Phenazine 5,10-Dioxide Derivatives as Hypoxic Selective Cytotoxins: Part II. Structure-Activity Relationship Studies

H. Cerecetto[1,*], M. González[1,*], M.L. Lavaggi[1], M.A. Aravena[2], C. Rigol[2], C. Olea-Azar[2], A. Azqueta[3], A. López de Cerain[3], A. Monge[3] and A.M. Bruno[4]

[1]*Departamento de Química Orgánica, Facultad de Química-Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, 11400 Uruguay,* [2]*Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Olivos 1007, 233 Santiago,* [3]*Centro de Investigación en Farmacobiología Aplicada, Universidad de Navarra, Irunlarrea s/n, 31080 Pamplona, Navarra, Spain and* [4]*Centro de Síntesis y Estudio de Nuevos Compuestos Antineoplásicos, Universidad de Buenos Aires, Buenos Aires, Argentina*

Abstract: The synthesis and evaluation as hypoxic selective cytotoxins of new derivatives of 2-amino or 2-hydroxyphenazine 5,10-dioxide are described. The compounds were developed as structural analogs of other bioreductive compounds and its *in vitro* cytotoxicities on V79 cells under hypoxic and aerobic conditions were determined. To gain insight into its mechanism of action electrochemical behavior, interaction with DNA experiments and QSAR studies were performed.

Key Words: Phenazine 5,10-dioxide derivatives, bioreductive compounds, DNA, cyclic voltammetry.

INTRODUCTION

Cancerous cells can become relatively isolated from the blood supply due to their rapid growth, turning increasingly difficult the diffusion of oxygen and resulting, frequently, in hypoxia [1]. Hypoxic cells are not accessible for conventional cytotoxic drugs in adequate concentrations due to the inadequate vascularization. On the other hand, conventional anticancer drugs in clinical use are antiproliferative agents that kill dividing cells, by attacking DNA (synthesis, replication or processing). However, in solid tumors where an important region is hypoxic conventional drugs resulted also ineffective because the majority of the cells are not dividing rapidly. Hypoxia appears to be a common and distinct property of cells in solid tumors that promotes an important mechanism for the specific activation of antitumoral prodrugs, namely bioreduction [2]. Compounds able to be selectively bioactivated, in absence of oxygen into the hypoxic tumor tissue, to active cytotoxic species have been developed as selective anti-tumor agents. On the basis that bioreduction is irreversible under hypoxic conditions, bioreductive agents [3], quinones, nitroderivatives, and *N*-oxides (Fig. (**1a**)) have been developed and are either in clinical use or entering in clinical trials [4]. For some *N*-oxide containing heterocycles (i.e. Tirapazamine, **1**, Fig. (**1a**)) the mechanism of cytotoxicity has been suggested to involve one-electron-reductive activation, enzymatic or non enzymatic process, or both, which could result in the production of ·OH [3c]. This radical would produce oxidative DNA cleavage, unlike quinones and nitrocompounds, without covalent binding to DNA and proteins. On the other hand, it was hypothesized that hybrid compounds (i.e. **2**, Fig. (**1a**)), which conjugate an *N*-oxide and a π DNA-stacking moieties, would be a new generation of bioreductive compounds. These could damage hypoxic cells generating ·OH as Tirapazamine and interacting with DNA before or after the bioreduction process [5]. This idea, together with our interest in the development of new hypoxic selective cytotoxic agents [6] (i.e. **3**, Fig. (**1a**)), encourages us to synthesize a first series of phenazine 5,10-dioxide derivatives (**4-17**, Fig. (**1a**)) structurally related to other bioreductive compounds and possessing potential π DNA-stacking properties [7]. Some derivatives from this family showed excellent biological behavior in hypoxic conditions, i.e. derivatives **6**, **8**, **10**, **12** and **14-16**. In this paper we present the activity of new synthesized derivatives developed as analogues of quinoxaline **3** (Fig. (**1b**)) and a structure-activity relationship study performed in order to find the structural requirements for optimal activity.

CHEMISTRY

In order to prepare the designed compounds (Fig. (**1b**)) we attempted two approaches. In one hand, we intend the preparation of the amides **18-20** (Scheme (**1**)) with ether and amine groups as linker. In Scheme (**1**) is shown the procedure used attempting to prepare these derivatives.

When we tried to condense the α–chloroamides with the parent compounds **4**, **7** and **11**, the starting material decomposed. At room temperature the nucleophilic substitutions did not take place and in the reflux conditions the phenazine dioxides decomposed completely. So, in another approach we designed derivatives **23-25** (Scheme (**2**)) where amide group was used as the linker moiety.

In this approach α–chloroamides **21** and **22** (Scheme (**2**)) were prepared with good results and further they were

*Address correspondence to this author at the Departamento de Química Orgánica, Facultad de Química-Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay; Tel: 598-2-5258618 (ext. 216); Fax: 598-2-525 07 49;
E-mail: hcerecet@fq.edu.uy, megonzal@fq.edu.uy

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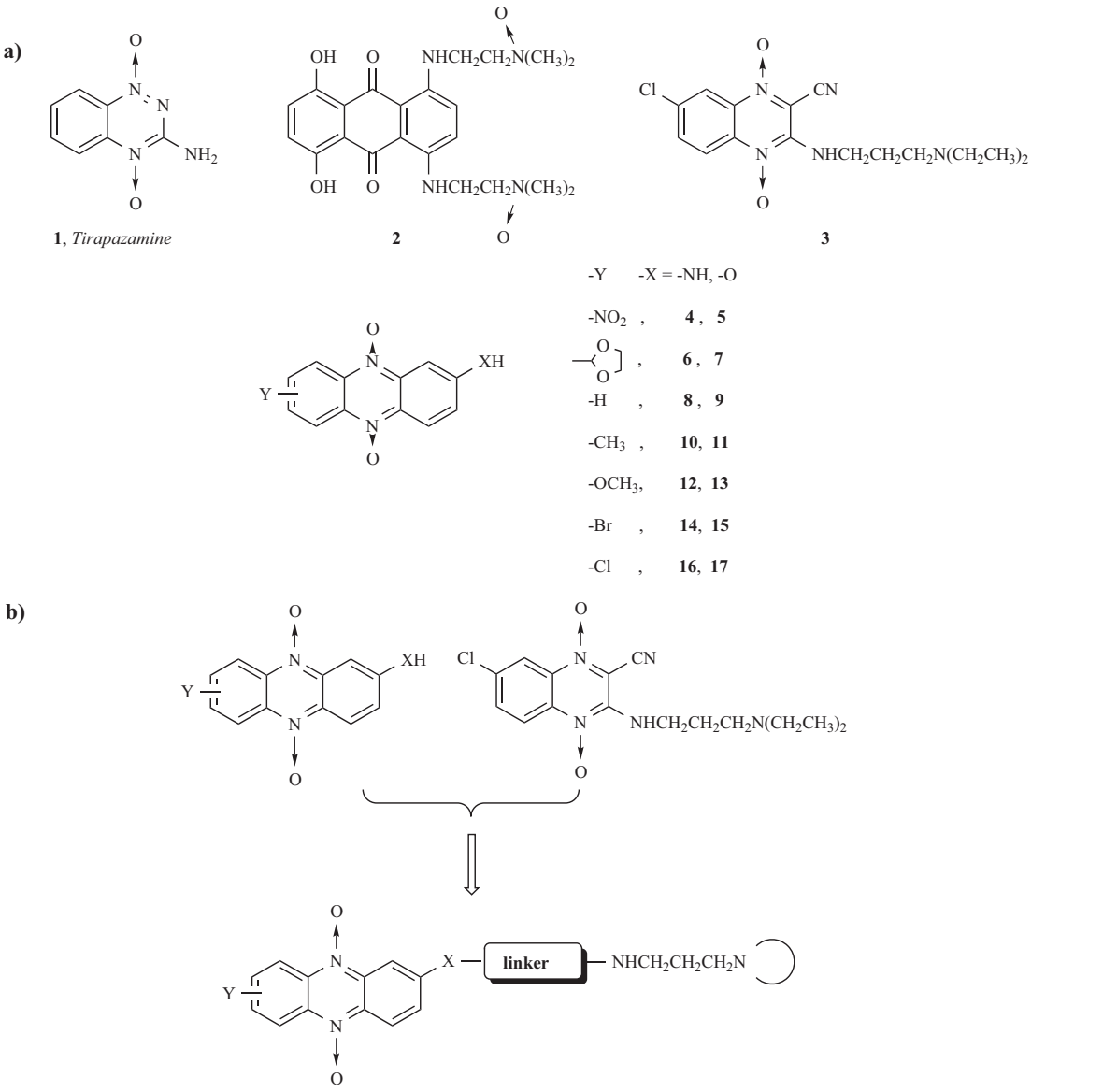
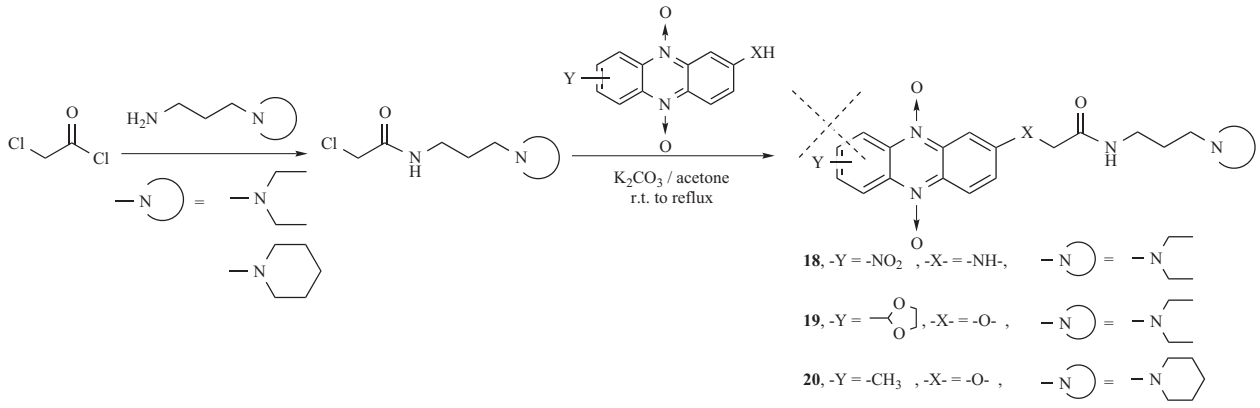
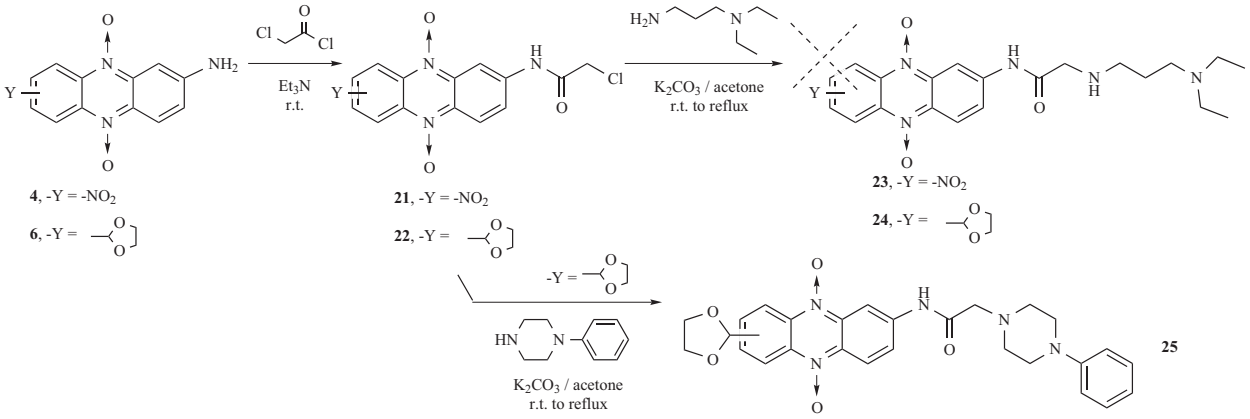


Fig. (1). a) Structures of first series of phenazine 5,10-dioxide derivatives developed. **b)** General structure of second series designed.



Scheme (1). Attempts to synthesize derivatives **18-20**.



Scheme (2). Attempts to synthesize derivatives **23** and **24** and preparation of derivative **25**.

reacted with some commercial diamines. However, when we tried these nucleophilic substitutions only with the secondary amine 1-phenylpiperazine was possible to generate the desired product (**25**). In the reaction with 3-(dimethylamino) propylamine complete decomposition of starting materials were observed. Further studies improving the synthetic results were not done.

BIOLOGICAL ACTIVITY

Aerobic and Hypoxic Cytotoxicity

In vitro selective cytotoxicity was evaluated by a clonogenic assay after two hours of treatment of V79 suspension cultures gassed with air or nitrogen at 20 μM [6,7]. When the products resulted cytotoxic the assayed concentrations were lowered down. The cytotoxic effects are expressed as cell survival fractions (SF), with respect to the control, in both conditions. For each of the tested derivatives the average SF values of two different experiments are reported in Table (**1**).

STUDY OF MECHANISM OF ACTION

In order to study of phenazine dioxide cytotoxic mechanism of action we performed some experiments related to DNA and oxidative damages. We studied *N*-oxides DNA-interaction capabilities and their electrochemical behavior.

DNA Binding Assay

Parent compounds and derivatives **21**, **22** and **25** were tested for their ability to bind to DNA, using the DNA-binding assay described previously [8]. Adequate aliquots of the test solutions were mixed with the DNA solution as described in Experimental Section. The binding capacity was tested by measuring the hypochromic and bathocromic effect of compound absorbance in the UV spectra in a 20 nm band centered on the maximal absorbance value of each compound (Fig. (**2**)). The classical procedure was improved by rotating the stirred DNA-drug mixture in a 5:1 ratio during 24 h. The method was validated by repeating the assays with

Table 1. Cytotoxic Effect in Oxia and Hypoxia on V-79 Cells of Phenazine 5,10-Dioxide Derivatives Developed

Parent compounds[a]		SF [b,c]			Derivatives			SF [c,d]	
Ref.	X	Y	air	hypoxia	Ref.	X	Y	air	hypoxia
4	NH	NO_2	1	2	21	$NCOCH_2Cl$	NO_2	0	0
			58 [e]	65 [e]				0 [e]	0 [e]
								0 [f]	0 [f]
6	NH	g	14	0	22	$NCOCH_2Cl$	g	0	0
			86 [e]	1 [e]				1 [e]	0 [e]
			100 [f]	36 [f]				9 [f]	0 [f]
					25	h	g	100	86
3 [i]	-	-	100 [f]	5 [f]					

[a] From reference [7]. [b] SF air= survival fraction in air at 20 μM. [c] Values are means of two different experiments. The assays were done by duplicate and using at least three repetitions, standard errors were not greater than 2 % for most assays. [d] SF hypoxia= survival fraction in hypoxia at 20 μM. [e] At 10 μM. [f] At 5 μM. [g] CH(1,3-dioxol-2-yl). [h] $NCOCH_2$(4-phenylpiperazine-1-yl). [i] Positive control (see Scheme (**1a**)).

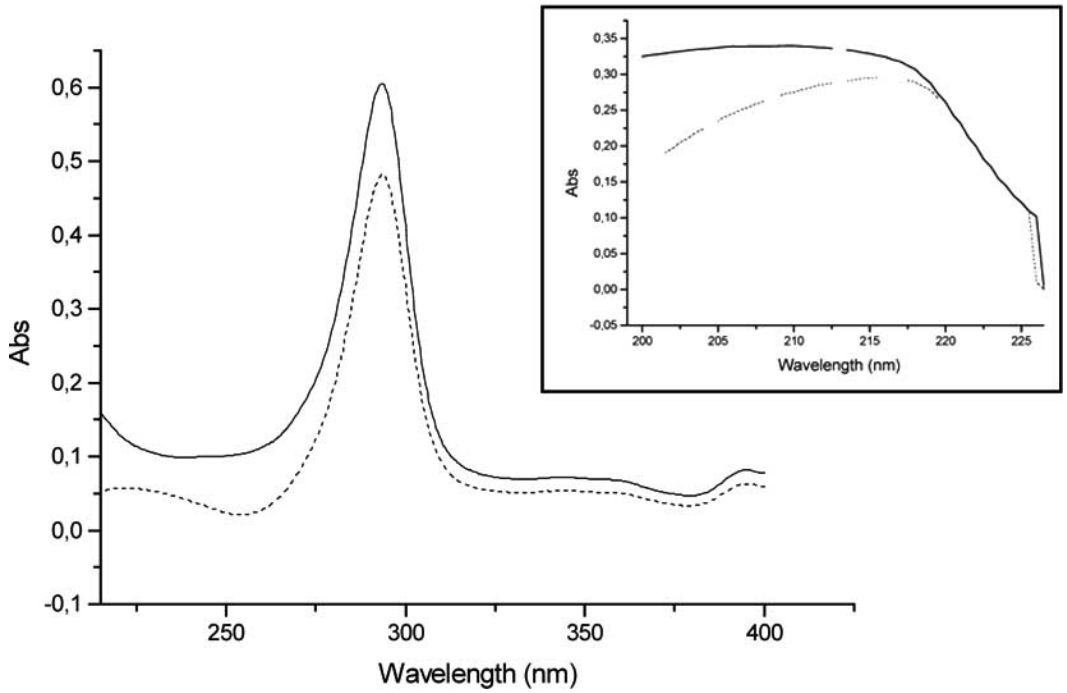


Fig. (2). UV spectra of parent compound **6**-DNA (0 h = solid line, 24 h = dash line). Inset: UV spectra of compound **22**-DNA (0 h = solid line, 24 h = dash line).

known intercalating agents (m-AMSA, ethidium bromide and mitoxantrone). The degree of interaction is expressed by the ratio between the final absorbance area (a_{24}) and the absorbance of the compound at equal concentration (a_0). Values of 1 or >1 indicate a total lack of affinity and a value of 0 indicate binding of the entire compound to DNA. The values of ratio a_{24}/a_0 are summarized in Table **2**.

Cyclic Voltammetry

In order to determine the electrochemical characteristic of the studied derivatives and its relationship with their cytotoxicity, experiments of cyclic voltammetry [9] were performed in organic medium. These *N*-oxide derivatives displayed comparable voltammetric behavior in DMSO, showing two to three reduction peaks and the anodic counterparts. Table **2** lists the values of the *N*-oxide cathodic peaks. Fig. (**3**) shows a selected voltammogram. The first wave corresponds to a quasireversible process.

STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

Molecular modeling studies were performed on the phenazine dioxide parent compounds, derivatives **4-17**, by calculating the stereoelectronic properties in order to understand its mechanism of action. These molecular properties were determined using DFT/B3LYP calculations [10-12]. The geometry of each compound was fully optimized by applying PM3/6-31G* in gas phase allowing to obtain acute results with low time of computational calculi. Then, single point B3LYP/6-31G* density functional calculation was performed. Total energy, solvation (water) energy, dipolar moment, volume, HOMO's and LUMO's energies, gap (E_LUMO – E_HOMO) and the logarithm of the partition coefficient of the non-ionized molecules (LogP) were determined and examined in this study. Theoretical LogP (cLogP) was calculated using Villar method, implemented in PC Spartan 04 package [13] at the PM3 semiempirical level on the optimized structures. In equation, *n* represents the number of data points, *r* is the root of correlation coefficient, *s* is the standard deviation of the regression equation, the *F* value is related to the F-statistic analysis (Fischer test) and r^2adj defines the cross-validated correlation coefficient. Only structure-activity models having a value of r^2adj above 0.5 were considered. Activity used in the structure-activity relationship studies was the survival fraction in air and hypoxia (SF_{air} and $SF_{hypoxia}$, respectively). Firstly, one-variable and multivariable regressions between both activities and the physicochemical properties (calculated descriptors and redox potential) were studied. The best equation was obtained when we analyzed the correlation between $SF_{hypoxia}$ and the

$$SF_{hypoxia} = 936(\pm258) + 239(\pm62)\ E_{pc} - 331(\pm119)\ \text{cLogP} + 40(\pm16)\ \text{cLogP}^2$$

r	r^2adj	s	p	F value	n
0.875	0.665	26.74	0.013	7.63	11

Used compounds: **7 – 17**

Equation 1

Table 2. DNA Biological Characterization, Reduction Potentials, and Theoretical Descriptors Used in the QSAR Study of Phenazine Dioxide Derivatives

Ref.	a_{24}/a_0 (solvent[a])	Epc vs. SCE [b] (V)	cLogP	E_{LUMO}[c]	E_{SHOMO}[c]	ΔE_K[c]
4	1.00 (MeOH)	nd [d]	3.51	-3.03	-0.67	2.36
6	0.79 (MeOH)	nd	2.37	-2.47	0.37	2.84
7	0.91 (MeOH)	-1.23	2.22	-2.59	0.31	2.90
8	1.00 (MeOH)	-1.00	3.66	-2.35	0.56	2.91
9	0.90 (MeOH)	-0.62	3.65	-2.58	0.49	3.07
10	0.98 (MeOH)	-1.00	4.80	-2.36	0.58	2.94
11	0.83 (MeOH)	-0.78	3.84	-2.48	0.51	2.99
12	0.89 (MeOH)	-1.03	3.31	-2.33	0.48	2.81
13	0.84 (MeOH)	-0.87	3.43	-2.45	0.52	2.97
14	1.00 (MeOH)	-0.92	4.40[e]	-2.64	0.24	2.88
15	1.00 (MeOH)	-1.14	4.80[e]	-2.76	0.16	2.92
16	0.99 (MeOH)	-0.96	3.83	-2.62	0.30	2.92
17	0.83 (MeOH)	-0.63	4.19	-2.74	0.23	2.97
21	0.89 (MeOH)	nd	nd	nd	nd	nd
22	0.79 (MeOH)	-0.80	nd	nd	nd	nd
25	0.80 (DMSO)	nd	nd	nd	nd	nd
m-AMSA	0.30 (EtOH)	-	-	-	-	-
Ethidium Bromide	0.50 (EtOH)	-	-	-	-	-
Mitoxantrone	0.00 (EtOH)	-	-	-	-	-

[a] Solvent used in the assays (no more than 5 %). [b] Peaks potentials (~± 0.01 V) measured at a scan rate of 2.00 V/s. [c] eV. [d] Not determined. [e] This value was estimated because parameters for bromine atom are not available into Villar method.

independent cLogP and E_{pc} (Equation 1). Besides, the correlation matrix for the used physicochemical descriptors was performed and cross-correlations between the descriptors used in this equation were not obtained. These parameters are therefore orthogonal, a fact that affords their use in the multilinear regression procedure [14].

On the other hand, non-statistical significant correlations were obtained when E_{pc} was included as independent variable, but it was observed a clear tendency between activities and redox potential, the most active compounds posses the most negative E_{pc}. This fact was studied dividing the phenazine dioxides into two groups of compounds, the actives in hypoxia ($SF_{hypoxia} < 50$ %) and the inactives in hypoxia ($SF_{hypoxia} \geq 50$ %). These two groups of activity resulted significantly different, for both, at 2×10^{-6} level (Fig. (**4**)). Then, the corresponding two groups of E_{pc}, for active and for inactive derivatives, were submitted to a t-Test analysis. The E_{pc} values, for both pre-defined populations of compounds, resulted significantly different at 1×10^{-3} level (Fig. (**4**)).

On other hand, it was qualitatively observed that dipolar moments of active derivatives are oriented in similar ways whereas the inactive ones oriented its dipolar moments in an opposite manner (Fig. (**5**)). The first ones oriented its dipolar moment to the 7-substituent whereas the dipolar moment for inactive derivatives, in general, were oriented to the hydroxyl or amine group or to the *N*-oxide moieties.

In order to compare the different capacities of these compounds to suffer reduction and produce free radical species, like *Tirapazamine*, the two processes shown in Scheme (**3**) were theoretically studied.

The process shown in Scheme (**3a**), the same that was studied experimentally using voltammetric experiments, was analyzed theoretically in thermodynamic and kinetic terms. In the first approach, the difference of energy (ΔE, Scheme (**3a**)) between free radical products and neutral phenazine dioxides was studied for the parent compounds, derivatives **4-17**. No correlations between these values and the biological activities were obtained. On the other hand, as kinetic study of these processes we studied the energetic differences between the highest occupied molecular orbital energy of the free radical products (E_{SHOMO}) and the lowest unoccupied molecular orbital energy of the reactants (E_{LUMO}). Like the E_{pc} non-statistical significant correlations were obtained when this difference was studied as independent variable,

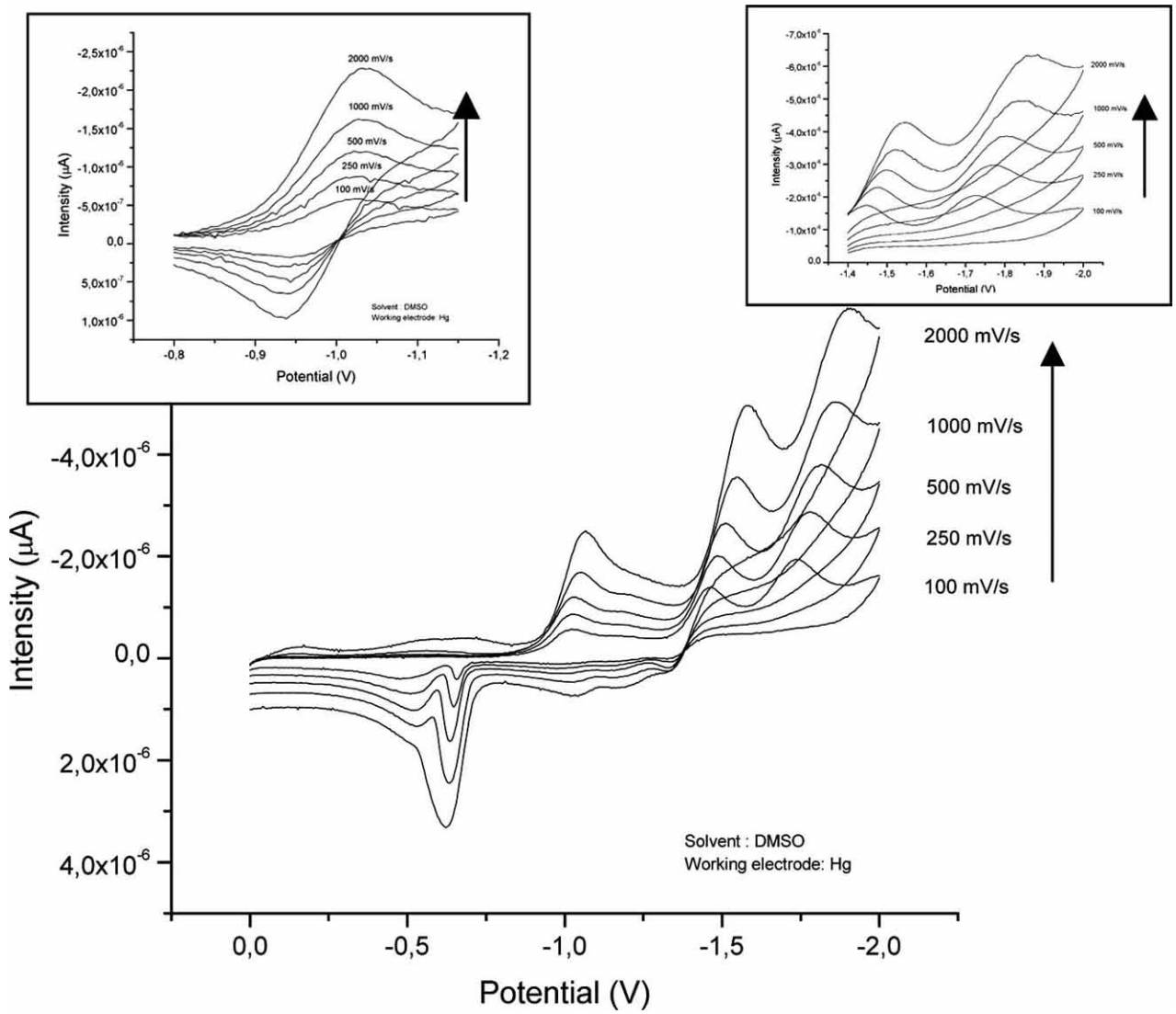


Fig. (3). Electrochemical behavior of compound **12**. Inset left: First reduction process. Inset right: second and third reduction peaks.

but it was observed a clear tendency between activities and ΔE_K ($E_{SHOMO} - E_{LUMO}$). So, ΔE_K was divided in two groups, according to the above pre-defined two populations of $SF_{hypoxia}$ and was submitted to a t-Test analysis. They were significantly different at a level lower than 0.02 (Fig. (**6a**)). Fig. (**6b**) shows, graphically, the distribution of the LUMO maps in some relevant phenazine dioxides and the distribution of the spin maps for the corresponding free radicals. In these graphics it is possible to observe the differences in the LUMO distribution between active and inactive derivatives and the electron delocalization positions for these derivatives.

When we analyzed the processes shown in Scheme (**3b**), in term of isodesmic reactions [11], we did not find energetic difference (ΔE differences) between active and inactive derivatives. The processes for all the parent compounds, actives and inactives, possessed similar values of ΔE (data not shown).

DISCUSSION

The new synthetic approaches produced complete decomposition of the parent phenazine dioxides allowing only preparing the amide **25** with the designed structure. However, when we compared with respect to the parent compound **6**, the biological response of this compound (Table (**1**)) a complete loss of activity at 20 μM was observed. The intermediate **21** and **22** resulted very cytotoxic but not selective in hypoxic conditions at 20 μM. Furthermore when lower concentrations were tested, 10 and 5 μM, they maintained similar cytotoxicity without selectivity. This fact could be the result of the presence of a very electrophile centre (α-chloroamide) in these molecules.

The DNA-binding studies indicate that the parent phenazines and the new compounds are not potentially toxic in oxic conditions by interaction with this bio-molecule. Especially the aerobic cytotoxicity of some derivatives (i.e. de-

Ref.	$SF_{hypoxia}$	E_{pc}
8		
10		
12	actives	actives
14	mean=5.7	mean=-1.01
15		
16	t=11.96	t=4.60
9	$p=2 \times 10^{-6}$	$p=1 \times 10^{-3}$
11	inactives	inactives[a]
13	mean=90.0	mean=-0.73
17		

[a] Outlier: derivative **7**.

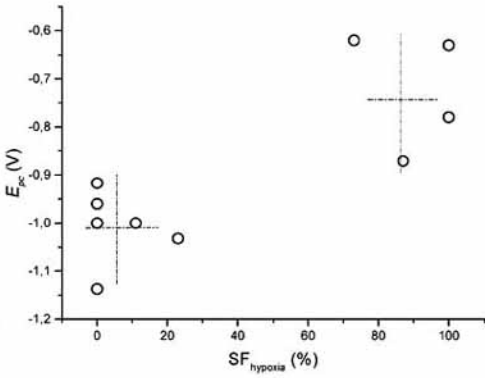


Fig. (4). Results of the t-Test analysis performed on the two phenazine dioxide populations and E_{pc}.

rivatives **4** and **21**) was not the result of a direct interaction with DNA. An interesting aspect observed in these experiments is that the best DNA-interacting phenazines are those with 1,3-dioxolan-2-yl substituent (see derivatives **6**, **22** and **25**, Table (**2**)). This structural feature would be taken in account in the design of new active derivatives. In this case, the presence of a protonated lateral chain, in derivative **25**, does not change the capacity of derivative **6** to interact with DNA.

The electrochemical studies performed permitted us to extract some relevant aspects related with the structural requirements for an adequate bioactivity. E_{pc} together with lipophilicity correlated to the fraction of cell survival in hypoxia (see equation 1). When redox potential of *N*-oxide moiety decreases (more negative) the hypoxic cytotoxicity increase (lower $SF_{hypoxia}$, see equation 1 and Fig. (**4**)). From equation 1 it was clear to understand the excellent activity of derivative **15**, the unique phenol derivative that resulted selectively cytotoxic in hypoxia. Derivative **15** possesses adequate E_{pc} (-1.14 V) and lipophilicity (one of the most lipophilic derivative evaluated). Derivative **7** E_{pc} is outlier from the t-Test analysis (Fig. (**4**)) however it is the most hydrophilic derivative include in the studies, so equation 1 that

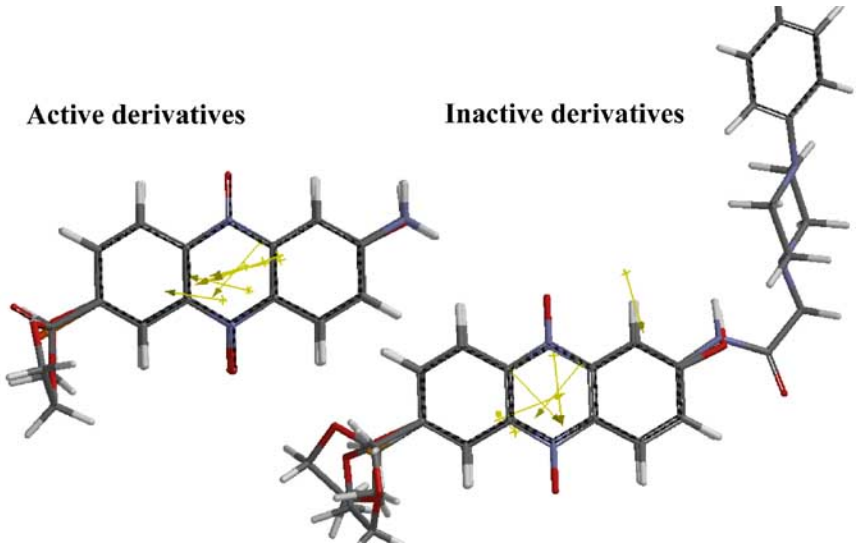
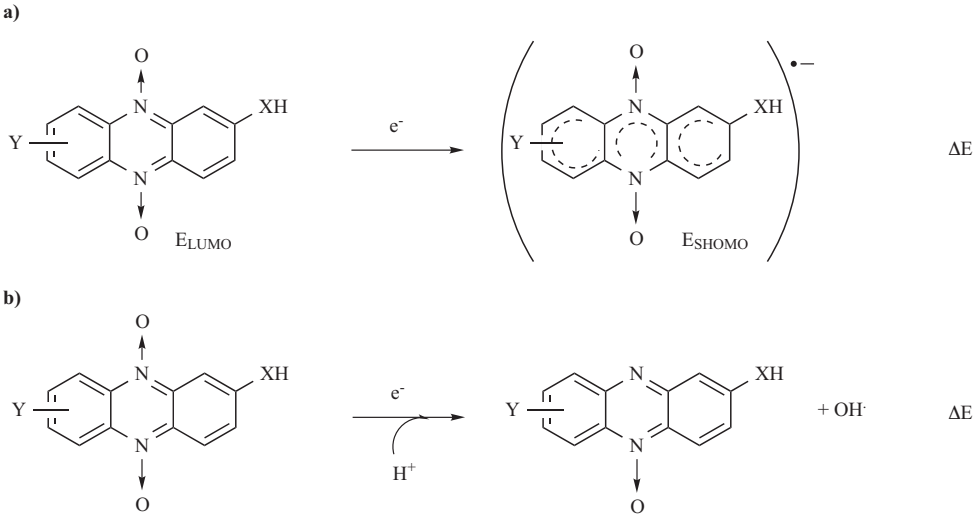


Fig. (5). Views of the dipolar moment of the studied phenazine dioxides (active derivatives: **4**, **6**, **8**, **10**, **12**, **14-16**; inactive derivatives: **7**, **9**, **11**, **13**, **17**, **25**).



Scheme (3). Processes studied theoretically in order to know the mechanism of action.

takes into account cLogP allow to explain its biological behavior. Also, the relevance of the monoelectronation processes in the displayed activities was confirmed analyzing the kinetic of these reactions throughout ΔE_K ($E_{SHOMO} - E_{LUMO}$) (Fig. (**6**)).

If the reduction processes were promoted by an enzymatic system the phenazines would fit adequately into the pocket of the biomolecule according to their electronic distributions. So, the orientation of the dipolar moment could play an important role in the bioactivities as we observed analyzing this property (Fig. (**5**)).

CONCLUSION

The biological and physicochemical information obtained herein allow us to redesign new phenazine 5,10-dioxides with the desired activity.

EXPERIMENTAL

Chemistry

Compounds **4**, **6-17** were prepared as previously reported [7]. All starting materials were commercially available research-grade chemicals and used without further purification. All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. The typical work-up included washing with brine and drying the organic layer with sodium sulfate before concentration. ^{1}H-NMR, ^{13}C-NMR spectra and HETCOR experiments were recorded on a Bruker DPX-400 (at 400 MHz and 100 MHz) instrument, with tetramethylsilane as the internal reference and in the indicated solvent. Mass spectra were recorded on a Shimadzu GC-MS QP 1100 EX (Kyoto, Japan) instrument using electron impact ionization at 70 eV.

N-(5,10-Dioxide-7(8)-nitrophenazin-2-yl)-2-chloroacetamide, 21

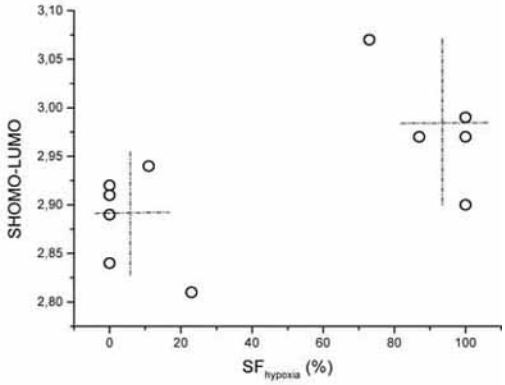
To a stirred solution of phenazine dioxide **4** (100 mg, 0.4 mmol) and triethylamine (60 μL) in toluene (5.0 mL), chloracetylchloride (35 μL) was added dropwise. The mixture was maintained at room temperature until phenazine **4** was not present (SiO_2, CH_2Cl_2:MeOH (9:1)). The mixture of reaction was extracted with EtOAc and saturated aqueous solution of $NaHCO_3$. After the work up the organic layer was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, CH_2Cl_2:MeOH (0-5 %)); 64 mg (50 %) as the mixture of 7- and 8-substituted derivatives (7-isomer: 8-isomer ratio = 4.0:6.0); red solid; MS, m/z (%): 348 ($M^{+\cdot}$, 0.4), 332 ($M^{+\cdot}$ - 16, 100.0), 316 ($M^{+\cdot}$ – 32, 57.6); Anal. ($C_{14}H_9ClN_4O_5$) C, H, N. 7-Substituted isomer: ^{1}H NMR (CD_3OD, 400MHz) δ: 4.31 (2H, s, CH_2), 6.77 (1H, d, H_3, J = 8.6 Hz), 7.50 (1H, m, H_4), 8.10 (1H, d, H_1, J = 2.1 Hz), 8.77 (1H, dd, H_8, J_1 = 9.7 Hz, J_2 = 1.7 Hz), 8.82 (1H, H_9, J = 9.6 Hz), 9.04 (1H, d, H_6, J = 2.2 Hz), 9.41 (1H, bs, NH). 8-Substituted isomer: ^{1}H NMR (CD_3OD, 400MHz) δ: 4.31 (2H, s, CH_2), 6.88 (1H, d, H_3, J = 8.3 Hz), 7.50 (1H, m, H_4), 8.10 (1H, d, H_1, J = 2.1 Hz), 8.46 (1H, dd, H_7, J_1 = 9.7 Hz, J_2 = 1.7 Hz), 8.73 (1H, H_6, J = 9.6 Hz), 9.04 (1H, d, H_9, J = 2.3 Hz), 9.41 (1H, bs, NH). ^{13}C NMR (HMQC-HMBC) (both isomers) (CD_3OD, 100MHz) δ: 54.40, 115.00, 117.00, 117. 70, 120.00, 122.50, 128.00, 129.50, 137.00, 141.50, 142.50, 145.00, 147.50, 150.00.

N-[5,10-Dioxide-7(8)-(1,3-dioxolan-2-yl)phenazin-2-yl]-2-chloroacetamide, 22

To a stirred solution of phenazine dioxide **6** (150 mg, 0.5 mmol) and triethylamine (78 μL) in DMF (5.0 mL) chloracetylchloride (45 μL) was added dropwise. The mixture was maintained at room temperature until phenazine **6** was not present (SiO_2, CH_2Cl_2:MeOH (9:1)). The mixture of reaction was concentrated *in vacuo* and the residue was purified by column chromatography (SiO_2, CH_2Cl_2:MeOH (0-5 %)); 94 mg (50 %) as the mixture of 7- and 8-substituted derivatives (7-isomer:8-isomer ratio = 5.5:4.5); red solid; MS, m/z (%): 375 ($M^{+\cdot}$, 3.1), 359 ($M^{+\cdot}$ - 16, 32.6), 343 ($M^{+\cdot}$ – 32, 70.7); Anal. ($C_{17}H_{14}ClN_3O_5$) C, H, N. 7-Substituted isomer: ^{1}H NMR (DMSO-d_6, 400MHz) δ: 4.05 (2H, d, -CH_2O, J = 2.1

a)

Ref.	$SF_{hypoxia}$	ΔE_K
8		
10		
12	actives	actives
14	mean=5.7	mean=2.89
15		
16	t=13.29	t=2.88
7	$p=3 \times 10^{-7}$	p=0.0163
9	inactives	inactives
11	mean=92.0	mean=2.98
13		
17		



b)

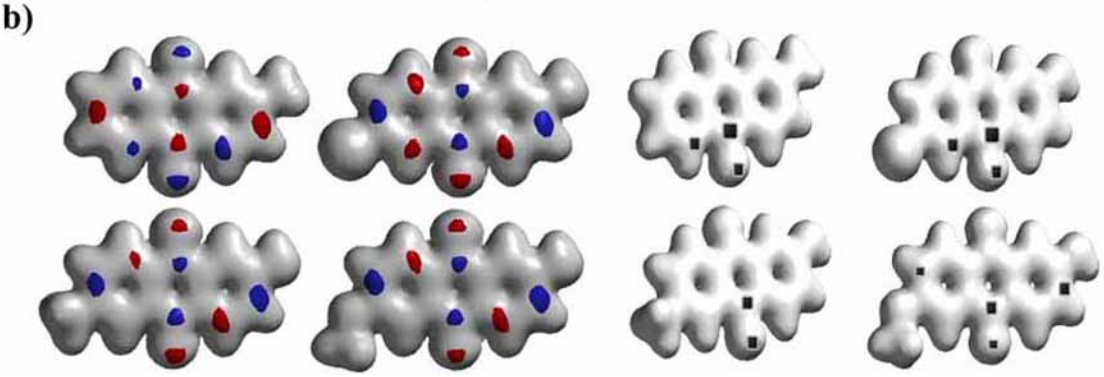


Fig. (6). a) Results of the t-Test analysis performed on the two populations of phenazine dioxides and ΔE_K. **b)** Left: electron density (solid grey, isovalue = 0.017) and LUMO (solid, isovalue = 0.030) isosurfaces for active derivatives **8** and **14** (up) and inactive derivatives **11** and **13** (down). Right: electron density (solid grey, isovalue = 0.025) and spin density (solid black, isovalue = 0.0175) isosurfaces for free radicals of active derivatives **8** and **14** (up) and free radicals of inactive derivatives **11** and **13** (down).

Hz), 4.13 (2H, d, -CH_2O, J = 2.1 Hz), 4.39 (2H, s, -CH_2CO), 6.05 (1H, s, -CHO_2), 7.35 (1H, dd, H_3, J_1 = 8.6 Hz, J_2 = 2.2 Hz), 7.71 (1H, d, H_1, J = 2.1 Hz), 7.93 (1H, dd, H_8, J_1 = 9.1 Hz, J_2 = 2.1 Hz), 8.58 (2H, m, H_4+H_9), 8.99 (1H, d, H_6, J = 2.0 Hz), 9.50 (1H, bs, NH). 8-Substituted isomer: ^{1}H NMR (DMSO-d_6, 400MHz) δ: 4.05 (2H, d, -CH_2O, J = 2.1 Hz), 4.13 (2H, d, -CH_2O, J = 2.1 Hz), 4.39 (2H, s, -CH_2CO), 6.05 (1H, s, -CHO_2), 7.37 (1H, dd, H_3, J_1 = 9.5 Hz, J_2 = 2.3 Hz), 7.68 (1H, d, H_1, J = 2.1 Hz), 7.90 (1H, dd, H_7, J_1 = 9.0 Hz, J_2 = 2.1 Hz), 8.58 (2H, m, H_4+H_6), 9.13 (1H, bs, H_9), 9.41 (1H, bs, NH). ^{13}C NMR (HMQC-HMBC) (both isomers) (DMSO-d_6, 100MHz) δ: 44.45, 66.08, 68.11, 102.59, 102.61, 106.64, 118.31, 118.35, 129.53, 129.64, 130.35, 132.40, 132.57, 132.67, 132.87, 136.84, 137.27, 138.00, 141.00, 141.79, 166.00.

N-[5,10-Dioxide-7(8)-(1,3-dioxolan-2-yl)phenazin-2-yl]-2-(4-phenylpiperazin-1-yl)acetamide, 25

To a stirred solution of **22** (100 mg, 0.3 mmol), K_2CO_3 (36 mg) in DMF (3.0 mL), 1-phenylpiperazine (41 μL) was added dropwise. The mixture was heated at 40ºC for 4 h. The mixture of reaction was treated with water and extracted with EtOAc. After the work up the organic layer was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, CH_2Cl_2:MeOH (0-5 %)); 68 mg (45 %) as the

mixture of 7- and 8-substituted derivatives (7-isomer:8-isomer ratio = 5.5:4.5); red solid; MS, m/z (%): 501 ($M^{+\cdot}$, 0.1), 485 ($M^{+\cdot}$ – 16, 6.1), 469 ($M^{+\cdot}$ – 32, 44.0); Anal. ($C_{27}H_{27}N_5O_5$) C, H, N. 7-Substituted isomer: ^{1}H NMR (DMSO-d_6, 400MHz) δ: 2.66 (2H, bs, -CH_2N), 3.15 (2H, bs, -CH_2N), 3.32 (2H, s, -CH_2CO), 4.11 (2H, d, -CH_2O, J = 2.2 Hz), 4.18 (2H, d, -CH_2O, J = 2.3 Hz), 6.05 (1H, s, -CHO_2), 6.83 (2H, bs, H_{phenyl}), 7.00 (1H, d, H_{phenyl}, J = 7.8 Hz), 7.23 (2H, d, H_{phenyl}, J = 7.2 Hz), 7.91 (1H, bs, H_3), 8.11 (1H, dd, H_8, J_1 = 9.0 Hz, J_2 = 2.4 Hz), 8.65 (2H, m, H_4+H_6), 8.67 (1H, bs, H_1), 9.11 (1H, bs, H_9), 10.13 (1H, s, NH). 8-Substituted isomer: ^{1}H NMR (DMSO-d_6, 400MHz) δ: 2.66 (2H, bs, -CH_2N), 3.15 (2H, bs, -CH_2N), 3.32 (2H, s, -CH_2CO), 4.11 (2H, d, -CH_2O, J = 2.2 Hz), 4.18 (2H, d, -CH_2O, J = 2.3 Hz), 6.05 (1H, s, -CHO_2), 6.83 (2H, bs, H_{phenyl}), 7.00 (1H, d, H_{phenyl}, J = 7.8 Hz), 7.23 (2H, d, H_{phenyl}, J = 7.2 Hz), 7.91 (1H, d, H_3, J_1 = 9.0 Hz), 8.11 (1H, dd, H_7, J_1 = 9.0 Hz, J_2 = 2.4 Hz), 8.65 (2H, m, H_4+H_6), 8.67 (1H, bs, H_1), 9.11 (1H, bs, H_9), 10.13 (1H, bs, NH). ^{13}C NMR (HMQC-HMBC) (both isomers) (DMSO-d_6, 100MHz) δ: 49.08, 53.33, 62.45, 65.80, 102.63, 106.47, 116.08, 116.16, 116.25, 119.43, 120. 57, 120.69, 128.84, 129.23, 129.29, 129.53, 137.29, 141.37, 141.78, 151.94, 169.62.

Bio-Reductive Activity

Cells

V79 cells (Chinese hamster lung fibroblasts) were obtained from ECACC (European Collection of Animal Cell Cultures) and maintained in logarithmic growth as subconfluent monolayer by trypsinization and subculture to (1-2) × 10^4 cells/cm^2 twice weekly. The growth medium was EMEM (Eagle's Minimal Essential Medium), containing 10% (v/v) fetal bovine serum (FBS) ad penicillin/streptomycin at 100 U/100 μg/mL. **Aerobic and hypoxic cytotoxicity: suspension cultures.** Monolayers of V79 cells in exponential growth were trypsinized, and suspension cultures were set up in 50 mL glass flasks: 2 × 10^4 cells/mL in 30 mL of EMEM containing 10% (v/v) FBS and HEPES (10 mM). The glass flasks were submerged and stirred in a water bath at 37 °C, where they were gassed with humidified air or pure nitrogen. **Treatment:** Compounds solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure DMSO (Aldrich) or sterilized distilled water. Thirty minute after the start of gassing, 0.2 mL of the stock compound solution was added to each flask, two flasks per dose. In every assay there was one flask with 0.2 mL of DMSO (negative control) and another with 7-chloro-3-[3-(*N, N*-dimethylamino)propylamino]-2-quinoxalinecarbonitrile 1, 4-dioxide hydrochloride (positive control). **Cloning:** After 2 h exposure to the compound, the cells were centrifuged and resuspended in plating medium (EMEM plus 10% (v/v) FBS and penicillin/streptomycin). Cell numbers were determined with a haemocytometer and 10^2-10^3 cells were plated in 6-well plates to give a final volume of 2 mL/30 mm of well. Plates were incubated at 37 °C in 5% CO_2 for 7 days and then stained with aqueous crystal violet. Colonies with more than 64 cells were counted. The plating efficiency (PE) was calculated by dividing the number of colonies by the number of cells seeded. The percent of control-cell survival for the compound-treated cultures (SFair and SFhypoxia) was calculated as $PE_{treated}/PE_{control}$ × 100. The compounds were tested at 20 μM in duplicate flasks both in aerobic and hypoxic conditions.

DNA Binding Assay

DNA Solution

Calf thymus DNA (12.5 mg) was slowly magnetically stirred in 5 mL Tris-HCl buffer (10 mM, pH 7.4) for 24 h at 4 ºC. From this solution, 0.6 mL was diluted with the same buffer to 25 mL. **Test compound solution:** it was prepared at 10^{-4} M concentration using a minimal volume of adequate solvent, no more than 5 %, and then diluted adding water to 2 × 10^{-5} M. No effect on DNA was observed by these concentrations of solvents. A 3.0 mL sample of this resulting solution was mixed with 3.0 mL of DNA solution described above. The mixtures were slowly rotated during 24 h and subsequently their UV spectra were recorded using a 1-cm cell at 20 ºC on a Jasco V-570 UV/VIS/NIR spectrophotometer. Areas were calculated automatically by the apparatus.

Cyclic Voltammetric Studies

DMSO (spectroscopy grade) was obtained from Aldrich. Tetrabutylammonium perchlorate (TBAP) used as supporting electrolyte was obtained from Fluka. Cyclic voltammetry was carried out using a Metrohm 693 VA instrument with a 694 VA Stand converter and a 693 VA Processor, in DMSO (*ca* 1.0 mM), under a nitrogen atmosphere at room temperature (TBAP, *ca* 0.1 mM) as supporting electrolyte. A three-electrode cell configuration was used, a mercury dropping working electrode, a platinum wire auxiliary electrode, and a saturated calomel reference electrode. Voltage scan rates ranged from 0.10 - 2.00 V/s.

QSAR Studies

All the compounds were built and analyzed as the 7-substituted isomers. The compounds were built with standard bond lengths and angles using the Spartan'04, 1.0.1 version, suite of programs and the geometry of each molecule was fully optimized by applying semiempirical PM3 method in gas phase from the most stable conformer obtained using molecular mechanics (MMFF) methods. Then, a single point calculation using density functional methodology (Becke's exchange functional (B) and Becke's three-parameter adiabatic connection (B3) hybrid exchange functional) were used in combination with the Lee-Yang-Parr correlation functional. The standard 6-31G* basis set of DZP quality was used for orbital expansion to solve the Kohn-Sham equations for first and second-row elements. Lipophilic properties of the compounds were included into the analyses, as cLogP (LogP calculated by Villar method at PM3 semiempirical level of calculi).

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