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SYNTHESIS AND ANTITUMOR ACTIVITY OF SOME 6-CHLORO- AND 6,7-DICHLORO-2,3-DISUBSTITUTED-QUINOXALINE DERIVATIVES

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Abstract

In this study, 6-chloro- and 6, 7-dichloro-2, 3-disubstituted-quinoxaline derivatives were synthesized and their cytotoxicity and antitumor activities were tested. 6-Chloro- and 6, 7-dichloro-2, 3-disubstituted-quinoxaline compounds were obtained by reacting 1, 2-dicarbonyl compound and substituted o-phenylenediamine under reflux for 4-5 hours in glacial acetic acid. The structure elucidation of the compounds was performed by IR, ^{1}H-NMR and Mass spectroscopic data and elemental analysis results. Antitumor activities and cytotoxicities of the compounds were examined.

Key Words: *6-Chloro- and 6, 7-dichloro-2, 3-disubstituted-quinoxaline, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Antitumor activity*

Bazı 6-kloro- ve 6,7-dikloro-2,3-disübstitüe-kinoksalin türevlerinin sentezi ve antitümör aktiviteleri

Bu çalışmada, 6-kloro- ve 6,7-dikloro-2,3-disübstitüe-kinoksalin türevleri sentezlenerek, sitotoksisiteleri ve antitümör etkileri araştırıldı. 6-Kloro- ve 6,7-dikloro-2,3-disübstitüe-kinoksalin bileşikleri, uygun 1,2-dikarbonil bileşiği ve sübstitüe o-fenilendiamin ile, derişik asetik asit içerisinde, 4-5 saat geri çeviren soğutucu altında kaynatılarak elde edildi. Sentezlenen bileşiklerin yapıları IR, ^{1}H-NMR ve Kütle spektroskopisi verileri ve elementel analiz sonuçları ile aydınlatıldı. Bileşiklerin antitümör aktiviteleri ve sitotoksisiteleri araştırıldı.

Anahtar Kelimeler: *6-Kloro- ve 6,7-dikloro-2,3-disübstitüe-kinoksalin, 3-(4,5-dimetiltiyazol-2-il)-2,5-difenil-2H-tetrazolyum bromür (MTT), antitümör aktivite*

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Introduction

DNA molecule contains the knowledge, which define the properties of the alive and ensure to transfer these knowledge to the next generations. In addition, DNA is functional on the cell protein synthesis (Ribonucleic asid). Proteins are produced according to the genetic knowledge on the DNA. As the increase in internal cell protein synthesis has stimulative effect on the cell proliferation, in the anticarcinogenic effect studies, the features that stop the DNA synthesis are looked for.

Quinoxaline structure selected for this aim has been studying since 1920's and its structure had been previously identified. This compound indicated antitumor (1-4), antibacterial (5-7), antimalarial (8), antimycotic (9), antispasmodic (10) and amebicidal (11) effects. Especially the novel studies on the antitumor effects in the literature had taken our attention. In these studies the antitumor and tremor making effects of quinoxaline and quinoxalinium ions, besides their interactions according to their chemical structures; were reported for the first time.

In view of these data we aimed the synthesis of some 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives for antitumor and cytotoxicity evaluation. The cytotoxicity (12,13) of 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxalines were determined by MTT assay. In the antitumor effect studies, the possible inhibitory effects of these compounds were measured on normal F2408 and H-ras active 5RP7 cancer cell line.

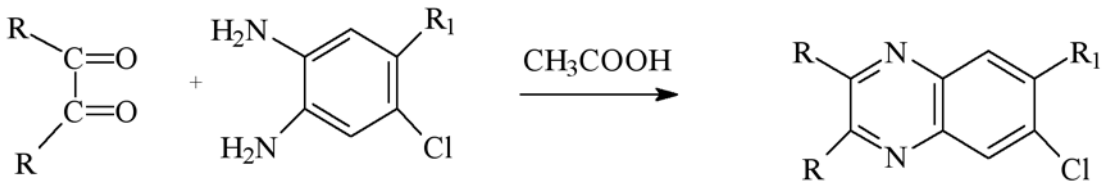
Experimental

Chemistry

Melting points of the compounds were determined by using Stuart Scientific Smpl Melting Point apparatus and reported uncorrected. Thin-Layer-Chromatography was performed on silica gel 60 GF_{254} and silica gel 60 G (Merck) (15:25) plates and spots were visualized by UV lamp. Spectroscopic data were recorded on the following instruments, IR: Schimadzu-435 IR spectrophotometer, ^{1}H-NMR: Bruker DPX 400 NMR spectrometer in DMSO-d_6 using TMS as internal standard, MS: VG Platform Mass spectrometer. Elemental analyses were performed by Carlo-Erba 1106. Analyses and results for C, H, N were within ±0.4% of the theoretical values. All the chemicals and solvents used in this study were of analytical grade (Merck, Fluka, Aldrich and Carlo-Erba).

6-Chloro- and 6,7-dichloro-2,3-disubstituted quinoxaline compounds were obtained by reacting 1,2-dicarbonyl compound and substituted o-phenylenediamine under reflux for 4-5 hours in glacial acetic acid.

The reaction is depicted in Scheme 1. Some characteristics of the compounds are given in Table 1. Spectral data of the compounds are given in Table 2.



Scheme 1

TABLE 1. Some characteristics of the compounds

Compound	R	R_1	M.p. (˚C)	Yield (%)	Formula	Molecular weight
1	Phenyl-	H	121-3[a]	55	$C_{20}H_{13}N_2Cl$	316.773
2	p-Tolyl-	H	163-5[b]	53	$C_{22}H_{17}N_2Cl$	344.833
3	p-Anisyl-	H	145-6[c]	60	$C_{22}H_{17}N_2O_2Cl$	376.833
4	2-Furyl-	H	115-7[d]	72	$C_{16}H_9N_2O_2Cl$	296.705
5	Acenaphtyl-	H	231-5	78	$C_{18}H_9N_2Cl$	288.725
6	Phenantryl-	H	251-3	85	$C_{20}H_{11}N_2Cl$	314.763
7	Phenyl-	Cl	154-6[e]	67	$C_{20}H_{12}N_2Cl_2$	351.23
8	p-Tolyl-	Cl	140-4	57	$C_{22}H_{16}N_2Cl_2$	379.28
9	p-Anisyl-	Cl	160-4	65	$C_{22}H_{16}N_2O_2Cl_2$	411.28
10	2-Furyl-	Cl	153-7	72	$C_{16}H_8N_2O_2Cl_2$	331.154
11	Acenaphtyl-	Cl	325-7	80	$C_{18}H_8N_2Cl_2$	323.174
12	Phenantryl-	Cl	271-3[f]	83	$C_{20}H_{10}N_2Cl_2$	349.21

[a] Lit. m.p. 123-4 ˚C (10, 14)
[b] Lit. m.p. 162 ˚C (10)
[c] Lit. m.p. 143-5 ˚C (10)
[d] Lit. m.p. 115 ˚C (10)
[e] Lit. m.p. 153 ˚C (15)
[f] Lit. m.p. 264 ˚C (15), 271-2 ˚C (16)

General procedure for the synthesis of 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxalines

Equimolar amounts (5 mmol) of substituted o-phenylenediamine and substituted dicarbonyl compound was heated under reflux for 4-5 hours in 50 ml glacial acetic acid. The reaction mixture was poured into ice water and neutralized with 25% aq. ammonia. The precipitate formed was filtered and recrystallized from ethanol.

TABLE 2. Spectral data of the compounds

Compound	IR(KBr) $\nu_{max}(cm^{-1})$	^{1}H-NMR (400 MHz) δ (ppm) (DMSO-d_6)	EI-MS: m/z
1	1630-1570 (C=N, C=C)	7.12-7.26 (10H, m, Ar-H), 7.67-7.70 (1H, d, quinoxaline C_8-H), 7.95-7.97 (1H, d, quinoxaline C_7-H), 8.02 (1H, s, quinoxaline C_5-H)	
2	1625-1565 (C=N, C=C)	2.00 (6H, s, Ar-CH_3), 7.80-8.20 (8H, d, Ar-H), 8.30-8.60 (2H, m, quinoxaline C_7-H and C_8-H), 9.10 (1H, s, quinoxaline C_5-H)	
3	1630-1575 (C=N, C=C), 1275-1150 (C-O)	3.30 (6H, s, Ar-OCH_3), 8.25-8.60 (10H, m, Ar-H), 9.10 (1H, s, quinoxaline C_5-H)	
4	1620-1550 (C=N, C=C), 1130-1075 (C-O-C cyclic)	8.10-8.70 (6H, m, furyl), 9.10 (1H, s, quinoxaline C_5-H), 9.40-9.50 (2H, d, quinoxaline C_7-H and C_8-H)	
5	1625-1560 (C=N, C=C)	7.89-8.62 (9H, m, Ar-H)	
6	1625-1555 (C=N, C=C)	7.78-8.52 (11H, m, Ar-H)	
7	1635-1575 (C=N, C=C)	7.37-7.49 (10H, m, Ar-H), 8.50 (2H, s, quinoxaline C_5-H and C_8-H)	
8	1630-1570 (C=N, C=C)	2.28 (6H, s, Ar-CH_3), 7.12-7.14 (4H, d, j: 7.95 Hz Ar-H), 7.32-7.34 (4H, d, j: 8.05 Hz Ar-H), 8.88 (2H, s, quinoxaline C_5-H and C_8-H)	
9	1635-1570 (C=N, C=C), 1280-1155 (C-O)	3.73 (6H, s, Ar-OCH_3), 6.88-6.90 (4H, d, j: 8.75 Hz Ar-H), 7.38-7.40 (4H, d, j: 8.73 Hz Ar-H), 8.32 (2H, s, quinoxaline C_5-H and C_8-H)	411(M^+), 135(100%), 133,103, 77, 63
10	1625-1555 (C=N, C=C), 1135-1070 (C-O-C cyclic)	6.51-7.73 (6H, m, furyl), 8.20 (2H, s, quinoxaline C_5-H and C_8-H)	
11	1630-1565 (C=N, C=C)	8.04-8.55 (6H, m, Ar-H), 8.61 (2H, s, quinoxaline C_5-H and C_8-H)	
12	1630-1560 (C=N,	8.03-8.56 (8H, m, Ar-H), 8.65 (2H, s, quinoxaline	

Antitumor Activity

Cell Culture

Normal rat embryo fibroblast-like cells (F2408) and H-ras oncogene activated rat embryo fibroblast-like cancer cell line (5RP7) were used. These two cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) and 100 U/ml penicillin/streptomycin in an atmosphere of 5% CO_2.

Cytotoxicity valuation

In order to evaluate the cytotoxic activity, 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives were assayed in F2408 cells. Individual wells of a tissue culture 96-well microtiter plate was inoculated with 200µl of 10% FCS medium containing $1x10^3$ cells /ml. After 24 h of exposure to the drug at various concentrations (0.01-0.05-0.10-0.20µM), these cells were used for the cytotoxicity evaluation.

The cytotoxicity response of these cells was determined with the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) method, using tetrazolium salt (17,18). 200µl of freshly prepared 5mg/ml MTT solution, was added to each well after drug treatment. The cells were incubated 2 h and washed with phosphate buffered saline (PBS). After removal of the wash solution, dimethylsulfoxide (DMSO) was added and suspended. The optical density was determined by using a Bio-Tek (ELx808-IU) ELISA reader at a wavelength of 540 nm. Each dose of compounds was repeated three times per experiment. The results were averaged and the SD within each experiment was always <10%.

The IC_{50} value was defined as the concentration of test compound resulting in a 50% reduction of absorbance in comparison with the untreated cells in the MTT assay.

Analysis of DNA Synthesis

Cell proliferation assay was performed in 96-well plates (Falcon, Beckton Dickinson) and the BrdU (5-bromo-2'-deoxyuridine) colorimetric kit (Boehringer Mannheim) was used to determine the DNA synthesis by the method as given by the manufacturer. F2408 and 5RP7 cells cultured as detailed above were detached with 0.25% trypsin/EDTA and $1x10^3$ cells/ml were transferred into each well.

To investigate the effects of 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives on DNA synthesis, the cells were incubated with various concentrations of compounds for 48 h that were chosen according to MTT assay as described above. After each time point, the cells were labeled with 10 μl BrdU solution at 37 °C for 2 hours and then fixed with the addition of fixdenaturation solution for 30 min at room temperature. After removing the fixdenaturation solution, cells were treated with 100 μl of anti-BrdU solution for 90 min at room temperature.

Then the cells were washed three times with PBS and incubated with substrate solution until the color is sufficient for photometric detection that was predetermined. The absorbance was measured in an ELISA reader (Organom, Technica) at 492 nm. DMSO as a negative control was added to the cells during the time course.

Statistic

The Dunnet t-test was used for statistical evaluation of the data. $P<0.05$ was considered as statistically significant.

Results and Discussion

Chemistry

6-Chloro-and 6,7-dichloro-2,3-disubstituted-quinoxaline compounds were obtained by reacting 1,2-dicarbonyl compound and substituted o-phenylenediamine under reflux for 4-5 hours in glacial acetic acid. The structure elucidation of the compounds was performed by IR, ^{1}H-NMR and mass spectroscopic data and elemental analyses results. In the IR spectra of the synthesized compounds, the bands originated from the amino and carbonyl groups of the starting materials were no longer present as expected. In the NMR spectra, protons of the quinoxaline ring system were resonated at 7.67-9.50 ppm region and all the other aromatic protons of phenyl, substituted phenyl, furyl, acenaphtyl and phenanthryl were resonated as multipled peaks at expected regions.

Antitumor Activity

The cytotoxicity of 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives was evaluated in F2408 cells.

The cytotoxic activity is expressed in terms of relative absorbance of drug-treated cells, in comparison to control cells. The results obtained from MTT assays are presented in Table 3 (IC_{50} at 24 h). Some 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives showed a dose- or time- dependent cytotoxic effects.

Results showed that compound **6** and **8** is quite toxic for F2408 cells in comparison with other compounds. However longer incubation with these compounds overcome the cytotoxic effects which seem to be time-dependent (data not shown). Except compound **6** and **8**, compounds **1-12** showed less toxicity.

Cytotoxicity difference of these compounds could be related to either the type or the position of the substituents on quinoxaline ring.

The effects of 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives on DNA synthesis in F2408 and 5RP7 cells were examined using a specific cell proliferation kit. Table 4

showed the effect of compounds on cell proliferation of normal and H-ras activated cancer cell lines. Results indicated that compound **1** and compound **6** at concentrations 10 nM had no effect on cell proliferation of either normal or cancer cell. The addition of methylphenyl and methoxyphenyl substituents (compound **2** and compound **3**, respectively) inhibited 5RP7 cell proliferation, but not F2408 cells, at 10 nM and 50 nM concentrations, respectively after 2 days incubation in comparison with untreated control cells. (Table 4). Compound **4**, **10** and **12** were also inhibitors in growing 5RP7 cancer cells after two days as compared to untreated cancer cells and normal cells. Compound **5** at 10 nM concentration and compound **11** at 50 nM concentration inhibited both F2408 and 5RP7 cell proliferation after two days, therefore this inhibitory effect does not seem to be specific for H-ras activated cancer cells. In contrast substantial inhibitory effects were obtained when 5RP7 cells treated with either compound **7**, **8** or **9**. As shown in Table 4, compound **7** at concentration 50 nM had a significant inhibitory effect on 5RP7 cell proliferation. Addition of methylphenyl-Cl (compound **8**) and methoxyphenyl-Cl (compound **9**) substituents to compound **7** reduced the inhibitory effects on these cell lines. F2408 cell proliferation was hardly affected by these compounds.

Some of these derivatives showed antitumor effects which seems to be dependent on the type or position of substituents.

TABLE 3. Cytotoxicity evaluation (IC_{50}) of 6-chloro- and 6,7-dichloro-2,3-disubstituted-quinoxaline derivatives in normal cells (μM)

Com. (μM)	**1**	**2**	**3**	**4**	**5**	**6**
Cell type F2408	0.10	0.07	0.06	0.10	0.10	0.02- 0.7
Com. (μM)	**7**	**8**	**9**	**10**	**11**	**12**
Cell type F2408	0.10	0.10	0.04-0.11	0.9	0.11	0.11

IC_{50}= 0.2-0.11μM for all compounds in F2408 fibroblast-like cell line.

TABLE 4. Percent proliferation of F2408 and 5RP7 cell lines *in vitro* by 6-chloro- and 6,7-dichloro-2,3-disubstituted quinoxaline derivatives

Concentration (nM)	F2408	5RP7
10	102 ± 7	105,5 ± 15
10	71 ± 9	39* ± 7
50	85 ± 13	43* ± 5
1	87 ± 10	15 ± 2
10	61 ± 15	59 ± 8
10	93 ± 13	95 ± 8
50	88 ± 9	18,8 ± 7
50	93 ± 12	57 ± 5
50	81 ± 9	57 ± 11
10	79 ± 6	25 ± 4
50	54 ± 10	61 ± 9
50	85 ± 11	27 ± 9

The cells were treated with quinoxaline derivatives and then their proliferative ability were determined after 48 h of incubation as described in materials and methods. Values are mean ± SD (n=3). Results are representative of two separate experiments. * $p < 0.05$

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