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Study of benzo[*a*]phenazine 7,12-dioxide as selective hypoxic cytotoxin-scaffold. Identification of aerobic-antitumoral activity through DNA fragmentation

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ABSTRACT

Phenazine 5,10-dioxides are prodrugs for antitumor therapy that undergo hypoxic-selective bioreduction to form cytotoxic species. Here we investigate the expanded system benzo[*a*]phenazine 7,12-dioxides as selective hypoxic cytotoxin-scaffold. The clonogenic survival of V79 cells on aerobic and anaerobic conditions, conduct us to study antiproliferative activity on Caco-2 tumoral cells in normoxia. Electrochemical, DNA-interaction and DNA-damage studies were performed to establish the mode of action. The results demonstrated the potential biological properties of the studied scaffold being derivatives **6–10** structural hits for further chemical-modifications to become into therapeutics for solid tumors. Compounds **6** and **8** with cytotoxicity against V79 cells in both conditions (aerobia and anaerobia) were also cytotoxic against Caco-2 tumoral cells in aerobiosis.

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1. Introduction

Since the 1960' it is well known the existence of hypoxic and necrotic regions in solid tumors[1] representing the hypoxic regions up to 20% of the total tumor mass. This situation is the result of the cancerous cells isolation from the blood supply, as consequence of their rapid growth and deficient vascularisation, decreasing the diffusion of molecular oxygen.[2] The hypoxic cells are associated with increased resistance to radiation and chemotherapy.[3] Conventional anticancer drugs in clinical use are antiproliferative agents that kill dividing cells, by attacking DNA (synthesis, replication or processing). These anticancer drugs could be ineffective because the hypoxic tumor cells are not dividing rapidly.

In addition, it has also been demonstrated that hypoxia in tumors alters cellular metabolism tending to select for a more malignant phenotype, increases mutation rates, increases expression of genes associated with angiogenesis and tumor invasion, and is associated with a more metastatic phenotype of human cancers.[4] By enhancing metastasis, hypoxia can compromise curability of tumors by surgery. This common feature of cancerous cells is used for the development of a distinct therapy for treating cancer. On the basis of using prodrugs, capable to be bioreduced under hypoxic conditions (bioreductive antitumor agents,[5] BAA) to further drugs that produce cytotoxic events causing different degrees of cancerous cells damage, hypoxic selective cytotoxins have been developed. Molecular oxygen reverts the bioreductive process in normal oxygenated tissues giving the selectivity to this process the redox properties of the drugs and the reductases enzymes levels.[6] Among the compounds classified as BAA N-oxide, nitro and quinone derivatives have been described.[7] This hypoxic tissues special condition has been employed as a strategy for delivering traditional antineoplastic agents. Consequently a great number of hybrid compounds have been described where N-oxide, nitro, azido, quinone, metal ion, 1,2-benzisoxazolyl and sulfoxide moieties have been employed as redox-activated trigger entities.[8]

We have recently reported[9] the first examples of a new group of N-oxide-hypoxia-activated prodrugs, compounds that display

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toxic selectivity to cells at low oxygen concentrations. Phenazine 5,10-dioxide derivatives (Scheme 1a), structurally related to other N-oxide bioreductive agents, were selected for their DNA–π-stacking structure that potentially interact with DNA after the corresponding bioreduction in hypoxic conditions. Amines **2**, and **4** displayed excellent selective cytotoxic properties while phenols, except the lead compound **1**, were non-cytotoxic in both of the studied conditions (i.e., **3** and **5**, Scheme 1b). Besides, some of the phenazine 5,10-dioxides displayed *in vitro* aerobic-antitumor activity against Caco-2 cells.[10] The selective anaerobic-reduction and its relation to bioreductive activity were proved using enzymatic mammal systems.[11] On the other hand, none of them were able to interact with DNA,[9] hence the proposed extra-mechanism is not functional in this system.

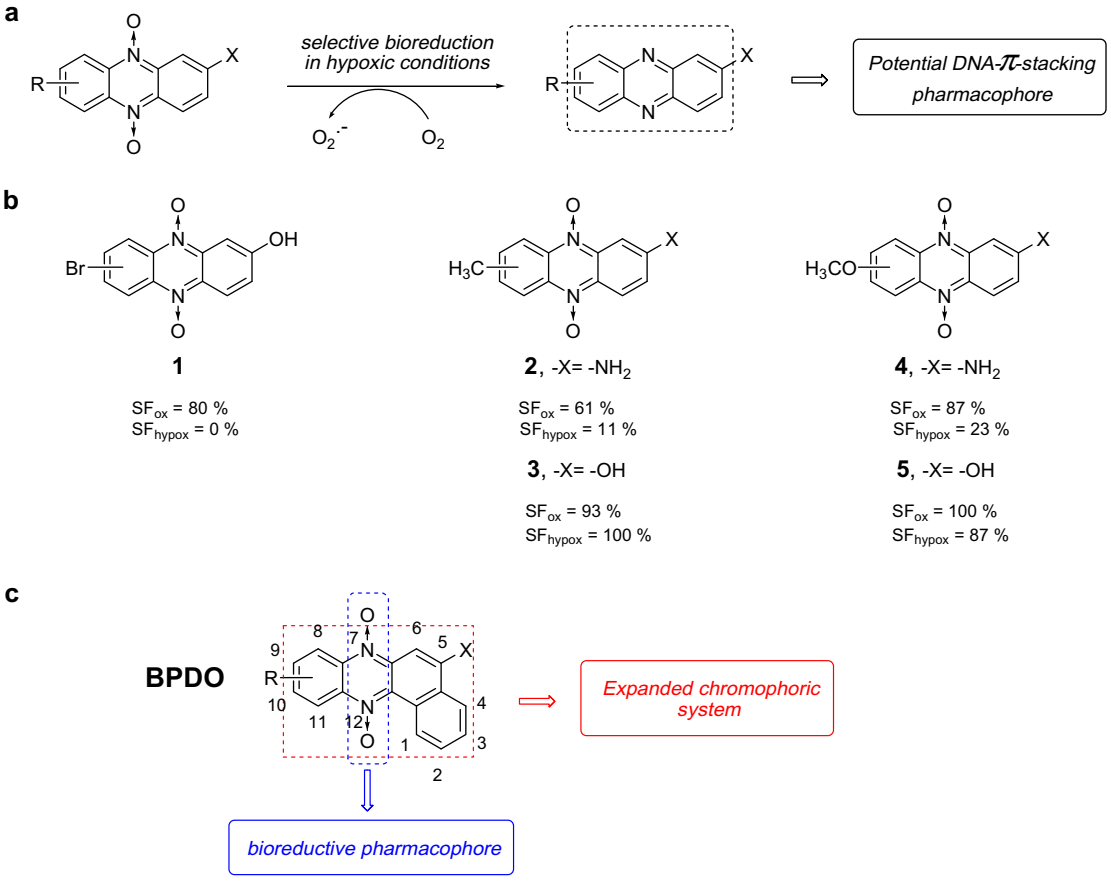
For that reason we investigated a new scaffold, the benzo[*a*]phenazine 7,12-dioxide system (BPDO, Scheme 1c), that potentially improves DNA-π-stacking properties, through the expanded chromophoric system, maintaining the bioreductive capability through the N-oxide pharmacophore. Therefore we have prepared a selected series of BPDO derivatives, *in vitro* evaluated its clonogenic capacity in normoxia and hypoxia and studied some aspects related to mechanism of action.

2. Methods and Results

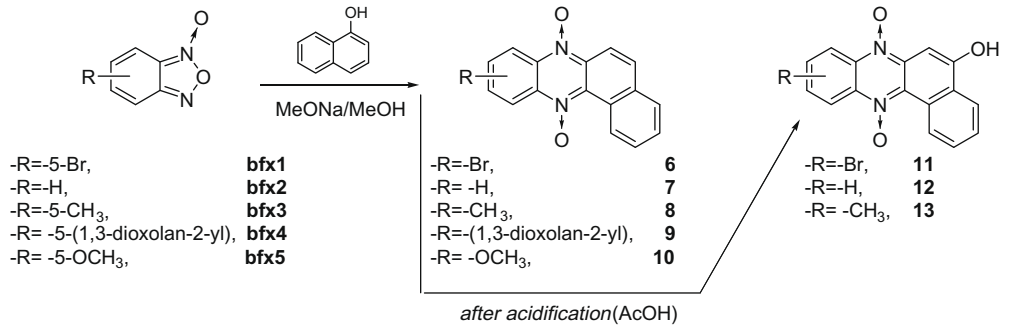
2.1. Chemistry

According to our previous biological results, we have prepared BPDOs containing as -R substituents the most relevant ones from the parent compound (**1**, **2**, and **4**, Scheme 1b). In this exploratory stage of the work we have selected one of the best described synthetic methodology to prepare the BPDO system (**6–13**, Scheme 2).[12] This procedure allowed us to prepare, besides the desired BPDO phenols after pH changes, 5-unsubstituted derivatives, being six of them no described previously (Scheme 2).

The compounds resulted from the *ortho* (**6–10**) and *para* (**11–13**) coupling procedures with the corresponding phenolate anion. We were enable to isolate, after acidification and by precipitation, the phenol derivatives when –R = –OCH_3 and –(1,3-dioxolan-2-yl). The acidified mother liquors showed a muddy complex mixture of products which after chromatographic resolution did not yield the desired phenols. All the structures were elucidated on the basis of assignment of the large chemical shift of the proton at 1-position in the NMR spectra (signal near to 10.50 ppm, Figure S1a, Supporting Information) and on the characteristic chemical shift of the *peri*-N-oxide proton at 11-position, then the complete proton and carbon assignments were performed in terms of NOE-diff, COSY, HSQC, and HMBC experiments (Figure S1b, Supporting Information). The previous described BPDO, that is, **7** and **12**,[12] were completely characterized in term of NMR spectroscopies. As we previously observed, using 5-methoxybenzofuroxan[9] as starting material only one of the two possible products, 9- or 10-substituted BPDO, was isolated by precipitation. According to the spectroscopic data derivative **10** was assigned as the 10-methoxy isomer. The other BPDOs were characterized as a non separable mixture of 9- and 10-isomers because they could not be isolated either by crystallization or by chromatographic methods (preparative-TLC, column chromatography, direct- and reverse-HPLC). The very close physicochemical behaviors of each positional isomer did not allow us to separate them by physicochemical methods. The purity of BPDOs was established by TLC and microanalysis.



Scheme 1. (a) Expected mechanism of action of the first series of developed phenazine 5,10-dioxides. (b) Selected derivatives, to exemplify was selected the data SF_{ox} (survival fraction in oxic conditions at 20 μM) and SF_{hypox} (survival fraction in hypoxic conditions at 20 μM). (c) Benzo[*a*]phenazine 7,12-dioxides studied in this work.



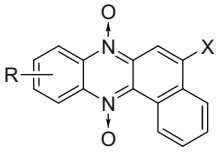
Scheme 2. Synthetic procedure used to prepare the benzo[*a*]phenazine 7,12-dioxides.

2.2. Biological characterization

2.2.1. In vitro normoxic and hypoxic cytotoxicity

All the BPDO were examined for its selective hypoxic-cytotoxicities, in a pre-established model of V-79 cells.[9,13,14] They were analyzed as a non separable mixture of 9- and 10-isomers. Previous results for similar chemical systems (quinoxaline 1,4-dioxides) demonstrated that no difference between both positional isomers were observed in the selective hypoxic cytotoxicities against V-79 cells.[13] The percentages of survival fractions (Table 1) were measured at 20 μM while derivative **6** was also evaluated at 10 μM. Except derivatives **10** and **12**, the BPDOs showed high to medium cytotoxicity in normoxia maintaining the 5-un-substituted derivatives **6–9** its cytotoxicity in hypoxia. These derivatives were classified as non-selective cytotoxins. The 5-hydroxy-substituted derivatives **11** and **13** showed very particular cytotoxic profiles having high oxic cytotoxicities they were not toxic in hypoxia. This particular toxicity could be the result of at least two different processes, the formation of toxic species, that is, superoxide anion (Scheme 1a), by the reverse reaction with molecular oxygen of the BPDO-free radical generated by the reductases (futile cycle) or the BPDO damage as result of its DNA–π-stacking property. Some experiments were performed in order to probe these hypotheses (see below). When derivative **6** was studied at lower doses it displayed similar cytotoxicity profile of derivatives **11** and **13**. The methoxy-derivative **10** was the only one with some degree of selective cytotoxicity in hypoxia having values of SF in both conditions similar to the methoxy-parent compound **4** (Scheme 1b), though it is not relevant.

Table 1
BPDO (**6**–**13**) cytotoxic effects in normoxia and hypoxia on V-79 cells and cytotoxic effects on tumoral Caco-2 cells for the normoxic-toxic BPDO on V-79 cells (**6**, **8**, **11**, and **13**)



Compd	–X	–R	SF[a,b,c,d]		VC_{50}[e,f] (μM)
			Norm	Hypox	
6	–H	–Br	0 (2)	0 (48)	23.0
7	–H	–H	40	45	nd[g]
8	–H	$-CH_3$	11	0	290.0
9	–H	–1,3-Dioxolan-2-yl	52	60	nd
10	–H	$-OCH_3$	88	44	nd
11	–OH	–Br	0	100	53.0
12	–OH	–H	100	93	nd
13	–OH	$-CH_3$	9	85	140.0

[a] SF norm = survival fraction in normoxia at 20 μM.
[b] SF hypox = survival fraction in hypoxia 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] Values in parentheses are at 10 μM.
[e] VC_{50} = concentration that causes 50% decrease of Caco-2 cell viability in normoxia, after 24 h of exposition.
[f] Values are means of three different experiments. The assays were done by triplicate and using at least three repetitions, standard errors were not greater than 5% for most assays.
[g] nd: not determined.

2.3. Normoxic cytotoxicity studies

To gain insight into BPDO mechanism of toxicity in oxic conditions, first of all we studied the BPDO electrochemical behavior in term of cyclic voltammetry in organic aprotic solvent (DMSO).[9] BPDO displayed comparable voltammetric behavior in this condition showing two to three reduction peaks and the anodic counterparts. Especially, we focus our attention in the first reduction potential because it could be used as a descriptor of the BPDO feasibility to be reduced in hypoxic conditions. Table 2 lists the values of the first N-oxide cathodic peaks which correspond to quasireversible processes. As expected the electron-donor-substituted derivatives, like the 5-hydroxy-substituted **11** and **13**, have more negative cathodic potential than the electron-withdrawing-substituted ones, that is, derivative **8** (E_{pc} = −1.36 V in **13** vs −0.95 V in **8**). However, the reduction potential does not seem to be correlated to the oxic toxicity since some of the most aerobic-toxic compounds, that is, derivatives **11** and **13**, have the lowest reduction potentials that are unfavorable to be reverted by molecular oxygen.

For this reason, secondly we analyze the BPDO capability to interact to DNA. As a first approach, we studied the binding capacity by measuring the hypochromic and bathochromic effect

Table 2
Reduction potentials and DNA interacting capability by UV studies of BPDO derivatives

Compd	E_{pc} versus SCE[a] (V)	a_{24}/a_0
6	nd[b,c]	0.88
7	nd[c]	1.00
8	−0.95	0.71
9	−0.85	nd[c]
10	−0.92	1.00
11	−1.22	0.94
13	−1.36	0.47
m-AMSA	—	0.30
Ethidium bromide	—	0.50
NSC 322921 (bis-benzimide)	—	0.57
Mitoxantrone	—	0.00

[a] Peaks potentials (~±0.01 V) measured at a scan rate of 2.00 V/s.
[b] Not determined.
[c] Solubility problems in the assay milieu do not allow to perform the study.

of BPDO absorbance in the UV spectra, in a 20 nm band centred on the maximal absorbance value of each compound, at 0 and 24 h.[15,16] The degree of interaction was expressed by the ratio between the final absorbance area (a_{24}) and the absorbance of the compound (a_0). Values of 1.00 or superior to 1.00 indicate a total lack of affinity and a value of 0 indicate binding of the entire compound to DNA (Table 2, Fig. S2a, Supplementary data). Derivatives with highest oxic-toxicity, that is, **6**, **8** and **13**, were the best DNA-interacting agents with values of a_{24}/a_0 between 0.47 and 0.88. The 9(10)-methyl-derivatives **13** and **8** have the highest capabilities of DNA-interactions being the 5-hydroxy-analogue, **13**, as interactive as NSC 322921, ethidium bromide or *m*-AMSA in our experimental conditions (Table 2).

To profoundly study the DNA-interaction processes derivatives **8** and **13** were selected to perform further interacting experiments. The degree of interaction by DNA-melting point changes promoted by the compounds was carried out.[16] Whereas the free DNA melted at 73.5 °C the systems DNA/**8** and DNA/**13** did at 75.5 and 78.5 °C, respectively (Fig. S2b, Supplementary data). The melting temperature displacements are in agreement with the obtained values a_{24}/a_0 (Table 2) showing that derivative **13** could interact with DNA by intercalation but in a minor way than others well known intercalators (i.e., proflavine, $\Delta T = 12.0$ °C[17]).

Moreover, we studied DNA structural parameters of **13**-modified DNA and unmodified (untreated) DNA via tapping mode atomic force microscopy (AFM).[18] We analyzed the effects on DNA structure of individual DNA molecules by increasing the amount of **13** (molar ratio **13**:DNA varying between 1:1 to 100:1). Measurements of **13**-modified DNA populations indicate significant changes respect to unmodified DNA control, in the contour length (cl) and at the end-to-end distance (eed) (Fig. 1). End-to-end distance shows clearly DNA shortening and eed/cl ratios were similar for the three studied reactants proportions, near to the unity, and significantly greater than with control DNA (~0.94 vs 0.51). This could be indicating that derivative **13** promotes DNA breakage yielding little DNA fragments. Also it could be suggested that the mechanism of interaction of derivative **13** with DNA is not by intercalation because no lengthening of DNA molecules is observed (Fig. 1b). Also condensate structures of DNA such as spheres and rods were observed (magnification Fig. 1b). Similar structures have been seen for other antitumoral DNA interacting agents such as some platinum complexes.[19]

This observation conducted us to study the aerobic antitumoral activity on Caco-2 cells.[20] Therefore, VC_{50}s (concentrations that caused 50% decrease of cell viability), for the oxic-cytotoxic BPDOs on V-79 cells, that is, **6**, **8**, **11**, and **13**, were determined (Table 1). The bromo-derivatives, **6** and **11**, were more toxics on Caco-2 cells than the methyl-ones, **8** and **11**, with the same cytotoxic-profile that observed on V-79 system (SF normoxia, Table 1).

Further the DNA damages, on Caco-2 cells, promoted by the less toxic derivatives, **8** and **13**, were evaluated. Gel electrophoresis results indicate that derivative **13** is able to induce DNA fragmentation in oxic conditions beginning at 50 μM (Fig. 2), a dose lower than the corresponding VC_{80}. Its effect is similar to the positive control used in this assay (staurosporine, Stau, at 10 μM). On the

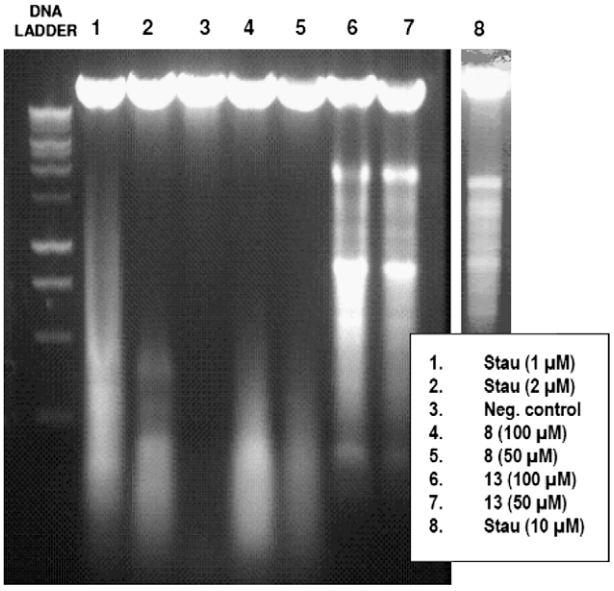
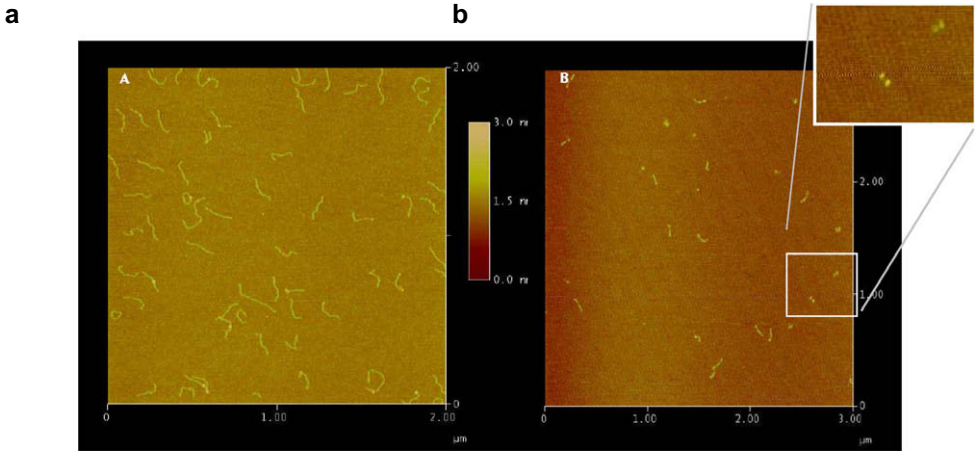


Figure 2. Genomic DNA analysis after exposure to BPDOs. Caco-2 cells were exposed for 24 h in oxic conditions to variable doses of **8** and **13**.



ratio BPDO 13:DNA	contour length (nm)	end-to-end distance (nm)	end-to-end distance/contour length
0:1	165	84	0.51
1:1	96	90	0.94
10:1	118	112	0.95
100:1	67	62	0.93

Figure 1. Top: (a) atomic force microscopy of unmodified linear DNA. (b) **13**-modified linear DNA (ratio 100:1). Up: length parameters of DNA in the different experimental conditions. In each case, contour lengths and end-to-end distances were measured for at least 100 molecules that could unequivocally be traced.

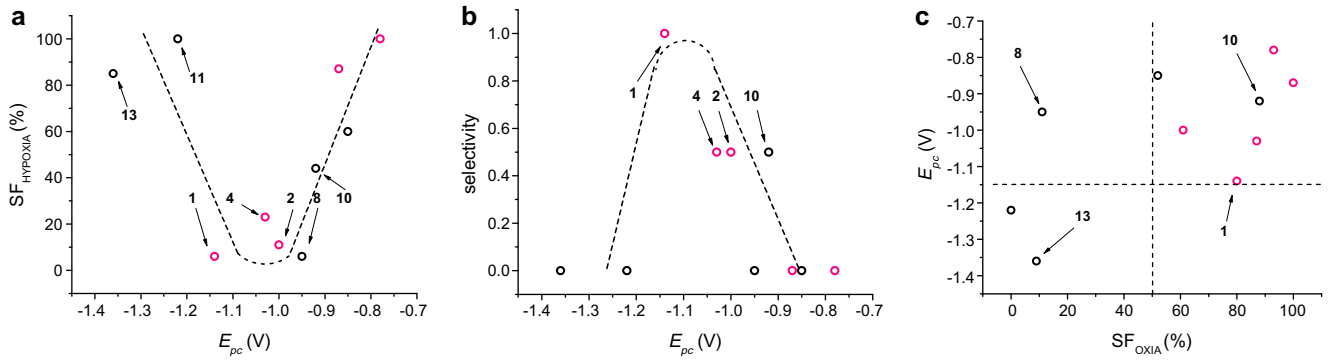


Figure 3. (a) E_{pc} versus $SF_{hypoxia}$ for phenazine 5,10-dioxide parent compounds (Scheme 1b) and the herein studied BPDO. Dot-lines show tendency. (b) E_{pc} versus selectivity for phenazine 5,10-dioxide parent compounds (Scheme 1b) and the herein studied BPDO. Categories of selectivity values were obtained by the relation between $SF_{hypoxia}$ and $SF_{normoxia}$ (category 1, ratio >6; category 0.5, 6 ⩾ ratio ⩾ 2; category 0, ratio <2). Dot-lines show tendency. (c) $SF_{normoxia}$ versus E_{pc} for phenazine 5,10-dioxide parent compounds (Scheme 1b) and the herein studied BPDO.

other hand, 5-un-substituted analogue, **8**, showed a different behavior at these doses accordingly to its lower DNA fragmentation, like Stau at 1 and 2 μM doses. These results are in agreement with the findings on the others DNA-studies.

3. Discussion

We report the study of benzo[*a*] phenazine 7,12-dioxides with potential use as cytotoxic-agents in normoxia. These derivatives were studied as bioreductive cytotoxins nevertheless only the methoxy-derivative, **10**, displayed some degree of hypoxic-selectivity at the studied doses (Table 1).

The profile of hypoxic cytotoxicity of these phenazine dioxides seems to be governed for the bio-reduction process. This fact could be realized in Figure 3a where hypoxia-cytotoxic compounds, that is, parent compounds **1**, **2** and **4**,[9] or BPDO derivatives **8** and **10**, have reduction potential (E_{pc}) between −0.92 to −1.14 V with the lowest hypoxic survival fractions ($SF_{hypoxia}$). In Figure 3b is possible to observe that selectivity reached a maximum and then declined with the increasing of E_{pc}. This kind of relationship has been previously described between air-to-nitrogen differential cytotoxicity and $E_{1/2}$ for benzotriazine di-N-oxides.[21] On the other hand, in reference to oxic-cytotoxicity compounds with E_{pc} higher than −1.14 V (value for the selective parent compound **1**, Scheme 1b) show $SF_{normoxia}$ higher than 50% (Fig. 3c). This result shows the enzymatic reduction processes for compounds with the least negative E_{pc} could be reverted by molecular oxygen consequently these dioxides could not be cytotoxic in oxic conditions. However the reduction of derivatives with more negative E_{pc}, that is, **11** and **13** (Table 2), could not be efficiently reverted by molecular oxygen and therefore they are cytotoxic in oxia. A special behavior is observed in BPDO **8**, according to its E_{pc} (−0.95 V, Table 2) it should be non-cytotoxic in oxic conditions; however $SF_{normoxia}$ for BPDO **8** is 11%. This fact could be clearly understood taking into account the capability of BPDO **8** to promote interaction to DNA ($a_{24}/a_0 = 0.71$, Table 2) and some degree of DNA-damage (Fig. 2) in oxic conditions. Besides, derivatives **6**, **8**, **11**, and **13**, were toxic in aerobic conditions against V-79 cells and showed antitumoral activity against Caco-2 cells (Table 1). On this cell line the least-toxic derivative **8** and the medium-toxic derivative **13** showed clear DNA-fragmentation capability at a sub-toxic dose (lower than the corresponding VC_{80}, Fig. S3, Supplementary data).

4. Conclusions

We have identified new benzo[*a*]phenazine 7,12-dioxides as promising antitumoral agents in oxic conditions taking this to defined a scaffolds of anticancer drugs not ever described. To our knowledge this is the first time that this kind of compounds is also studied as bioreductive cytotoxins. Further structural modifications on the benzo[*a*]phenazine 7,12-dioxide scaffold, QSAR studies, and in vivo activities are currently underway.

5. Experimental

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. Benzofuroxan **bfx1–5** were prepared according to procedures previously described.[22] 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). Most chemicals and solvents were analytical grade and used without further purification.

5.1. General procedure for the preparation of the benzo[*a*]phenazine 7,12-dioxide derivatives 6–13

To a solution of 7.3 mmol of NaOMe and 1.1 mL of MeOH, at −5 °C and under nitrogen atmosphere, was added a solution of 20.0 mmol of α-naphthol and 7.3 mmol of the corresponding benzofuroxan in 7.3 mL of MeOH. After stirring at room temperature for 24 h and maintaining at −4 °C for 24 h, the resulting precipitate was filtered to yield the corresponding 5-unsubstituted-benzo[*a*]phenazine 7,12-dioxide (**6–10**, –X = –H, Scheme 2). The mother liquor from the reaction mixture was acidified with AcOH and cooled at −4 °C for 24 h, to yield the corresponding 5-hydroxy-benzo[*a*]phenazine 7,12-dioxide (**11–13**, –X = –OH, Scheme 2).

5.2. 9(10)-Bromobenzo[*a*]phenazine 7,12-dioxide (6)

Yellow solid (15%). Proportion of 9 and 10-isomers 49:51. ^{1}H NMR (DMSO-d_6) δ: *9-isomer*, 7.80–7.90 (3H, m), 8.05 (1H, dd, $J_1 = 9.4$, $J_2 = 2.2$), 8.30 (1H, s), 8.40 (1H, d, $J = 9.2$), 8.60 (2H, m), 10.57 (1H, d, $J = 9.5$); *10-isomer*, 7.80–7.90 (3H, m), 8.05 (1H, dd, $J_1 = 9.4$, $J_2 = 2.2$), 8.30 (1H, s), 8.40 (1H, m), 8.90 (2H, m), 10.57 (1H, d, $J = 9.5$). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: *9- and 10-isomers,* 118.5, 119.7, 120.5, 121.6, 122.9,

124.2, 125.0, 126.7, 128.7, 129.4, 130.2, 132.9, 134.0, 134.8. MS, *m/z* (%): *9- and 10-isomers,* 340 ($M^{+\cdot}$, 28), 326 (47), 310 (62). (Found: C, 56.0; H, 2.4. $C_{16}H_9BrN_2O_2$ requires C, 56.3; H, 2.7).

5.3. Benzo[*a*]phenazine 7,12-dioxide (7)

Yellow solid (35%), mp = 178.5–181.5 °C (lit. 179.0–180.0 °C).[12] ^{1}H NMR (DMSO-d_6) δ: 7.93 (2H, m), 8.06 (2H, m), 8.19 (1H, dd, J_1 = 9.3, J_2 = 2.1), 8.24 (1H, d, J = 9.2), 8.52 (1H, d, J = 9.2), 8.71–8.82 (2H, m), 10.71 (1H, d, J = 9.3). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: 119.8, 120.4, 122.4, 122.8, 125.2, 128.3, 128.4, 128.8, 130.4, 130.9, 132.1, 133.8, 135.0, 135.2. MS, *m/z* (%): 262 ($M^{+\cdot}$, 100), 246 (75), 230 (23). (Found: C, 73.2; H, 3.9. $C_{16}H_{10}N_2O_2$ requires C, 73.3; H, 3.8).

5.4. 9(10)-Methylbenzo[*a*]phenazine 7,12-dioxide (8)

Yellow solid (35%). Proportion of 9 and 10-isomers 65:35. ^{1}H NMR (DMSO-d_6) δ: *9-isomer*, 2.66 (3H, s), 7.88 (3H, m), 8.19 (2H, m), 8.50 (1H, m), 8.58 (2H, m), 10.66 (1H, d, J = 9.5); *10-isomer*, 2.66 (3H, s), 7.88 (3H, m), 8.19 (2H, m), 8.50 (1H, m), 8.69 (2H, m), 10.66 (1H, d, J = 9.5). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: *9- and 10-isomers,* 23.0, 23.1, 110.4, 113.0, 118.6, 121.6, 122.9, 123.9, 125.9, 126.8, 128.9, 129.0, 131.5, 132.9, 134.4, 135.1, 145.0. MS, *m/z* (%): *9- and 10-isomers,* 276 ($M^{+\cdot}$, 57), 260 (100), 244 (36). (Found: C, 73.7; H, 4.2. $C_{17}H_{12}N_2O_2$ requires C, 73.9; H, 4.4).

5.5. 9(10)-(1,3-Dioxolan-2-yl)benzo[*a*]phenazine 7,12-dioxide (9)

Yellow solid (12%). Proportion of 9 and 10-isomers 52:48. ^{1}H NMR (DMSO-d_6) δ: *9-isomer*, 4.15 (4H, m), 6.03 (1H, s), 7.74 (1H, dd, J_1 = 8.8, J_2 = 1.6), 7.76–8.00 (4H, m), 8.51 (1H, d, J = 8.8), 8.62 (1H, d, J = 1.6), 8.63 (1H, m), 10.59 (1H, m); *10-isomer*, 4.15 (4H, m), 6.05 (1H, s), 7.58 (1H, dd, J_1 = 9.2, J_2 = 1.2), 7.76–8.00 (4H, m), 8.24 (1H, d, J = 8.0), 8.73 (1H, d, J = 9.2), 8.82 (1H, d, J = 2.1), 10.59 (1H, m). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: *9- and 10-isomers,* 66.5, 68.3, 102.6, 103.7, 121.5, 123.4, 124.0, 125.4, 128.3, 128.8, 129.6, 130.4, 131.0, 131.5, 132.6, 134.3, 134.7. MS, *m/z* (%): *9- and 10-isomers,* 335 ($M^{+\cdot}$ + H$^{\cdot}$, 3), 318 (5), 302 (5), 301 (1). (Found: C, 68.0; H, 4.4. $C_{19}H_{14}N_2O_4$ requires C, 68.3; H, 4.2).

5.6. 10-Methoxybenzo[*a*]phenazine 7,12-dioxide (10)

Yellow solid (6%); mp >240.0 °C. ^{1}H NMR (DMSO-d_6) δ: 4.07 (3H, s), 7.69 (1H, dd, J_1 = 9.6, J_2 = 2.4), 7.92 (2H, m), 8.00 (1H, d, J = 2.4), 8.19 (1H, dd, J_1 = 7.6, J_2 = 2.0), 8.26 (1H, d, J = 9.2), 8.55 (1H, d, J = 9.6), 8.73 (1H, d, J = 9.6), 10.68 (1H, d, J = 9.3). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: *9- and 10-isomers,* 57.7, 106.3, 108.6, 119.4, 120.7, 122.3, 122.8, 124.7, 127.9, 128.5, 128.9, 130.7, 131.9, 133.6, 135.2, 135.5, 145.3. MS, *m/z* (%): *9- and 10-isomers,* 292 ($M^{+\cdot}$, 98), 276 (54), 260 (15). (Found: C, 68.6; H, 3.9. $C_{17}H_{12}N_2O_3$ requires C, 68.9; H, 4.1).

5.7. 9(10)-Bromo-5-hydroxybenzo[*a*]phenazine 7,12-dioxide (11)

Proportion of 9 and 10-isomers 55:45. Yellow solid (12%). ^{1}H NMR (DMSO-d_6/D_2O) δ: *9-isomer*, 6.78 (1H, s), 7.70–7.87 (2H, m), 7.86 (1H, dd, J_1 = 9.3, J_2 = 2.1), 8.27 (1H, d, J = 9.3), 8.48 (2H, m),, 10.60 (1H, m); *10-isomer*, 6.78 (1H, s), 7.70–7.87(2H, m), 7.60 (1H, dd, J_1 = 9.3, J_2 = 2.1), 8.48 (2H, m), 8.69 (1H, d, J = 9.3), 10.60 (1H, m). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: *9- and 10-isomers,* 119.9, 120.8, 122.0, 122.9, 125.6, 126.7, 128.3, 128.7, 130.4, 131.0, 132.6, 133.0, 135.0, 142.5. MS, *m/z* (%): *9- and 10-isomers,* 356 ($M^{+\cdot}$, 24), 340 (67), 324 (16). (Found: C, 53.9; H, 2.3. $C_{16}H_9BrN_2O_3$ requires C, 53.8; H, 2.5).

5.8. 5-Hydroxybenzo[*a*]phenazine 7,12-dioxide (12)

Yellow solid (35%), mp >240.0 °C (lit. 250.0–253.0 °C dec.).[12] ^{1}H NMR (DMSO-d_6/$D_{2O)}$ δ: 7.40–7.85 (5H, m), 8.40–8.61 (3H, m), 10.67 (1H,, d, J = 9.3). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: 119.8, 120.4, 122.4, 122.8, 125.2, 128.3, 128.4, 128.8, 130.4, 130.9, 132.1, 133.8, 135.0, 135.2. MS, *m/z* (%): 278 ($M^{+\cdot}$, 28), 262 (47), 246 (62). (Found: C, 68.9; H, 3.3. $C_{16}H_{10}N_2O_3$ requires C, 69.1; H, 3.6).

5.9. 5-Hydroxy-9(10)-methylbenzo[*a*]phenazine 7,12-dioxide (13)

Yellow solid (15%). Proportion of 9 and 10-isomers 53:47. ^{1}H NMR (DMSO-d_6/D_2O) δ: *9-isomer*, 2.68 (3H, s), 7.82 (1H, s), 7.90 (3H, m), 8.40–8.50 (3H, m), 10.73 (1H, d, J = 9.5); *10-isomer*, 2.68 (3H, s), 7.82–7.90 (4H, m), 8.50–8.55 (3H, m), 10.73 (1H, d, J = 9.5). ^{13}C NMR (from the HMQC and HMBC experiments) (DMSO-d_6) δ: *9- and 10-isomers,* 22.8, 23.0, 108.3, 116.0, 118.4, 120.2, 122.1, 124.4, 126.0, 127.4, 129.5, 129.5, 131.0, 133.7, 134.0, 135.5, 144.2. MS, *m/z* (%): *9- and 10-isomers,* 292 ($M^{+\cdot}$, 10), 276 (93), 260 (100). (Found: C, 70.0; H, 3.9. $C_{17}H_{12}N_2O_3$ requires C, 69.9; H, 4.1).

5.10. Biology

5.10.1. Bio-reductive activity[9,14] 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) and 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 minutes 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 (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.

5.11. Antiproliferative activity on Caco-2 cells[10,23] cells

Caco-2 cells (colorectal adenocarcinoma, ATCC/HTB-37), provided by the American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Prat de Llobregat, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS, Gibco, Prat de Llobregat, Barcelona, Spain) and 1% antibiotic (10.000 U/mL penicillin and 10.000 μg/mL streptomycin, Gibco, Prat de Llobregat, Barcelona, Spain). Cells were maintained as monolayer cultures at 37 °C in a humidified atmosphere with 5% CO_2. *Treatment*: The assay was performed using the method described in the literature[23] slightly modified. Samples containing 200 μL cell suspension (2×10^4 cells/mL) were plated in 96 well-flat-bottomed microtiter plates. After adherence of the cells within 24 h of incubation at 37 °C, studied derivatives at concentrations ranging from 1 to 1000 μM were added to different wells (3–4 per concentration). After additional incubation time (24 and 48 h) at 37 °C in a humidified incubator with 5% CO_2, MTT dissolved in PBS and sterile filtered was added to all the wells at a final concentration of 1 mg/mL. Following 1 h of incubation, the generated formazan was dissolved with 100 μL DMSO per well. The optical density was measured using an ELISA plate reader (Merck ELISA system MIOS version 3.2.) at 550 nm. The derivatives concentrations that caused 50% decrease of cell viability (VC_{50}) was calculated. The experiments were performed in triplicate.

5.12. Cyclic voltammetric studies[24]

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 convertor 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 to 2.00 V/s.

5.13. DNA interaction studies

5.13.1. UV–visible spectroscopy[9]

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 °C. 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. *Study*: 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 °C on a Jasco V-570 UV/VIS/NIR spectrophotometer. Areas were calculated automatically by the apparatus.

5.14. Changes in DNA melting point[17]

Thermal denaturation experiments were carried out by monitoring absorbance variations with temperature at 260 nm. The ratio of DNA:BPDO was 1:1 for all experiments, where the DNA was prepared as in the previous described experiment. The absorbance of the mixture was using a 1-cm cell on a Jasco V-570 UV/VIS/NIR spectrophotometer.

5.15. AFM experiments[18]

DNA was prepared from a pGEM-3-Zf(−)-derived plasmid containing the duplicated multiple-cloning site of pBEND2 (pGB). The linear 350 bp fragment was dissolved in 10 mM Tris–HCl (pH 7.5), and aliquots were allowed to react with studied BPDO for 20 min at 37 °C. The BPDO was previously dissolved in DMSO and added to the DNA solution to give a final DMSO concentration of 20% (v/v). The ratio BPDO:DNA was varied between 100:1 and 1:1. The solution was adjusted to 0.1 M NaCl, and hydrolysis products were removed by multiple extractions with ethyl acetate and ether, followed by ethanol precipitation and ether extraction of the precipitate. Measurements: DNA samples were diluted in HM1 buffer [20 mM HEPES/KOH (pH 7.6) and 10 mM magnesium acetate] to a concentration of 2–3 ng/μL. Approximately 8 ng of DNA was adsorbed to a freshly cleaved mica surface (glued to steel disks), allowed to incubate at ambient temperature, then gently washed with Milli-Q water, and dried under a gentle stream of filtered, dry nitrogen. In some cases, the samples were further dried under vacuum. AFM imaging was performed in tapping mode on a Vecco-Digital Instruments microscope, model Multimode (MMAFM) NanoScope IIIa-Quadrex, with a vertical J scanner having a maximal lateral range of approximately 150 μm. Standard silicon cantilevers 125 μm in length were used for all tapping in air images. The cantilever oscillation frequency was tuned to 280–320 nm, and samples were scanned at 3–5 lines/s. Images were processed by flattening (using NanoScope software) to remove background slope. All images that are shown were analyzed by tapping in air.

5.16. Agarose gel electrophoresis studies[10]

DNA fragmentation was determined using 'Real Pure Extraction Kit'. In short, 2×10^6 Caco-2 cells/well were cultured in 100-mm plates and treated for 24 h with BPDO between 50 μM and the corresponding VC_{50} doses. Chromosomal DNA was isolated (Real, Durviz, Valencia, Spain) and analyzed in a 1% agarose gel visualizing with ethidium bromide staining. This assay was performed on four separate occasions. Staurosporine was used as positive control (1–10 μM).[20]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.074.

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