Synthesis and Biological Evaluation of New 2-Arylcarbonyl-3-trifluoromethylquinoxaline 1,4-Di-*N*-oxide Derivatives and Their Reduced Analogues

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As a continuation of our research in the quinoxaline 1,4-di-*N*-oxide new series of 2-arylcarbonyl-3-trifluoromethylquinoxaline, 1,4-di-*N*-oxide derivatives have been synthesized and evaluated in a full panel of 60 human tumor cell lines. Selective reductions were carried out on two compounds which allowed us to determine the compound structures by comparison of the ^{1}H NMR spectra. In general, all the di-*N*-oxidized compounds showed good cytotoxic parameters. The best activity was observed in derivatives with electron-withdrawing groups in position 6 or 7 on the quinoxaline ring and in the unsubstituted analogues, whereas loss of one or two oxygens reduced the cytotoxicity. The best five compounds were selected for evaluation for the in vivo hollow fiber assays. In vitro studies reveal that compound **5h** efficiently generates reactive oxygen species via redox cycling in the presence of the NADPH/cytochrome P450 enzyme system, providing a plausible molecular mechanism for the observed aerobic cytotoxicity of these quinoxaline *N*-oxides.

Introduction

Quinoxaline derivatives are a class of substances possessing a broad spectrum of pharmacological activities, such as antibacterial, antiviral, anticancer, antifungical, antihelmintic, and insecticidal.[1] Because of a strong interest in the biological activities associated with these compounds, the synthesis of new quinoxaline derivatives have been performed by our group.[2−5] Some of these derivatives showed good in vitro anticancer activity as well as efficient cytotoxicity against hypoxic cells in solid tumors.[6−10]

Recently, our research group has reported in vitro anticancer activity of 3-trifluoromethyl-2-carbonylquinoxaline di-*N*-oxide derivatives, leading to the identification of 2-benzoyl-6,7-difluoro-3-trifluoromethylquinozaline 1,4-di-*N*-oxide (Figure 1), with a GI_{50}[a] mean value of 0.15 in the in vitro inhibitory assay against 60 human tumor cells lines, as a lead compound.[10] Unfortunately, the compound did not show significant cytotoxicity in the in vivo hollow fiber assay performed by the National Cancer Institute (NCI). Nonetheless, these studies highlighted two characteristics of 2-carbonylquinoxaline 1,4-di-*N*-oxide derivatives with in vitro activity against cancer cell lines: first, electron withdrawing groups in positions 6 or/and 7 or their unsubstituted analogues showed an excellent activity; second, the cytotoxic activity of the compounds was influenced by the nature of the group directly joined to the carbonyl group; thus, the derivatives containing a phenyl group showed better activity than those with alkyl chains.[10] To improve the previous antitumoral activity, we explored the influence of different substituents in position 6,7 of the quinoxaline 1,4-di-*N*-oxide ring as well as the importance of the aryl group attached to the 2-carbonyl position. Moreover, some initial assays were carried out to evaluate the in vitro DNA damage caused by these compounds.

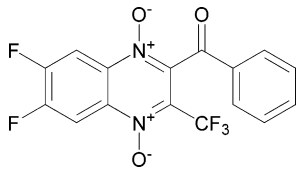

Figure 1. 2-Benzoyl-6,7-difluoro-3-trifluoromethylquinoxaline 1,4-di-*N*-oxide.

Chemistry. Phenyl, 2-naphthyl, 2-thienyl, and 2-furyl carbonyl-3-trifluoromethyl-quinoxaline 1,4-di-*N*-oxide derivatives were prepared according to the synthetic process illustrated in Scheme 1.[11] The starting compounds, 5-substituted or 5,6-disubstituted benzofuroxanes were obtained by previously described methods.[6]

As previously described,[10] the formation of isomeric quinoxaline 1,4-di-*N*-oxides was observed in the case of monosubstituted benzofuroxanes. In most cases, it was observed that one of the isomers predominated, and workup and purification of the reaction mixture afforded the major isomer. Only one isomer is formed in the case of a methoxy substituent.[4] When the Beirut reaction was carried out with 5-trifluoromethylbenzofuroxan (**2i**) and the corresponding 4,4,4-trifluoro-1-(2-aryl)-1,3-butanedione a mixture of positional isomers was obtained in a ratio of 35:65. In these cases, both isomers were isolated and purified by flash chromatography.

To further characterize these materials, compounds **6i** and **6j** were mono- and di-reduced as shown in Scheme 2. Selective monodeoxygenations were performed on these compounds, as a model of the series. As described by others,[12,13] the trimethyl phosphite in refluxing alcohol appears to be useful for selective deoxygenation at the nitrogen adjacent to carbon bearing an electron-withdrawing group in quinoxaline 1,4-di-*N*-oxide.

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[a] Abbreviations: NCI, National Cancer Institute; BEC, Biological Evaluation Committee for Cancer Drugs; MG_MID, mean graph midpoints; SOD, superoxide dismutase; CYP P450, cytochrome P450; GI_{50}, 50% growth-inhibitory concentration; TGI, total inhibition concentration; LC_{50}, 50% lethal concentration; IP, intraperitoneal; SC, subcutaneous.

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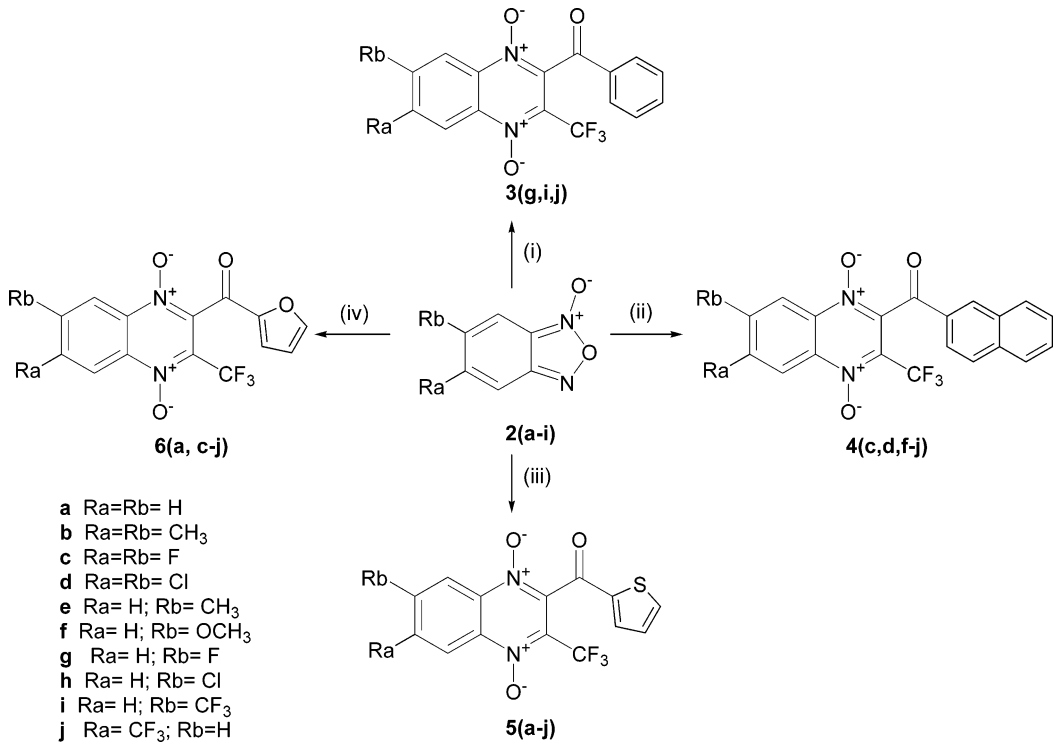
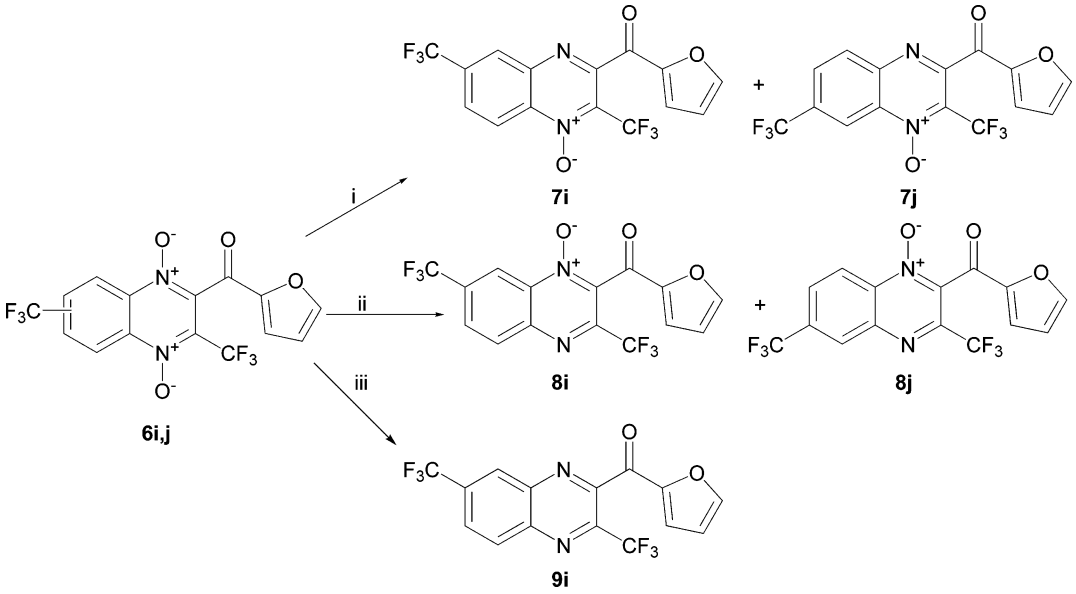
Scheme 1. Synthesis of 2-Arylcarbonyl-3-trifluoromethylquinoxaline 1,4-Di-*N*-oxide Derivatives **3g**,**h**,**i**, **4c**,**d**,**f**−**j**, **5a**−**j** and **6a**,**c**−**j**[a]



[a] Conditions at room temperature: (i) 4,4,4-trifluoro-1-phenyl-1,3-butanedione, $CHCl_3$, EtN_3; (ii) 4,4,4-trifluoro-1-(2-naphthyl)-1,3-butanedione, $CHCl_3$, EtN_3; (iii) 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione, $CHCl_3$, EtN_3; (iv) 4,4,4-trifluoro-1-(2-furyl)-1,3-butanedione, $CHCl_3$, EtN_3.

Scheme 2. Reduction Methods Used for the Synthesis of the Mono- and Di-reduced Derivatives[a]



[a] Conditions: (i) $P(OCH_3)_3$ PrOH, reflux; (ii) refluxed in methanol solution; (iii) methanol 70 °C, $Na_2S_2O_4$.

Although these compounds have an electron-withdrawing group adjacent to *N*-1 and *N*-4, fortunately, a single product was obtained in each case, with the *N*-oxide to the carbon bearing the carbonyl group (*N*-1-oxide) undergoing selective reduction. In contrast, when 1,4-di-*N*-oxide compounds were refluxed in methanol solution only *N*-4-oxide was reduced. These selectively monoreductions led us to the determination of the compound structures by comparison of the di-*N*-oxide and the monoreduced ^{1}H NMR spectra.[12]

The trends observed in the chemical shifts in the quinoxaline dioxides are those expected for the shielding effects resulting from oxidation at *N*-1 or *N*-4. Comparison of the corresponding monoxide and dioxide pairs shows that the proton next to the nitrogen has experienced downfield shifts in the monoxide. The signal that experiences displacement in the spectrum, when compound **6i** was reduced with trimethyl phosphite in refluxing alcohol to give compound **7i**, was the singlet. This resonance was shifted downfield to a value of δ 8.52, markedly different from the value of δ 8.91 found in the 1,4-dioxide (**6i**). However, when the reaction was carried out with compound **6j** the displacement was observed in the doublet (δ 8.75 for **6j)** of the corresponding derivative **7j** (δ 8.35). On the other hand, when the reactions were carried out in refluxing methanol the shifted signal was the doublet corresponding to H-5 proton, compound

Table 1. Gi_{50},[a] Tgi,[b] and Lc_{50}[c] μM Mean Graph Midpoints (MG_MID)[d] of the in Vitro Inhibitory Activity Test for Compounds against 60 Human Tumor Cells Lines[e]

compd	GI_{50}	TGI	LC_{50}	compd	GI_{50}	TGI	LC_{50}
3g	0.78	2.51	9.12	**5i**	0.72	1.91	5.50
3i	0.51	1.23	3.89	**5j**	0.18	0.47	1.48
3j	1.29	2.95	8.32	**6a**	0.41	5.13	26.92
4c	2.19	6.46	23.44	**6c**	0.38	1.48	7.76
4d	1.58	3.16	6.17	**6d**	0.63	3.09	10.96
4g	1.15	2.82	8.32	**6e**	0.66	3.47	17.78
4h	1.23	2.63	5.50	**6f**	2.82	18.62	74.13
4i	0.72	1.91	5.50	**6g**	1.62	4.27	15.85
4j	1.58	3.47	9.33	**6h**	0.32	2.57	10.00
5a	0.98	3.89	11.22	**6i**	0.60	1.48	5.01
5b	3.47	17.38	61.66	**6j**	0.47	1.20	3.89
5c	0.37	1.62	9.33	**7i**	45.71	97.72	>100
5d	0.74	3.02	10.96	**7j**	47.86	95.50	>100
5e	0.55	5.50	33.11	**8i**	37.15	83.18	>100
5f	5.01	29.51	48.98	**8j**	46.77	89.13	>100
5g	1.20	2.88	9.33	**9i**	87.10	97.72	>100
5h	0.07	0.50	2.19				

[a] GI_{50} growth inhibition activity. [b] TGI cytostatic activity. [c] LC_{50} cytotoxic activity. [d] (MG_MID) means graph midpoints, the average sensitivity of all of the cell lines toward the test agent. [e] From the NCI.

6i versus **8i** ($\Delta\delta = 0.38$), and the singlet on **6j** (δ 8.99) versus **8j** of the proton H-5 (δ 8.65). These results indicate that the position of the trifluoromethyl group is 7 for the derivatives of series **i** and 6 on series **j.** The 3,7-bis-trifluoromethyl-2-furylcarbonylquinoxaline compound (**9i**) was obtained by heating to 70 °C a solution of the di-*N*-oxide derivative and adding $Na_2S_2O_4$.[14] The identity of the mono-*N*-oxide derivatives was further confirmed by preparation of the fully reduced quinoxaline derivative (**9i**) for which the H-8 and H-5 proton resonances were observed at δ 8.59 and δ 8.48, respectively.

Results and Discussion

Cytotoxic Activity. The compounds **3g**,**i**,**j**, **4c**,**d**,**f**−**j**, **5a**−**j**, and **6a**,**c**−**j**, **7i**,**j**, **8i**,**j**, **9i** (Table 1) were selected by the National Cancer Institute (NCI, Bethesda) for an anticancer evaluation which was performed following the in vitro disease-oriented antitumor screening program.[15] The compounds were evaluated in the three-cell line one dose primary anticancer assay consisting of MCF7 (breast), NCI-H460 (lung), and SF-268 (CNS). The compounds, which passed the criteria set by the NCI for activity in this assay, were automatically scheduled for evaluation against the full panel of 60 tumor cell lines representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney.[16] The results of the active compounds were referred to the Biological Evaluation Committee for Cancer Drugs (BEC), and the compounds selected as leads from the large scale in vitro cell line screening were referred for preliminary testing in the hollow fiber-based screening.

All of the compounds analyzed in the one-dose primary screening in three cell lines passed the criteria set by the NCI for activity in the primary anticancer assay except 7-methoxy 2-naphthylcarbonyl derivative (**4f**). This compound showed a reduction of percentage of growth less than 32% in the three-cell line panel. Although compound **6g** had low inhibition capacity in two of the three cell lines, 72 and 82 percentage of growth in MCF7 and SF-268, respectively, the compound was selected for further screening assays. Some compounds were directly evaluated in 60 cell lines.

Mean GI_{50}, TGI, and LC_{50} values on midpoints graph of **3g**,**i**,**j**, **4c**,**d**,**g**−**j**, **5a**−**j**, **6a**,**c**−**j**, **7i**,**j**, **8i**,**j**, and **9i** compounds against 60 human tumor cell lines are summarized in Table 1. In general, all of the di-*N*-oxidized compounds showed good cytotoxic parameters with mean GI_{50} between 5.01 and 0.07 μM. The activity seems to be influenced when the phenyl group,[10] found in compounds **3 g**,**i**−**j**, is replaced by a naphthyl group to yield compounds **4**, whose mean GI_{50} values were greater than 0.72 μM, worse than the activity showed by the phenyl derivatives, Table 1. However the cytotoxic activity is preserved when the phenyl moiety is replaced by various heteroaromatic groups. Within these derivatives the best activity was observed in those with electron-withdrawing groups in one of both positions 6 and 7 on the quinoxaline ring and in the unsubstituted analogues.

In regards to the compounds having an additional trifluoromethyl rest in the quinoxaline ring, the activity appeared to be influenced by position 6 or 7 of this group when an aromatic ring (phenyl and naphthyl group) is introduced in position 2 (see compounds **3i** vs **3j** and **4i** vs **4j**, Table 1), whereas the cytotoxicity of compounds with an heteroaromatic ring in position 2 was less influenced by the position of the trifluoromethyl group (compounds **5i**, **5j**, **6i**, and **6j**).

The loss of one or two oxygens of the 1,4-di-*N*-oxide quinoxalines gave compounds (**7i**,**j**, **8i**,**j**, **9i**) with reduced activity, the mean GI_{50} being 37.15−87.10 μM.

Table 2 shows the in vitro anticancer mean data recorded on each subgroup of cancer for these compounds. On average, 18 out of 28 di-*N*-oxidized compounds showed the best cytotoxic activity against leukemia lines. This patron could not be observed among the mono- or di-reduced compounds. It is worth noting the activity of the compounds **5d** and **5j** in the leukemia cell lines. Derivative **5d** showed GI_{50} values less than 0.01 μM in CCRF-CEM, K-562, MOLT-4, and SR cell lines; **5j** compound gave GI_{50} values of 0.07, 0.02, and 0.02 μM in CCRF-CEM, MOLT-4, and SR cell lines, respectively, and less than 0.01 μM in HL-60. On the other hand, nonsmall lung cells were more resistant to the cytotoxic effect of these compounds.

In Vivo Activity. Some of the most active compounds were referred to the BEC to be analyzed in hollow to fiber screening. The hollow fiber assay is used as the initial in vivo experience for compounds having a reproducible activity in the in vitro anticancer drug screen and provides quantitative indices of drug efficacy.[17] The results are summarized in Table 3. Compounds with a combined ip + sc score ≥20, a sc score ≥8, or a net cell kill of one or more cell lines were considered active. Although none of the compounds showed a total score ≥20, compounds **5c** and **6d** displayed a sc score of 10 when they were tested against leukemia and lymphoma cell lines, and **5h** derivative showed a sc score of 8 against the complete cell-line panel. Moreover, compounds **5c** and **5h** killed net cell of one or more cell lines, therefore, these compounds can be evaluated for further in vivo testing. The furyl derivative **6h** was able to kill cells at a concentration of 18 mg/kg/dose, whereas it was not able to kill cells at lower concentrations. Derivative **5d** has the highest total score, but it did not have the required values in this assay for it to be considered an active derivative by NCI. The thienyl derivatives (**5c**, **5h**) that combine a sc score ≥8 and kill cell lines have the best activity.

In Vitro DNA Damage. Several structural classes of heterocyclic *N*-oxides are known to derive anticancer properties through their abilities to cause DNA damage. For example, a number of heterocyclic *N*-oxides have been examined for their ability to selectively kill oxygen-poor (hypoxic) tumor cells.[6,18−25] However, because the cell culture experiments reported here were conducted under standard aerobic conditions, we are primarily concerned with considering potential chemical mechanisms underlying the cytotoxicity of *N*-oxides under aerobic

Table 2. Cytotoxic Activities (mean Gi_{50} μM Inhibition Cell Growth) against Tumoral Subgroup Cell Lines of Quinoxaline Derivatives

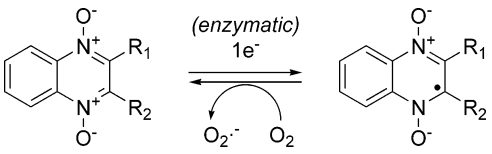
compd	leukemia	nonsmall lung	colon	CNS	melanoma	ovarian	renal	prostate	breast
3g	0.23	0.80	0.54	1.45	0.95	1.34	1.14	0.53	0.73
3i	0.24	0.64	0.46	0.69	1.13	0.58	0.33	0.27	0.54
3j	0.77	1.66	1.04	1.71	1.23	1.18	1.47	1.32	1.41
4c	0.77	3.47	1.71	2.51	2.50	3.80	2.18	1.46	2.36
4d	1.58	1.70	1.55	1.63	1.57	1.51	1.64	1.55	1.62
4g	0.44	1.54	0.74	1.61	1.12	1.20	1.48	1.62	1.70
4h	0.65	1.51	1.29	1.47	1.23	1.20	1.38	1.48	1.15
4i	0.26	1.42	0.66	0.95	0.97	0.47	0.58	0.57	0.99
4j	1.20	1.78	1.73	1.72	1.48	1.69	1.55	1.51	1.67
5a	13.93	0.35	0.83	1.45	1.39	0.75	0.48	0.40	0.85
5b	2.71	2.81	5.31	3.05	3.99	2.34	4.62	3.89	3.08
5c	0.05	0.83	0.27	0.44	0.39	0.53	0.50	0.51	0.41
5d	0.02	2.04	0.59	0.67	0.94	1.16	1.60	0.79	0.79
5e	0.49	0.56	0.88	0.75	0.90	0.27	0.25	0.89	0.56
5f	4.43	3.93	7.08	6.24	5.89	4.99	5.11	2.48	4.92
5g	0.27	1.68	0.92	1.63	1.51	1.17	1.65	1.51	1.19
5h	0.07	0.07	0.04	0.09	0.12	0.16	0.03		0.08
5i	0.20	0.45	0.38	0.48	0.90	0.43	0.31	0.20	0.50
5j	0.03	0.30	0.15	0.18	0.33	0.20	0.24	0.15	0.18
6a	0.07	0.35	0.51	0.40	0.67	1.45	0.33	0.20	0.76
6c	0.43	0.73	0.19	0.37	0.56	0.39	0.36	0.42	0.20
6d	0.27	0.28	0.50	0.79	1.57	1.65	0.31	0.33	1.38
6e	0.26	0.47	1.17	0.74	0.88	0.85	0.58	0.24	0.66
6e	3.96	3.49	2.51	3.20	3.03	2.51	1.74	3.16	2.88
6g	0.30	3.13	1.06	2.35	1.56	2.03	2.41	2.60	1.33
6h	0.19	0.36	0.21	0.77	0.49	0.56	0.20	0.11	0.28
6i	0.21	0.76	0.57	0.91	1.11	0.72	0.43	0.42	0.57
6j	0.13	0.48	0.28	0.81	0.86	0.65	0.49	0.19	0.48
7i	51.09	48.14	41.41	45.53	42.90	42.49	60.60	51.88	32.04
7j	48.42	41.93	75.11	35.48	46.11	42.99	49.69	35.08	59.11
8i	88.10	39.41	49.79	25.31	31.52	29.29	28.59	38.46	34.33
8j	43.65	47.74	85.39	24.45	80.58	40.43	34.57	44.67	38.52
9i	53.95	81.52	100.00	75.86	100.00	90.85	100.00	100.00	84.00

Table 3. Activity in the in Vivo Hollow Fiber Assay

compd	concentration (mg/kg/dose)	total score	IP score	SC score	cell kill
5c	4.5	8	4	4	no
	2.4[a]	14	4	10	yes
5d	18	16	12	4	no
5h	4.5	14	6	8	yes
6d	6	12	10	2	no
	6[a]	14	4	10	no
6h	18	14	8	6	yes
	5.5	10	4	6	no
	5[a]	8	2	6	no

[a] The testing results reported represent the activity against six-cell line panel consisting of leukemia and lymphoma cell lines.

Scheme 3. Enzyme-Driven Redox Cycling by Quinoxaline 1,4-Dioxides



conditions. In this regard, it is noteworthy that triazine, quinoxaline, and phenazine *N*-oxides can undergo efficient redox cycling under aerobic conditions.[6,18−29] This process involves enzymatic one-electron reduction of the heterocycle, followed by aerobic back-oxidation of the resulting radical to generate superoxide radical and the starting drug molecule (Scheme 3).[18,30] Under physiological conditions, a superoxide radical decomposes to yield hydrogen peroxide and a hydroxyl radical as shown in the (unbalanced) eq 1, where M represents a transition metal.[31] In general, intracellular generation of bx;1superoxide radical causes a host of deleterious effects including oxidative damage to DNA, proteins, and lipids and cytotoxicity.[31−38] In the context of the studies described here, it is important to note that the hydroxyl radical is the actual agent responsible for spontaneous strand cleavage induced by superoxide.[31,39] Interestingly, there is a growing belief that some cancer cell lines may succumb more easily to oxidant-induced stress than do normal cells.[40]

$$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 + M^{n+} \rightarrow HO^{\bullet} + M^{(n+1)+} \qquad (1)$$

Here we made use of a plasmid-based DNA strand cleavage assay to assess the ability of the quinoxaline di-*N*-oxide **5h** to generate reactive oxygen species via enzyme-driven redox cycling under aerobic conditions. This assay capitalizes on the fact that oxidative DNA strand cleavage causes conversion of the supercoiled plasmid to the open circular form.[41−44] These two forms of plasmid DNA are easily separated and quantitated using agarose gel electrophoresis, followed by staining with DNA-binding dyes such as ethidium bromide. We employed the NADPH/cytochrome P450 reductase enzyme system to carry out one-electron reduction of the heterocyclic *N*-oxide. NADPH/cytochrome P450 reductase is important in the in vivo reduction of heterocyclic *N*-oxides[45−47] and has been used successfully as a reagent for the one-electron reduction of *N*-oxides in a variety of in vitro studies.[20−22,30,47−49]

We observe that **5h** generates efficient single-strand cleavage that is dependent upon the presence of NADPH and the enzyme NADPH/cytochrome P450 reductase (Table 4). To further investigate the chemical mechanism of this strand cleavage process, a series of reactions were carried out in the presence of various additives. Specifically, the effect of agents that interact with various species shown in eq 1 was examined. Superoxide dismutase (SOD) destroys superoxide radical, catalase destroys hydrogen peroxide, the trace metal chelator desferal

Table 4. Effect of Additives on DNA Cleavage by Compound **5h**[a]

reaction/additive	*S*-value[b]
5h (25 μM, no enzyme)	0.03 ± 0.02
CYP P450 reductase/NADPH	0.05 ± 0.03
standard cleavage reacn: 403.02 + enzyme system[a]	0.45 ± 0.03
standard cleavage reacn + additive:	
SOD (10 μg/mL)	0.16 ± 0.04
catalase (100 μg/mL)	0.18 ± 0.05
desferal (1 mM)	0.11 ± 0.05
methanol (500 mM)	0.09 ± 0.00
ethanol (500 mM)	0.05 ± 0.01
t-butanol (500 mM)	0.10 ± 0.00
DMSO (500 mM)	0.04 ± 0.00
mannitol (500 mM)	0.07 ± 0.00

[a] In a typical reaction, plasmid DNA (33 μg/mL), **5h** (25 μM), NADPH (500 μM), and NADPH/cytochrome P450 reductase (1 mU) were incubated in sodium phosphate buffer (50 mM, pH 7.0, containing 2.5% acetonitrile v/v) at 24 °C for 2 h. [b] The *S*-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation $S = -\ln f_I$, where f_I is the fraction of uncut, form I DNA remaining. Values are corrected to account for the small amount of nicked DNA in the untreated plasmid.

Table 5. Effect of Additives on DNA Cleavage by the Known Redox Cycling Agent, Menadione[a]

reaction/additive	*S*-value[b]
menadione (25 μM, no enzyme)	0.02 ± 0.01
CYP P450 reductase/NADPH	0.04 ± 0.00
standard cleavage reacnn:	0.55 ± 0.11
menadione + enzyme system[a]	
standard cleavage reacn + additive:	
SOD (10 μg/mL)	0.04 ± 0.00
catalase (100 μg/mL)	0.04 ± 0.03
desferal (1 mM)	0.06 ± 0.05
methanol (500 mM)	0.04 ± 0.03
ethanol (500 mM)	0.03 ± 0.01
t-butanol (500 mM)	0.11 ± 0.01
DMSO (500 mM)	0.01 ± 0.00
mannitol (500 mM)	0.04 ± 0.01

[a] In a typical reaction, plasmid DNA (33 μg/mL), menadione (25 μM), NADPH (500 μM), and NADPH/cytochrome P450 reductase (1 mU) were incubated in sodium phosphate buffer (50 mM, pH 7.0, containing 2.5% acetonitrile) at 24 °C for 2 h. [b] The *S*-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation $S = -\ln f_I$, where f_I is the fraction of uncut, form I DNA remaining. Values are corrected to account for the small amount of nicked DNA in the untreated plasmid.

sequesters trace metals in a redox-inactive form, and radical scavenging agents such as DMSO, methanol, ethanol, *t*-butanol, and mannitol quench hydroxyl radical (HO•).[31,50,51] As shown in Table 4, we find that DNA cleavage by **5h** is inhibited by the presence of SOD (64%), catalase (60%), desferal (76%), and radical scavengers (76−91%). The pattern of inhibition observed for these various additives is diagnostic for DNA cleavage arising from the cascade of reactions involving a superoxide radical, hydrogen peroxide, and a hydroxyl radical (eq 1), in which a hydroxyl radical is the ultimate DNA-cleaving agent.[31,52] The enzyme-driven DNA cleavage seen here for **5h** mirrors that generated by the archetypal biologically active, redox-cycling agent menadione which is known to generate superoxide radical following enzymatic reduction (Table 5).[53−57] Intracellular generation of superoxide radical is known to have cytotoxic consequences.[31−38] Thus, the redox-cycling properties observed here for compound **5h** provide a plausible chemical mechanism to explain the cytotoxic properties of the group of quinoxaline di-*N*-oxides reported here. Although the focus here is on the aerobic cytotoxicity, it is interesting to note that, under *anaerobic* conditions, **5h** causes DNA strand scission by an oxygen-independent mechanism that cannot be quenched by SOD or catalase, likely involving release of a hydroxyl radical or a functionally equivalent diffusible oxidant (data not shown), in a manner analogous to a number of other heterocyclic *N*-oxides.[6,18−25] This chemistry may also occur, to a certain extent, under aerobic conditions, thus accounting for the component of DNA strand cleavage that is quenched by radical scavengers but not quenched by SOD or catalase (Table 4).

Conclusions

We report here the cytotoxic activity of new 2-arylcarbonyl-3-trifluoromethylquinoxaline 1,4-di-*N*-oxide derivatives. The in vitro activity seems to be influenced by the aryl group introduced in position 2, now that the naphthyl derivatives showed GI_{50} values grater than phenyl derivatives. When a heteroaromatic group replaced the phenyl moiety the activity was preserved. The electron-withdrawing groups in position 6 and/or 7 on the quinoxaline ring and the unsubstituted analogues showed the best activity. When an additional trifluoromethyl group was added in the quinoxaline ring the Beirut reaction produced a mixture of positional isomers in a ratio of 35:65. These compounds were characterized by selective monodeoxygenations which led us to determine the compound structures by comparison of the ^{1}H NMR spectra. The additional trifluoromethyl moiety in position 6 or 7 produces an influence on the activity when a phenyl or naphthyl group is introduced in the carbonyl group. The loss of one or two oxygens of the 1,4-di-*N*-oxide quinoxalines gave compounds (**7i**,**j**, **8i**,**j**, **9i**) with reduced activity, the mean GI_{50} being 37.15−87.10 μM. In regards to the activity on each subgroup of cancer, the best cytotoxicity was shown against leukemia lines.

The hollow fiber assay used as the initial in vivo experience showed some positive activity for compounds **6d**, **5c**, and **5h**.

In vitro studies reveal that compound **5h** efficiently generates the reactive oxygen species via redox cycling in the presence of the NADPH/cytochrome P450 enzyme system. This provides a plausible molecular mechanism for the observed aerobic cytotoxicity of these quinoxaline *N*-oxides.

Experimental Section

General. The ^{1}H NMR spectra were recorded on a Bruker 400 Ultrashield (400 MHz) (Rheinstetten, Germany), using TMS as internal standard and $CDCl_3$ as solvent; the chemical shifts are reported in ppm (δ) and coupling constant (J) values are given in Hertz (Hz). Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quadruplet), dd (double doublet), and m (multiplet). The IR spectra were performed on Thermo Nicolet FT-IR Nexus Euro (Madison, WI) using KBr pellets; the frequencies are expressed in cm^{-1}. Elemental microanalyses were obtained on an Elemental Analyzer (LECO CHN-900, Michigan) from vacuum-dried samples. The analytical results for C, H, and N were within ±0.4 of the theoretical values.

Alugram SIL G/UV254 (layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG. Postfach 101352. D-52313 Düren, Germany) was used for thin-layer chromatography and silica gel 60 (0.040−0.063 mm) for column flash chromatography (Merck).

Chemicals were purchased from E. Merck (Darmstadt, Germany), Scharlau (F.E.R.O.S.A., Barcelona, Spain), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma-Aldrich Química, S.A. (Alcobendas, Madrid), Acros Organics (Janssen Pharmaceuticalaan 3a, 2440 Geel, België), and Lancaster (Bischheim-Strasbourg, France).

Synthesis of 2-Arylcarbonyl-3-trifluoromethyl-quinoxaline 1,4-Di-*N*-oxide Derivatives (3g,i,j, 4c,d,f−j, 5a−j, 6a,c−j). The compounds under study were previously synthesized and reported by our group.[11] The synthesis of compounds was carried out by the classical Beirut reaction (Scheme 1). The appropriate benzofuroxane, (**2a**−**i**) obtained by previously described methods,[6,58] and 4,4,4-trifluoro-1-(2-aryl)-1,3-butanediones were dissolved in dry chloroform in the presence of triethylamine, which acts as catalyst.

When the reaction was finished, the solvent was evaporated to dryness and yellow crude solid or brown oil was obtained. Each compound was purified by either recrystallization or flash chromatography.

3,7-Bis-trifluoromethyl-2-furylcarbonylquinoxaline-4-*N*-oxide (7i). A mixture of 100 mg of 3,7-bis-trifluoromethyl-2-furylcarbonylquinoxaline 1,4-di-*N*-oxide (0.26 mmol), 0.033 mL of trimethylphosphite (0.28 mmol), and 5 mL of 1-propanol was refluxed for 1 h. After evaporation to dryness under pressure, the crude was purified by flash chromatography on silica gel, using toluene as the mobile phase. Yield 66%. IR (KBr): ν 1331, 1681, 1143 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.72 (dd, $J_{4'-3'} = 3.51$, $J_{4'-5'} = 1.63$ Hz, 1H, H-4′), 7.41 (d, 1H, H-3′), 7.75 (d, 1H, H-5′), 8.08 (d, $J_{6-5} = 9.07$ Hz, 1H, H-6), 8.53 (s, 1H, H-8), 8.78 (d, 1H, H-5) ppm. Anal. ($C_{15}H_6F_6N_2O_3$) C, H, N.

3,6-Bis-trifluoromethyl-2-furylcarbonylquinoxaline-4-*N*-oxide (7j). This compound was obtained in 60% yield from quinoxaline 1,4-di-*N*-oxide **6j** following the procedure previously described. IR (KBr): ν 1668, 1328, 1142 cm^{-1}, ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.71 (dd, $J_{4'-3'} = 3.68$, $J_{4'-5'} = 1.66$ Hz, 1H, H-4′), 7.40 (d, 1H, H-3′), 7.74 (d, 1H, H-5′), 8.16 (d, $J_{7-8} = 8.78$ Hz, 1H, H-7), 8.35 (d, 1H, H-8), 8.95 (s, 1H, H-5) ppm. Anal. ($C_{15}H_6F_6N_2O_3$) C, H, N.

3,7-Bis-trifluoromethyl-2-furylcarbonylquinoxaline-1-*N*-oxide (8i). A 100 mg portion of 3,7-bis-trifluoromethyl-2-furylcarbonylquinoxaline 1,4-di-*N*-oxide was dissolved in 75 mL of methanol and 10 mL of water. The solution was stirred at reflux for 1.5 h. The solvent was evaporated under pressure; the solid was precipitated and washed by adding diethyl ether affording a white solid **8i**. Yield 50%. IR (KBr): ν 1662, 1368, 1162 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.71 (dd, $J_{4'-3'} = 3.69$, $J_{4'-5'} = 1.62$ Hz, 1H, H-4′), 7.48 (d, 1H, H-3′), 7.64 (d, 1 H, H-5′), 8.19 (d, $J_{6-5} = 8.75$ Hz, 1H, H-6), 8.47 (d, 1H, H-5), 8.90 (s, 1H, H-8) ppm. Anal. ($C_{15}H_6F_6N_2O_3$) C, H, N.

3,6-Bis-trifluoromethyl-2-furylcarbonylquinoxaline-1-*N*-oxide (8j). This compound was obtained in 45% yield from 3,6-bis-trifluoromethyl-2-furylcarbonylquinoxaline 1,4-di-*N*-oxide following the procedure described for compound **8i**. IR (KBr): ν 1655, 1365, 1140 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.71 (dd, $J_{4'-3'} = 3.74$, $J_{4'-5'} = 1.68$ Hz, 1H, H-4′), 7.47 (dd, $J_{3'-5'} = 0.65$ Hz, 1H, H-3′), 7.64 (dd, 1H, H-5′), 8.11 (d, $J_{7-8} = 9.10$ Hz, 1H, H-7), 8.65 (s, 1 H, H-5), 8.72 (d, 1H, H-8) ppm. Anal. ($C_{15}H_6F_6N_2O_3$) C, H, N.

3,7-Bis-trifluoromethyl-2-furylcarbonylquinoxaline (9i). A 108 mg portion (0.28 mmol) of 3,7-bis-trifluoromethyl-2-furylcarbonylquinoxaline 1,4-di-*N*-oxide was heated in 30 mL of methanol. When the solution reached 70 °C a solution of 256 mg of $Na_2S_2O_4$ in 4 mL of water was added dropwise. The mixture was stirred for 1 h. The obtained white solid was washed with water. Yield 70%. IR (KBr): ν 1655, 1139 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.71 (dd, $J_{4'-3} = 3.65$, $J_{4'-5'} = 1.62$ Hz, 1H, H-4′), 7.39 (d, 1H, H-3′), 7.80 (d, 1H, H-5′), 8.19 (d, $J_{6-5} = 8.78$ Hz, 1H, H-6), 8.48 (d, 1H, H-5), 8.90 (s, 1H, H-8) ppm. Anal. ($C_{15}H_6F_6N_2O_2$) C, H, N.

In Vitro Primary Anticancer Assay. The compounds were evaluated in vitro against a three-cell line panel consisting of MCF7 (breast), NCI-H460 (lung), and SF-268 (CNS). In this protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents were then added at a single concentration (100 μM), and the culture was incubated for 48 h. End-point determinations were made with alamar blue sulforhodamine B,[59] a protein-binding dye. Results for each test agent were reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduced the growth of any one of the cell lines to 32% or less were considered active and passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range.

In Vitro Anticancer Screening against a Panel of 60 Human Tumor Cell Lines. The antitumor activity of tested compounds is reported for each cell line by three parameters: The 50% growth-inhibitory concentration (GI_{50}) describes the concentration of the compound required to cause 50% inhibition of net cell growth. The total inhibition concentration (TGI) represents the concentration of the compound resulting in total inhibition of net cell growth. The 50% lethal concentration (LC_{50}) indicates the concentration of the compound leading to 50% net cell death. Values are calculated for each of these three parameters if the level of effect is reached. If the effect is not reached or is exceeded, the value is expressed as greater than the maximum or less than the minimum drug concentration. Furthermore, a meangraph midpoint (MG_MID) is calculated for each of the mentioned parameters, giving a mean activity parameter over cell lines. For calculation of the MG_MID, insensitive cell lines are included with the highest concentration tested. The experimental procedures have been described in detail previously.[16,60]

Hollow Fiber Assay for Preliminary in Vivo Testing. Each compound was tested against a standard panel of 12 human tumor cell lines including NCI-H23, NCI-H522, MDA-MB-231, MDA-MB-435, SW-620, COLO 205, LOX IMVI, UACC-62, OVCAR-3, OVCAR-5, U251, and SF-295. The cell lines were cultivated in RPMI-1640, containing 10% FBS and 2 mM glutamine. A total of three different tumor lines were prepared for each experiment so that each mouse would receive three intraperitoneal (IP) implants (one from each tumor line) and three subcutaneous (SC) implants (one from each tumor line). A value of 2 was assigned to each compound dose which resulted in a 50% or greater reduction in viable cell mass. Compounds with a combined ip + sc score of 20, a sc score of 8, or a net cell kill of one or more cell lines were referred for further studies.[17] Additionally some compounds were tested against a six-cell line panel consisting of leukemia and lymphoma cell lines K-562, MOLT-4, HL-60(TB), SR, RPMI-8226, and CCRF-CEM.

DNA Cleavage Studies. In a typical reaction, supercoiled plasmid DNA (33 μg/mL, pGL-2 Basic), **5h** or menadione (25 μM), NADPH (500 μM), and NADPH/cytochrome P450 reductase (1 mU) were incubated in sodium phosphate buffer (50 mM, pH 7.0) containing 2.5% acetonitrile under aerobic conditions at 24 °C for 2 h. Compound **5h** and menadione were introduced as stock solutions in acetonitrile. Following incubation, the reactions were stopped by the addition of 50% glycerol loading buffer (5 μL), and the resulting mixture was loaded onto a 0.9% agarose gel. The topological forms of plasmid DNA were separated by electrophoresis at 85 V in 1XTAE buffer and then stained by immersing the gel in a solution of aqueous ethidium bromide (0.3 μg/mL) for 2 h. DNA in the gel was visualized by UV-transillumination, and the amount of DNA in each band was quantified using an Alpha Innotech IS-1000 digital imaging system. DNA cleavage assays containing SOD, catalase, desferal, and radical scavengers were carried out in a manner identical to the standard reaction, except with additives present prior to the addition of NADPH and NADPH/cytochrome P450 reductase.

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Supporting Information Available: Results from elemental analysis, primary anticancer assay, and the ^{1}H NMR spectra of compounds **6i**, **7i**, **8i**, **9i**, **6j**, **7j**, and **8j**. This material is available free of charge via the Internet at http://pubs.acs.org.

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