Synthesis and cytotoxic activity of 3-phenyl-2-thio-quinoxaline 1,4-dioxide derivatives in hypoxia and in normoxia

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ABSTRACT: A series of novel 3-phenyl-2-ethylthio/ethylsulfinyl/ethylsulfonyl/phenylthio/ phenylsulfonyl-quinoxaline 1,4-dioxide derivatives were synthesized and screened for their cytotoxicity *in vitro* on human leukaemia cell line HL-60, human esophagus cancer cell line ECA-109, human prostate cancer cell line PC-3, human gastric carcinoma cell line SGC-7901, and human breast cancer cell line MCF-7 in hypoxia and in normoxia. Half of tested compounds showed higher cytotoxic activity both in hypoxia and in normoxia. The mechanism of one potent compound, 67, in hypoxia showed that the mitochondria pathway is involved in the antitumor activity of this class of compounds.

Key Words: Quinoxaline 1,4-dioxides, antitumor, hypoxia and normoxia

Introduction

Hypoxic tumor cells in a solid tumor cause resistance to radiotherapy and chemotherapy (*1-6*). Traditional chemotherapeutic agents have no or little effect on hypoxic tumor cells. Bioreductive prodrugs can effectively kill this kind of cell. One of the most promising bioreductive prodrugs is quinoxaline 1,4-dioxide (*7-10*), and the known compound 3-amino-2-carbonitrile quinoxaline 1,4-dioxide (TPZCN) is an important lead compound with beneficial biological activity *in vitro* (*11*). The 3-methyl-2-phenylthio-quinoxaline 1,4-dioxides were reported to have several forms of beneficial biological activity such as antimycobacterial and anticandidal activity (*12-14*). There are, however, no reports on the antitumor activity of this kind of compound. 3-phenyl-quinoxaline 1,4-dioxide derivatives should be effective antitumor agents in hypoxia since they contain the quinoxaline 1,4-dioxide pharmacore. Therefore, a series of novel 3-phenyl-2-thio-quinoxaline 1,4-dioxides were synthesized and screened for their cytotoxic activity in hypoxia and in normoxia.

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Materials and Methods

Chemistry

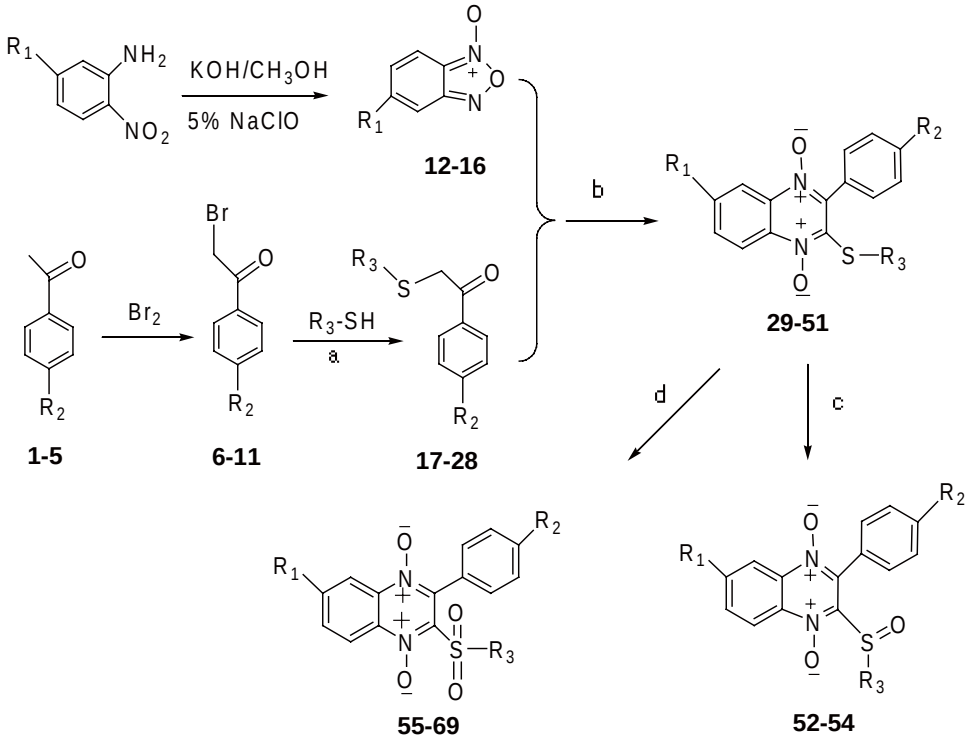
The synthetic pathway of the target compounds **20-59** is shown in Scheme 1. Compounds **12-16** were prepared by reaction of 2-nitroanilines with 5% sodium hypochlorite solution in the presence of KOH. Cyclocondensation of compounds **12-16** with appropriate 2-ethylthio (or phenylthio)-1-phenyl ethanone by the well-known Biuret reaction afforded 3-phenyl-2-ethylthio/phenylthio-quinoxaline 1,4-dioxides **29-51** (*15*). Compounds **29-51** were oxidized by different amounts of m-chloroperoxybenzoic acid (MCPBA) to produce target compounds 3-phenyl-2-ethylsulfinyl-quinoxaline 1,4-dioxides **52-54** or 3-phenyl-2-ethylsulfonyl/ phenyl sulfonyl-quinoxaline 1,4-dioxides **55-69**, respectively. All of the prepared compounds were confirmed by spectral data including IR, ^{1}H NMR, and MS (*16*).

X-ray analysis

In order to identify the structures of the class of compounds, the single-crystal structure of **55** was determined by X-ray crystallography as illustrated in Figure 1. In **55**, all H atoms were placed in geometrically idealized positions. The quinoxaline 1,4-dioxide system is almost planar. The quinoxaline 1,4-dioxide and phenyl planes are approximately perpendicular, with a dihedral angle of 85.8.

Biological evaluation

All of the prepared compounds were evaluated for their cytotoxic activity *in vitro* on human leukaemia cell line



29 R_1=R_2=H, R_3=CH_2CH_3;
30 R_1=H, R_2=F, R_3=CH_2CH_3;
31 R_1=H, R_2=Cl, R_3=CH_2CH_3;
32 R_1=H, R_2=Br, R_3=CH_2CH_3;
33 R_1=H, R_2=CH_3, R_3=CH_2CH_3;
34 R_1=H, R_2=OCH_3, R_3=CH_2CH_3;
35 R_1=Cl, R_2=H, R_3=CH_2CH_3;
36 R_1=Cl, R_2=F, R_3=CH_2CH_3;
37 R_1=Cl, R_2=Cl, R_3=CH_2CH_3;
38 R_1=Cl, R_2=Br, R_3=CH_2CH_3;
39 R_1=CH_3, R_2=H, R_3=CH_2CH_3;
40 R_1=CH_3, R_2=F, R_3=CH_2CH_3;
41 R_1=CH_3, R_2=Cl, R_3=CH_2CH_3;
42 R_1=CH_3, R_2=Br, R_3=CH_2CH_3;
43 R_1=R_2=H, R_3=Ph;
44 R_1=Cl, R_2=H, R_3=Ph;
45 R_1=CH_3, R_2=H, R_3=Ph;
46 R_1=H, R_2=Cl, R_3=Ph;
47 R_1=Cl, R_2=Cl, R_3=Ph;
48 R_1=CH_3, R_2=Cl, R_3=Ph;
49 R_1=H, R_2=Br, R_3=Ph;
50 R_1=Cl, R_2=Br, R_3=Ph;
51 R_1=CH_3, R_2=Br, R_3=Ph;
52 R_1=R_2=H, R_3=CH_2CH_3;
53 R_1=Cl, R_2=H, R_3=CH_2CH_3;
54 R_1=CH_3, R_2=H, R_3=CH_2CH_3;
55 R_1=R_2=H, R_3=CH_2CH_3;
56 R_1=Cl, R_2=H, R_3=CH_2CH_3;
57 R_1=CH_3, R_2=H, R_3=CH_2CH_3;
58 R_1=OCH_3, R_2=H, R_3=CH_2CH_3;
59 R_1=H, R_2=F, R_3=CH_2CH_3;
60 R_1=Cl, R_2=F, R_3=CH_2CH_3;
61 R_1=CH_3, R_2=F, R_3=CH_2CH_3;
62 R_1=H, R_2=Cl, R_3=CH_2CH_3;
63 R_1=Cl, R_2=Cl, R_3=CH_2CH_3;
64 R_1=CH_3, R_2=Cl, R_3=CH_2CH_3;
65 R_1=H, R_2=Br, R_3=CH_2CH_3;
66 R_1=Cl, R_2=Br, R_3=CH_2CH_3;
67 R_1=CH_3, R_2=Br, R_3=CH_2CH_3;
68 R_1=H, R_2=CH_3, R_3=CH_2CH_3;
69 R_1=R_2=H, R_3=Ph

Scheme 1. The synthetic route of the compounds **29–69**. Reagents and conditions: (a) K_2CO_3, THF, reflux, 12 h; (b) NH_3; (c) 2.0 equiv of MCPBA, Chloroform; (d) 4.0 equiv of MCPBA, Chloroform.

HL-60, human esophagus cancer cell line ECA-109, human prostate cancer cell PC-3, human gastric-carcinoma cell line SGC-7901, and human breast cancer cell line MCF-7 in hypoxia and in normoxia according to reported methods (*17*). The IC_{50} values of the tested compounds in normoxia and in hypoxia are summarized in Table 1.

Results and Discussion

SAR studies

As shown in Table 1, half of the tested compounds displayed higher cytotoxic activity on all tested cancer cell lines than the reference drug both in hypoxia and in normoxia. Obviously, the cytotoxic potency of tested compounds on these five cancer cell lines was highly dependent on structures of the 2-position side chains. When 2-position was occupied by an ethylthio group (*e.g.*, **29-42**) or phenylthio group (*e.g.*, **43-51**),

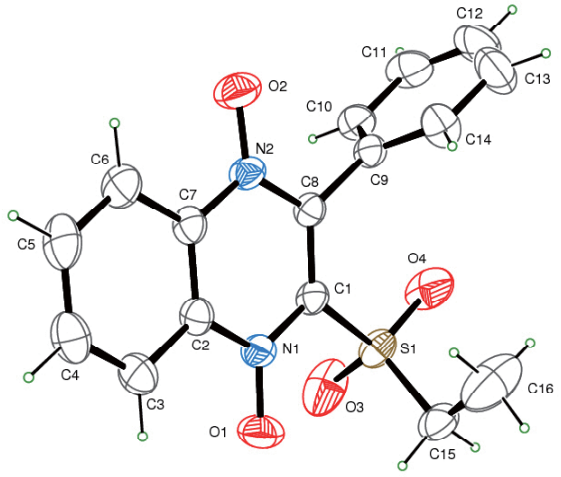


Figure 1. Single-crystal structure of **55**.

the compound showed almost no or weak activity. For the 2-phenylthio series, the substituents both at the 5-position of quinoxaline and on the 3-benzene ring can slightly affect cytotoxic activity. Compounds with electron-withdrawing substituents (**44, 46, 47, 50**) such as fluorine, chlorine, and bromine showed higher activity than those with electron-donating substituents (**45**). On the other hand, 3-phenyl-2-ethylsulfinyl/ethylsulfonyl/phenyl-sulfonyl-quinoxaline 1,4-dioxides (**52-69**) exhibited impressive cytotoxic activity on most tested cancer cell lines. Most traditional antitumor agents are useless on hypoxic cells, while the current class of compounds showed higher activity both in hypoxia and in normoxia may thus be effective agents in tumor therapy.

Mechanism studies

Further study of the mechanisms of cytotoxic activity

Table 1. Cytotoxicity of quinoxaline 1,4-di-N-oxides derivatives (**29-69**) on five human cancer cell lines in hypoxia and in normoxia *in vitro*

Comd.	Cytotoxicity (IC_{50}, μM)[a]									
	K562		Eca109		SGC7901		PC3		SMMC7721	
	H[b]	N[c]	H	N	H	N	H	N	H	N
29	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
30	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
31	>50	>50	>50	>50	>50	>50	>50	44.9	>50	>50
32	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
33	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
34	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
35	18.6	>50	>50	>50	>50	>50	>50	>50	>50	>50
36	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
37	6.5	27.8	11.9	8.8	43.1	29.1	39.6	34.8	>50	>50
38	10.0	>50	>50	30.6	38.3	19.6	>50	>50	34.9	>50
39	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
40	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
41	>50	>50	>50	>50	>50	>50	>50	>50	>50	40.4
42	>50	38.5	>50	>50	>50	>50	46.7	>50	>50	>50
43	47.6	23.2	>50	>50	>50	>50	46.1	4.6	>50	>50
44	12.9	3.5	18.8	38.2	35.5	33.2	18.0	>50	>50	28.2
45	>50	45.8	>50	>50	>50	>50	>50	>50	>50	>50
46	15.0	14.9	26.2	19.9	>50	35.9	40.0	43.6	33.1	38.9
47	4.7	3.9	13.5	2.6	14.2	12.5	4.9	4.3	21.4	9.9
48	12.7	0.5	>50	43.4	>50	32.4	>50	16.1	35.9	>50
49	5.0	10.0	>50	>50	>50	34.2	29.3	23.0	31.4	38.7
50	3.0	3.2	23.9	31.8	27.9	18.3	39.0	42.3	>50	>50
51	6.2	6.5	26.0	49.4	41.8	11.3	31.1	15.5	>50	19.0
52	1.6	0.6	8.6	23.3	5.8	5.3	7.0	6.2	31.3	19.3
53	1.2	0.3	6.0	7.6	3.1	1.7	3.4	2.4	8.8	3.6
54	1.2	1.3	10.9	14.1	13.0	22.7	7.0	11.4	5.3	4.2
55	1.3	1.0	4.7	9.7	6.5	11.8	2.5	5.1	>50	29.2
56	1.5	0.6	3.4	6.4	2.9	1.3	8.4	4.1	4.7	5.9
57	1.8	1.2	5.2	6.8	6.9	8.2	4.3	2.3	13.5	6.3
58	1.6	1.3	9.6	3.3	5.2	5.5	6.8	3.8	18.5	6.0
59	1.1	1.3	7.0	9.5	8.8	2.9	0.9	1.0	1.6	0.7
60	3.2	0.1	5.2	5.0	2.2	4.9	0.7	0.8	2.6	3.7
61	1.0	1.4	10.6	4.4	5.1	6.2	4.4	1.8	12.0	7.5
62	1.8	0.5	9.7	7.9	7.9	9.7	0.7	5.3	11.9	5.0
63	3.7	4.7	4.9	3.1	1.8	1.4	1.6	>50	2.6	3.1
64	3.8	4.2	4.0	1.4	4.5	2.9	0.5	3.2	10.2	12.2
65	0.8	2.5	1.8	1.2	3.1	0.6	13.3	3.1	2.2	4.6
66	0.8	0.6	6.2	2.2	1.5	1.5	13.8	1.0	0.1	16.1
67	0.2	0.3	6.4	0.5	8.9	6.8	5.0	8.7	2.2	3.5
68	1.9	0.7	7.1	3.1	3.6	20.8	13.9	1.1	8.1	8.3
69	1.2	0.5	4.7	4.4	3.4	9.4	7.6	1.9	14.4	16.3

[a]Each experiment was independently performed three times; [b]H=Hypoxia: 3% oxygen; [c]N=Normoxia: 20% oxygen

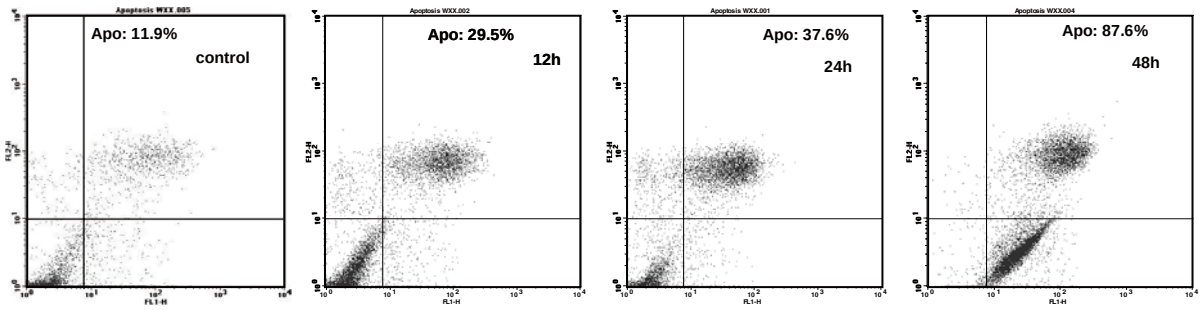


Figure 2. Compound **67** induced apoptosis in K562 cells. K562 cells were treated with 6.0 μM **67** in hypoxia for 0, 12, 24 and 48 h. Apoptosis was assessed by Annexin V-FITC/Propidium iodide (PI) staining.

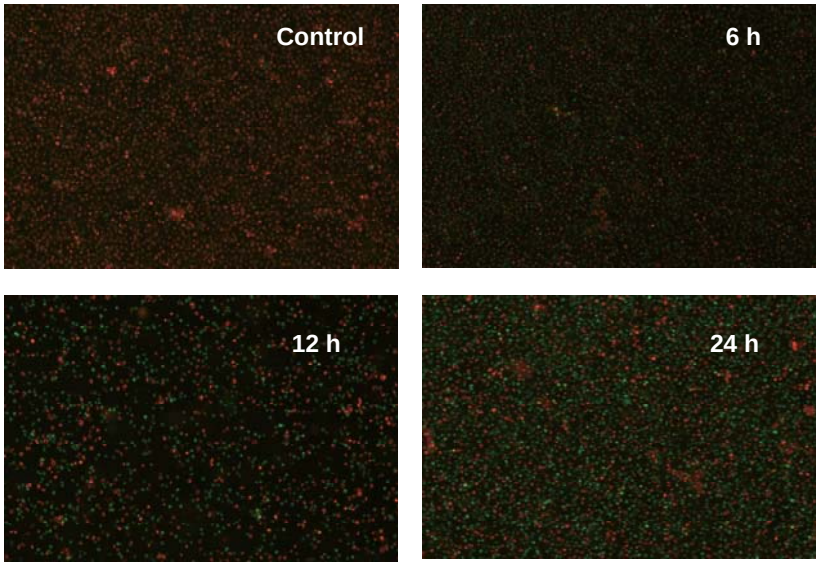


Figure 3. Compound **67** induced ΔΨm loss in K562 cells. K562 cells were treated with 6.0 μM **67** in hypoxia for 0, 6, 12 and 24 h. ΔΨm loss was assessed by JC-1 staining in which mitochondria depolarization is indicated by an increase in the green-to-red fluorescence intensity ratio.

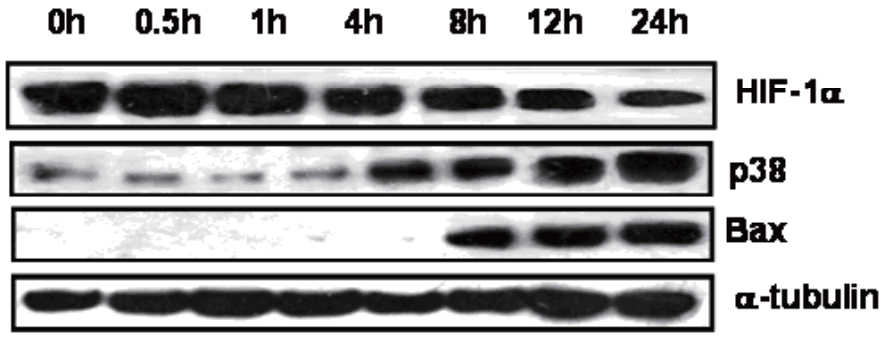


Figure 4. Protein expressions of HIF-1α, P38, and Bax in K562 cells treated with 6.0 μM compound **67** in hypoxia for 0.5, 1, 4, 8, 12 and 24 h. Each lane was loaded with 40 μg of protein.

in hypoxia was performed with one potent compound (**67**) in K562 cells. K562 cells were treated with tested compound for 0 h, 6 h, 12 h or 24 h respectively, and then apoptosis, the mitochondrial membrane potential (ΔΨm), and protein expression were determined according to reported methods (*17*). The results are shown in Figures 2-4. All experiments were repeated three times.

K562 cells were cultured in complete medium with 6.0 μM **67** for 0-48 h in 3% O_2. Every six hours the cells were collected and the apoptotic percentage was analyzed by flow cytometry (Figure 2). As shown in Figure 2, an apoptotic phenomenon was observed at 12

h after cells were exposed to **67**. After K562 cells were incubated with **67** for 0, 12, 24 and 48 h, the percentage of apoptotic cells was 11.9%, 29.5%, 37.6% and 87.6%, respectively. This increase occurred in a time-dependent manner, indicating that the apoptotic pathway was involved in the mechanisms of compound **67**-mediated cytotoxic activity.

To investigate the pathway of apoptosis induced by tested compounds, ΔΨm loss and protein expression of HIF-1α, Bax, and P38 in K562 cells treated with 6.0 μM 67 for 0-24 h in hypoxia were determined. With JC-1 staining, mitochondria depolarization is specifically indicated by a fluorescence emission shift from red to green. ΔΨm loss in K562 cells was reduced by **67** after 0-24 h treatment; corresponding data are shown in Figure 2. Compared to the control, K562 cells treated with **67** exhibited a mass of green fluorescence, suggesting that **67** might possess highly potent cytotoxic activity *via* a mitochondrial pathway.

P38, Bax, and HIF-1α were the key proteins involved in cell apoptosis and DNA damage. The expression of P38, Bax, and HIF-1α in K562 cells treated with **67** was performed by Western blot analysis. As shown in Figure 3, 6.0 μM **67** increased P38 and Bax levels in K562 cells after 24 h of exposure in hypoxia and reduced the HIF-1α protein level. The data obtained confirmed that higher cytotoxicity of **67** was related to the P38 and Bax-mediated apoptosis pathway.

Conclusions

In summary, a new series of novel 2-substituted-phenyl-3-ethylthio/ethylsulfinyl/ethyl sulfonyl/phenylthio/phenylsulfonyl-quinoxaline 1,4-dioxides were synthesized and screened for their antitumor activity *in vitro* on five cancer cell lines in hypoxia and in normoxia. Half of the tested compounds showed higher antitumor activity both in hypoxia and in normoxia. When treated with compound **67**, K562 cells exhibited overexpression of P38 and Bax, and K562 cells also induced down-regulation of HIF-1α, suggesting modulation of protein and mitochondria pathways involved in the anti-cancer activity of compound **67**.

Acknowledgments

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16. All compounds gave satisfactory spectroscopic data in accordance with their proposed structures. Selected data are as follows: compound **67** Mp: 209-211°C; MS (ESI): 425 (M+H); ^{1}H NMR ($CDCl_3$) δ: 8.51 (d, 1H, *J* = 8.8 Hz, Ar-H), 8.44 (s, 1H, Ar-H), 7.79 (d, 1H, *J* = 8.8 Hz, Ar-H), 7.68 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.28 (d, 2H, *J* = 8.4 Hz, Ar-H), 3.78~3.83 (m, 2H, CH_2), 2.67 (s, 3H, CH_3), 1.33 (t, 3H, *J* = 7.6 Hz, CH_3); IR (KBr): 3082, 1610, 1492, 1457, 1395, 1312, 1219, 1182, 1128, 1089, 1012, 935, 916, 843, 781, 760, 716.
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