than the reference drug Acyclovir [6].

Many short-term genotoxicity tests are employed quickly and inexpensively for predicting the carcinogenicity and mutagenicity of industrial chemicals, drugs, environmental samples [9,10]. The umu test is a widely known assay system used for the detection of genotoxicity of compounds and it is based on the induction of SOS response against DNA damaging agents. Therefore, cross-linking double-strand breaks and intercalation, all yield positive responses within a relatively shorter experimental time with conventional apparatus. Tester strains posses a plasmid carrying the fused gene *umuC'–'lacZ* and enable the monitoring of the expression levels of the *umu* operon by measuring the activity of β-gal produced in the cells

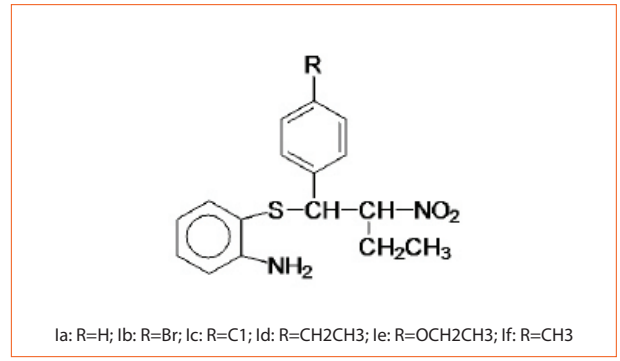

Figure 1. Molecular structure of potent antiviral nitrobutane derivatives.

by the fusion gene. Umu test involves specifically developed strains for detection of mutagenicity of nitroarene and aromatic amines [7,11]. Nitro compounds usually do not require exogenous metabolic activation and it appears that they are reduced to the hydroxylamine by cytosolic reductases. In the activation of nitroarenes, the resulting N-hydroxylamines are then esterified by *O*- sulfonylation, *N,O*-transacetylation or *O*-acetylation [12,13].

Materials and Methods

Bacterial strains

S. typhimurium NM1011 that overexpresses NR and *S. typhimurium* NM2009 that overexpresses O-AT strains were kindly provided by Dr. Y. Oda. The tester strains were constructed by introducing a vector plasmid pACY184 carrying only the NR gene (NM1011) or only the O-AT gene (NM2009) into the parent strain *S. typhimurium* TA1535/pSK1002 harboring the *umuC'–'lacZ* fusion gene [14].

umu-microplate test

The umu-microplate test was performed as recommended by Oda et al. (2004). NM1011 and NM2009 were grown in Luria-Bertani (LB) broth containing chloramphenicol (5 μg/mL) and ampicillin (25 μg/mL) at 37°C for about 16 h with vigorous shaking. The overnight cultures were then diluted 100-fold with TGA medium (1% Bacto tryptone, 0.5% NaCl and 0.2% glucose) containing ampicillin (20 μg/mL), and cultured until the absorbance at 600 nm reached about 0.25-0.3. The test chemicals were dissolved in DMSO and diluted. Each concentration (4 μL) was transferred to the well of a microplate. An exponentially growing culture (96 μL) of the appropriate tester strains were then added to each well. The contents of the wells were mixed for a few minutes using shaker. After incubation at 37°C for 2 h in an incubator-shaker, bacterial growth was measured as turbidity at 595 nm, with a microplate reader. Subsequently, 10 mL of the culture was transferred to a new microplate; 90 μL aliquots of Z-buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.01 M $MgSO_4.7H_2O$ and 0.05 M β-mercaptoethanol) and 50 μL 0.1% sodium dodecyl sulfate (SDS) were added to the wells. A further 10 μL of

Table 1. The induction of the *umu*C gene in S. *typhimurium* NM1011 and NM2009 strains with CPRG substrate in the umu test

		NM1011			NM2009		
Chemical	Conc (μM)	Cell growth (A_{595})	CPRG[c] (A_{570})	RGA[b]±SD A_{570}/A_{595}	Cell growth (A_{595})	CPRG[c] (A_{570})	RGA[b]±SD A_{570}/A_{595}
4NQO	0	0.253	0.677	2.631±0.02	0.233	0.813	3.304±0.08
	1.25	0.210	0.895	4.261±0.14	0.200	1.089	5.445±0.26
	2.5	0.200	0.947	4.745±0.41	0.201	1.565	7.804±0.32
	5	0.194	1.160	5.993±0.40	0.206	1.919	9.336±0.78
	10	0.202	1.453	7.209±0.67	0.200	2.271	11.439±0.54
Ia	0	0.258	0.713	2.763±0.19	0.249	0.736	2.955±0.44
	10^{-7}	0.288	0.736	2.561±0.44	0.286	0.718	2.515±0.38
	10^{-6}	0.267	0.683	2.560±0.83	0.268	0.716	2.672±0.15
	10^{-5}	0.266	0.721	2.713±0.42	0.267	0.733	2.743±0.11
	10^{-4}	0.273	0.675	2.470±0.31	0.265	0.720	2.721±0.19
	10^{-3}	0.272	0.632	2.323±0.44	0.271	0.692	2.552±0.21
	10^{-2}	0.270	0.700	2.596±0.47	0.275	0.696	2.532±0.12
	10^{-1}	0.266	0.628	2.363±0.29	0.270	0.634	2.348±0.40
	1	0.291	0.668	2.293±0.29	0.282	0.726	2.578±0.13
	10	0.264	0.704	2.669±0.36	0.277	0.706	2.549±0.09
	100	0.289	0.698	2.420±0.36	0.283	0.683	2.411±0.25
	1000	0.274	0.694	2.533±0.51	0.275	0.642	2.332±0.12
Ib	0	0.252	0.537	2.131±0.78	0.247	0.526	2.129±0.94
	5.10^{-6}	0.286	0.582	2.035±0.71	0.286	0.500	1.749±0.62
	5.10^{-5}	0.270	0.603	2.236±0.62	0.263	0.541	2.057±1.01
	5.10^{-4}	0.272	0.523	1.924±0.47	0.259	0.488	1.882±0.51
	5.10^{-3}	0.271	0.579	2.135±0.59	0.265	0.562	2.125±0.79
	5.10^{-2}	0.268	0.582	2.169±0.71	0.259	0.543	2.095±0.71
	5.10^{-1}	0.258	0.545	2.114±0.66	0.257	0.500	1.948±0.58
	5	0.259	0.576	2.226±0.62	0.261	0.494	1.895±0.53
	50	0.267	0.556	2.078±0.62	0.272	0.521	1.920±0.63
	500	0.259	0.546	2.113±0.54	0.252	0.488	1.936±0.60
	2500	0.261	0.545	2.090±0.29	0.251	0.537	2.141±0.19
	5000	0.242	0.466	1.922±0.47	0.230	0.385	1.674±0.41
	7500	0.238	0.391	1.639±0.30	0.244	0.381	1.557±0.12
	10 000	0.294	0.382	1.299±0.22	0.282	0.463	1.643±0.27
Ic	0	0.249	0.476	1.911±0.44	0.292	0.471	1.617±0.78
	10^{-8}	0.264	0.528	2.001±0.61	0.296	0.526	1.776±1.08
	10^{-7}	0.270	0.509	1.885±0.64	0.275	0.532	1.937±0.94
	10^{-6}	0.260	0.481	1.851±0.24	0.297	0.486	1.637±0.85
	10^{-5}	0.263	0.474	1.807±0.41	0.293	0.505	1.722±0.83
	10^{-4}	0.270	0.460	1.707±0.62	0.299	0.502	1.677±0.92
	10^{-3}	0.279	0.476	1.708±0.41	0.299	0.496	1.657±0.88
	10^{-2}	0.277	0.455	1.641±0.45	0.297	0.494	1.667±0.97
	10^{-1}	0.267	0.445	1.664±0.35	0.299	0.469	1.571±0.83
	1	0.268	0.469	1.749±0.27	0.301	0.455	1.514±0.70
	10	0.278	0.477	1.715±0.51	0.299	0.466	1.559±0.82
	100	0.276	0.448	1.625±0.52	0.294	0.432	1.471±0.67
	1000	0.273	0.487	1.784±0.33	0.299	0.451	1.509±0.67

Table 1. (continued) The induction of the *umu*C gene in S. *typhimurium* NM1011 and NM2009 strains with CPRG substrate in the umu test

Chemical	Conc (μM)	NM1011 Cell growth (A_{595})	NM1011 CPRG[c] (A_{570})	NM1011 RGA[b]±SD A_{570}/A_{595}	NM2009 Cell growth (A_{595})	NM2009 CPRG[c] (A_{570})	NM2009 RGA[b]±SD A_{570}/A_{595}
Id	0	0.257	0.381	1.484±0.48	0.262	0.363	1.383±0.47
	10^{-3}	0.266	0.281	1.057±0.36	0.244	0.250	1.025±0.36
	10^{-2}	0.255	0.287	1.125±0.39	0.245	0.261	1.063±0.36
	10^{-1}	0.264	0.310	1.173±0.51	0.246	0.274	1.111±0.47
	1	0.258	0.284	1.101±0.32	0.250	0.267	1.068±0.36
	10	0.262	0.277	1.057±0.52	0.245	0.253	1.034±0.35
	100	0.256	0.288	1.121±0.29	0.245	0.255	1.043±0.32
	1000	0.258	0.294	1.138±0.29	0.240	0.294	1.226±0.47
	2500	0.265	0.296	1.117±0.21	0.230	0.365	1.590±0.20
	5000	0.250	0.237	0.948±0.19	0.226	0.244	1.075±0.42
	7500	0.234	0.250	1.068±0.30	0.205	0.318	1.555±0.33
	10 000	0.227	0.276	1.213±0.37	0.217	0.282	1.300±0.49
	12 500	0.220	0.303	1.380±0.15	0.194	0.372	1.920±0.28
	15 000	0.207	0.320	1.545±0.23	0.198	0.359	1.814±0.63
	17 500	0.189	0.393	2.076±0.24	0.170	0.460	2.510±0.70
	20 000	0.176	0.394	2.243±0.57	0.165	0.381	2.312±1.07
Ie	0	0.245	0.376	1.533±0.46	0.260	0.425	1.633±0.30
	10^{-2}	0.243	0.257	1.057±0.36	0.219	0.265	1.213±0.34
	10^{-1}	0.239	0.249	1.042±0.26	0.209	0.328	1.567±0.30
	1	0.245	0.264	1.080±0.35	0.202	0.286	1.418±0.35
	5	0.263	0.366	1.390±0.11	0.223	0.395	1.771±0.31
	10	0.240	0.250	1.043±0.25	0.202	0.257	1.268±0.24
	50	0.270	0.338	1.252±0.21	0.237	0.385	1.624±0.36
	100	0.252	0.287	1.140±0.30	0.212	0.335	1.583±0.32
	250	0.265	0.330	1.243±0.21	0.217	0.409	1.887±0.30
	500	0.247	0.307	1.241±0.22	0.227	0.366	1.612±0.31
	1000	0.230	0.308	1.339±0.30	0.211	0.345	1.637±0.37
	2500	0.223	0.300	1.346±0.46	0.217	0.334	1.535±0.42
	5000	0.224	0.313	1.401±0.49	0.222	0.354	1.591±0.42
	7500	0.199	0.329	1.651±0.44	0.170	0.374	2.200±0.34
	10 000	0.204	0.322	1.582±0.49	0.181	0.357	1.972±0.87
If	0	0.209	0.400	1.913±0.26	0.163	0.398	2.440±0.33
	10^{-3}	0.213	0.380	1.787±0.25	0.163	0.370	2.265±0.58
	10^{-2}	0.222	0.351	1.584±0.27	0.160	0.327	2.039±0.35
	10^{-1}	0.221	0.354	1.601±0.33	0.165	0.339	2.059±0.44
	1	0.215	0.354	1.644±0.22	0.163	0.353	2.161±0.46
	10	0.215	0.373	1.734±0.12	0.166	0.363	2.182±0.17
	100	0.218	0.329	1.507±0.18	0.165	0.344	2.087±0.31
	1000	0.222	0.355	1.596±0.04	0.168	0.339	2.020±0.33
	2500	0.214	0.365	1.709±0.35	0.172	0.330	1.920±0.16
	5000	0.218	0.351	1.610±0.00	0.207	0.359	1.731±0.37
	10 000	0.258	0.403	1.563±0.11	0.222	0.367	1.653±0.38
	15 000	0.246	0.489	1.987±0.36	0.208	0.419	2.018±0.34

[a]Each value is the mean ± S.D. of four replicates from each of four separate experiments. 4NQO was used as a positive control; [b]RGA, relative β-galactosidase activity. RGA ≥ 2-fold over the control levels is considered positive (Oda et al., 2004); [c]CPRG and ONPG are used as substrates; New synthesized 1-[(2-aminophenyl)thio]-1-phenyl-2 nitrobutane (I) derivatives contain H, Br, Cl, ethyl, ethoxy, methyl groups are Ia, Ib, Ic, Id, Ie and If , respectively.

Table 2. The induction of the *umu*C gene in S. *typhimurium* NM1011 and NM2009 strains with ONPG substrate in the umu test

		NM1011			NM2009		
Chemical	Conc (μM)	Cell growth (A_{595})	ONPG[c] (A_{415})	RGA[b]±SD A_{415}/ A_{595}	Cell growth (A_{595})	ONPG$_c$ (A_{415})	RGA[b]±SD A_{415}/ A_{595}
4NQO	0	0.262	0.260	0.992±0.01	0.229	0.285	1.245±0.08
	1.25	0.209	0.304	1.456±0.23	0.190	0.413	2.177±0.16
	2.5	0.202	0.374	1.854±0.13	0.203	0.511	2.521±0.25
	5	0.210	0.436	2.079±0.14	0.199	0.624	3.141±0.21
	10	0.213	0.510	2.398±0.24	0.212	0.785	3.709±0.40
Ia	0	0.267	0.242	0.904±0.09	0.249	0.244	0.983±0.27
	10^{-7}	0.277	0.271	0.979±0.18	0.262	0.263	1.003±0.44
	10^{-6}	0.277	0.229	0.827±0.19	0.255	0.241	0.944±0.37
	10^{-5}	0.264	0.233	0.884±0.14	0.256	0.231	0.904±0.36
	10^{-4}	0.259	0.218	0.840±0.13	0.249	0.228	0.916±0.37
	10^{-3}	0.271	0.206	0.759±0.24	0.258	0.214	0.830±0.37
	10^{-2}	0.272	0.215	0.790±0.17	0.272	0.226	0.831±0.34
	10^{-1}	0.262	0.220	0.838±0.18	0.264	0.229	0.866±0.37
	1	0.274	0.219	0.799±0.18	0.263	0.222	0.844±0.34
	10	0.270	0.213	0.789±0.18	0.266	0.250	0.942±0.36
	100	0.273	0.214	0.783±0.17	0.256	0.223	0.869±0.37
	1000	0.266	0.237	0.891±0.18	0.278	0.228	0.821±0.33
Ib	0	0.253	0.184	0.729±0.23	0.258	0.181	0.703±0.11
	5.10^{-6}	0.271	0.172	0.635±0.16	0.276	0.187	0.677±0.04
	5.10^{-5}	0.263	0.184	0.700±0.19	0.268	0.184	0.686±0.14
	5.10^{-4}	0.267	0.182	0.682±0.12	0.263	0.211	0.803±0.17
	5.10^{-3}	0.248	0.181	0.728±0.17	0.260	0.197	0.758±0.14
	5.10^{-2}	0.257	0.168	0.654±0.12	0.257	0.176	0.686±0.09
	5.10^{-1}	0.240	0.175	0.730±0.14	0.260	0.187	0.720±0.16
	5	0.247	0.161	0.653±0.15	0.261	0.177	0.677±0.11
	50	0.256	0.170	0.665±0.15	0.263	0.176	0.668±0.14
	500	0.238	0.166	0.698±0.13	0.257	0.165	0.640±0.15
	2500	0.252	0.158	0.625±0.05	0.255	0.146	0.571±0.06
	5000	0.219	0.147	0.668±0.14	0.227	0.138	0.608±0.10
	7500	0.235	0.114	0.484±0.03	0.246	0.115	0.470±0.06
	10 000	0.278	0.118	0.424±0.18	0.252	0.119	0.472±0.07
Ic	0	0.257	0.131	0.511±0.01	0.277	0.132	0.476±0.14
	10^{-8}	0.263	0.150	0.573±0.03	0.293	0.144	0.490±0.05
	10^{-7}	0.288	0.133	0.464±0.04	0.298	0.133	0.445±0.07
	10^{-6}	0.264	0.129	0.489±0.04	0.299	0.128	0.427±0.03
	10^{-5}	0.277	0.122	0.442±0.09	0.298	0.127	0.428±0.03
	10^{-4}	0.267	0.126	0.473±0.03	0.314	0.133	0.423±0.04
	10^{-3}	0.275	0.123	0.447±0.03	0.304	0.138	0.454±0.05
	10^{-2}	0.273	0.126	0.463±0.03	0.299	0.136	0.456±0.11
	10^{-1}	0.268	0.127	0.476±0.00	0.314	0.145	0.461±0.04
	1	0.269	0.125	0.467±0.01	0.303	0.137	0.452±0.03
	10	0.281	0.128	0.457±0.11	0.307	0.139	0.454±0.03
	100	0.262	0.128	0.489±0.05	0.303	0.138	0.455±0.01
	1000	0.276	0.128	0.464±0.11	0.300	0.133	0.444±0.07

Table 2. (continued) The induction of the *umu*C gene in S. *typhimurium* NM1011 and NM2009 strains with ONPG substrate in the umu test

		NM1011			NM2009		
Chemical	Conc (μM)	Cell growth (A_{595})	ONPG[c] (A_{415})	RGA[b]±SD A_{415}/A_{595}	Cell growth (A_{595})	ONPG$_c$ (A_{415})	RGA[b]±SD A_{415}/A_{595}
Id	0	0.261	0.104	0.399±0.09	0.235	0.098	0.418±0.10
	10^{-3}	0.272	0.098	0.361±0.11	0.243	0.094	0.388±0.13
	10^{-2}	0.269	0.095	0.352±0.11	0.257	0.085	0.332±0.13
	10^{-1}	0.275	0.093	0.339±0.10	0.255	0.087	0.342±0.10
	1	0.269	0.095	0.354±0.09	0.257	0.091	0.354±0.12
	10	0.272	0.095	0.351±0.10	0.254	0.090	0.353±0.16
	100	0.261	0.091	0.348±0.11	0.262	0.091	0.350±0.10
	1000	0.259	0.089	0.342±0.09	0.248	0.091	0.367±0.12
	2500	0.254	0.068	0.269±0.03	0.215	0.084	0.393±0.07
	5000	0.252	0.083	0.328±0.08	0.234	0.079	0.340±0.07
	7500	0.238	0.073	0.309±0.05	0.194	0.089	0.459±0.02
	10 000	0.233	0.091	0.391±0.15	0.214	0.093	0.434±0.13
	12 500	0.226	0.075	0.333±0.02	0.186	0.088	0.472±0.04
	15 000	0.213	0.100	0.468±0.12	0.202	0.099	0.489±0.07
	17 500	0.193	0.089	0.461±0.01	0.167	0.106	0.633±0.05
	20 000	0.190	0.111	0.586±0.17	0.168	0.111	0.661±0.22
Ie	0	0.233	0.103	0.445±0.05	0.236	0.127	0.540±0.12
	10^{-2}	0.231	0.085	0.365±0.03	0.219	0.090	0.410±0.17
	10^{-1}	0.232	0.085	0.364±0.02	0.216	0.094	0.436±0.13
	1	0.235	0.089	0.380±0.02	0.204	0.092	0.451±0.20
	5	0.280	0.079	0.284±0.02	0.230	0.087	0.378±0.03
	10	0.225	0.083	0.370±0.01	0.204	0.091	0.446±0.18
	50	0.280	0.082	0.293±0.03	0.225	0.090	0.399±0.04
	100	0.251	0.079	0.315±0.06	0.228	0.085	0.371±0.05
	250	0.271	0.074	0.273±0.01	0.226	0.085	0.377±0.05
	500	0.277	0.071	0.256±0.03	0.220	0.094	0.429±0.09
	1000	0.238	0.085	0.356±0.09	0.213	0.097	0.455±0.04
	2500	0.217	0.093	0.429±0.05	0.209	0.098	0.470±0.04
	5000	0.219	0.090	0.413±0.09	0.214	0.104	0.486±0.05
	7500	0.235	0.077	0.327±0.04	0.191	0.092	0.480±0.04
	10 000	0.196	0.101	0.519±0.11	0.200	0.111	0.554±0.14
If	0	0.231	0.140	0.604±0.09	0.171	0.115	0.672±0.01
	10^{-3}	0.214	0.125	0.583±0.14	0.170	0.113	0.664±0.03
	10^{-2}	0.213	0.131	0.614±0.09	0.161	0.108	0.672±0.01
	10^{-1}	0.218	0.123	0.562±0.19	0.169	0.113	0.668±0.15
	1	0.210	0.115	0.545±0.05	0.165	0.101	0.612±0.07
	10	0.215	0.104	0.484±0.06	0.164	0.113	0.691±0.02
	100	0.220	0.113	0.512±0.08	0.166	0.104	0.626±0.05
	1000	0.217	0.106	0.487±0.06	0.174	0.101	0.577±0.03
	2500	0.223	0.108	0.485±0.02	0.178	0.106	0.594±0.03
	5000	0.231	0.116	0.500±0.08	0.197	0.113	0.572±0.20
	10 000	0.251	0.110	0.437±0.06	0.214	0.120	0.561±0.11
	15 000	0.265	0.144	0.544±0.11	0.192	0.136	0.708±0.15

[a]Each value is the mean±S.D. of four replicates from each of four separate experiments. 4NQO was used as a positive control; [b]RGA, relative β-galactosidase activity. RGA≥2-fold over the control levels is considered positive (Oda et al., 2004); [c]CPRG and ONPG are used as substrates; New synthesized 1-[(2-aminophenyl) thio]-1-phenyl-2 nitrobutane (I) derivatives contain H, Br, Cl, ethyl, ethoxy, methyl groups are Ia, Ib, Ic, Id, Ie and If , respectively.

either ONPG (4 mg/mL) or CPRG (4 mg/mL) were then added. This microplate was then agitated gently and incubated at 37°C for 15 min. After incubation, 100 µL of stop solution (1 M Na_2CO_3) was added to each well, and the plate again was agitated gently. Absorbance at A415 and A570 was then measured with a microplate reader for ONPG and CPRG, respectively.

The Relative β-gal Activity (RGA) was calculated as described previously using the following equations:

$$\text{RGA (units)} = \text{A415(ONPG)}/\text{A595 (growth turbidity)} \quad \text{(I)}$$

$$\text{A570(CPRG)}/\text{A595} \quad \text{(II)}$$

The values in these equations were corrected by subtracting the value of the absorbance blank. A twofold increase in RGA above the control levels was considered positive [7]. Each value is the mean of four replicates from each of four separate experiments. In the test, 4NQO was used as positive control.

Results and Discussion

Table 1 and 2 show RGA values for both strains and both substrates with nitrobutane (I) derivatives that contain H, Br, Cl, ethyl, ethoxy and methyl groups on benzene ring (Ia, Ib, Ic, Id, Ie, If, respectively). For all compounds, the induction of umuC gene expression was found to be almost the same for the strains that overexpress NR and O-AT enzymes. In addition, the β-gal activities by using CPRG were three fold higher than ONPG, for both strains in all tested compounds. These findings also demonstrate that CPRG is a more effective substrate than ONPG for detection β-gal activity. Since the RGA values for all chemicals did not show at least a twofold increase as compared to the control (4NQO), they were not evaluated as mutagenic. The attachment of H, Br, Cl, ethyl, ethoxy and methyl groups to the aromatic ring does not lead to any change of the mutagenicity of the compound. The nitro groups are regarded as mutagenic and carcinogenic in several drugs, like in the case of metronidazole. On the other hand, nitazoxanide, has a wide range antiviral activity, was found to be weak positive in only Ames tester strain TA102, whereas non-mutagenic in other Ames tester strains [15,16]. Nowadays, antiviral therapies utilize combination therapies involving generally nucleoside analogs such as zidovudine (AZT) and lamivudine (3TC). Being widely used these antiviral drugs induce SOS response by inhibiting DNA replication and are shown to be genotoxic [17]. Besides, the appearing resistance against certain antiviral drugs such as acyclovir [18] requires the design of novel antiviral drugs.

The bacterial reverse mutation test (Ames test) is one of the standard battery test systems used for mutajenicity screening of pharmaceutics [19,20]. However, based on the structural knowledge of the chemicals to be tested, suitable strain/cell, exogeneous activation systems and tests should be chosen for genotoxicity screening [15,21]. In this study, since 1-[(2-aminophenyl)thio]-1-phenyl-2-nitrobutane (I) derivates involve nitro groups, makes advantageous the use of the umu-test, using NM2009 and NM1011 strains especially designed for detecting the mutagenicity of nitroarenes. The umu test had been standardized by the German Institute for Standardization [22] and also by ISO [23] to detect genotoxins in various compounds including drug candidates [8,24]. An excellent correlation between the results of the umu test and those of the Ames test was observed with a concordance of 90%. The umu test has several advantages. The assay time is relatively short, and the assay is easy to handle. It has a high-throughput methodology by using 96-well plates. These advantages allow rapid screening of genotoxicity using minimum amounts of compounds [25].

In conclusion, the results of the present study have very important data making these agents potential drug candidates. Bacterial mutagenicity and genotoxicity tests are primary and significant steps of this process. An important principle in drug development is to perform safety tests of previously determined significant drug activity in in-vitro assays. This may be even more crucial than its efficiency in terms of experimental conditions, since it is important in chemotherapy to treat without risk for the patient. So, our results showed that, this drug agents can be suitable candidates for clinical trials in the way to be a drug with potent antiviral activity. Detection of all relevant genotoxic effects is difficult to be achieved by a single test. Further studies are needed for more detailed characterization of the genotoxicity of these drug candidates.

Conflict of Interest

There are no conflicts of interest among the authors.

References

[1] Custer LL, Sweder KS. The role of genetic toxicology in drug discovery and optimization. Curr Drug Metab 2008; 9(9):978-85.

[2] Brambilla A, Martelli A. A review of the genotoxicity of marketed pharmaceuticals. Mutat Res 2007; 635;17-52.

[3] Buschini A, Ferrarini L, Franzoni S, Galati S, Lazzaretti M, et al. Genotoxicity revaluation of three commercial nitroheterocyclic drugs: nifurtimox, benznidazole, and metronidazole. J Parasitol Res 2009; 2009:463575.

[4] Chung MC, Bosquesi PL, dos Santos JL. A prodrug approach to improve the physico-chemical properties and decrease the genotoxicity of nitro compounds. Curr Pharm Des 2011; 17(32):3515-26.

[5] Dihl RR, Bereta MS, do Amaral VS, Lehmann M, Reguly ML, et al. Nitropolycyclic aromatic hydrocarbons are inducers of mitotic homologous recombination in the wing-spot test of Drosophila melanogaster. Food Chem Toxicol 2008; 46(7):2344-8.

[6] Gökçe M, Özçelik B, Bakır G, Karaoğlu T, Berçin E, et al. Antiviral and antimicrobial activities of new nitrobutane derivatives. Arzneimittelforschung Drug Res 2004; 12;891-7.

[7] Oda Y, Nakamura S, Oki I, Kato T, Shinagawa H. Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. Mutat Res 1985; 147(5):219-29.

[8] Yasunaga K, Kiyonari A, Nakagawa M, Yoshikawa K. Investigation into the ability of the Salmonella umu test to detect DNA damage using antitumor drugs. Toxicol In Vitro 2006; 20(5):712-28.

[9] Gatehouse D. Bacterial mutagenicity assays: test methods. Meth-

ods Mol Biol 2012; 817:21-34.

[10] Benigni R, Bossa C, Tcheremenskaia O, Giuliani A. Alternatives to the carcinogenicity bioassay: in silico methods, and the in vitro and in vivo mutagenicity assays. Expert Opin Drug Metab Toxicol 2010; 6(7):809-19.

[11] Hamer B, Bihari N, Reifferscheid G, Zahn RK, Müller WE, et al. Evaluation of the SOS/umu-test post-treatment assay for the detection of genotoxic activities of pure compounds and complex environmental mixtures. Mutat Res 2000; 466(2):161-71.

[12] McCoy EC, McCoy GD, Rosenkranz HS. Esterification of arylhydroxylamines: evidence for a specific gene product in mutagenesis. Biochem Biophys Res Commun 1982; 108(3):1362-7.

[13] Rosenkranz HS, Mermelstain R. Mutagenicity and genotoxicity of nitroarenes. All nitro-containing chemicals were not created equal. Mutat Res 1983; 114;217-67.

[14] Oda Y, Funasaka K, Kitano M, Nakama A, Yoshikura T. Use of a high-throughput umu-microplate test system for rapid detection of genotoxicity produced by mutagenic carcinogens and airborne particulate matter. Environ Mol Mutagen 2004; 43;10-9.

[15] Ku WW, Bigger A, Brambilla G, Glatt H, Gocke E, et al. Strategy for genotoxicity testing-metabolic considerations. Mutat Res 2007; 627;59-77.

[16] Rossignol JF. Thiazolides: A new class of antiviral drugs. Expert Opin Drug Metab Toxicol 2009; 5;667-74.

[17] Poirier MC, Olivero OA, Walker DM, Walker VE. Perinatal genotoxicity and carcinogenicity of anti-retroviral nucleoside analog drugs. Toxicol Appl Pharmacol 2004; 199(2):151-61.

[18] Arif JM, Kunhi M, Subramanian MP, Bekhit AA, El-Sayed OA, et al. Cytotoxic and genotoxic potentials of newly synthesized antiviral aminopyrazoquinoline derivatives. Med Chem Res 2008; 17;297-304.

[19] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), S2A: Spesific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Available at: http://www.ifpma.org/ich1.html.

[20] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), S2B: A Standart battery for Genotoxicity Testing of Pharmaceuticals. Available at: http://www.ifpma.org/ich1.html.

[21] Atterwill CK, Wing MG. In vitro preclinical lead optimization technologies (PLOTs) in pharmaceutical development. Toxicol Lett 2002; 127;143-51.

[22] German Institute for Standardization(DIN). (1996). DIN 38415-3, Part 3 Determination of the mutagenic potential of water using the umu-test (T3). Berlin, Germany.

[23] ISO. ISO13829. Water quality-determination of the genotoxicity of water and waste water using the umu test. Geneva, Switzerland. 2002.

[24] Ghazali AR, Sipain Y, Rajab FN, Ling SE, Ramli N, et al. Genotoxic Potential of Shrimp Pastes (Belacan) Extracts Using Umu Test. Food and Nutrition Sciences 2012; 3;522-5.

[25] Takamura-Enya T, Ishii R, Oda Y. Evaluation of photo-genotoxicity using the umu test in strains with a high sensitivity to oxidative DNA damage. Mutagenesis 2011; 26(4):499-505.