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1,2,4-Triazine *N*-oxide Derivatives: Studies as Potential Hypoxic Cytotoxins. Part III

New 5-(2-arylethenyl)-1,2,4-triazine *N*-oxide and *N,N′*-dioxide derivatives were synthesized in order to obtain compounds as selective hypoxic cell cytotoxins. The desired products were obtained when the 5-methyl heterocycle reacted with the corresponding iminium electrophiles. The new compounds were tested for their cytotoxicity in oxia and hypoxia. Some of them proved to be less active in hypoxic conditions than Tirapazamine, 3-aminobenzo[1,2-*e*]1,2,4-triazine N^1,N^4-dioxide. Derivative **11**, 6-methyl-5-[2-(5-nitrofuryl)ethenyl)-1,2,4-triazine N_4-oxide, was the most cytotoxic compound, but it was non-selective. Some derivatives were studied as DNA-binding agents in oxic conditions showing poor affinity for this biomolecule. This result showed that the cytotoxic activity in oxia is DNA damage not dependent. Electrochemical and ESR spectroscopy studies were performed in order to determine the ability of compounds to produce radicals and the relation of these in the mechanism of cytotoxicity.

Keywords: 1,2,4-Triazine *N*-oxide; Bioreductive compounds; Redox properties.

Introduction

Several *N*-oxides of aromatic heterocyclic amines have been described as hypoxia-selective cytotoxic agents (bioreductive drugs) [1-3]. One of the most interesting compound is Tirapazamine (3-aminobenzo[1,2-*e*]1,2,4-triazine N^1,N^4-dioxide, **1**, Figure 1). This drug possesses differential hypoxic cytotoxicity for a great number of cell lines of hamster, mouse and human beings, with a hypoxic cytotoxicity ratio (HCR, relationship between concentration of drug in air and concentration of drug in hypoxia that produce the same level of cell killing) of approximately 25-200 for the different cellular lines [4-6]. In order to describe the behavior of 1,2,4-triazine *N*-oxide as bioreductive agent, we described recently the biological characterization of 1,2,4-triazine N^4-oxide and N^1,N^4-dioxide derivatives as hypoxia selective cytotoxins (i.e. derivatives **2**, **3**, and **4**, Figure 1) [7, 8]. These derivatives showed poor cytotoxic selectivity against V79 cells in hypoxic conditions, however, compound **4** (Figure 1) displayed excellent V79 cell-growth inhibition even at 5 μM in air and hypoxia, i.e. as potent as Tirapazamine. In order to obtain more active compounds and to gain insight into the molecular requirements for bioreductive activity we included different arylethenyl substituents in the 5-position of the 1,2,4-triazine N^4-oxide and N^1,N^4-dioxide system (Scheme 1).

In this paper, we report the synthesis of some selected 5-(2-arylethenyl)-1,2,4-triazine *N*-oxide derivatives. We analyzed the cytotoxic activity of the new compounds in oxia and hypoxia against V79 cells and studied some of them for their aerobic DNA-affinity properties. Furthermore, we present the electrochemical and ESR spectroscopic studies that permit to explain the observed activity.

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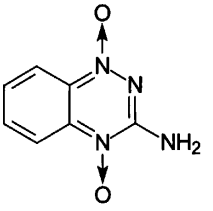
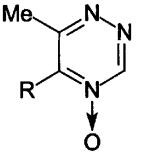
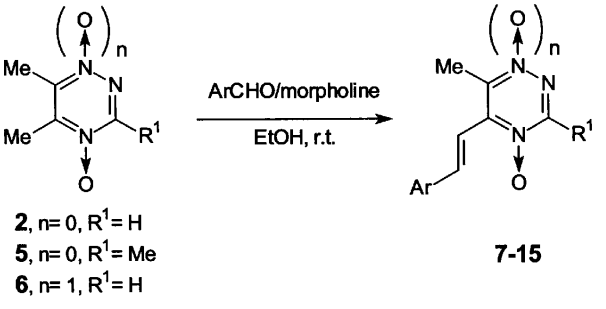




No	-R
2	-Me
3	-CH=CHPh
4	-CH=CH-(5-nitrothienyl)

Figure 1. Structures of Tirapazamine and 1,2,4-triazine N4-oxide early developed.



No	R^1 (n=0)	Ar-
7	H	4-Cl-phenyl
8		4-MeO-phenyl
9		4-Me$_2$N-phenyl
10		3,4-methylenedioxyphenyl
11		5-nitro-2-furyl
12	Me	4-MeO-phenyl
13		4-Me$_2$N-phenyl
	R^1 (n=1)	
14	H	4-MeO-phenyl
15		4-Me$_2$N-phenyl

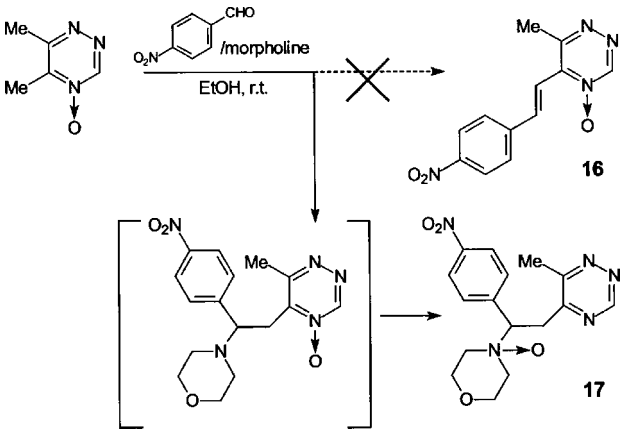
Scheme 1. Preparation of 5(2-arylethenyl)-1,2-tiazine N-oxide and N,N′-dioxide derivatives.

Results

Chemistry

In order to generate the desired derivatives we used a Mannich protocol previously described by us (Scheme 1) for the preparation of compounds **3** and **4** [8]. Choosing the Mannich-iminium electrophile as reactant and triazine **2**, **5** and **6** as the heterocyclic-nucleophiles, the condensation products **7**−**15** were separated as a precipitate from the reaction medium (Scheme 1). The *N*-oxide moiety did not enhance the heterocycle nucleophilicity in the 3-methyl-position (for compound **5**) or in the 6-methyl-position (for compound **6**), resulting thus exclusively in 5-methyl-nucleophilic condensation products [8].

Attempts to obtain derivative **16** in the same manner as compounds **7**−**15** rendered product **17** (Scheme 2). Derivative **17** was the addition product, following an *N*-oxide isomerization to the morpholine cycle. The



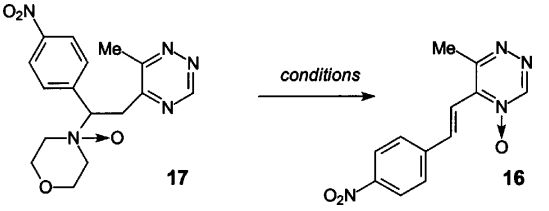
Scheme 2. Generation of compound **17**.

product **17**, unlike the other addition products, was insoluble in EtOH, so it precipitated in the reaction medium. Attempts to obtain the desired derivative **16**, improving the solubility of **17**, conducted us to change the reaction solvent to DMF or DMSO. In the first case, **17** precipitated not reacting for several days. Although product **17** was soluble in DMSO, it was impossible to transform it into compound **16**. Compound **17** was submitted to the action of different bases under different conditions but with unsuccessful results, it was not possible to obtain derivative **16** in any case (see assayed experimental conditions and results in Table 1).

All new compounds were identified by IR, MS, and one- and two-dimensional ^{1}H NMR, ^{13}C NMR experiments, and their purity was established by TLC and microanalysis. In all cases, the *E*-isomer of the olefin was the unique stereo species identified in solution by ^{1}H NMR and ^{13}C NMR experiments from the isolated compounds. The olefinic coupling constant, near to 16 Hz for all derivatives, was used to assign the stereochemistry.

The unequivocal condensation or addition site (compound **17**) was determined by means of HMQC for one-bond correlation and from sequences of HMBC for long distance/carbon correlation. Thus, 2′-CH coupled exclusively with the heterocyclic quaternary 5-carbon, which was determined by its correlation with the 3-H-heterocycle (determined by HMBC for compounds **7**−**11**, **14**−**15**, and **17**, Figure 2A). In derivatives **12**−**13** the HMBC-correlations and the characteristic chemical shifts of 3-, 5-, and 6-methyl protons and chemical shifts of the heterocyclic 5-carbon in parent compound **5** allowed us to assign the reaction site (Figure 2B).

Table 1. Experimental conditions assayed in the attempts to obtain compound **17**.



Base	Solvent	Temperature	Results
Et_3N	Et_3N	r. t.	NR
		Reflux	NR
Piperidine	DMSO	r. t.	NR
		Reflux	NR
EtONa	EtOH	r. t.	NR
		Reflux	Decomposition of **17**

NR − no reaction, r.t. − room temperature.

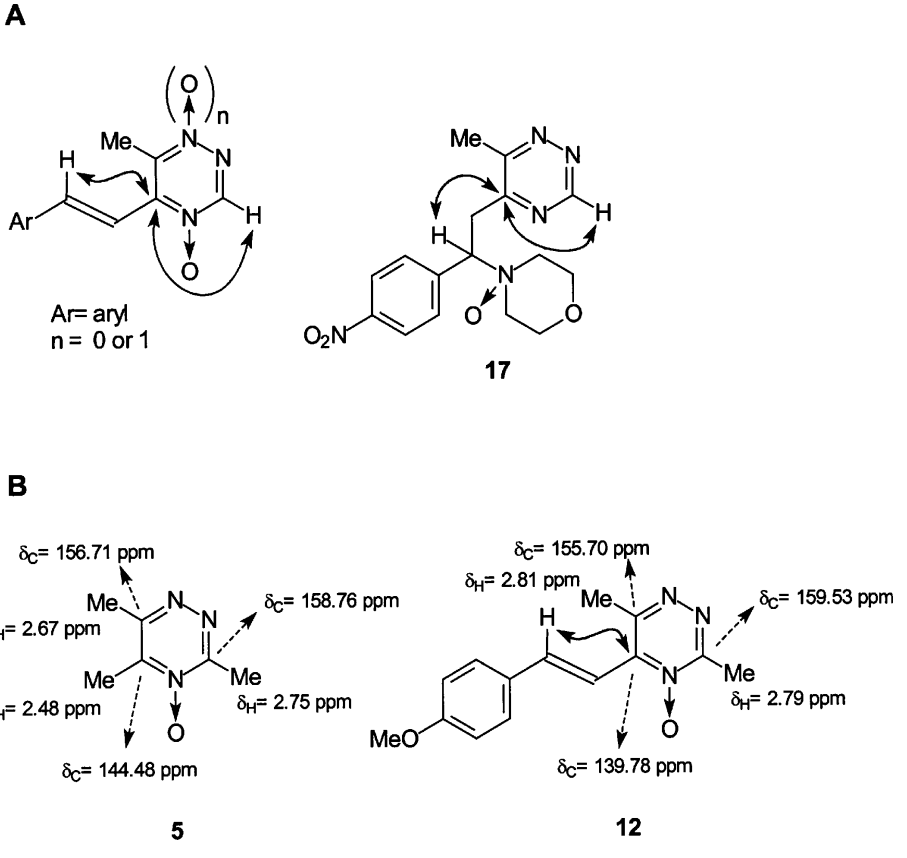


Figure 2. Correlations observed in HMBC experiment.

Biological studies

Aerobic and hypoxic cytotoxicity

Compounds were subjected to a preliminary cytotoxic evaluation on V79 cells under hypoxic and aerobic conditions using a cloning assay as previously described [7−9]. All the compounds were tested at 20 μM and derivative **11** was tested at 10 μM, 5 μM, 1 μM and 0.1 μM concentration. The surviving fractions of the two conditions (SFair and SFhipox) were determined. The results obtained are summarized in Table 2. Derivatives **7**-**10**, **12**-**15** and **17** showed less cytotoxicity and selectivity than the parent compounds (**2**, **5** and **6**) [7, 8]. Although derivative **11** was more cytotoxic than the parent compounds, it was not selective for hypoxic conditions.

DNA binding assay

Compounds **3**, **4**, **8**−**10** and **17** were tested for their ability to bind to DNA, using the DNA-binding assay described previously [7, 10−13]. Test compound solutions were prepared at a concentration of 10^{-4} M using the minimal volume of a suitable solvent (see Table 2) and were then diluted with water to 2×10^{-5} M. No effect on DNA was observed by these concentrations of solvents. Adequate aliquots of the test solutions were mixed with the DNA solution described in Experimental Section. The binding capacity was tested by measuring the hypochromic and bathocromic effect of compound absorbance in the UV spectra in a 20-nm band centered on the maximal absorbance value of each compound (see Table 2). The classical procedure was improved by rotating the stirred DNA-drug mixture in a 5:1 ratio during 24 h. The method was validated by repeating the assays with known intercalating agents (*m*-AMSA, ethidium bromide and mitoxantrone) and a compound binding closely to the minor groove (bis-benzimide [12], Hoechst No. 33258, Buenos Aires, Argentina). The degree of interaction was expressed by the ratio between the final absorbance area (a_{24}) and the absorbance of the compound at equal concentration (a_0). Areas were calculated automatically by the apparatus. Values of 1 or >1 indicated a total lack of affinity and a value of 0 the binding of

Table 2. Biological characterization and reduction potential of 1,2,4-triazine derivatives.

Compound	Biological characterization				Epc *vs* SCE[§] (Epc_{NO2}/Epc_{NOx}) (V)
	Doses (μM)	SFair (%)[#,†]	SFhypox (%)[†,‡]	a_{24}/a_0 (solvent)	
1	20	100 ± 0	0 ± 0	ND	− / −0.90[□]
2[∩]	20	75 ± 16	74 ± 8	1.25 (EtOH)	− / −1.61
3[€]	20	100 ± 0	86 ± 11	0.96 (DMF)	− / −1.12
4[¿]	5	0 ± 0	0 ± 0		
	1	69 ± 10	100 ± 0	0.96 (DMF)	−0.56 / −1.03
	0.1	92 ± 6	100 ± 0		
7	20	100 ± 5	90 ± 5	ND	− / −1.15
8	20	69 ± 10	100 ± 0	0.96 (DMF)	− / −1.28
9	20	100 ± 5	100 ± 5	0.96 (DMF)	− / −1.30
10	20	55 ± 11	78 ± 10	0.96 (DMF)	− / −1.18
11	20	0 ± 0	0 ± 0		
	10	0 ± 0	0 ± 0		
	5	0 ± 0	0 ± 0	ND	−0.63 / −0.94
	1	0 ± 0	0 ± 0		
	0.1	70 ± 10	100 ± 0		
12	−	ND		ND	−
13	20	100 ± 5	71 ± 12	ND	− / −1.36
14	20	100 ± 0	90 ± 5	ND	− / −1.12
15	20	100 ± 5	80 ± 10	ND	− / −1.16
17	20	100 ± 0	100 ± 0	0.93 (DMF)	−0.98 / −1.27
Reference	5	100 ± 0	0 ± 0	ND	−
***m*-AMSA**	−	−	−	0.30 (EtOH)	−
Ethidium bromide	−	−	−	0.50 (EtOH)	−
Mitoxantrone	−	−	−	0.00 (EtOH)	−
Bis-benzimide	−	−	−	0.57 (EtOH)	−

[#] Survival fraction in air; [†] Tests were carried out in duplicate; [‡] Survival fraction in hypoxia; [§] Peaks potentials (~± 0.01 V) measured at a scan rate of 0.50 V/s; ND−Not determined; [∩] From reference [14]; [€] From reference [7]; [¿] From reference [8]; Reference: 7-Chloro-3-[3-(*N,N*-dimethylamino)propylamino]-2-quinoxalinecarbonitrile hydrochloride.

the entire compound to DNA. The values of coefficient a_{24}/a_0 are summarized in Table 2.

Cyclic voltammetry

In order to determine the electrochemical characteristic of the studied derivatives, and its relationship with their cytotoxicity, experiments of cyclic voltammetry [14, 15] were performed in organic medium. These *N*-oxide derivatives displayed comparable voltammetric behavior in DMF, showing one reduction peak and the anodic counterpart for the N^4-oxide derivatives and two reduction waves for the N^1,N^4-dioxide derivatives. However, in the case of another electroactive moiety in the molecule (i.e. nitro moiety in derivatives **4**, **11** or **17**), a new wave appeared at a less negative potential. Table 2 lists the values of the nitro cathodic peaks (Epc_{NO2}) and the *N*-oxide cathodic peaks (EpcNOx) for the compounds biologically evaluated. Figure 3 shows some selected voltamograms. The first wave corresponds to a quasireversible process; this step was studied by ESR spectroscopy.

ESR Spectroscopy

The anion radicals produced in the electrochemical process was characterized for compound **2** by ESR spectroscopy [16, 17]. ESR spectra were recorded in

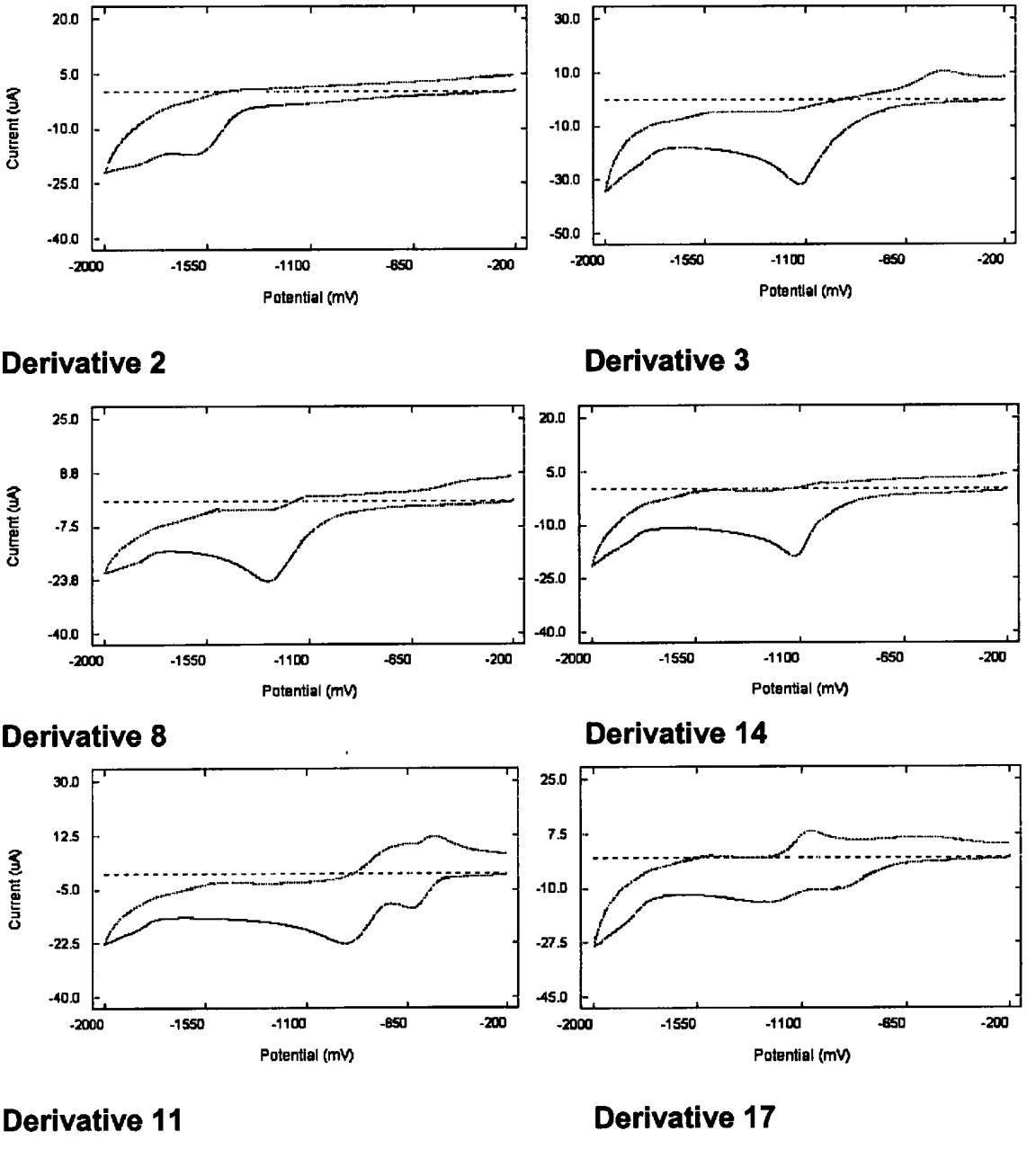


Figure 3. Cyclic voltammetry of some selected triazine derivatives in DMF at 0.5 V/s and at room temperature.

the X band (9.85 GHz) using a Bruker ECS 106 (Rheinstetten, Germany) spectrometer with a rectangular cavity and 50 KHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G. ESR spectra of the *N*-oxide anion radical was obtained in the electrolysis solution. Figure 4 shows the ESR spectrum for derivative **2**.

Discussion

Ten new 1,2,4-triazine *N*-oxide derivatives were prepared and evaluated as selective hypoxic cytotoxins. Some compounds were less active in hypoxic conditions at the dose assayed, than Tirapazamine. Compound **11** showed good cytotoxic activity, displaying

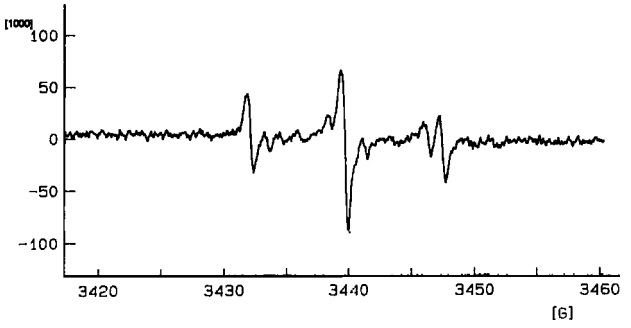


Figure 4. ESR experimental spectrum of the radical-anion of derivative **2** generated by electrochemical reduction in DMSO and at room temperature. Spectrometer conditions: microwave frequency 9.68 GHz, microwave power 20 mW, modulation amplitude 0.2 G, scan rate 1.25 G/s, time constant 0.5 s, number of scans 15.

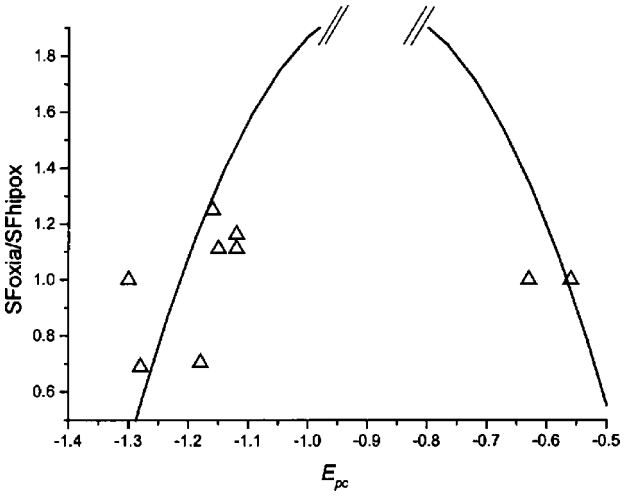


Figure 5. First reduction potential (E_{pc}) *vs.* SFoxia/SFhypoxia ratio for the arylethenyl-1,2,4-triazine derivatives **3**, **4**, **7**−**11**, **14**, and **15**.

excellent V79 cell-growth inhibition even at 1 μM being as potent as the reference compound. However, **11** proved to be non-selective to hypoxia.

This fact could be explained, in terms of a bioreductive process, in which 5-nitrothienyl and 5-nitrofuryl reductions are more facile than those of the *N*-oxide moiety. Thus, cytotoxic free radical-events would be produced in major proportions. However, the reduction potential of these nitro groups resulted to be less negative than the optimum for an adequate bioreductive compound. Qualitative correlation of the first reduction potential for the arylethenyl-1,2,4-triazine *N*- and *N,N′*-dioxides and the ratio SFair/SFhipox resulted a like-quadratic correlation (Figure 5), such as observed for Tirapazamine and derivatives [18, 19]. The decrease in the hypoxic ratio for the more electron-affine derivatives results from an increased toxicity toward aerobic cells compared to hypoxic cells. This may indicate an increased rate of drug reduction, even under aerobic conditions.

The DNA-binding studies indicate that the compounds are not potentially toxic in oxic conditions by interaction with this bio-molecule. Especially the aerobic cytotoxicity of some derivatives (i.e. derivatives **4** or **11**) was not the result of a direct interaction with DNA. This fact is in agreement with the speculative bioreduction pathway discussed before.

The electrochemical studies performed permitted us to extract some relevant aspects related with the structural requirements for an adequate redox potential. On one hand, the incorporation of arylethenyl moiety in position 5 of triazine heterocycle improved the redox potential (compare redox potential of *N*-oxide moiety in derivative **3** and the corresponding value in derivative **2**). On the other hand, the electronic effect of the aryl substituent affects the redox potential of *N*-oxide moiety, so the values were shifted to a less negative potential in presence of an electron-withdrawing group (compare values for derivatives **11** and **3**) while an electron donor group produced the opposite effect (compare redox potential of *N*-oxide moiety for derivatives **8**−**9** and **3**). The substituents in the triazine heterocycle could be affecting this physicochemical property. Furthermore, the presence of the methyl group, an electron donor group, in position 3 produced a shift of the reduction potential to more negative values (compare potential of derivative **9** with potential of derivative **13**) while the presence of another *N*-oxide moiety, as cationic nitrogen electron withdrawing group, shifted the peaks to less negative potentials (compare derivative **9** with derivative **15**).

The electrochemical reductions to the radical forms (*in situ*) in DMSO were carried out applying the potential corresponding to the first wave for derivative **2**, as obtained from the cyclic voltammetric experiments. The interpretation of the ESR spectra by means of a simulation process allowed the determination of the coupling constants for all magnetic nuclei; derivative **2** was analyzed in terms of (i), one triplet being due to the nitrogen of the *N*-oxide group, 8.5 G; (ii) one doublet due to the 3-hydrogen corresponding to a triazine ring, 8.5 G; (iii) two triplets due to the 1- and 2-nitrogen of the triazine ring, 1.45 G and 1.35 G respectively.

The biological and physicochemical information obtained with these derivatives allow us to re-designed new structures with the potential desired activity.

Acknowledgments

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Experimental

Chemistry

All starting materials were commercially available research-grade chemicals and used without further purification. All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. The typical work-up included washing with brine and drying the organic layer with sodium sulfate before concentration. Melting points were determined using a Leitz Microscope Heating Stage Model 350 (Wetzlar, Germany) apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3−4 mm Hg, 24 h at room temperature), performed on a Fisons EA 1108 CHNS-O analyzer (Valencia, USA), and were within ± 0.4% of theoretical values. Infrared spectra were recorded on a Perkin Elmer 1310 (Jügesheim, Germany) apparatus, using potassium bromide tablets; the frequencies are expressed in cm^{-1}. ^{1}H-NMR, ^{13}C-NMR spectra and HETCOR experiments were recorded on a Bruker DPX-400 (at 400 MHz and 100 MHz) instrument, with tetramethylsilane as the internal reference and in the indicated solvent. Mass spectra were recorded on a Shimadzu GC-MS QP 1100 EX (Kyoto, Japan) instrument using electron impact ionization at 70 eV. The compounds **2**−**6** were prepared as reported previously [7, 8].

*General procedure for the synthesis of derivatives **7**−**15** and **17***

A solution of triazine (**2**, **5** or **6**, 1.0 equiv.) in EtOH as solvent was added dropwise to a stirred mixture of morpholine (1.0 equiv.) and the corresponding aldehyde (1.0 equiv.) in EtOH as solvent. The mixture was maintained at room temperature. For all derivatives, except for derivative **12**, the precipitate was filtered off, washed with ethanol and purified as indicated. For derivative **12**, the mixture of reaction was concentrated *in vacuo* and the residue was purified by column chromatography (SiO_2, CH_2Cl_2: MeOH (0−5%)).

*5-[2-(4-Chlorophenyl)ethenyl]-6-methyl-1,2,4-triazine N_4-oxide **7***

Time of reaction 5 d, yellow-brown needles (18%); mp 159.5−160.0°C (petroleum ether:ethyl acetate). IR ν: CH 3040, C=C 1603, 1501, NO 1287 cm^{-1}. − ^{1}H NMR ($CDCl_3$) δ: 2.85 (s, 3H, -CH_3), 7.04 (d, 1H, =C*H*, *J* = 16.0 Hz), 7.28 (d, 2H, phenyl H, *J* = 6.3 Hz), 7.57 (d, 2H, phenyl H, *J* = 6.3Hz), 9.04 (d, 1H, =C*H*, *J* = 16.0 Hz), 9.14 ppm (s, 1H, -C_3-*H*).- ^{13}C NMR ($CDCl_3$) δ: 20.82 (-*C*H_3), 113.66 (=$C_{1'}$H), 129.64 (phenyl-*C*), 129.81 (phenyl-*C*), 134.84 (phenyl-*C*), 137.08 (phenyl-*C*), 140.14 (C_5), 143.79 (=$C_{2'}$H), 150.19 (C_3), 157.29 ppm (C_6).- MS; *m/z* (%): 247 (100) [M^+], 231 (16), 77 (11). Calculated for $C_{12}H_{10}ClN_3O$: C, 58.19; H, 4.07; N, 16.97. Found: C, 58.10; H, 4.02; N, 16.78.

*5-[2-(4-Methoxyphenyl)ethenyl]-6-methyl-1,2,4-triazine N_4-oxide **8***

Time of reaction: 1 d, yellow needles (74%); mp 164.0−164.5°C (petroleum ether:ethyl acetate). IR ν: CH 3042, C=C 1592, 1499, NO 1270 cm^{-1}. − ^{1}H NMR ($CDCl_3$) δ: 2.83 (s, 3H, -CH_3), 3.87 (s, 3H, O-CH_3), 6.95 (m, 3H, =C*H* + phenyl H), 7.61 (d, 2H, phenyl H, *J* = 8.7Hz), 9.09 (d, 1H, =C*H*, *J* = 15.9 Hz), 9.12 ppm (s, 1H, -C_3-*H*). − ^{13}C NMR ($CDCl_3$) δ: 20.87 (-*C*H_3), 55.86 (O-*C*H_3), 110.76 (=$C_{1'}$H), 115.06 (phenyl-*C*), 129.07 (phenyl-*C*), 130.38 (phenyl-*C*), 141.15 (C_5), 145.32 (=$C_{2'}$H), 150.14 (C_3), 157.08 ppm (C_6), 162.34 (phenyl-*C*). − MS; *m/z* (%): 243 (100) [M^+], 227 (39), 77 (31). Calculated for $C_{13}H_{13}N_3O_2$: C, 64.19; H, 5.39; N, 17.27. Found: C, 64.08; H, 5.21; N, 17.32.

*5-[2-(4-Dimethylaminophenyl)ethenyl]-6-methyl-1,2,4-triazine N_4-oxide **9***

Time of reaction: 1 d, red needles (40%); mp 242.5−243.1°C (ethyl acetate:methanol). IR ν: CH 3040, C=C 1619, 1501, NO 1281 cm^{-1}. − ^{1}H NMR ($CDCl_3$) δ: 2.81 (s, 3H, -CH_3), 3.07 (s, 6H, N-CH_3), 6.73 (d, 2H, phenyl H, *J* = 9.0Hz), 6.88 (d, 1H, =C*H*, *J* = 15.7 Hz), 7.57 (d, 2H, phenyl H, *J* = 9.0Hz), 9.09 (s, 1H, -C_3-*H*), 9.15 ppm (d, 1H, =C*H*, *J* = 15.7 Hz). − ^{13}C NMR ($CDCl_3$) δ: 20.94 (-*C*H_3), 40.40 (N-*C*H_3), 107.58 (=$C_{1'}$H), 112.18 (phenyl-*C*), 124.08 (phenyl-*C*), 130.68 (phenyl-*C*), 141.07 (C_5), 146.46 (=$C_{2'}$H), 150.05 (C_3), 152.64 (phenyl-*C*), 156.77 ppm (C_6).- MS; *m/z* (%): 256 (55) [M^+], 240 (99), 77 (13). Calculated for $C_{14}H_{16}N_4O$: C, 65.61; H, 6.29; N, 21.86. Found: C, 65.55; H, 6.30; N, 21.82.

*6-Methyl-5-[2-(3,4-methylendioxyphenyl)ethenyl]-1,2,4-triazine N_4-oxide **10***

Time of reaction: 1 d, yellow needles (60%); mp 213.3-214.8 (d)°C (methanol:dimethylformamide). IR ν: CH 3048, C=C 1598, 1501, NO 1287 cm^{-1}. − ^{1}H NMR ($CDCl_3$) δ: 2.84 (s, 3H, -CH_3), 6.02 (s, 2H, O-CH_2), 6.85 (m, 2H, =C*H* + phenyl H), 7.12 (m, 2H, phenyl H), 9.03 (d, 1H, =C*H*, *J* = 15.8 Hz), 9.10 ppm (s, 1H, -C_3-*H*).- ^{13}C NMR ($CDCl_3$) δ: 20.73 (-*C*H_3), 102.15 (O-*C*H_2), 106.52 (phenyl-*C*), 109.20 (=$C_{1'}$H), 111.06 (phenyl-*C*), 125.65 (phenyl-*C*), 130.77 (phenyl-*C*), 140.58 (C_5), 145.37 (=$C_{2'}$H), 149.08 (phenyl-*C*), 150.08 (C_3), 150.59 (phenyl-*C*), 157.18 ppm (C_6).- MS; *m/z* (%): 257 (45) [M^+], 241 (57), 240 (100). Calculated for $C_{13}H_{11}N_3O_3$: C, 60.70; H, 4.31; N, 16.33. Found: C, 60.62; H, 4.28; N, 16.33.

*6-Methyl-5-[2-(5-nitrofuryl)ethenyl)-1,2,4-triazine N_4-oxide **11***

Time of reaction: 5 d, brown solid (15%); mp 233.6-234.2°C. IR ν: CH 3096, C=C 1607, 1568, NO_2 1316, NO 1279 cm^{-1}. − ^{1}H NMR (acetone-d_6) δ: 2.86 (s, 3H, -CH_3), 7.30 (d, 1H, furyl H, *J* = 3.9 Hz), 7.39 (d, 1H, =C*H*, *J* = 15.9 Hz), 7.64 (d, 1H, furyl H, *J* = 3.9 Hz), 8.99 (d, 1H, =C*H*, *J* = 15.9 Hz), 9.27 ppm (s, 1H, -C_3-*H*). − ^{13}C NMR (acetone-d_6) δ: 20.52 (-*C*H_3), 114.30 (furyl-*C*), 116.75 (furyl-*C*), 117.61 (=$C_{1'}$H), 127.25 (=$C_{2'}$H), 135.97 (C_5), 150.29 (C_3), 151.00 (furyl-*C*), 152.00 (furyl-*C*), 157.25 ppm (C_6).- MS; *m/z* (%): 248 (6) [M^+], 232 (83), 51 (100). Calculated for $C_{10}H_8N_4O_4$: C, 48.39; H, 3.25; N, 22.57. Found: C, 48.01; H, 2.97; N, 22.18.

*3,6-Dimethyl-5-[2-(4-methoxyphenyl)ethenyl]-1,2,4-triazine N_4-oxide **12***

Time of reaction: 1 d, yellow oil (30%). ^{1}H NMR ($CDCl_3$) δ: 2.79 (s, 3H, -CH_3), 2.81 (s, 3H, -CH_3), 3.87 (s, 3H, O-CH_3), 6.97 (m, 3H, =C*H* + phenyl H), 7.59 (d, 2H, phenyl H, *J* = 8.7 Hz), 9.05 ppm (d, 1H, =C*H*, *J* = 15.9 Hz).- ^{13}C NMR ($CDCl_3$)

δ: 17.79 (-*C*H_3), 20.86 (-*C*H_3), 55.84 (O-*C*H_3), 111.57 (=$C_{1'}$H), 115.02 (phenyl-*C*), 129.90 (phenyl-*C*), 130.20 (phenyl-*C*), 139.78 (C_5), 144.63 (=$C_{2'}$H), 155.70 (C_6), 159.53 ppm (C_3), 162.00 (phenyl-*C*).- MS; *m/z* (%): 257 (100) [M^+], 241 (35), 77 (28). Calculated for $C_{14}H_{15}N_4O_2$: C, 65.36; H, 5.88; N, 16.33. Found: C, 65.12; H, 6.00; N, 15.99.

3,6-Dimethyl-5-[2-(4-dimethylaminophenyl)ethenyl]-1,2,4-triazine N_4-oxide ***13***

Time of reaction: 1 d, red-brown solid (31%); mp 203.5-204.0 °C. IR ν: CH 3040, C=C 1584, 1505, NO 1268 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 2.80 (s, 3H, -C*H*$_3$), 2.81 (s, 3H, -C*H*$_3$), 3.08 (s, 6H, N-C*H*$_3$), 6.74 (d, 2H, phenyl H, *J* = 8.9Hz), 6.91 (d, 1H, =C*H*, *J* = 15.7Hz), 7.57 (d, 2H, phenyl H, *J* = 8.9Hz), 9.14 ppm (d, 1H, =C*H*, *J* = 15.7 Hz). ^{13}C NMR ($CDCl_3$) δ: 17.85 (-*C*H_3), 20.94 (-*C*H_3), 40.50 (N-*C*H_3), 108.43 (=$C_{1'}$H), 112.40 (phenyl-*C*), 124.36 (phenyl-*C*), 130.47 (phenyl-*C*), 140.63 (C_5), 145.81 (=$C_{2'}$H), 152.47 (phenyl-*C*), 155.46 (C_6), 159.65 ppm (C_3). MS; *m/z* (%): 270 (100) [M^+], 254 (69), 77 (20). Calculated for $C_{15}H_{18}N_4O$: C, 66.65; H, 6.71; N, 20.73. Found: C, 66.30; H, 6.80; N, 20.34.

5-[2-(4-Methoxyphenyl)ethenyl]-6-methyl-1,2,4-triazine N_1,N_4-dioxide ***14***

Time of reaction: 1 d, yellow solid (62%); mp 227.0-227.9 °C. IR ν: CH 3087, C=C 1595, 1512, NO 1367, 1269 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 2.70 (s, 3H, -C*H*$_3$), 3.89 (s, 3H, O-C*H*$_3$), 6.97 (m, 3H, =C*H* + phenyl H), 7.62 (d, 2H, phenyl H, *J* = 8.8Hz), 8.74 ppm (m, 2H, =C*H* + -C_3-*H*). ^{13}C NMR ($CDCl_3$) δ: 15.15 (-*C*H_3), 55.89 (O-*C*H_3), 111.19 (=$C_{1'}$H), 115.10 (phenyl-*C*), 128.36 (phenyl-*C*), 130.53 (phenyl-*C*), 139.95 (C_6), 145.94 (=$C_{2'}$H), 147.78 (C_5), 148.19 (C_3), 162.63 ppm (phenyl-*C*). MS; *m/z* (%): 259 (27) [M^+], 243 (90), 77 (29). Calculated for $C_{13}H_{13}N_3O_3$: C, 60.23; H, 5.05; N, 16.21. Found: C, 59.91; H, 4.98; N, 15.89.

5-[2-(4-Dimethylaminophenyl)ethenyl]-6-methyl-1,2,4-triazine N_1,N_4-dioxide ***15***

Time of reaction: 1 d, red-wine needles (80%); mp 250.9-251.8 °C (methanol:dimethylformamide). IR ν: CH 3060, C=C 1584, 1505, NO 1350, 1250 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 2.67 (s, 3H, -C*H*$_3$), 3.08 (s, 6H, N-C*H*$_3$), 6.71 (d, 2H, phenyl H, *J* = 8.9Hz), 6.86 (d, 1H, =C*H*, *J* = 15.7Hz), 7.55 (d, 2H, phenyl H, *J* = 8.9Hz), 8.69 (s, 1H, -C_3-*H*), 8.89 ppm (d, 1H, =C*H*, *J* = 15.7 Hz). ^{13}C NMR ($CDCl_3$) δ: 18.82 (-*C*H_3), 40.48 (N-*C*H_3), 107.77 (=$C_{1'}$H), 112.34 (phenyl-*C*), 123.39 (phenyl-*C*), 130.97 (phenyl-*C*), 139.20 (C_6), 147.23 (C_3), 148.01 (C_5), 148.12 (=$C_{2'}$H), 152.91 ppm (phenyl-*C*). MS; *m/z* (%): 272 (16) [M^+], 256 (46), 77 (16). Calculated for $C_{14}H_{16}N_4O_2$: C, 61.75; H, 5.92; N, 20.57. Found: C, 61.75; H, 5.83; N, 20.49.

6-Methyl-5-[2-(4-nitrophenyl)-2-(morpholine-4'-yl)ethyl]-1,2,4-triazine $N_{4'}$-oxide ***17***

Time of reaction: 7 d, white solid (7%); mp 203.0-203.5 °C. IR ν: CH 3061, NO_2 1312, NO 1269 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.99 (s, 3H, -C*H*$_3$), 3.02 (dd, 1H, Ar-C*H*$_2$, J_1 = 11.9Hz, J_2 = 7.5Hz), 3.08 (dd, 1H, Ar-C*H*$_2$, J_1 = 12.0Hz, J_2 = 7.3Hz), 3.43 (m, 4H, N-C*H*$_2$), 3.61 (m, 4H, O-C*H*$_2$), 5.05 (m, 1H, N-C*H*-Ar), 7.52 (d, 2H, phenyl H, *J* = 8.7Hz), 8.14 (d, 2H, phenyl H, *J* = 8.7Hz), 11.31 ppm (s, 1H, -C_3-*H*). ^{13}C NMR (DMSO-d_6) δ: 13.06 (-*C*H_3), 34.25 (Ar-*C*H_2), 47.50 (-*C*H_2-morpholine), 67.00 (-*C*H_2-morpholine), 70.50 (Ar-*C*H), 123.77 (phenyl-*C*), 127.86 (phenyl-*C*), 147.17 (phenyl-*C*), 154.54 (phenyl-*C*), 156.00 (C_6), 156.33 (C_5), 160.07 ppm (C_3). MS; *m/z* (%): 345 (3) [M^+], 328 (3), 86 (100). Calculated for $C_{16}H_{19}N_5O_4$: C, 55.65; H, 5.55; N, 20.28. Found: C, 55.85; H, 5.90; N, 19.90.

Biology

Bioreductive evaluation

Cells: V79 cells (Chinese hamster lung fibroblasts) were obtained from ECACC (European Collection of Animal Cell Cultures) and maintained in logarithmic growth as subconfluent monolayers by trypsinization and subculture to $(1-2) \times 10^4$ 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: Compound solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure DMSO (Aldrich, Milwaukee, USA) 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 each 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 d and then stained with aqueous crystal violet. Colonies with more than 64 cells were counted. The plating efficiency (PE) was calculated by dividing the number of colonies by the number of cells seeded. The percent of control-cell survival for the compound-treated cultures (SFair and SFhipox) 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-affinity assay

DNA solution: Calf thymus DNA (12.5 mg) was slowly magnetically stirred in 5 mL Tris-HCl buffer (10mM, pH 7.4) for 24 h at 4 °C. From this solution, 0.6 mL were 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 and then diluted adding water to 2×10^{-5} M. 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 7850 (Easton, USA) spectrophotometer.

Cyclic voltammetry and ESR studies

DMF (spectroscopy grade) was obtained from Aldrich. Tetrabutylammonium perchlorate (TBAP) used as supporting electrolyte was obtained from Fluka (Buchs, Switzerland). Cyclic voltammetry was carried out using a BAS-Epsilon EC (Bioanalytical Systems, Inc., West Lafayette, USA) instrument in a BAS C3 cell, in *N,N*-dimethylformamide with tetrabutylammonium perchlorate (ca. 0.1 mol/mL) as the supporting electrolyte and purged with nitrogen at room temperature. A three-electrode cell configuration was used, with a platinum working electrode, with a platinum wire auxiliary electrode, with a saturated calomel reference electrode. Voltage scan rates ranged from 0.05-1.0 V/s. The *N*-oxide radicals were generated by electrolytic reduction *in situ* at room temperature.

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