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Phenazine *N*,*N*′-dioxide scaffold as selective hypoxic cytotoxin pharmacophore. Structural modifications looking for further DNA topoisomerase II-inhibition activity†

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Phenazine-5,10-dioxides have been identified as prodrugs for antitumour therapy that undergo hypoxic-selective bioreduction, in the solid tumour cells, to form cytotoxic species. We investigated structural modifications of the phenazine-5,10-dioxide scaffold attempting to find new selective hypoxic cytotoxins with additional ability to inhibit DNA topoisomerase II. Four series of new phenazine-5,10-dioxides aryl-substituted connected by different linkers were prepared. The clonogenic survivals of V79 cells on aerobic and anaerobic conditions were determined, and studies of oxic DNA-interaction and hypoxic DNA topoisomerase II-inhibition, for the most relevant derivatives, were performed. Four new hypoxic-selective cytotoxins were identified at the assayed doses. In some of them were operative the DNA-interaction and/or the inhibition of DNA topoisomerase II. For one of the unselective cytotoxin biotransformation studies were performed on aerobic and anaerobic conditions, explaining the lack of selectivity.

Introduction

It is well known that the existence of hypoxic and necrotic regions in solid tumours[1] is consequence of the rapid growth of cancerous cells and their deficient vascularisation that produces molecular oxygen diffusion decreasing.[2] The hypoxic regions are associated to refractory radio- and chemotherapy.[3] Additionally, the hypoxia in tumours alters cellular metabolism tending to select for a more malignant phenotype, increasing mutation rates, and the expression of genes associated with angiogenesis and tumour invasion, and it is associated with a more metastatic phenotype of human cancers.[4] By enhancing metastasis, hypoxia can compromise curability of tumours by surgery. This common feature of cancerous cells, hypoxia, is used for the development of a distinct therapy for treating cancer, the use of bioreductive antitumour agents (BAA).[5] BAA are prodrugs, capable to be bioreduced under hypoxic conditions to further drugs that produce cytotoxic events causing different degrees of cancerous cells damage. *N*-oxide derivatives have been described among the compounds classified as BAA.[6,7] We have reported phenazine *N*,*N*′-dioxide (PDO) as a hypoxia-activated prodrug pharmacophore that potentially interacts with DNA after the corresponding bioreduction in hypoxic conditions.[8] The PDO **1** (Scheme 1a) displayed excellent *in vitro* selective cytotoxic property and *in vivo* antitumoral activity.[9] For this, selective anaerobic-reduction and its relation to bioreductive activity, and the ability to release ˙OH in hypoxia were proved using enzymatic mammal systems.[9,10] Furthermore, we have tried to improve the PDO DNA interaction by generating new derivatives with increased planar π-conjugation[8d] or flexible moieties that increase DNA-stacking.[11] PDO **2** (Scheme 1a) is an example of the latter mentioned approach that partially improve, comparing to PDO **1**, the interaction with DNA (see UV results, Scheme 1a) but with the concomitant loss of cytotoxic activity. On the other hand, the PDOs **3–5** were toxic in both conditions, non-selective, at the assayed dose (20 μM) (Scheme 1b).[8a,11f] Besides, some non-selective PDO cytotoxins displayed *in vitro* aerobic-antitumor activity against Caco-2 cells.[12] Recently, we also described QSAR-models that provided structural information for the design of new PDO derivatives with improved potency.[13]

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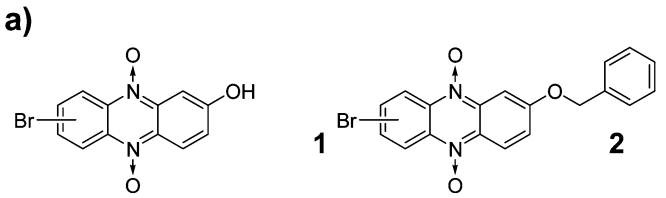
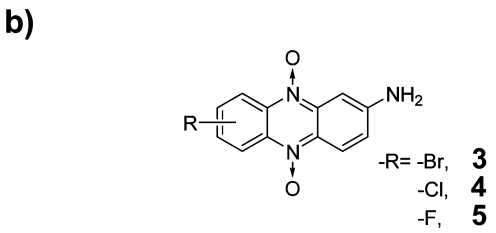


Cpd.	a_{24}/a_0
1	1.00
2	0.88
m-AMSA	0.30
mtx	0.00

m-AMSA: amsacrine
mtx: mitoxantrone
a_{24}/a_0 : compound absorbance, in DNA presence at t=0 (a_0) or t=24 h (a_{24}) (values lower than 1.00 indicate better interaction with DNA)



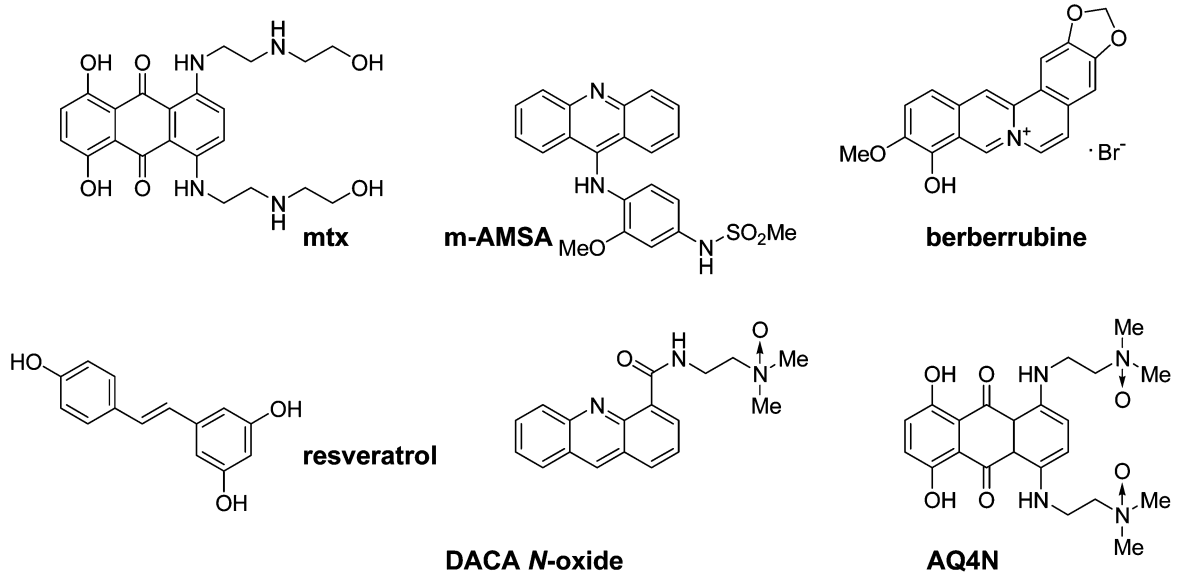
Cpd.	SF_{oxia} (%)	$SF_{hypoxia}$ (%)
1	80	0
2	86	90
3	12	0
4	0	0
5	0	0

SF: survival fraction of V-79 cells treated with PDOs at 20 μM

Scheme 1 Phenazine *N*,*N*′-dioxide (PDO) previously developed as hypoxia-activated prodrugs.

DNA topoisomerase II, an enzyme that manipulates DNA topology, such as knots, tangles, and catenanes, remaining on DNA after replication or transcription, has been described as an excellent target for the development of anticancer drugs.[14] A series of DNA topoisomerase II inhibitors are currently used in therapy, such as epipodophyllotoxins, antracyclines, antraquinones (*i.e.* mitoxantrone, mtx, Scheme 2), and acridines (*i.e.* amsacrine, *m*-AMSA, Scheme 2), for the treatment of different kind of cancers. The investigation in the DNA topoisomerase II inhibitors development is currently very wide ranging from natural products to synthetic compounds. Among the natural products, we have paid attention in the molecular structures of berberrubine and resveratrol (Scheme 2). For the synthetic products, we have been interested in certain *N*-oxides which combine in their structures pharmacophores moieties for hypoxic-cytotoxicity and, after bioreduction, become DNA topoisomerase II inhibitors, *i.e.* DACA *N*-oxide and AQ4N (Scheme 2).[15]

Taking into account our developed PDOs, the structural exingencies from QSAR-models,[13] and some DNA topoisomerase II inhibitors we planned PDOs aryl-substituted modifying the linker moiety (*series a*, *b*, *c*, and *d*, Scheme 3). The



Scheme 2 DNA topoisomerase II inhibitors used as template in the current design.

rationality was to: (i) use PDOs **1** and **3–5** as parent compounds; (ii) include an extra π-interacting moiety, like in PDO 2, with certain flexibility to improve the DNA-interaction capability or the DNA topoisomerase II inhibition, like in *m*-AMSA which stacks the acridinyl ring into the AT-base sequences and orientates the flexible anilino side-chain in the DNA-minor groove[11a,b] or binds, *via* the methanesulfonylanilino moiety, at the interface of DNA topoisomerase II and DNA[11c–e] (*series a* and *d*); (iii) use sulfonamide moiety as linker like in the DNA topoisomerase II *m*-AMSA (*series b*); (iv) use ethenyl moiety as linker like in the DNA topoisomerase II resveratrol (*series c*); (v) include in the aryl position a benzo[*d*]dioxolyl moiety like in the DNA topoisomerase II berberrubine (*series a* and *c*); (vi) include in the aryl substituent a guanidinium moiety that could reinforce the interaction with DNA (*series d*).[16] Additionally, the biological effects of all the new compounds was analyzed by use of a clonogenic assay with V79 cells in simulated oxic and hypoxic conditions. The PDOs DNA-interaction capability and DNA topoisomerase II inhibition ability of some of these prodrugs were also studied. For one of the unselective PDO bioreduction studies, on aerobic and anaerobic conditions, were performed in order to explain the lack of selectivity.

Results and discussion

Design and synthesis of new PDOs

The starting material for the synthesis of the new PDOs belonging to *series a* and *b*, **6–12** (Scheme 4a), were the parent compounds **1**, **3**, and **4** that were used as nucleophiles through its phenol or amino groups, respectively. Nucleophilic substitution reactions[11f] using phenol **1** and benzylic-derivatives with traditional heating were incomplete being the main products the corresponding de-oxygenated analogues of the parent compounds, leading to very low yield of the desired ethers (see examples in Table 1). However, we were able to improve slightly the yields of these reactions using microwave irradiation, as energy source, minimizing the presence of de-oxygenated products (Table 1). In the case of amines **3–5**, reacting with benzylic-derivatives, only derivative **11**, from parent compound **4**, was generated in very low yield and under microwave irradiation (Table 1). The amine **3** only was able to react with the good electrophile *p*-toluenesulfonyl chloride generating sulfonamide **12** in a very low yield.

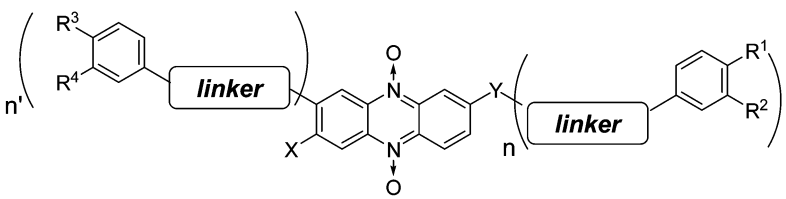
The syntheses of the new PDOs belonging to *series c*, **13–18**, were obtained *via* Beirut processes (Scheme 4b) using benzofuroxans **(I)–(III)**[17] and the nucleophiles *p*-aminophenol or *p*-hydroquinone in moderate yields. Derivatives from *series d*, **19–22**, were prepared using as starting material 4,5-difluoro-2-nitroaniline (Scheme 4c) through a nucleophilic reaction followed by a transformation to the corresponding benzofuroxan, **(IV)**, which was expanded *via* Beirut processes to **19** or **20**. Product **19**, after its purification, was obtained mixed with the corresponding imine, as result of the secondary reaction between the compound and *p*-aminophenol (see Fig. 1S in ESI†). Then, in order to include in the lateral chain a moiety that strengthens the interaction with DNA at the acidic biological pH,[18] the aldehydes **19**, mixed with the imine, and **20** were reacted with aminoguanidine producing, the aminoguanidones **21** and **22** in good yields.

Additionally, in order to confirm the relevance of the *N*-oxide moiety in the biological response of these prodrugs, deoxygenated derivatives were developed (**23–25**, Scheme 5). In order to simulate the bioreductive process, chemical reductions were carried out with sodium dithionite, considered as an analogue of reductive enzymes (Scheme 5).[19] Derivative **12** produced, in the assayed conditions, the mono-reduced derivative **23**, N^{10}-oxide while derivative **20**, in the assayed conditions, produced the mono-oxygenated derivative **24**, isolated as mixture of N^5- and N^{10}-oxide isomers, and phenazine **25**.

In all cases, the products **6–25** were characterized and evaluated as a non-separable mixture of 7- and 8-isomers because we could not separate them neither by crystallization nor by chromatographic methods. The isomeric proportion, determined by ^{1}H NMR, is shown in Table S1 (see ESI†). All the proposed structures were established by ^{1}H-, and ^{13}C NMR spectroscopies (see examples in ESI†), using COSY, HMQC or HSQC, and HMBC experiments, and MS. The purity was analyzed and established by TLC and microanalysis, respectively.

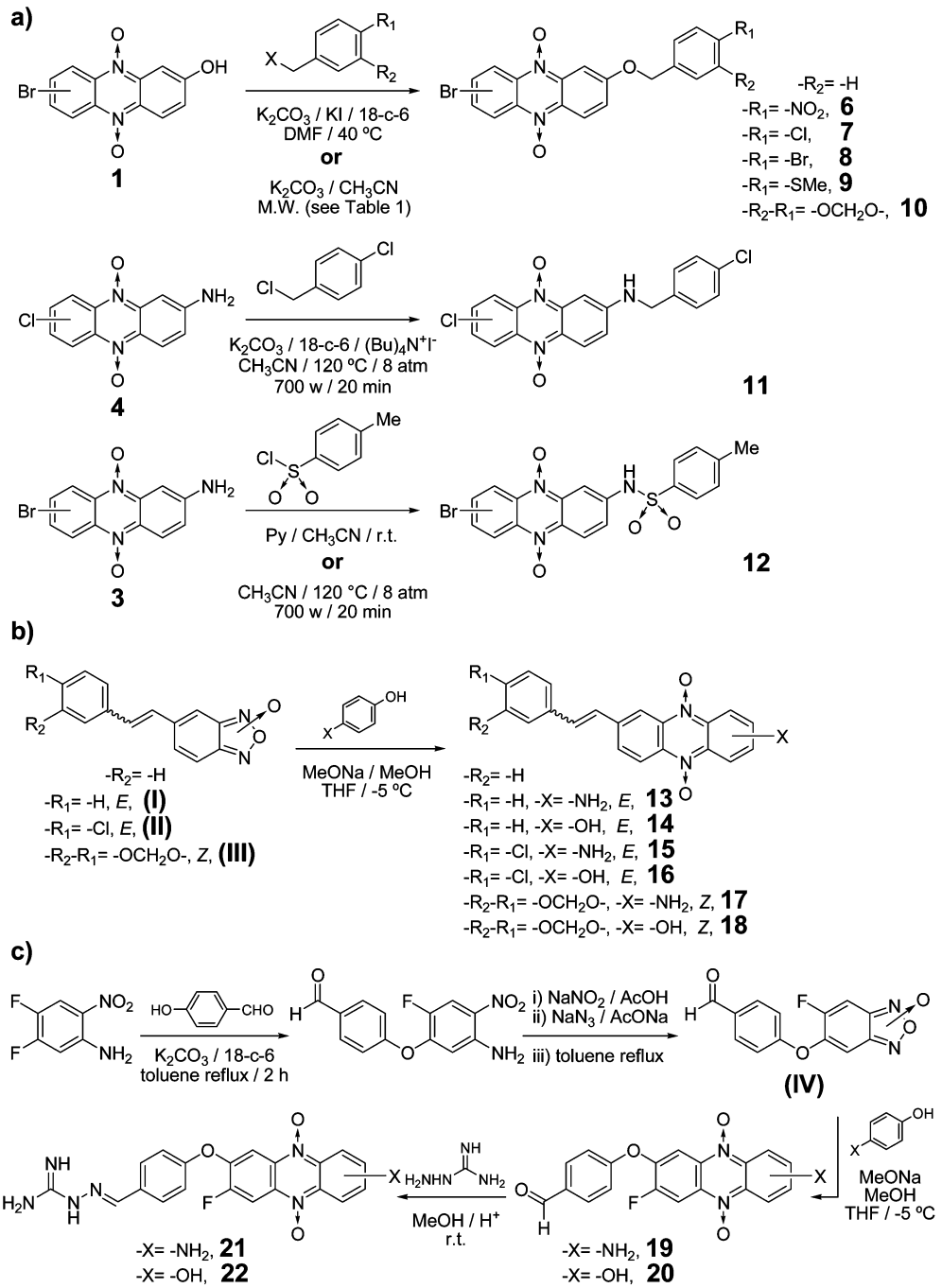
In vitro normoxic and hypoxic cytotoxicity

The PDOs and the reduced derivatives were examined for their selective hypoxic-cytotoxicities in a pre-established model of V79 cells.[8,11f,20] They were analysed as a non-separable mixture of 7- and 8-isomers. Previous results for similar chemical systems



series a	series b	series c	series d
n= 1, n'= 0	n= 1, n'= 0	n= 0, n'= 1	n= 0, n'= 1
-X= -Br or -Cl, -Y-= -O- or -NH-	-X= -Br, -Y-= -NH-	-X= -H, -Y= -OH or -NH_2	-X= -F, -Y= -OH or -NH_2
linker= -CH_2-	*linker*= -SO_2-	*linker*= -CH=CH-	*linker*= -O-
-R^1, -R^2= variable	-R^1, -R^2= variable	-R^3, -R^4= variable	-R^3, -R^4= variable

Scheme 3 General structure of the four series of designed PDOs herein.



Scheme 4 Synthetic procedure used for preparation of PDO **6–22**.

(quinoxaline 1,4-dioxides) demonstrated that no difference between both positional isomers was observed in the selective hypoxic cytotoxicities against V79 cells.[20] The percentages of survival fractions (SF, Table 2) were measured at 20.0 μM while derivative **8** was also evaluated at 10.0 μM. This compound, from *series a*, showed high cytotoxicity in both conditions, normoxia and hypoxia, at 20 μM but when the dose was lowered the toxicity disappeared without selectivity. Furthermore, three of six compounds in *series a* displayed some degree of selectivity, *i.e.* **6**, **7**, and **9**, lower than that of compound **1** but clearly better than that of parent compound **2**. The remaining studied compounds displayed not relevant biological activities, except derivative **17**, belonging to *series c*, and the intermediate selectivity derivatives **18**, from *series c*, **22**, from *series d*, and **24**, a *N*-oxide derivative. Additionally, the complete de-oxygenated derivative, **25**, showed some degree of cytotoxicity in both conditions. This result confirmed that after parent compound **20** is metabolised to the metabolite, **25**, could harm the cell *via* its own mechanism. The best behaviours as selective hypoxic cytotoxins were observed in compounds **7** and **17**.

Table 1 Assayed conditions in the synthesis of *series a* derivatives

Starting material	Product[a]	Conditions	T (°C)	Time (h)	Yield[b] (%)	Main product
1	**6**	K_2CO_3/KI/18-c-6[c]/DMF	25	48	—	No reaction
		K_2CO_3/KI/18-c-6/DMF	40	24	2	Deoxygenated product
		5 atm/300 w[d,e]	50	0.7	—	No reaction
		CH_3CN/5 atm/300 w[d]	120	0.4	<1	Deoxygenated product
		K_2CO_3/CH_3CN/5 atm/300 w[d]	50	0.5	<1	Deoxygenated product
		K_2CO_3/CH_3CN/5 atm/300 w[d]	120	0.4	10	Deoxygenated product
1	**7**	K_2CO_3/KI/18-c-6/DMF	40	24	5	Deoxygenated product
		K_2CO_3/CH_3CN/5 atm/300 w[d]	50	0.3	—	No reaction
		K_2CO_3/CH_3CN/18-c-6/TBAI 8 atm/700 w[d,f]	120	0.3	11	Deoxygenated product
4	**11**	K_2CO_3/KI/18-c-6/DMF	40	48	—	No reaction
		K_2CO_3/CH_3CN/5 atm/300 w[d]	50	0.3	—	No reaction
		K_2CO_3/CH_3CN/18-c-6/TBAI 8 atm/700 w[d]	120	0.3	7	Deoxygenated product

[a] See Scheme 4 for structures. [b] After isolation by chromatography column. [c] 18-c-6: crown ether 18-crown-6. [d] Microwave conditions. [e] Without solvent. [f] TBAI: tetrabutylammonium iodide.

Study of lack of selectivity of PDO 12

PDO **12** was one of the non-selective derivative towards both conditions, normoxia and hypoxia. For that reason, we selected it to perform an *in vitro* bio-reduction study. We studied both the metabolism in hypoxia and in normoxia of derivative **12** using S9 fraction and rat liver microsomal and cytosolic fractions.[10] For the hypoxic bioreductions nitrogen atmosphere were used while normoxic conditions were simulated in air atmosphere. The processes were monitored by chromatography (TLC) using mono-deoxygenated derivative **23** as standard of one of the possible metabolites.

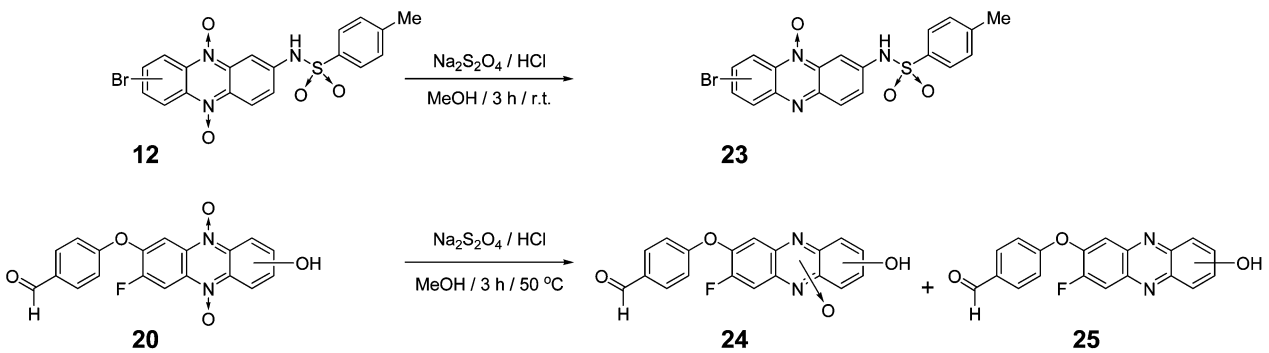
Derivative **12** suffered bioreduction in both gassing conditions (see representative chromatograms in Fig. 1 and Fig. 2S in ESI†) and similar bioreductive behaviour in the three studied protein fractions. The corresponding phenazine **23** was identified as one of the main metabolic product. Also, other metabolic products were evidenced and, according to their higher R_f values, it is possible to establish that one of these could be the corresponding phenazine analogue. Efforts to elucidate the chemical structures of these metabolic products were not done.

These results are completely in agreement with the behaviour of compound **12** in the cellular model (Table 2).

DNA interaction capability

We analyzed the capability to interact with DNA, in oxic conditions, of selected PDO prodrugs and some reduced derivatives. For that, we used the intrinsic changes in the fluorescence of phenazine while varying calf thymus DNA (CT DNA) concentrations after 30 min of incubation.[11f,21] Because it is the static quenching of DNA to compounds, the quenching constant (K_q) is considered as the formation constant of compounds and DNA complexes,[22] *i.e.*, the binding constant of compounds with DNA. The Stern–Volmer plots provided the K_q of studied compounds at pH 6.0 (see examples in Fig. 3S in ESI,† Table 3).[23] For PDO **7**, it was also evaluated its DNA-interaction ability measuring the hypochromic and bathochromic effect of compound-absorbance in the UV spectra, in a 20 nm band centered on the maximal absorbance value of each compound, at 0 and 24 h,[24] in order to compare with parent PDOs **1** and **2** (Table 3).

Firstly, the studied PDOs were able in different degrees to interact with DNA showing in some cases, derivatives **7**, **14**, **15**, and **16**, similar interacting capabilities compared with the reference compound, toluene blue (TB, Table 3). Secondly, two PDOs displayed better DNA-interacting properties than TB, they



Scheme 5 Synthetic procedure used for preparation of deoxygenated PDO **23–25**.

Table 2 PDO and deoxygenated PDO cytotoxic effects in normoxia and hypoxia on V79 cells

		$SF^{a,b,c,d}$				$SF^{a,b,c,d}$	
Series	Compd	Norm	Hypox	Series	Compd	Norm	Hypox
a	**6**	89 ± 10	58 ± 4	**d**	**19**[e]	77 ± 3	77 ± 5
	7	100 ± 6	44 ± 5		**20**	100 ± 7	95 ± 5
	8	0(86 ± 5)	0(100 ± 4)		**21**	100 ± 7	100 ± 10
	9	100 ± 6	55 ± 3		**22**	100 ± 8	73 ± 4
	10	100 ± 7	100 ± 8				
	11	85 ± 5	85 ± 4	Deoxygenated derivatives of **20**			
b	**12**	57 ± 7	66 ± 6		**24**	98 ± 2	61 ± 4
					25	67 ± 3	61 ± 5
c	**13**[f]	50 ± 3	100 ± 10				
	14[f]	50 ± 4	100 ± 7				
	15[f]	100 ± 10	100 ± 7				
	16[f]	55 ± 4	100 ± 5				
	17	100 ± 10	45 ± 4				
	18	100 ± 7	75 ± 2				

[a] SF norm = survival fraction in normoxia at 20 μM. [b] SF hypox = survival fraction in hypoxia at 20.0 μM. [c] Values are means of two different experiments. The assays were done by duplicate and using at least three repetitions. [d] Values in parenthesis are at 10.0 μM. [e] Evaluated as mixture together with the imine by-product. [f] Some compound solubility problems in the assay milieu, and both conditions, were observed.

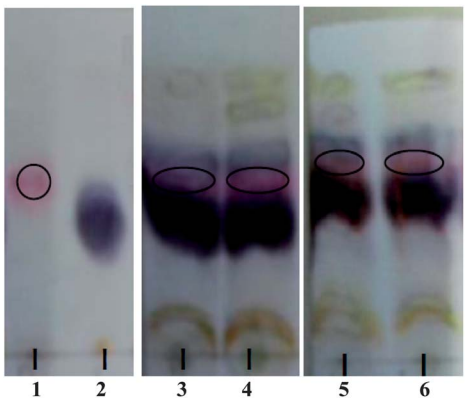


Fig. 1 TLC chromatograms (see Experimental section for experimental conditions) taken after 30 min of incubation of PDO **12** with different protein fractions and in different gasification conditions. Lanes: (1) PDO **23**; (2) PDO **12**; incubations in hypoxic conditions with cytosolic (3), and microsomal (4) fractions; incubations in normoxic conditions with cytosolic (5), and microsomal (6) fractions.

Table 3 DNA interacting capability by fluorescence-studies of PDOs, phenazine mono-oxide and phenazine

Series	Compound	Ratio K_q[a]
a	**6**	1.52 ± 0.37[b]
	7	0.92 ± 0.37 (0.63)[c]
b	**12**	1.37 ± 0.28
c	**13**	1.94 ± 0.23
	14	1.13 ± 0.15
	15	0.80 ± 0.16
	16	1.21 ± 0.17
	17	0.22 ± 0.19
	18	0.74 ± 0.19
d	**20**	0.66 ± 0.17
	21	0.01 ± 0.01
	22	0.01 ± 0.01
	24	0.24 ± 0.15
	25	1.42 ± 0.21
	TB	1.00 ± 0.19
	1	(1.00)
	2	(0.88)
	m-AMSA	(0.30)
	mtx	(0.00)
	maph[d]	0.48 ± 0.22[e]

[a] Ratio K_q = ratios of Stern–Volmer fluorescence quenching constants of the compounds and the reference compound (toluene blue, TB). [b] See text for comments. [c] Values in parenthesis are ratios of a_{24} and a_0 (see Scheme 1). [d] **maph**: 6-methyl-2-aminophenazine. [e] From ref. 11*f*.

were phenylethenyl-derivative **13** and sulfonamide-derivative **12**. It is clear the relationship between their oxic-cytotoxicities (see values of SFnorm, Table 2) and DNA-interaction abilities. This observation, in general, is a rule for all the population of studied compounds, *i.e.* the worst DNA-interacting PDOs, **21** and **22**, did not display oxic-cytotoxicities (Fig. 2a). Thirdly, the level of phenazine oxygenation and DNA-interaction capability are noticeably related, *i.e.* the deoxygenated PDO **25** interacted better than the PDO parent compound **20** (Table 3, Fig. 2b). Finally, *p*-nitrophenyl derivative **6** showed some particular behavior. The fluorescence of PDO increased when DNA concentrations was increased to 40 μM and after this, for higher DNA concentration, the effect of the quencher was evident promoting the decreasing of the PDO fluorescence (see Fig. 4Sa in ESI†). This phenomenon could be explained in term of aggregation in the assay milieu of compound **6** at low DNA concentration that was abolished at higher concentrations of the biomolecule. The values of K_q for this compound (see Fig. 4Sb and c,† Table 3) was calculated.

From a structural point of view we could draw attention to: (i) the inclusion in *series a*, *b*, and *d* of an extra π-interacting moiety with some flexibility, like in *m*-AMSA,[11a–c] improved the DNA-interaction capability (compare PDOs **6**, **7**, **12** and **20** with

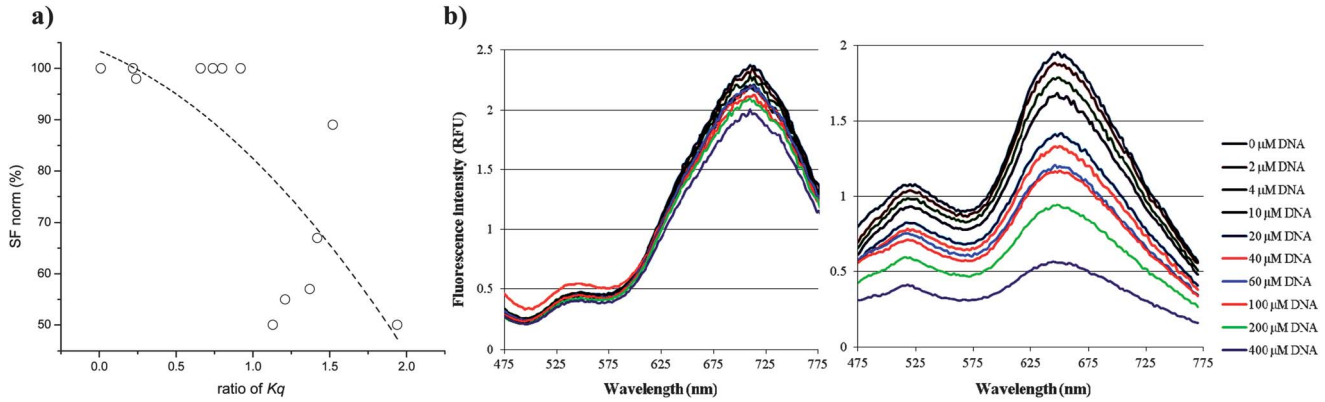


Fig. 2 (a) Ratio of K_q *vs.* survival fractions in normoxia. The dot-line is only indicative. (b) Fluorescence spectra of PDO **20** (left) and the corresponding deoxygenated derivative **25** (right) in PBS (50 mM, pH 6.0) after addition of different amounts of CT DNA.

parent compound **1**); (ii) the sulfonamide moiety, like in *m*-AMSA, improve DNA interaction more than the benzyl moiety (compare PDOs **12** and **7** or **12** and **20**); (iii) the incorporation of a *trans*-ethenylaryl moiety in *series c*, like resveratrol, contributed to increase the DNA-interaction capacity producing the *cis*-ethenylaryl substitution compounds with lower K_q than those with *trans*-arrangement (compare PDOs **13–16** with **17–18**); (iv) the aryloxy moiety, *series d*, improved the DNA interaction when it was substituted by a 4-formyl group (*i.e.* PDO **20**, compare deoxy-derivative **25** with maph, Table 3); (v) guanidinium moiety did not reinforce the interaction with DNA (compare PDO **20** with **21** and **22**).

DNA topoisomerase II inhibition ability

We analyzed the ability to inhibit DNA topoisomerase II, in microaerobic conditions,[25] hypoxia, of selected PDO prodrugs. For that, we used a yeast survival test where wild type strains, SC7K(Lys2-3) or JN362a, and mutant, ts14-16 or JN362at2-1, strains were employed as models due to the second ones are mutated at the top2-1 locus underexpressing DNA topoisomerase II.[26] We selected one derivative belonging to each series to study in these assays. However members of *series c* and *d* were completely unsoluble in the yeast culture. Consequently, we included in the studies parent compound **1**, the selective PDO **6** from *series a*, the unselective PDO **12** from *series b* and etoposide as reference compound. All the compounds were incubated with cells during 60 min and in doses of 1 mM. As previously described for the well known hypoxic-cytotoxin tirapazamine,[25a] DNA topoisomerase II inhibition could be one of the mechanisms of action of parent PDO **1** (Fig. 3a), due to its high surviving fraction in the mutant yeast ts14-16 underexpressing this enzyme. Similarly, although to a lesser extent, PDO **6** showed, in the assayed conditions (Fig. 3a), a behaviour like PDO **1**. Conversely, sulfonamide **12** did not produce a significant difference in the percentages of survival on the wild type JN362a and mutant JN362at2-1 (Fig. 3b) indicating that, in these conditions, the mechanism of DNA topoisomerase II inhibition was not operative.

These results demonstrated, at a first time, that the excellent selective hypoxic cytotoxin PDO **1** acts, at least in part, *via* DNA topoisomerase II inhibition. Additionally, the incorporation of the benzylic moiety, in derivative **6**, maintained this property but the sulfonamido group, in derivative **12**, does not contribute to enzymatic inhibition.

Experimental section

Chemistry

General methods. Some 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. Starting materials **1**, **3**, **4**, and **(I)–(III)** were prepared following synthetic procedures previously reported.[8a,17] The microwave-assisted synthesis were performed using a reactor WX-4000 (EU Chemical instruments). Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer at 298 K. The chemical shifts values are expressed in ppm (δ) relative to tetramethylsilane as internal standard and the J in Hertz. Mass spectra were determined on a MSD 5973 Hewlett–Packard spectrometer using electronic impact ionization. Microanalyses were performed on a Fisons EA 1108 CHNS-O instrument and were within (0.4% of the calculated compositions). Column chromatography was carried out using Merck silica gel (60–230 mesh).

General procedure for the synthesis of PDOs 6, 7 and 11. To a solution of the parent PDO (**1** or **4**, 1 eq.) in acetonitrile (25 mL mmol^{-1} of **23**), the corresponding benzyl halide (1 eq.), and K_2CO_3 (1 eq.) were added. In the cases of **7** and **11** preparation, 18-crown-6 ether (1 eq.) and TBAI (0.1 eq.) were also added. The reaction mixture was heated at 120 °C using microwave energy at the potency, pressure and time indicated in Table 1. Then the crude reaction mixture was partitioned between EtOAc and aqueous HCl (10%). After the work up the organic layer was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, petroleum ether : EtOAc (4 : 6)).

7(8)-Bromo-2-(4-nitrobenzyloxy)phenazine-5,10-dioxide (6). Brown-orange solid (10%). ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ

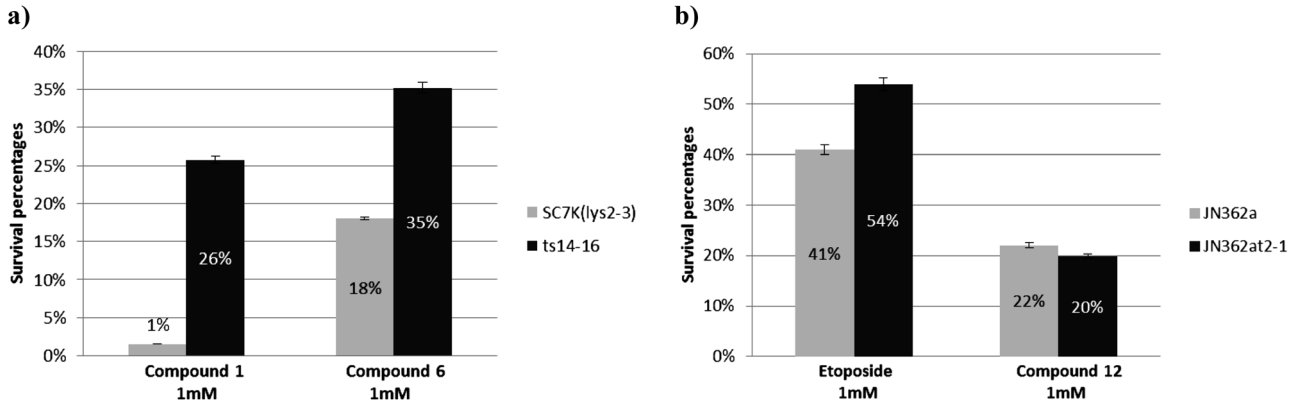


Fig. 3 (a) Survival percentages corresponding to strains SC7K(lys2-3) (gray) and ts14-16 (black) exposed 60 min to 1 mM of parent compound **1** (left) or benzylic-PDO **6** (right). (b) Survival percentages of strains JN362a (gray) and JN362at2-1 (black) exposed 60 min to 1 mM of sulfonamido-PDO **12**. Behaviour of etoposide at 1 mM was also showed.

(ppm): *7-isomer*, 5.58 (s, 2H), 7.74 (d, 2H, $J = 7.9$ Hz), 7.95 (s, 1H), 8.04 (m, 3H), 8.46 (m, 2H), 8.70 (m, 2H); *8-isomer*, 5.58 (s, 2H), 7.72 (d, 2H, $J = 7.9$ Hz), 7.96 (s, 1H), 8.06 (m, 3H), 8.53 (m, 2H), 8.54 (m, 2H). ^{13}C NMR (from HMQC and HMBC experiments) (DMSO-d_6, 100 MHz) δ (ppm): *7- and 8-isomers*, 70.1, 99.3, 122.2, 122.8, 122.7, 124.6, 129.3, 131.6, 135.4, 137.7, 144.7, 148.1. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 443/441 ($M^{+\cdot}$, 0.2), 426 (1), 410 (1). (Found: C, 51.5; H, 2.56; N, 9.2. $C_{19}H_{12}BrN_3O_5$ required C, 51.6; H, 2.74; N, 9.5%).

7(8)-Bromo-2-(4-chlorobenzyloxy)phenazine-5,10-dioxide (7). Red solid (11%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): *7-isomer*, 5.42 (s, 2H), 7.50 (m, 1H), 7.52 (d, 2H, $J = 7.8$ Hz), 7.55 (m, 3H), 7.75 (m, 2H), 8.03 (m, 2H); *8-isomer*, 5.40 (s, 2H), 7.61 (m, 2H), 7.68 (d, 2H, $J = 7.7$ Hz), 7.93 (s, 1H), 8.53 (m, 3H), 8.51 (m, 2H). ^{13}C NMR (from HMQC and HMBC experiments) (DMSO-d_6, 100 MHz) δ (ppm): *7- and 8-isomers*, 72.9, 99.1, 108.3, 122.2, 122.6, 126.2, 126.3, 128.8, 129.0, 130.7, 131.3, 132.5, 133.4, 134.3, 135.7, 161.5, 161.7. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 432/430 ($M^{+\cdot}$, 1.3/1), 429 (4), 413 (7). (Found: C, 53.0; H, 2.66; N, 6.4. $C_{19}H_{12}BrClN_2O_3$ required C, 52.9; H, 2.80; N, 6.5%).

7(8)-Chloro-2-(4-chlorobenzylamino)phenazine-5,10-dioxide (11). Violet solid (7%). ^{1}H NMR (CD_3OD : D_2O (9 : 1), 400 MHz) δ (ppm): *7-isomer*, 5.07 (s, 2H), 7.54 (m, 1H), 7.57 (m, 1H), 7.93 (m, 3H), 8.08 (m, 2H), 8.27 (m, 1H), 8.54 (d, 1H, $J = 9.2$ Hz), 8.69 (m, 2H); *8-isomer*, 5.07 (s, 2H), 7.29 (m, 1H), 7.57 (m, 1H), 7.64 (m, 2H), 7.87 (dd, 1H, $J_1 = 9.12$, $J_2 = 2.3$ Hz), 8.08 (m, 2H), 8.27 (m, 1H), 8.69 (m, 1H). EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 371/369 ($M^{+\cdot} - O$, 1/1.6), 257 (1), 245 (3). (Found: C, 58.9; H, 3.07; N, 11.2. $C_{19}H_{13}Cl_2N_3O_2$ required C, 59.1; H, 3.39; N, 10.9%).

General procedure for the synthesis of PDOs 8–10. To a solution of **1** (1 eq.) in DMF (5 mL per 0.03 mmol of **1**) was added the corresponding benzyl chloride (1 eq.), K_2CO_3 (1 eq.), 18-crown-6 ether (1 eq.), and KI (0.5 eq.). The mixture was heated at 40 °C for 24 h. Then the crude reaction mixture was partitioned between EtOAc and aqueous HCl (10%). After the work up the organic layer was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, petroleum ether : EtOAc (4 : 6)).

7(8)-Bromo-2-(4-bromobenzyloxy)phenazine-5,10-dioxide (8). Red solid (29%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): *7-isomer*, 5.40 (s, 2H), 7.53 (m, 2H), 7.76 (s, 1H), 8.01 (m, 3H), 8.59 (m, 2H), 8.63 (m, 2H); *8-isomer*, 5.38 (s, 2H), 7.51 (m, 2H), 7.56 (m, 2H), 7.76 (s, 1H), 7.75–7.95 (m, 3H), 8.51 (m, 2H). ^{13}C NMR (from HMQC and HMBC experiments) (DMSO-d_6, 100 MHz) δ (ppm): *7- and 8-isomers*, 70.1, 99.3, 122.2, 122.8, 122.7, 124.6, 128.8, 129.0, 130.7, 131.3, 132.5, 133.4, 134.3, 135.7, 161.5, 161.7. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 478/476/474 ($M^{+\cdot}$, 0.5/1/0.5), 459 (1), 443 (6). (Found: C, 47.7; H, 2.33; N, 5.6. $C_{19}H_{12}Br_2N_2O_3$ required C, 47.9; H, 2.54; N, 5.9%).

7(8)-Bromo-2-(4-methylthiobenzyloxy)phenazine-5,10-dioxide (9). Brown-orange solid (23%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): *7-isomer*, 2.73 (s, 3H), 5.43 (s, 2H), 7.32 (d, 2H, $J = 8.9$ Hz), 7.33 (s, 1H), 7.54 (m, 3H), 8.46 (m, 2H), 8.66 (m, 2H); *8-isomer*, 2.70 (s, 3H), 5.40 (s, 2H), 7.29 (d, 2H, $J = 8.9$ Hz), 7.35 (s, 1H), 7.64 (m, 3H), 8.52 (m, 2H), 8.80 (m, 2H). ^{13}C NMR (from HMQC and HMBC experiments) (DMSO-d_6, 100 MHz) δ (ppm): *7- and 8-isomers*, 15.2, 72.3, 109.4, 121.1, 121.2, 121.5, 126.7, 126.9, 127.1, 128.3, 129.6, 131.5, 132.2, 132.4, 132.6, 135.5, 135.9, 138.1, 147.9. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 444/442 ($M^{+\cdot}$, 1), 430 (3), 414 (7). (Found: C, 53.9; H, 3.25; N, 6.0; S, 7.1. $C_{20}H_{15}BrN_2O_3S$ required C, 54.2; H, 3.41; N, 6.3; S, 7.2%).

7(8)-Bromo-2-(benzo[*d*][1,3]dioxol-5-ylmethyloxy)phenazine-5,10-dioxide (10). Brown-orange solid (23%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): *7-isomer*, 4.60 (s, 2H), 6.1 (s, 2H), 7.47 (d, 2H, $J = 8.9$ Hz), 7.96 (s, 1H), 8.14 (m, 2H), 8.48 (m, 2H), 8.71 (m, 2H); *8-isomer*, 4.52 (s, 2H), 6.0 (s, 2H), 7.45 (m, 2H, $J = 8.9$ Hz), 7.35–7.64 (m, 3H), 8.52 (m, 2H), 8.80 (m, 2H); ^{13}C NMR (from HMQC and HMBC experiments) (DMSO-d_6, 100 MHz) δ (ppm): *7- and 8-isomers*, 72.9, 99.1, 108.3, 122.2, 122.6, 123.6, 126.8, 130.0, 134.5, 136.8, 138.6, 138.8. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 440 ($M^{+\cdot}$, 1), 426 (4), 410 (7). (Found: C, 54.1; H, 3.00; N, 5.9. $C_{20}H_{13}BrN_2O_5$ required C, 54.4; H, 2.97; N, 6.3%).

7(8)-Bromo-2-(4-methylphenylsulfonylamino)phenazine-5,10-dioxide (12). A mixture of the parent PDO **1** (0.16 mmol) and *p*-toluenesulfonyl chloride (0.16 mmol) in acetonitrile (3 mL) was heated at 120 °C using microwave energy at 700 watts of potency and 8 atm of pression during 20 min. Then the crude reaction

mixture was purified by column chromatography (SiO_2, petroleum ether : EtOAc (3 : 7)). Orange solid (15%). ^{1}H NMR (DMSO-d_6 : D_2O (1 : 1), 400 MHz) δ (ppm): *7-isomer*, 2.38 (s, 3H), 7.40 (m, 2H), 7.53 (m, 2H), 7.71 (dd, 1H, $J_1 = 9.1, J_2 = 2.0$ Hz), 7.86 (d, 2H, $J = 8.4$ Hz), 7.92 (m, 1H), 8.29 (m, 2H); *8-isomer*, 2.35 (s, 3H), 7.40 (m, 4H), 7.92 (m, 4H), 8.29 (m, 1H), 8.54 (d, 1H, $J = 2.0$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (1 : 1), 100 MHz) δ (ppm): *7- and 8-isomers*, 21.5, 128.9, 129.0, 129.1 (four carbons), 122.2 (two carbons), 129.9 (two carbons), 130.0 (two carbons), 131.0, 131.1, 131.2, 131.3, 131.4, 131.5. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 461/459 (M^+, 5), 445 (1), 304 (100), 288 (32). (Found: C, 49.5; H, 3.17; N, 8.9; S, 6.9. $C_{19}H_{14}BrN_3O_4S$ required C, 49.6; H, 3.07; N, 9.1; S, 7.0%).

General procedure for the preparation of the PDOs 13–20. To a solution of 3.9 mmol of metallic sodium in anhydrous MeOH (25.0 mL), at −5 °C and under nitrogen atmosphere, was added a solution of 20.0 mmol of the corresponding phenol (*p*-aminophenol or *p*-hydroquinone) and 20.0 mmol of the corresponding benzofuroxan, **(I)**, **(II)**, **(III)** or **(IV)** (see ESI for detailed experimental procedures and spectroscopic characterization of benzofuroxan **(IV)**†), in 5.0 mL of anhydrous MeOH and 30.0 mL of anhydrous THF. After stirring at room temperature for 24 h and maintaining at −20 °C for 24 h, the resulting precipitate was filtered, washed with THF yielding the desired product.

2-Amino-7(8)-(*E*-2-phenylethenyl)phenazine-5,10-dioxide (13). Black solid (47%). ^{1}H NMR (DMSO-d_6 : D_2SO_4 (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.20 (d, 1H, $J = 7.5$ Hz), 7.26 (d, 1H, $J = 8.1$ Hz), 7.32 (m, 4H), 7.37 (d, 1H, $J = 15.1$ Hz), 7.40 (d, 1H, $J = 17.3$ Hz), 7.46 (s, 2H), 7.47 (d, 1H, $J = 15.8$ Hz), 7.49 (d, 1H, $J = 17.5$ Hz), 7.60 (d, 2H, $J = 6.9$ Hz), 7.63 (d, 1H, $J = 7.2$ Hz), 8.06 (d, 1H, $J = 9.3$ Hz), 8.14 (d, 1H, $J = 9.3$ Hz), 8.15 (d, 2H, $J = 9.5$ Hz), 8.25 (d, 1H, $J = 10.0$ Hz), 8.34 (d, 1H, $J = 8.7$ Hz), 8.40 (s, 1H), 8.43 (s, 1H). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6, 100 MHz) δ (ppm): *7- and 8-isomers*, 100.3, 101.5, 113.0, 113.1, 116.9, 117.2, 120.8, 122.9, 123.0, 125.7, 125.9, 126.4, 126.5, 126.7, 127.1, 127.4, 127.6, 127.9, 129.0, 129.2, 133.3, 133.4, 133.5, 134.2, 135.4, 134.5, 136.3, 136.7, 137.3, 138.3, 139.0, 144.7, 150.0, 150.1, 157.2, 157.6. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 329 (M^+, 2), 313 (37), 297 (47). (Found: C, 73.0; H, 4.56; N, 12.8. $C_{20}H_{15}N_3O_2$ required C, 72.9; H, 4.59; N, 12.8%).

2-Hydroxy-7(8)-(*E*-2-phenylethenyl)phenazine-5,10-dioxide (14). Black solid (36%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.34 (d, 1H, $J = 7.2$ Hz), 7.35 (d, 1H, $J = 7.2$ Hz), 7.43 (m, 4H), 7.58 (d, 1H, $J = 16.4$ Hz), 7.60 (d, 1H, $J = 17.3$ Hz), 7.63 (d, 1H, $J = 16.8$ Hz), 7.68 (s, 1H), 7.69 (s, 1H), 7.64 (d, 1H, $J = 18.0$ Hz), 7.72 (d, 2H, $J = 7.2$ Hz), 7.73 (d, 2H, $J = 7.3$ Hz), 8.18 (dd, 1H, $J_1 = 9.6, J_2 = 1.4$ Hz), 8.26 (dd, 1H, $J_1 = 9.4, J_2 = 1.4$ Hz), 8.44 (d, 1H, $J = 9.6$ Hz), 8.45 (d, 1H, $J = 9.7$ Hz), 8.47 (d, 1H, $J = 9.0$ Hz), 8.49 (d, 1H, $J = 9.3$ Hz), 8.54 (d, 1H, $J = 1.2$ Hz), 8.55 (d, 1H, $J = 9.3$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 100.0, 100.1, 112.8, 116.7, 117.2, 117.6, 120.1, 120.5, 122.0, 122.1, 127.5, 127.6, 129.2, 129.3, 131.0, 131.5, 131.8, 132.8, 133.3, 133.4, 133.8, 134.5, 135.3, 136.4, 136.7, 136.9, 137.0, 137.3, 137.7, 139.0, 140.5, 141.5, 149.1, 150.0, 162.2, 162.3. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 330 (M^+, 1), 314 (14), 297 (100). (Found: C, 72.4; H, 4.11; N, 8.6. $C_{20}H_{14}N_2O_3$ required C, 72.7; H, 4.27; N, 8.5%).

2-Amino-7(8)-[*E*-2-(4-chlorophenyl)ethenyl]phenazine-5,10-dioxide (15). Black solid (79%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.42 (dd, 1H, $J_1 = 9.6, J_2 = 2.9$ Hz), 7.43 (dd, 1H, $J_1 = 9.6, J_2 = 3.0$ Hz), 7.48 (d, 2H, $J = 8.5$ Hz), 7.49 (d, 2H, $J = 8.3$ Hz), 7.56 (d, 1H, $J = 16.7$ Hz), 7.58 (d, 1H, $J = 16.7$ Hz), 7.60 (d, 1H, $J = 2.8$ Hz), 7.64 (d, 1H, $J = 16.7$ Hz), 7.66 (d, 1H, $J = 3.0$ Hz), 7.67 (d, 1H, $J = 16.3$ Hz), 7.73 (d, 2H, $J = 8.5$ Hz), 7.74 (d, 2H, $J = 8.5$ Hz), 8.16 (dd, 1H, $J_1 = 9.3$, $J_2 = 1.6$ Hz), 8.23 (dd, 1H, $J_1 = 9.5, J_2 = 1.4$ Hz), 8.42 (d, 1H, $J = 9.6$ Hz), 8.43 (d, 1H, $J = 9.6$ Hz), 8.47 (d, 1H, $J = 9.4$ Hz), 8.49 (d, 1H, $J = 9.4$ Hz), 8.53 (d, 1H, $J = 1.3$ Hz), 8.55 (d, 1H, $J = 1.2$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 99.9, 100.0, 117.4, 117.8, 120.1, 120.6, 121.9, 122.0, 126.2, 126.5, 127.5, 128.1, 128.2, 129.0, 129.1, 129.2, 129.3, 129.4, 130.9, 131.3, 131.4, 131.9, 133.2, 133.3, 133.4, 134.3, 135.3, 135.9, 135.9, 136.3, 137.4, 137.8, 138.6, 140.2, 162.8, 162.9. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 365/363 (M^+, 1.6/5), 347 (30), 331 (39). (Found: C, 65.8; H, 3.55; N, 11.2. $C_{20}H_{14}ClN_3O_2$ required C, 66.0; H, 3.88; N, 11.5%).

7(8)-[*E*-2-(4-Chlorophenyl)ethenyl)-2-hydroxyphenazine-5,10-dioxide (16). Black solid (77%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.42 (dd, 1H, $J_1 = 9.6, J_2 = 2.5$ Hz), 7.43 (dd, 1H, $J_1 = 9.6, J_2 = 2.5$ Hz), 7.49 (d, 2H, $J = 8.5$ Hz), 7.50 (d, 2H, $J = 8.6$ Hz), 7.57 (d, 1H, $J = 16.4$ Hz), 7.60 (d, 1H, $J = 16.8$ Hz), 7.64 (d, 1H, $J = 2.8$ Hz), 7.65 (d, 1H, $J = 16.5$ Hz), 7.65 (d, 1H, $J = 2.5$ Hz), 7.66 (d, 1H, $J = 16.4$ Hz), 7.74 (d, 2H, $J = 8.6$ Hz), 7.75 (d, 2H, $J = 8.6$ Hz), 8.17 (dd, 1H, $J_1 = 9.3$, $J_2 = 1.4$ Hz), 8.24 (dd, 1H, $J_1 = 9.2, J_2 = 1.5$ Hz), 8.43 (d, 1H, $J = 9.6$ Hz), 8.44 (d, 1H, $J = 9.6$ Hz), 8.48 (d, 1H, $J = 9.8$ Hz), 8.50 (d, 1H, $J = 9.7$ Hz), 8.54 (d, 1H, $J = 1.2$ Hz), 8.56 (d, 1H, $J = 1.2$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 98.1, 98.7, 116.2, 116.6, 119.3, 120.2, 123.5, 123.8, 125.2, 125.3, 126.0, 126.4, 127.6, 127.9, 128.0, 128.2, 129.0, 129.1, 129.2, 129.5, 130.1, 130.2, 131.3, 131.7, 132.2, 132.5, 133.3, 134.3, 134.6, 134.8, 136.1, 136.9, 139.5, 141.3, 161.2, 161.9. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 366/364 (M^+, 1.3/4), 347 (17), 332 (64). (Found: C, 65.6; H, 3.48; N, 7.8. $C_{20}H_{13}ClN_2O_3$ required C, 65.8; H, 3.59; N, 7.7%).

2-Amino-7(8)-[*Z*-2-(benzo[*d*][1,3]dioxol-5-yl)ethenyl]phenazine-5,10-dioxide (17). Black solid (30%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 6.01 (s, 1H), 6.02 (s, 1H), 6.77 (s, 2H), 6.78 (d, 2H, $J = 8.4$ Hz), 6.79 (d, 2H, $J = 7.7$ Hz), 6.80 (d, 2H, $J = 7.8$ Hz), 6.85 (d, 2H, $J = 8.5$ Hz), 7.33 (dd, 1H, $J_1 = 9.5, J_2 = 2.2$ Hz), 7.34 (dd, 1H, $J_1 = 9.4, J_2 = 2.3$ Hz), 7.36 (d, 1H, $J = 2.3$ Hz), 7.38 (d, 1H, $J = 2.3$ Hz), 7.50 (dd, 1H, $J_1 = 9.2, J_2 = 1.6$ Hz), 7.61 (dd, 1H, $J_1 = 9.3, J_2 = 1.7$ Hz), 8.30 (d, 1H, $J = 9.0$ Hz), 8.31 (d, 1H, $J = 10.0$ Hz), 8.32 (d, 1H, $J = 9.0$ Hz), 8.33 (d, 1H, $J = 9.1$ Hz), 8.36 (d, 1H, $J = 1.5$ Hz), 8.38 (d, 1H, $J = 1.5$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 94.1, 94.3, 98.5, 101.6, 109.0, 109.2, 118.5, 119.1, 119.3, 120.0, 121.3, 121.4, 123.5, 123.6, 125.0, 125.2, 127.3, 127.6, 127.7, 129.8, 130.1, 130.4 (three carbons), 132.1, 132.2, 132.9, 133.2,

133.4, 134.0, 134.9, 136.0, 137.6, 137.9, 138.0, 140.5, 147.4, 147.5, 147.6, 147.8, 152.3, 152.4. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 373 (M^+, 2), 357 (44), 341 (42). (Found: C, 67.5; H, 3.99; N, 10.9. $C_{21}H_{15}N_3O_4$ required C, 67.6; H, 4.05; N, 11.2%).

7(8)-[*Z*-2-(Benzo[*d*][1,3]dioxol-5-yl)ethenyl]-2-hydroxyphenazine-5,10-dioxide (18). Black solid (19%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 6.03 (s, 1H), 6.04 (s, 1H), 6.68 (d, 1H, $J = 6.2$ Hz), 6.71 (d, 2H, $J = 6.0$ Hz), 6.75 (s, 2H), 6.79 (d, 1H, $J = 8.1$ Hz), 6.80 (d, 1H, $J = 7.8$ Hz), 6.81 (d, 1H, $J = 7.6$ Hz), 6.82 (d, 1H, $J = 8.4$ Hz), 6.85 (d, 1H, $J = 7.0$ Hz), 6.87 (d, 1H, $J = 6.5$ Hz), 7.31 (dd, 1H, $J_1 = 9.6$, $J_2 = 1.9$ Hz), 7.33 (dd, 1H, $J_1 = 9.6$, $J_2 = 2.0$ Hz), 7.50 (d, 1H, $J = 2.0$ Hz), 7.52 (d, 1H, $J = 2.0$ Hz), 7.89 (dd, 2H, $J_1 = 7.3$, $J_2 = 2.0$ Hz), 8.14 (d, 1H, $J = 7.6$ Hz), 8.16 (d, 1H, $J = 8.1$ Hz), 8.18 (d, 1H, $J = 9.3$ Hz), 8.26 (d, 1H, $J = 9.1$ Hz), 8.27 (d, 1H, $J = 7.9$ Hz), 8.32 (s, 1H). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 94.6, 94.9, 98.0, 99.0, 109.5, 110.3, 117.0, 117.9, 118.5, 119.3, 120.2, 120.7, 121.8, 122.4, 125.2, 125.4, 125.9, 126.2, 127.0, 127.3, 128.5, 128.8, 129.2, 129.6, 130.7, 130.9, 131.5, 132.1, 132.3, 133.5, 134.1, 135.7, 136.1, 136.2, 136.9, 137.2, 146.5, 146.8, 147.8, 147.9, 151.1, 151.3. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 374 ($M^{+\cdot}$, 4), 357 (21), 341 (46). (Found: C, 67.1; H, 3.65; N, 7.3. $C_{21}H_{14}N_2O_5$ required C, 67.4; H, 3.77; N, 7.5%).

7(8)-Fluoro-8(7)-(4-formylphenyloxy)-2-hydroxyphenazine-5,10-dioxide (20). Red solid (57%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.45–7.50 (two dd, 6H, $J_1 = 8.8$, $J_2 = 2.4$ and $J_1 = 7.2$, $J_2 = 2.4$ Hz), 7.66 (d, 1H, $J = 2.4$ Hz), 7.72 (d, 1H, $J = 2.4$ Hz), 7.95 (d, 1H, $J = 7.6$ Hz), 8.03 (d, 1H, $J = 8.4$ Hz), 8.05 (d, 2H, $J = 8.8$ Hz), 8.07 (d, 2H, $J = 7.2$ Hz), 8.39 (d, 1H, $J = 9.6$ Hz), 8.43 (d, 1H, $J = 10.4$ Hz), 8.44 (d, 1H, $J = 9.6$ Hz), 8.46 (d, 1H, $J = 10.8$ Hz), 10.01 (s, 1H), 10.02 (s, 1H). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6: D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 99.9, 100.0, 106.8, 107.0, 108.6, 109.8, 119.5, 119.9, 122.1, 122.1, 125.5, 125.7, 131.5, 132.0, 132.7, 132.8, 133.4, 133.5, 133.6, 133.8, 137.3 (two carbons), 147.0, 147.1, 148.4, 148.6, 154.9, 157.5, 160.0, 160.3, 161.1, 161.2, 192.2, 192.3. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 366 (M^+, 7), 350 (100), 334 (55). (Found: C, 62.5; H, 2.99; N, 7.7. $C_{19}H_{11}FN_2O_5$ required C, 62.3; H, 3.03; N, 7.6%).

General procedure for the preparation of the phenazine-5,10-dioxide derivatives 21 and 22. To a solution of the corresponding aldehyde (**19**, mixed with the imine by-product, or **20**) (1 eq.) and aminoguanidine bicarbonate (1 eq.) in methanol (5 mL per mmol of aldehyde), was added concentrated hydrochloric acid, two drops. After stirring at room temperature for 8 h ethyl ether (2 mL per mmol of aldehyde) was added. The precipitate was filtered *in vacuo* and washed with ethyl ether.

2-Amino-7(8)-fluoro-8(7)-(4-guanidinoiminomethylphenyloxy)phenazine-5,10-dioxide (21). Black solid (59%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.31 (dd, 1H, $J_1 = 9.6$, $J_2 = 1.6$ Hz), 7.35 (dd, 1H, $J_1 = 9.4$, $J_2 = 1.7$ Hz), 7.40 (d, 4H, $J = 8.2$ Hz), 7.49 (d, 1H, $J = 1.8$ Hz), 7.53 (d, 1H, $J = 2.1$ Hz), 7.65 (d, 1H, $J = 8.4$ Hz), 7.72 (d, 1H, $J = 8.0$ Hz), 8.02 (d, 2H, $J = 7.6$ Hz), 8.04 (d, 2H, $J = 8.2$ Hz), 8.19 (s, 1H), 8.21 (s, 1H), 8.25 (d, 1H, $J = 9.8$ Hz), 8.27 (d, 1H, $J = 9.6$ Hz), 8.29 (d, 1H, $J = 9.1$ Hz), 8.32 (d, 1H, $J = 9.8$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 98.7, 99.1, 105.3, 106.1, 106.2, 106.4, 120.1, 120.5, 121.2, 121.9, 122.0, 123.8, 125.2, 125.3, 128.6, 129.0, 129.3, 129.7, 129.9, 130.4, 131.2, 133.0, 135.9, 136.4, 145.2, 148.5, 150.1, 150.2, 152.8, 154.0, 154.7, 155.1, 159.5, 160.3. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 421 (M^+, 2), 373 (4), 363 (13). (Found: C, 56.8; H, 3.56; N, 23.0. $C_{20}H_{16}FN_7O_3$ required C, 57.0; H, 3.83; N, 23.3%).

7(8)-Fluoro-8(7)-(4-guanidinoiminomethylphenyloxy)-2-hydroxyphenazine-5,10-dioxide (22). Orange solid (55%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.40 (d, 2H, $J = 8.0$ Hz), 7.43 (d, 2H, $J = 8.6$ Hz), 7.50 (dd, 2H, $J_1 = 9.3$, $J_2 = 1.5$ Hz), 7.68 (d, 2H, $J = 2.3$ Hz), 7.76 (d, 1H, $J = 8.2$ Hz), 7.83 (d, 1H, $J = 8.0$ Hz), 8.05 (d, 2H, $J = 7.1$ Hz), 8.07 (d, 2H, $J = 8.0$ Hz), 8.24 (s, 1H), 8.25 (s, 1H), 8.38 (d, 1H, $J = 9.7$ Hz), 8.42 (d, 1H, $J = 9.9$ Hz), 8.44 (d, 1H, $J = 9.3$ Hz), 8.45 (d, 1H, $J = 10.7$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 99.9, 100.0, 106.2, 106.9, 107.1, 107.3, 120.4, 120.7, 121.0, 122.0, 122.1, 123.9, 125.3, 125.6, 130.5, 130.9, 131.0, 131.3, 131.5, 131.7, 131.9, 133.7, 137.1, 137.3, 146.3, 148.7, 150.0, 150.1, 153.6, 155.8, 156.2, 156.5, 161.0, 161.2. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 420 ($M^{+\cdot}$-2H, 1), 364 (6), 348 (2). (Found: C, 56.7; H, 3.43; N, 20.0. $C_{20}H_{15}FN_6O_4$ required C, 56.9; H, 3.58; N, 19.9%).

7(8)-Bromo-2-(4-methylphenylsulfonylamino)phenazine N^{10}-oxide (23). PDO **12** (0.087 mmol) was dissolved in concentrated hydrochloric acid (0.06 mL) and methanol (2.7 mL). Then sodium dithionite (0.35 mmol) was added at room temperature. The mixture was stirred at room temperature for 3 h. The solvent was evaporated *in vacuo* and the residue was partitioned between EtOAc (30 mL) and saturated solution of sodium bicarbonate (30 mL). The organic phase was dried and evaporated *in vacuo*. The crude mixture was purified by column chromatography (SiO_2, petroleum ether : EtOAc (3 : 7)). Yellow-orange solid (45%). ^{1}H NMR (DMSO-d_6 : D_2O (1 : 1), 400 MHz) δ (ppm): *7-isomer*, 2.34 (s, 3H), 7.37 (m, 3H), 7.65 (m, 1H), 7.76 (m, 1H), 7.93 (m, 5H); *8-isomer*, 2.37 (s, 3H), 7.38 (m, 3H), 7.65 (m, 1H), 7.93 (m, 5H), 8.28 (d, 1H, $J = 2.4$ Hz). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (1 : 1), 100 MHz) δ (ppm): *7- and 8-isomers*, 22.5, 126.0, 127.5, 127.8, 128.5, 129.2, 129.5, 129.7, 129.9, 130.0, 130.3, 130.4, 131.3, 131.4, 131.5 (two carbons), 133.8, 134.0, 134.3. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 445/443 (M^+, 100), 289 (32), 124 (8). (Found: C, 51.5; H, 3.16; N, 9.1; S, 6.9. $C_{19}H_{14}BrN_3O_3S$ required C, 51.4; H, 3.18; N, 9.5; S, 7.2%).

Synthesis of 7(8)-fluoro-8(7)-(4-formylphenyloxy)-2-hydroxyphenazine *N*-oxide (24) and 7(8)-fluoro-8(7)-(4-formylphenyloxy)-2-hydroxyphenazine (25). PDO **20** (0.2 mmol) was dissolved in concentrated hydrochloric acid (0.1 mL) and methanol (7 mL). Then sodium dithionite (0.8 mmol) was added at room temperature. The mixture was stirred at 50 °C for 3 h. The solvent was evaporated *in vacuo* and the residue was partitioned between EtOAc (30 mL) and saturated solution of sodium bicarbonate (30 mL). The organic phase was dried and evaporated *in vacuo*. The crude was purified by column

chromatography (SiO_2, petroleum ether : EtOAc (4 : 6)). The first eluted product corresponded to derivative **25** and the second to derivative **24**.

7(8)-Fluoro-8(7)-(4-formylphenyloxy)-2-hydroxyphenazine *N*-oxide (24). Yellow solid (24%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *as a mixture of at least four isomers*, 7.27 (s, 1H), 7.31 (s, 1H), 7.34–7.59 (m, 12H), 7.65 (s, 1H), 7.71 (s, 1H), 7.78 (d, 1H, $J = 8.1$ Hz), 7.95 (d, 1H, $J = 8.1$ Hz), 7.97–8.13 (m, 12H), 8.19 (d, 1H, $J = 10.6$ Hz), 8.24 (d, 1H, $J = 11.2$ Hz), 8.38–8.47 (m, 4H), 9.98–10.04 (m, 4H). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *as a mixture of at least four isomers*, 98.3, 98.4, 102.7, 103.9, 105.1, 105.3, 106.0, 107.3, 108.8, 108.9, 114.8, 115.5, 115.8, 115.9, 118.5, 118.9, 119.2, 119.3, 119.7, 120.2, 120.3, 120.5, 120.6, 120.8, 120.9, 121.0, 125.7, 125.8, 126.5 (two carbons), 126.9, 127.2, 129.3, 129.9, 131.0, 131.1, 132.4 (two carbons), 132.6, 132.7 (two carbons), 133.0, 133.6, 133.9, 134.6, 134.8 (two carbons), 135.5, 136.3, 136.7, 137.0 (two carbons), 140.5, 140.8 (two carbons), 141.2, 141.7, 141.8, 143.1, 143.2, 143.9, 144.0, 147.6, 147.8, 148.0, 148.1, 153.5, 160.4, 160.9, 161.0, 161.2, 192.2, 192.3, 192.4. EI-MS: *m*/*z* (abundance, %): *as mixture of isomers*: 350 (M^+, 1), 279 (9), 263 (1). (Found: C, 64.9; H, 3.05; N, 8.0. $C_{19}H_{11}FN_2O_4$ required C, 65.1; H, 3.17; N, 8.0%)

7(8)-fluoro-8(7)-(4-formylphenyloxy)-2-hydroxyphenazine (25). White solid (19%). ^{1}H NMR (DMSO-d_6 : D_2O (9.5 : 0.5), 400 MHz) δ (ppm): *7- and 8-isomers*, 7.30 (d, 1H, $J = 2.8$ Hz), 7.34 (d, 1H, $J = 2.4$ Hz), 7.40 (d, 1H, $J = 8.4$ Hz), 7.43 (m, 1H), 7.43 (d, 2H, $J = 8.8$ Hz), 7.60 (dd, 1H, $J_1 = 9.9$, $J_2 = 2.6$ Hz), 7.80 (d, 1H, $J = 8.8$ Hz), 7.93 (d, 1H, $J = 8.8$ Hz), 8.01 (d, 2H, $J = 8.8$ Hz), 8.03 (d, 2H, $J = 8.8$ Hz), 8.08 (d, 1H, $J = 9.6$ Hz), 8.12 (d, 1H, $J = 9.2$ Hz), 8.15 (d, 1H, $J = 12.4$ Hz), 8.20 (d, 1H, $J = 11.6$ Hz), 9.99 (s, 1H), 10.01 (s, 1H). ^{13}C NMR (from HSQC and HMBC experiments) (DMSO-d_6 : D_2O (9.5 : 0.5), 100 MHz) δ (ppm): *7- and 8-isomers*, 118.3, 118.8, 127.1 (two carbons), 131.0 (two carbons), 132.6 (two carbons), 132.8, 133.0, 138.2, 138.3, 139.1 (two carbons), 139.6, 139.7, 141.5 (two carbons), 145.0 (two carbons), 146.2, 147.1, 147.2, 150.2, 153.5 (two carbons), 155.9, 156.0, 160.3 (two carbons), 160.9, 161.0, 192.1, 192.2. EI-MS: *m*/*z* (abundance, %): *7- and 8-isomers*: 334 (M^+, 1), 317 (1), 279 (11). (Found: C, 68.0; H, 3.10; N, 8.2. $C_{19}H_{11}FN_2O_3$ required C, 68.3; H, 3.32; N, 8.4%).

Biology

Bio-reductive activity.[8,9,11f]

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) $\times$ 10^4 cells per cm^2 twice weekly. The growth milieu was EMEM (Eagle's Minimal Essential Milieu), containing 10% (v/v) foetal bovine serum (FBS) and penicillin/streptomycin at 100 U per 100 μg mL^{-1}.

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 per 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, **6–22**, **24**, and **25**, were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure DMSO (Aldrich) or sterilized distilled water. Thirty min 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 milieu (EMEM plus 10% (v/v) FBS and penicillin/streptomycin). Cell numbers were determined with a haemocytometer and 10^2 to 10^3cells were plated in 6-well plates to give a final volume of 2 mL per 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 (SFnormoxia and SFhypoxia) was calculated as $PE_{treated}/PE_{control} \times 100$. The compounds were tested at 20 μM in duplicate flasks both in aerobic and hypoxic conditions.

DNA interaction studies.[21]

DNA solutions. Calf thymus DNA (CT DNA, 12.5 mg, Sigma Chemical Co., USA) was slowly magnetically stirred in 5 mL phosphate buffer solution (PBS) (50 mM, pH 6.0) for 24 h at 4 °C. From this solution, the further necessary dilutions were performed using the same buffer. Test compound solution: It was prepared at 20 μM concentration using PBS (50 mM, pH 6.0) and 20% of DMSO. No effect on DNA was observed by these concentrations of solvents.

Study. Fluorescence spectra were recorded on a Varioskan Flash 2.4.1 spectrometer. The fluorescence quenching titrations with CT DNA were performed by keeping the compounds concentrations constants (20 μM) and varying the nucleic acid concentrations (0–400 μM) maintaining the total volume of the solution constant. Fluorescence emission spectra were recorded in the wavelength range of 320–840 nm by exciting the compounds at the corresponding wavelength with the excitation slit widths of 12 nm. The intrinsic binding constants of compounds with CT DNA were determined by fluorescence titrations. The data were plotted according to the Stern–Volmer equation:[27] $I_0/I = 1 + K_q[Q]$, where, I_0 and I are the fluorescence intensities in the absence and presence of CT DNA, respectively. K_q is the Stern–Volmer fluorescence-quenching constant. [Q] is the concentration of quencher.

***In vitro* bio-reduction study.**[9,10] The S9 fraction of Sprague-Dawley rats pretreated with Aroclor 1254 was obtained from Moltox (Boone, North Carolina, USA).

Preparation of the rat liver microsomal and cytosolic fractions. Livers were obtained from male Sprague-Dawley rats (198–202 g) from "Centro de Investigaciones Nucleares" (UdelaR, Montevideo, Uruguay). The animals were allowed for food and water *ad libitum*. The experimental protocols with animals were

adhered to the Principles of Laboratory Animal Care.[28] The animals were sacrificed by cervical dislocation and the livers, maintained in a ice bath, were perfused *in situ* with an ice-cold KCl (0.9%) solution and washed with 3 volumes of Tris–HCl (0.05 M)-sucrose (0.25 M) pH = 7.4 in a Potter-Elvehjem glass-Teflon homogeniser. The homogenates were centrifuged for 30 min at 900 g at 4 °C and the supernatant fraction was centrifuged at 10000 g for 1 h at 4 °C. The pellet was discarded and the supernatant fraction was further centrifuged at 100 000*g* for 1 h at 4 °C. Metabolic assays were carried out with microsomes and cytosol either fresh or frozen in Tris–HCl buffer and stored at −80 °C.

Incubation of PDO 12 with rat microsomal and rat cytosolic fractions and S9 fraction in normoxia and hypoxia. Our procedure was adapted from published methods.[29] The standard incubation mixture, in nitrogen or air purged flask, contained $MgCl_2$ (1.3 mM), NADPH generating system ($NADP^+$, 0.4 mM; glucose 6-phosphate, 3.5 mM; 0.5 U mL^{-1} glucose 6-phosphate dehydrogenase) in a 0.1 M potassium phosphate buffer (pH 7.4) containing EDTA (1.5 mM) and the corresponding PDO (40 mM) dissolved in dimethylsulfoxide (DMSO). Nitrogen gas was passed through a silica gel trap. The experiments were performed by triplicate. After pre-equilibration of the mixture at 37 °C, gassing with nitrogen or air, appropriate volume of microsomal, cytosolic or S9 suspension was added to give a final protein concentration of 1 mg mL^{-1}. The mixtures were incubated for 30–120 min at 37 °C. Three control incubations were used: (1) 0.1 M potassium phosphate buffer (pH 7.4) (C1); (2) (C1) + NADPH-generating system (C2); 3) (C2) + heat-inactivated cytosolic or microsomal fractions (C3).

TLC monitoring of metabolites. The incubated mixtures were extracted with EtOAc (3 × 400 μL) and the organic layer was evaporated to dryness *in vacuo*. The residue was treated twice with EtOAc, 500 μL each. TLC experiments were performed using Al_2O_3 as solid phase and CH_2Cl_2 : MeOH (99 : 1). The spots were visualised using UV-light (240 nm), directly for its characteristic colours (PDO, orange; phenazine monoxides, yellow) or by spraying with a solution of *p*-anisaldehyde : H_2SO_4(c) : EtOH (95 : 4 : 1) followed by heating.

DNA topoisomerase II inhibition studies. Yeast cells were grown in liquid nutrient milieu YPD [1% yeast extract (USB, Cleveland, OH), 2% bactopeptone (USB), and 2% dextrose (Sigma, St Louis, MO)]. Cells were grown and maintained at 30 °C for 1–7 days with aeration by shaking, without added nutrients. Thereafter, samples of these cultures were grown in nutrient YPD up to the exponential phase (2–5 × 10^7 cells per mL). Solid used nutrient milieu was YPDA (YPD + 2% Agar, USB).[26a]

Treatment. Compounds solutions, were prepared just before dosing. Stock solutions, 20-fold more concentrated, were prepared in pure DMSO (Aldrich) or in sterilized distilled water. Cells (1.5 mL from exponential phase cultures) were incubated with 0.1 mL of compounds solutions in closed flasks, shaked for sixty min, two flasks per dose. In every assay there was one flask with 0.1 mL of DMSO (negative control) and another with etoposide (positive control) at 30 °C (SC7K(lys2-3), JN362a and JN362at2-1) and at 22 °C for ts14-16. After treatment, cells were centrifuged and washed twice with sterilized distilled water and resuspended in sterilized distilled water. Cells were plated in Petri dishes at a number of 10^2 for negative control and 10^2 to 10^3 per plate for treated cells. Plates were incubated for 3–6 days and number of cell colonies was determinated. Negative control cell growth was 100% and treated cells surviving fraction was calculated as (surviving $cells_{treated}$/surviving $cells_{control}$) × 100. Statistical analysis was performed with the method of binomial distribution, with a confidence interval of 95%.

Conclusions

In conclusion, we designed and prepared a new series of phenazine dioxide derivatives as potential selective hypoxic cytotoxins. Different moieties were included in the phenazine scaffold trying to increase the DNA or DNA topoisomerase II interaction capabilities. Four of the new derivatives, PDOs **6**, **7**, **9**, and **17**, showed promising *in vitro* profiles. One of them, PDO **6**, together with the parent compound, PDO **1**, showed capability to inhibit DNA topoisomerase II in hypoxia. The compounds DNA interaction abilities, in oxia, were related to the oxic cytotoxicities.

According to our results, the incorporation of a benzyl-group, *i.e.* in derivatives **6**, **7**, and **9**, was related to the desired bioactivity. In contrast, the arylethenyl-moieties were, in general, responsible of normoxic cytotoxicity and the sulfonamido-group produced non-selective compound.

The present results can inspire the synthesis of new bioreductive agents.

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