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RESEARCH PAPER

Toxicological Evaluation of Benzothiazole Derivatives Carrying Piperazine Ring

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ABSTRACT

Benzothiazole-piperazine derivatives prepared previously, **1h** and **1j** are potential anticancer agents. Since the mutagenic and genotoxic properties of anticancer drugs compose an essential issue to be researched, this study is focused on the analysis of the mutagenicity and genotoxicity of these molecules. The mutagenicity of **1h** and **1j** were determined by Ames test performed on *Salmonella* TA98 and TA100 strains. Sample **1j** was mutagenic on TA98 bacterial strain. However, compound **1h** was not mutagenic in bacterial strains TA98 and TA100 with and without S9 activation. The genotoxicity of **1h** was evaluated by the chromosomal aberration assay on human lymphocytes. Compound **1h** was also not genotoxic in human lymphocytes *in vitro*. All results revealed that, **1h** was not mutagenic in the two *Salmonella* strains tested and was not genotoxic in chromosomal aberration assay. Therefore, results demonstrate that the described molecule is promising as a new anticancer drug without mutagenicity. Also, after performing Ames test with other recommended bacterial strains and *in vivo* experiments can be used safely for the development of new structures exhibiting anticancer activities.

Key Words: Benzothiazole, piperazine, anticancer, mutagenicity assay, genotoxicity, *in vitro* chromosomal aberration assay

INTRODUCTION

Cancer as a very common health problem is a disease of cellular mutation, proliferation and abnormal cell growth (1). Anticancer drug development aims the generation of chemical structures that can control the growth of cancerous cells efficiently.

The aim of targeting cell proliferation is to arrest the cell cycle or cause cancer cell death using cytotoxic compounds. DNA is one of the main targets of these therapies because DNA replication is an essential phase of the cell cycle (2). Many of the cytotoxic agents commonly used to treat cancer patients cause high levels of DNA damage which initiate cell cycle checkpoints, leading to cell cycle arrest and/or cell death (3). As designed for inducing considerable damage to genetic material in cancer cells, anticancer drugs have to be studied for their genotoxic effects on normal cells in order to estimate their relative potencies to promote the growth of secondary malignancies (4).

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Drugs used in cancer chemotherapy have serious side effects, selectivity problems and potential to develop tolerance. Thus, discovering new anticancer drugs is crucial under these circumstances.

Benzothiazole derivatives have been extensively studied as potential anticancer drug candidates (5-10) and several attempts were made to modify the benzothiazole nucleus improving their antitumor activities. Modifications on the benzothiazole core have resulted in a more complex structure to develop a relationship between their structures and the antineoplastic activity (11).

In the previous studies of Gürdal *et al.,* synthesis, characterization and anticancer activities of several benzothiazole-piperazine derivatives were presented. Among these compounds, aroyl substituted **1h** and **1j** were found to be active against HUH-7 (hepatocellular), MCF-7 (breast) and HCT-116 (colorectal) cancer cell lines. Although their direct mechanism of action has not been clarified yet, it was shown that these compounds cause apoptosis by cell cycle arrest at subG1 phase by Hoechst staining and Fluorescence Activated Cell Sorting (FACS) assays (12). Many studies focused on the evaluation of the genotoxicity of widely used anticancer agents in Turkey and around the world. For example; cisplatin (13-15), poly polymerases (16) and paclitaxel (17, 18) genotoxicity potential on somatic and/or germ cells were studied previously.

In this study, we aimed to determine the mutagenic and genotoxic properties of **1h** and **1j** using the *Salmonella*/microsome assay on TA98 and TA100 strains and human lymphocytes *in vitro* since optimization studies of these compounds led to the discovery of a new family of benzothiazole compounds with promising anticancer properties.

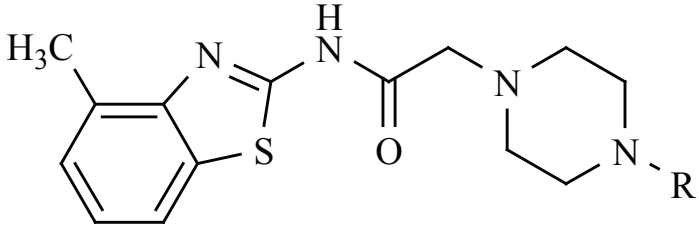
MATERIALS AND METHODS

Chemicals

Sodium azide (SA), 4-nitro-*o*-phenylenediamine (NPD), biotin, histidine, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, ampicillin trihydrate, mitomycin C (MMC), colchicine and agar were supplied from Sigma Aldrich Chemical Company (St Louis, Missouri, USA). Dimethyl sulfoxide (DMSO) was purchased from Acros (New Jersey, USA). 2-Aminofluorene (2-AF) was from Merck (Hohenbrunn, Germany) and nutrient broth was supplied from Hi Media laboratories Ltd (Mumbai, India). Peripheral blood karyotyping medium was purchased from the Gibco (Life Technologies, USA).

Synthesis of 1h and 1j

Compounds **1h** and **1j** were synthesized according to the procedure described by Gürdal *et al.* (12) (Figure 1).



Compounds	R	Cancer Cell Line (GI_{50}, µM)					
		HCT-116	R^2	MCF-7	R^2	HUH-7	R^2
1h	Benzoyl	0.9	1.0	9.2	1.0	0.7	0.9
1j	2-Furoyl	1.3	0.7	4.3	0.8	4.5	0.9
5-Fluorouracil		30.66		3.51		18.67	

Figure 1. Structures and cytotoxic activities of compounds **1h** and **1j** (12).

Bacterial strains

Mutagenic activity was tested by the *Salmonella*/microsome assay, using the *Salmonella typhimurium* tester strains TA98 and TA100 provided with and without metabolization activity. TA98 detects frameshift mutagens and TA100 detects base pair mutagens. These strains were primarily recommended by Maron and Ames (19) for routine mutagenicity assays. This test is an important tool for screening substances for potential carcinogenicity. The TA98 strain was provided by the Toxicology Department of the Faculty of Pharmacy of Gazi University (Ankara, Turkey) and the TA100 strain was received from the Marmara Research Center of the Turkish Scientific and Technological Research Council (Gebze, Turkey).

Genetic analysis of *Salmonella typhimurium* strains for Ames test

Salmonella typhimurium strains were checked before the experiment. The following steps were performed for a complete strain check (20, 21).

Histidine dependence

Bacteria from overnight culture grown in nutrient broth were plated on minimal glucose agar plates and histidine/biotin plaques. Plates were left to incubate overnight at 37°C (Binder, USA). Growing was observed in histidine/biotin plaques but no growth was observed in minimal glucose agar plates, indicating that both *Salmonella* strains were histidine dependent.

Rfa marker

The presence of the Rfa marker was defined by the crystal violet sensitivity test. For this, 0.1 mL of bacterial culture was placed in 2 mL of top agar and kept at 45°C. Then, the bacterial-top agar mixture spread over nutrient agar. After solidification of the agar, a sterile filter paper disk was placed in the center of the plate and 10 µL of a sterile 0.1% crystal violet solution was applied on filter paper disk. Plates were incubated for 12 hours at 37°C. Both *Salmonella* strains showed a zone of growth inhibition surrounding the disk.

UVrB deletion

This mutation was identified by a sensitivity test to ultraviolet rays. Cultured bacteria were plated on nutrient agar. Two petri dishes were prepared with the same cultured bacteria. These plates were irradiated in a 15 W power with a UV lamp at a distance of 33 cm for 8 sec. Then, the plates were incubated for 24 hours at 37°C. Bacterial growth was not observed in the plate which was exposed to UVB. However, bacteria that were not exposed to UVB light showed proliferation.

R Factor Presence

A loopful of the plasmid carrying *Salmonella* culture was streaked across a minimal glucose agar plate supplemented with histidine/biotin and 24 mg/mL ampicillin. The plates were kept at 37°C for 24 hours. Growth was observed with tested strains TA98 and TA100.

Preparation of S9 Fraction

The rat liver homogenate was prepared according to methods of Ames *et al.* (22), Garner *et al.* (23) and Halder *et al.* (24). Two Sprague–Dawley male rats weighing 150–200 g were provided from the Yeditepe University Medical School Experimental Research Center (YUDETAM) and the experimental protocol was approved by the ethical committee of Yeditepe University. Animals were fed with 0.1% phenobarbital in their drinking water for 7 days. On day 6, no food was provided to rats. The following day, the animals were sacrificed and the rat liver homogenate (S9) was prepared by centrifugation at 9000g following the method of Maron and Ames (19). Approximately 2 mL of S9 fractions were distributed in different small sterile cryo vials and quickly frozen and stored at -80°C.

Mutagenicity assays

Standard mutagenicity assays in plate incorporation tests were carried out by following the method of Maron and Ames procedure (19). Compounds **1h** and **1j** were dissolved in DMSO and different concentrations (0, 1, 10, 100, 1000 and 5000 µg/plate) in 50 mL of DMSO were used for mutagenicity assay. Plates were co-incubated with the bacterial strains and the different concentrations of samples, inverted and placed at 37°C for 48 h in dark and revertant colonies were counted after incubation. To evaluate the impact of **1h** and **1j** metabolites, similar experiments were also carried out by incubating bacteria and chemicals with liver S9. Four plates were used for each concentration tested with and without S9 experiments.

Five different concentrations of the test materials were used with 10 fold intervals between test points and the maximum test concentration was 5000 μg/plate as recommended by the Organization for Economic Cooperation and Development's (OECD) guideline (25).

In vitro chromosomal aberration assay in human lymphocyte culture

For chromosomal aberration (CA) analysis, human blood samples were obtained from one male and one female healthy volunteers (aged between 25 and 35 years, non-smoker, non-alcoholic, not under drug therapy and with no recent history of exposure to mutagens). Blood samples were collected in the heparinized vials, and 0.5 mL of blood was added to 5 mL of Karyomax medium and incubated at 37°C. After 24 h, three different concentrations of **1h** (1.5, 3 and 6 μg / mL of culture) were added to the blood culture. The stock solution was prepared by dissolving **1h** in DMSO first, this solution is subsequently diluted with Karymax medium to be added to the culture media in the concentrations mentioned above. Two cultures were treated with each concentration of chemical (one from male and one from female blood samples) as recommended by the OECD guideline (26). Two sets of cultures were also treated with only DMSO and MMC (0.50 mg/mL) and were used as negative and positive controls. After 70 h of the blood culture, colchicine was added to the culture media to arrest the metaphase cells. At 72 h, cultures were harvested and slides were prepared for metaphase chromosomes following the method of De Chaudhuri *et al.* (27) and also OECD test guideline (26). All slides were coded, and 100 well-spread metaphase cells (46±2 chromosomes) per culture were scored for CA. A total of 200 metaphase cells were scored for each concentration of chemical and for both negative and positive controls. For the mitotic index (MI) analysis, 1000 cells/culture were scored, and the MI was expressed in percentages. CA was scored according to the World Health Organization (28) and OECD guideline (26). Frequency of aberrations per cell for chromatid type and chromosome types were calculated. Gaps were recorded as indicated in the OECD guideline but were not included neither as percentage of aberrant cells nor as frequency of aberrations per cell.

Statistical analysis

The differences between the groups were compared using SPSS 20 program. Experimental results were expressed as mean ± deviation. Dunnett's multiple comparison test was carried out for mutagenicity CA analysis (29). The values of $p < 0.05$ was considered statistically significant.

RESULTS

The results of the mutagenicity assays induced by **1h** and **1j** in the TA98 and TA100 *Salmonella* strains with and without metabolic activation was given in Table 1. As expected, the positive control showed very high frequencies of revertant colonies when compared to the negative control.

Neither TA98 nor TA100 strains treated with **1h** concentrations of 1–5000 μg/plate exhibited significant increase in revertant colonies number when compared with the negative control, suggesting no mutagenicity to the tested strains. For TA100 strain, toxicity was observed to appear for a **1h** concentration of 1000 μg/plate of culture with and without S9 activation. For TA100, 5000 μg/plate was found to be a totally toxic concentration. In TA98 strain **1h** did not influence indicator bacteria viability, suggesting no toxicity in the tested strain at concentrations up to ≤ 5000 μg/plate with or without S9 metabolic system. On the other hand, **1j** increased the revertant colonies significantly when compared to negative control ($p<0.05$) indicating the mutagenic effect on TA98 strain with or without S9 activation. In the TA100 strain treated with **1j** concentrations of 1–5000 μg/plate did not induce significant revertant colonies when compared with the negative control, and toxicity was observed to appear at concentration of 1000 and 5000 μg/plate of culture (Table 1).

Genotoxicity results of compound **1h** measured by the CA assay are shown in Table 2. Knowing that **1h** exhibited half maximal inhibitory concentration (IC_{50}) value of around 1 μM (1.5 μg/mL) when evaluated on various cancer cells, the CA assays were chosen to be carried out at three concentrations centered on this value. Thus, the experiments were conducted at concentrations of 1.5, 3 and 6 μg/mL.

No significant CA (percentage of aberrant cells) were observed in tested concentrations when compared to DMSO control culture. Results of the MI showed a slightly reduced MI for the highest dose even if not statistically significant suggesting cytotoxic/cytostatic effect at the highest dose (6 μg/mL) (Table 2). The positive mutagen MMC showed very high frequencies of CA, which indicated that lymphocytes were cultured under proper conditions.

Table 1. Results of mutagenicity assay induced by **1h** and **1j** in *Salmonella* strains TA98 and TA100 with and without S9 activation.

		Number of revertant / plate			
	Dose (µg/plate)	**+ S9[a]**	**- S9[a]**	**+ S9[a]**	**- S9[a]**
TA98	Positive control	569.5 ± 34.6	505.8 ± 32.5		
	Negative control	36.3 ± 7.9	26.8 ± 5.1		
		1h		**1j**	
	1	35.8 ± 12.0	26.5 ± 6.2	35.8 ± 6.0	26.5 ± 4.2
	10	32.8 ± 11.1	26.3 ± 7.6	36.0 ± 7.3	27.0 ± 5.1
	100	34.5 ± 8.9	27.0 ± 6.7	*52.8 ± 7.5	*42.0 ± 5.5
	1000	35.5 ± 7.0	27.3 ± 5.0	*77.5 ± 3.9	*80.5 ± 9.6
	5000	33.4 ± 9.4	25.0 ± 7.1	*92.0 ± 8.1	*98.9 ± 6.2
TA100	Positive control	865.5 ± 62.6	1023.8 ± 195.9		
	Negative control	190.5 ± 22.5	173.5 ± 23.3		
		1h		**1j**	
	1	189.8 ±2 4.6	183.8 ± 25.7	194.5 ± 16.1	174.3 ± 30.6
	10	185.5 ± 23.3	178.5 ± 30.7	196.0 ± 18.4	176.5 ± 30.6
	100	188.0 ± 26.3	176.0 ± 22.0	193.5 ± 21.6	173.8 ± 33.4
	1000	*138.0 ± 14.3	*46.0 ± 12.9	*108.5 ± 11.8	*66.8 ± 18.1
	5000	*53.8 ± 5.7	*12.3 ± 6.8	*62.2 ± 9.1	*26.9 ± 5.0

*P<0.05; negative control vs treatment. Dunnett Multiple Comparisons Test. DMSO (50 µL/plate) was used as negative control. 2-aminofluorene (2-AF) (5 µg/plate) was used as positive mutagen for both TA98 and TA100 strains with S9 experiment; 4-Nitro-o-phenylenediamine (NPD) (20 µg/plate) was used as positive direct mutagen (positive control) in the absence of S9 mix for S. typhimurium TA98 strain. Sodium azide (SA) (1 µg/plate) was used as positive direct mutagen (positive control) in the absence of S9 mix for S. typhimurium TA100

Table 2. Chromosomal aberrations induced by 1h in human lymphocytes *in vitro*.

Treatment	Metaphase cells scored	Chromatid type abberation/cells	Chromosome type abberations/cells	Aberrant cells % (Mean ± S.D.)	Mitotic indices (Mean±S.D.)
Negative Control	200	0.070	0.080	7.50 ± 2.12	3.57 ± 0.74
1h (1.5 µg/mL)	200	0.060	0.080	7.50 ± 1.41	3.03 ± 0.22
1h (3.0 µg/mL)	200	0.010	0.090	7.00 ± 0.71	3.07 ± 0.18
1h (6.0 µg/mL)	200	0.011	0.070	8.00 ± 1.41	2.18 ± 0.71
Positive Control	200	0.250	0.200	19.50 ± 0.71*	3.22 ± 1.95

Results at each dose were compared to those of negative control using Dunnett's multiple comparison test. *$p < 0.05$: Positive control versus negative control using Dunnett's multiple comparisons. Mitomycin C (MMC) 0.5 µg/mL was used as a positive control. DMSO (50µL) was used as a negative control.

DISCUSSION

Benzothiazole is a heterocyclic ring having varied biological activities and still of great scientific interest (30). Piperazine is another important ring structure also investigated for the drug development intensively. Some studies reported that combination of benzothiazole and piperazine rings might lead to biologically active new structures with antimicrobial (31), antidepressant (32) and anticancer (33) effects. Recently, we have synthesized ten new benzothiazole-piperazine derivatives as anti-tumor agents for the generation of a new class of antiproliferative compounds. Among them, **1h** and **1j** were found to be the most active structures, and herein we aimed to investigate the safety of these molecules analyzing their possible mutagenicity and genotoxicity. Firstly, the possible mutagenic effects of these anticancer drug candidates were investigated by Ames mutagenicity assay carried out on *Salmonella typhimurium* strains. Ames test is a rapid bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances capable of causing DNA damage leading to gene mutation that is used commonly as an initial screen to estimate the mutagenic potential of chemicals and drugs. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen sample is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. A positive result indicates that the chemical or drug is mutagenic and hence may have a carcinogenic potential, because cancer is often linked to mutation (21). It should be considered important to clarify the structure-activity relationship of a novel molecule for mutagenicity. According to Hsu *et al.*, all of the substituted structures derived from major mutagenic scaffolds were also mutagenic and the parent scaffolds with lack of mutagenicity may or not produce mutagenic derivatives depending on the attached substituent (34). Our results showed that (substituted – benzoyl) benzothiazol-piperazine was not mutagenic on both tested strains, whereas (substituted – 2-furoyl) benzothiazol-piperazine was found to be strongly mutagenic to TA98 strain with or without S9 activation. It should be noted that anticancer agents that have been shown to be mutagenic in the *Salmonella* test yield a high risk of primary or secondary cancers following exposure to these drugs (35). The difference in the hydrophobicity of moieties is one of the causative factors for mutagenicity of chemical structures. Considering a possible effect of permeability of these investigated benzothiazol-piperazine derivatives across the cell membrane of the tested bacteria strain on their mutagenic activity, benzoyl residue was expected to across the membrane easily because of its more hydrophobic property compared to 2-furoyl moiety. Results of present study suggest that mutagenicity of these compounds cannot be attributed to a difference in the membrane permeability. On the basis of our observations, the cause of developing mutagenicity may be explained by the nature of each aromatic ring.

As seen in the Ames assay, **1h** was found to be not mutagenic on TA98 and TA100 strains, so as recommended by the OECD guideline, (26) the *in vitro* CA assay was used to ensure the safety of **1h** and to find out that this compound does not

cause genotoxicity. CA analysis is utilized to assess potential genotoxic hazard of test substances. Mammalian cells are cultured *in vitro*, exposed to a test substance, harvested, and the frequency of asymmetrical structural chromosome aberrations is measured (36).

Two and four times higher concentrations than the average IC_{50} value previously determined on cancer cell lines were investigated for CA assay. Results revealed that **1h** was not genotoxic even at the four times higher concentration than IC_{50} value in human lymphocytes culture. Many of the anticancer drugs are described to be genotoxic because they are designed to alter the DNA structure or its function. Since genotoxicity can result in the rise of secondary cancers, studying the generation of structures that do not exhibit any genotoxicity constitutes a trending research topic (37-39).

Taken together, the compound **1h** is not mutagenic in bacterial strains TA98 and TA100 and also not genotoxic in human lymphocytes *in vitro*. Though some more studies on mutagenicity assays using another *Salmonella* tester strains together with *in vivo* studies are necessary before coming to a final decision, given the present mutagenicity and genotoxicity data, the use of (substituted–benzoyl) benzothiazole-piperazine analog seems to be suitable for the development of non-genotoxic anticancer agents. This structure can be optimized and designed without causing any genotoxic damage thus may be used safely in the future.

CONFLICT OF INTEREST

No conflict of interest

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Piperazin halkası taşıyan benzotiyazol türevlerinin toksikolojik değerlendirilmesi

ÖZET

Önceki çalışmalardan elde edilen **1h** ve **1j** adlı bileşikler potansiyel antikanser aktiviteye sahiptir. Antikanser ilaçların mutajenik ve genotoksik özelliklerinin bu ilaçların araştırılmasında temel konuyu oluşturması sebebiyle, çalışmada söz konusu moleküllerin mutajenite ve genotoksisite analizleri üzerinde durulmuştur. **1h** ve **1j** Moleküllerinin mutajeniteleri *Salmonella* TA98 ve TA100 suşları ile Ames testi uygulanarak belirlenmiştir. **1j** Bileşiği TA98 bakteri suşu üzerinde mutajen bulunmuştur. Ancak, **1h** bileşiğinin TA 100 ve TA98 suşları ile S9 varlığında ve yokluğunda mutajenik aktiviteye sahip olmadığı gösterilmiştir. **1h** Bileşiğinin genotoksisitesi insan lenfositleri üzerinde yapılan kromozomal aberasyon testi ile değerlendirilmiş olup genotoksik bir risk oluşturmadığı saptanmıştır. Bütün sonuçlar değerlendirildiğinde, **1h**'nin iki farklı *Salmonella* suşu üzerinde yapılan çalışmada mutajenik aktivitesi olmadığı ve kromozom aberasyon testinde genotoksik olmadığı saptanmıştır. Bu nedenle, sonuçları belirtilen molekülün mutajenik ve genotoksik etkileri olmadığı için yeni bir antikanser ilaç olarak umut verici olduğu görülmüştür. Güvenle kullanılabilecek antikanser aktivite sergileyen yeni bir molekülün varlığını göstermek için yukarıdaki analizlere ek olarak Ames testi tavsiye edilen diğer bakteri suşlarıyla ve *in vivo* deneylerle desteklenmelidir.

Anahtar Kelimeler: Benzotiyazol, piperazin, antikanser, mutajenite deneyi, genotoksisite, *in vitro* kromozomal aberasyon deneyi

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