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Synthesis of new pyrazolo[5,1-*c*][1,2,4] benzotriazines, pyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4] triazines and their open analogues as cytotoxic agents in normoxic and hypoxic conditions

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ABSTRACT

The synthesis and antitumor activity in normoxic and hypoxic conditions of a series of pyrazolo[5,1-*c*][1,2,4]benzotriazine and its related analogues are reported. All compounds were tested on human colorectal adenocarcinoma cell line HCT-8 and for compounds **15** and **20**, which show to have selective cytotoxicity in hypoxic and in normoxic conditions respectively, ROS production, cell cycle, and DNA fragmentation were measured. This preliminary study encouraged us to consider **15** and **20** as interesting leads for further optimization.

1. Introduction

In the last 50 years, the extensive in vitro and in vivo screening of natural products and chemical compounds has led to the development of the anticancer drugs currently utilized in the clinic. Despite many therapeutic successes, cancer is the second-most-frequent cause of death[1,2] and may become the most common in the near future. Thus, the search for new drugs continues to be a great challenge for medical science.

In this work, the attention has been focused on solid tumours, whose common condition is the presence of hypoxic cells,[3–5] which not only accelerate malignant progression and increase metastasis, but also negatively influence responsiveness to radiotherapy and chemotherapy.[6,7]

The approach proposed by Sartorelli's group[8] in the early 1970s that 'tumour hypoxia can be turned to advantage by exploiting it to activate bioreductive prodrugs within tumours', has led to the development of several hypoxia selective agents. The main categories of these bioreductive drugs belong to nitroaromatics (**I**),[5,6,9,10] quinones (MMC),[11] aromatic (**II–V**),[12–17] or aliphatic-*N*-oxide (**VI**)[18–20] derivatives and metal complexes (**VII**)[21–24] (Chart 1). Since several bioreductive agents contain nitro and/or N-oxide group, in their aromatic or heteroaromatic planar system, it could be very attractive to explore if the pyrazolo[5,1-*c*][1,2,4]benzotriazine system possesses a similar activity.

Our research group has been involved in designing derivatives of the pyrazolo[5,1-*c*][1,2,4] benzotriazine system which have been extensively studied over a period of time in our laboratory for the synthesis of benzodiazepine receptor ligands. These studies have led us to individuate high affinity ligands endowed with inverse agonist pharmacological efficacy,[25] anxioselective agents,[26] and selective anticonvulsant activity.[27] On the other hand this planar system can be considered a 'useful chemical space' for various biological activity too. In fact, in previous papers[27,28] we pointed out that the 3-nitropyrazolo[5,1-c*]*[1,2,4]benzotriazine system, 7,8-disubstituted possesses cytotoxic activity in the micromolar range in normoxic conditions, on leukemia cell lines more then against solid tumours.

The aim of the present study was to synthesize and develop a set of new compounds containing the pyrazolo[5,1-*c*][1,2,4]benzotriazine and pyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazine system (**A**) and their open analogues (**B** and **C**, Chart 2) in order to evaluate their potential anticancer properties in normoxic and hypoxic conditions on human colorectal adenocarcinoma cell line HCT-8. In this study, two previously synthesized compounds, 8-iodopyrazol-

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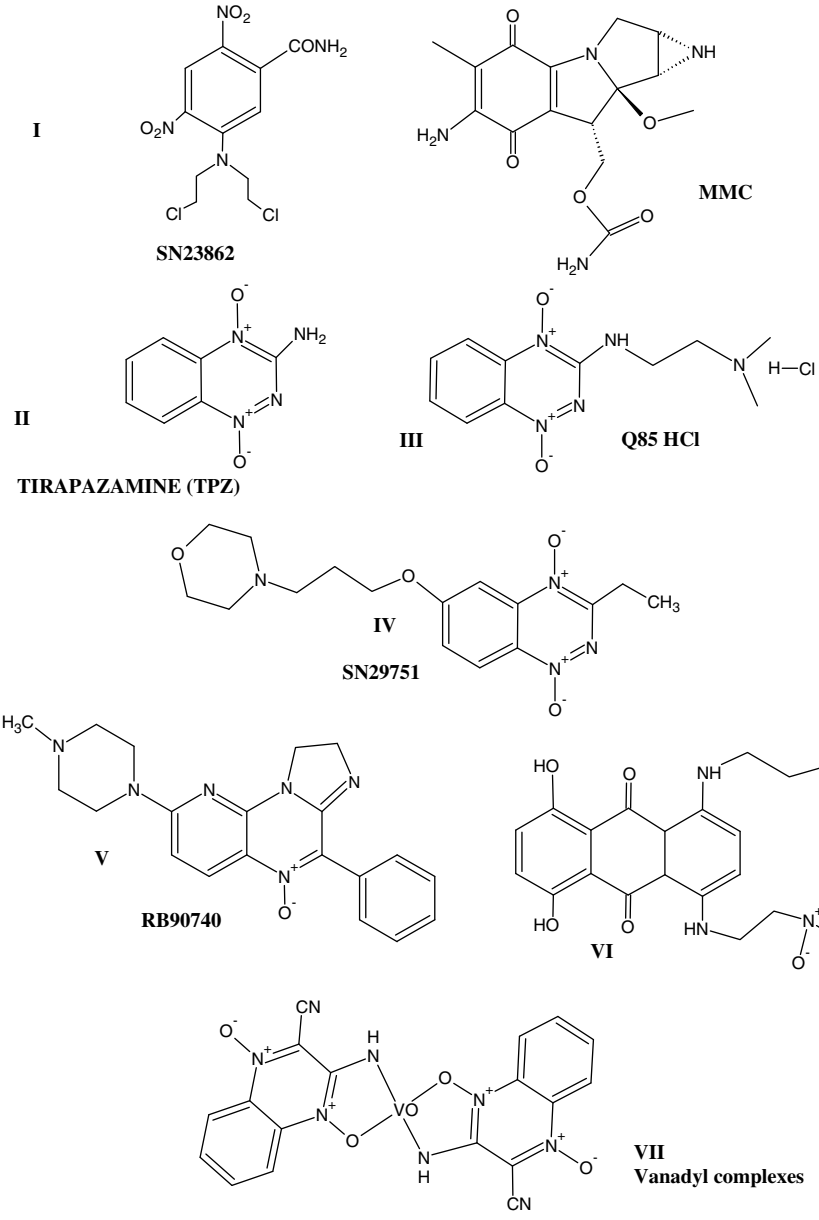


Chart 1.

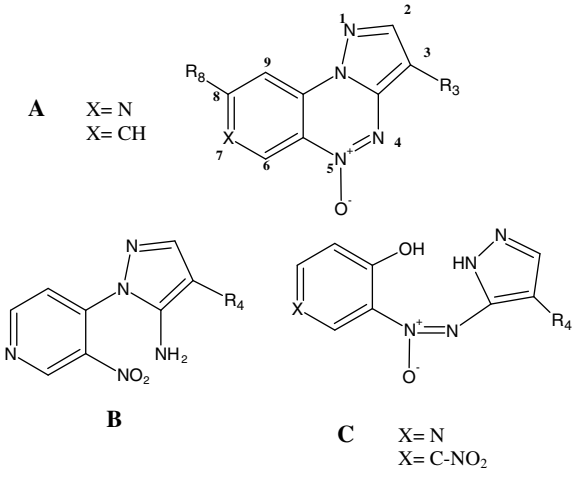


Chart 2.

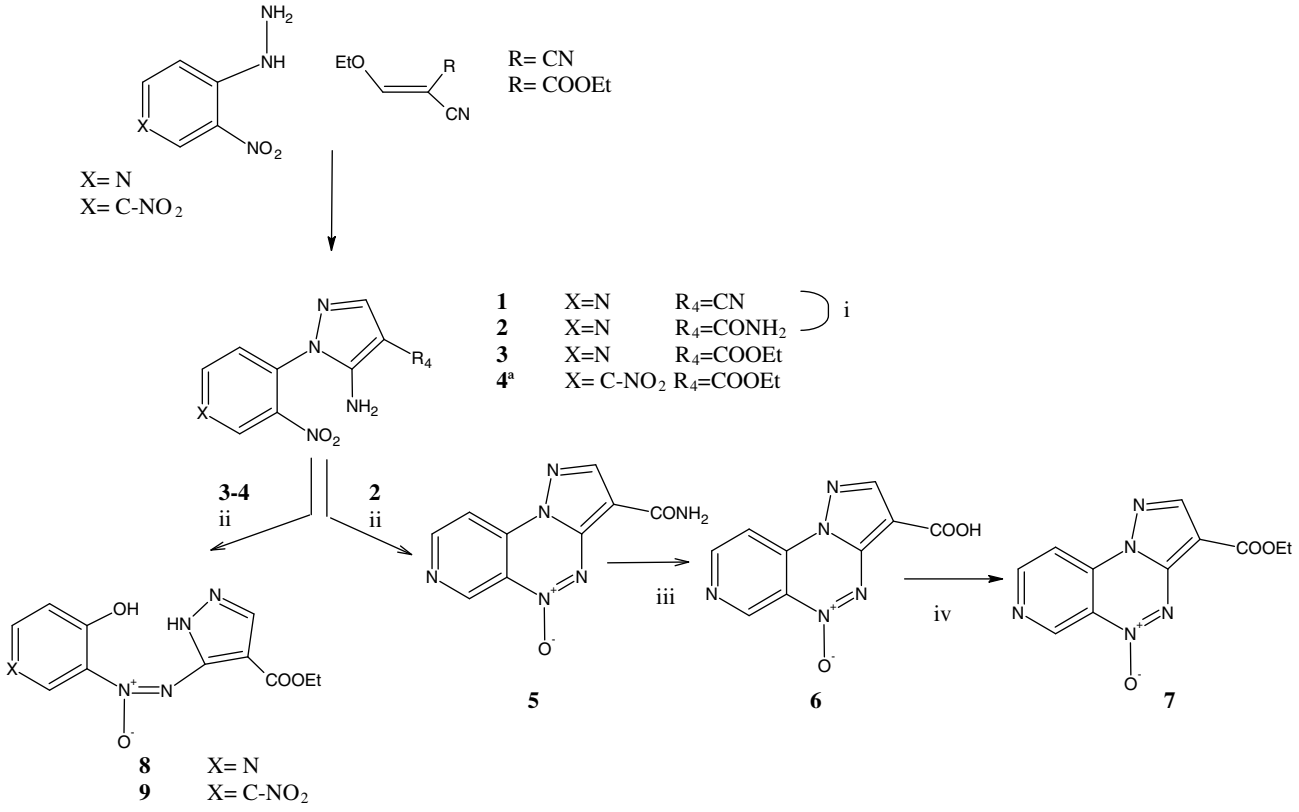
o[5,1-*c*][1,2,4]benzotriazine 5-oxide, **a43**[29] and 8-hydroxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **a45**[30], were tested for the structure activity relationships (SARs) discussion.

In this preliminary stage, reactive oxygen species (ROS) production, cell cycle and DNA damage in normoxic and hypoxic conditions for the most interesting compounds, were evaluated. Mitomicyn C (MMC) was our reference compound in all performed tests.

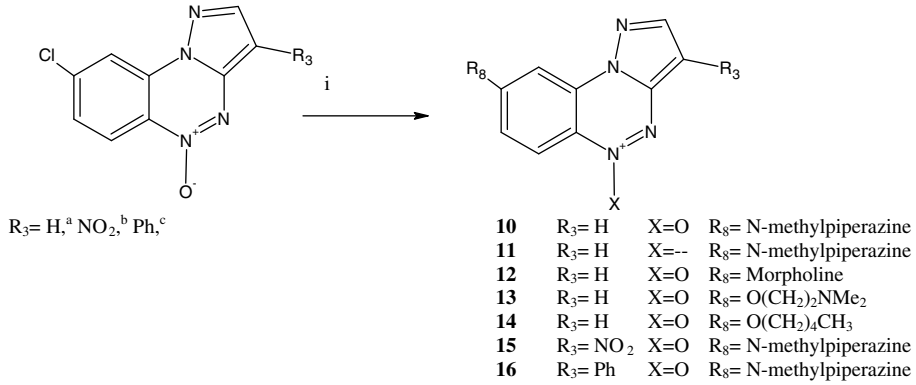
2. Chemistry

The synthetic pathways to obtain desired final compounds (**1**–**3**, **5**–**20**) are illustrated in Schemes 1–3 and chemical-physical data are shown in Tables 1 and 2.

Compounds **1** and **3** were achieved from 3-nitro-4-hydrazinopyridine[31] and 2-cyano-3-ethoxypropeneate or 2-ethoxy-1,1-ethenedicarbonitrile in ethanol. The previously synthesized compound **4**,[30] utilized as starting material in this study, was obtained from 2,4-dinitrophenylhydrazine. Compound **1**, 5-aminopyrazole-



Scheme 1. Reagents: (i) H_2SO_4, 60 °C, ammonia; (ii) NaOH 10%; (iii) $NaNO_2/H_2SO_4$; (iv) EtOH/ H_2SO_4; (a) see Ref. 30.

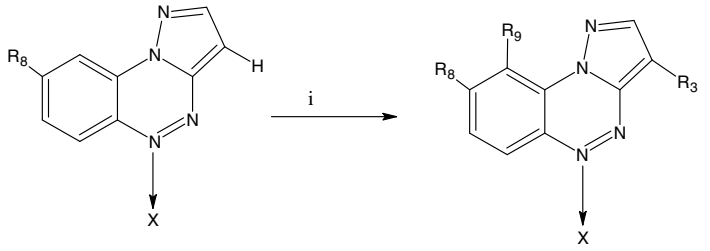


Scheme 2. Reagents: (i) *N*-methylpiperazine or morpholine in ethoxyethanol (for compounds **10** and **12**); *N*-methylpiperazine as reagent/solvent and N2 (for compound **11**); dimethyletanolamine and sodium hydroxide 10% (for compound **13**), pentanol, sodium hydroxide 40% and *tetra*-butylamonium bromide in dichloromethane (for compound **14**); *N*-methylpiperazine, potassium carbonate in toluene (for compounds **15** and **16**), (a) see Ref. 30; (b) see Ref. 27; (c) see Ref. 35.

4-carbonitrile derivative, was easily converted into 4-carboxyamide, **2**, for simple treatment with concentrated sulfuric acid at 60 °C and then precipitated with ammonia solution.

The next treatment with sodium hydroxide solution of the 5-aminopyrazoles **2**-**4** gave two different types of derivatives. Compound **2**, the 5-aminopyrazole-4-carboxyamide derivative, underwent the ring closure to pyrazolobenzotriazine 5-oxide system following the traditional intramolecular cyclization between the nitro and amino group under basic conditions.[32,33,30] The obtained product **5** was in turn transformed into 3-carboxyderivative **6** and then into 3-ethoxycarbonylderivative **7**, following a previously described method. Instead the 4-ethoxycarbonyl-5-aminopyrazoles **3** and **4**,[30] in addition to the expected condensation between the nitro and the amino group, underwent the nucleophilic attack of the OH^- species at the heteroaromatic-/aromatic-C1 and a cleavage of Het/Ar-N (pyrazole) bond has produced. The recovered products were 5-(4-hydroxypyridin-3-yl-*ONN*-azoxy)-1*H*-pyrazole and 5-(3-nitro-6-hydroxyphenyl-*ONN*-azoxy)-1*H*-pyrazole 4-substituted, **8**–**9**. This type of reactivity was previously observed on analogues[30] and the fact that **3** behaves in the same manner could be due to electronic feature of the pyridine ring that resembles, in terms of σ parameters, a 2,4-dinitrophenyl ring, see Scheme 1.

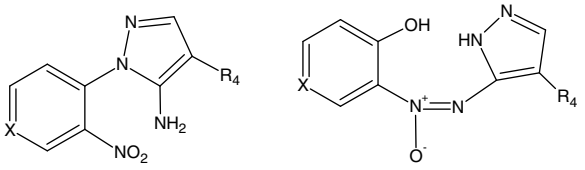
Compounds **10**–**16** were obtained by exploiting the reactivity of the chlorine atom at position 8 to nucleophilic aromatic substitution, as previously reported.[30,34] The starting material, 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide variously substituted at position 3[30,27,35] was reacted with nucleophilic reagents (*N*-methylpiperazine, morpholine, dimethylethanolamine, and pentanol) in different conditions able to permit the nucleophilic substitution (see Section 6, for details). In particular, when the *N*-methylpiper-



a43[a]	R_8= I	X=O	**17**	R_8= Phenyl	X=O	R_3= H R_9= H
			18	R_8= *p*-OMe-Phenyl	X=O	R_3= H R_9= H
			19	R_8= Thienyl	X=O	R_3= H R_9= H
11	R_8= N-methylpiperazine	X=--	**20**	R_8= N-methylpiperazine	X=--	R_3= NO_2 R_9= NO_2

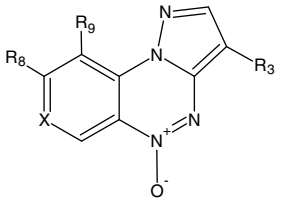
Scheme 3. Reagents: (i) for compounds **17–19,** Suzuki coupling conditions: $ArB(OH)_2$, sodium carbonate, tetrakis ($Pd(PPh_3)_4$), tetrahydrofurane; (a) see Ref. 29. (i) For compound **20**: HNO_3 65%/H_2SO_4.

Table 1
Chemical data of 5-aminopyrazoles **1–3** and –ONN-azoxy derivatives, **8-9**



Compounds	R_4	X	MF (MW)	Yield (%)	Mp °C (recryst. solvent)
1	CN	N	$C_9H_6O_2N_6$ (230.18)	35	198–199 (ethanol)
2	$CONH_2$	N	$C_9H_8O_3N_6$ (248.20)	80	220–221 (methoxyethanol)
3	COOEt	N	$C_{11}H_{11}O_4N_5$ (277.24)	40	158–159 (ethanol 80%)
8	COOEt	N	$C_{11}H_{11}O_4N_5$ (277.24)	45	260–262 (boiled in acetone)
9	COOEt	C-NO_2	$C_{12}H_{11}O_6N_5$ (321.25)	43	251–252 (water)

Table 2
Chemical data of pyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazines 5-oxides and pyrazolo[5,1-*c*] [1,2,4]benzotriazines 5-oxides



No.	R_3	X	R_8	R_9	MF (MW)	Yield (%)	Mp °C (recryst. solvent)
5	$CONH_2$	N	H	H	$C_9H_6O_2N_6$ (230.17)	60	>300 (water)
6	COOH	N	H	H	$C_9H_5O_3N_5$ (231.17)	70	>300 (water)
7	COOEt	N	H	H	$C_{11}H_9O_3N_5$ (259.22)	50	197–198 (ethanol)
10	H	CH	*N*-Methylpiperazine	H	$C_{14}H_{16}ON_6$ (284.32)	25	238–239 (ethanol)
11[a]	H	CH	*N*-Methylpiperazine	H	$C_{14}H_{16}N_6$ (268.32)	20	176–177 (ethoxyethanol)
12	H	CH	Morpholine	H	$C_{13}H_{13}O_2N_5$ (271.28)	85	226–227 (ethanol)
13	H	CH	$O(CH_2)_2NMe_2$	H	$C_{13}H_{15}O_2N_5$ (273.29)	25	68–69 (cyclohexane)
14	H	CH	$O(CH_2)_4CH_3$	H	$C_{14}H_{16}O_2N_4$ (272.30)	35	93–94 (ethanol)
15	NO_2	CH	*N*-Methylpiperazine	H	$C_{14}H_{15}O_3N_7$ (329.31)	65	264–265 (methoxyethanol)
16	Ph	CH	*N*-Methylpiperazine	H	$C_{20}H_{20}ON_6$ (360.41)	20	210–211 (ethanol)
17	H	CH	Ph	H	$C_{15}H_{10}ON_4$ (262.27)	75	175–176° (ethanol)
18	H	CH	*p*-OMe-Ph	H	$C_{16}H_{12}O_2N_4$ (292.29)	90	195–196° (ethanol)
19	H	CH	2-Tienyl	H	$C_{13}H_8ON_4S$ (268.30)	87	209–210° (ethanol)
20[a]	NO_2	CH	*N*-Methylpiperazine	NO_2	$C_{14}H_{14}O_4N_8$ (358.31)	50	245–246° (methoxyethanol)

[a] N_5-deoxide compounds.

azine was used as solvent/reagent in nitrogen atmosphere, the starting material 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[30] underwent both the 8-nucleophilic substitution and the N-5 deoxidation and derivative **11** was recovered; it is plausible

that the *N*-methylpiperazine behaves as a reducing agent, see Scheme 2.

Scheme 3 depicts the synthetic approach either to obtaining derivatives **17**–**19** or compound **20**. The Suzuki cross-coupling reaction has been applied on starting product **a43**, 8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide;[29] its reaction with phenylboronic-, *p*-OMe-phenylboronic-, and 2-thienylboronic acid respectively gave, with good yield, the desired compounds **17**–**19**, useful, as **a43**, for the next biological screening.

Finally, 3,9-dinitroderivative **20**, was obtained by nitration with concentrated nitric acid/sulfuric acid from **11** (Scheme 3).

3. Results

3.1. In vitro normoxic and hypoxic cytotoxicity

Previously synthesized compounds **a43**[29] and **a45**[30] and newly compounds **2**–**3**, **6**–**10**, **12**–**20**, were evaluated for cytotoxicity on oxygenated HCT-8 colon carcinoma cells, using the sulforhodamine B (SRB) colorimetric assay as previously described.[36] All compounds were tested in normoxic conditions at 10 and 100 μM following 72 h of incubation and the percentages of cell inhibition are reported in Table 3.

Before studying the cytotoxic effects of our compounds in hypoxia, the effect of hypoxia on HCT-8 cell proliferation (data not shown), was investigated after 72 h incubation using SRB test.[36] Cell proliferation was slightly less than normal for the first 24 h of hypoxic treatment, which was chosen for drug exposure with drug washout (DW) and reoxygenation at 24–48 h.

Figure 1 reports the cytotoxic effects under normoxia and hypoxia of our compounds at 10 μM following 24 h drug exposure and 48 h drug washout (48 h DW). In Figure 1 and Table 3, all compounds exhibit a time-related cytotoxicity, with the exception of compound **20** that does not follow this trend (% inhib. 70.25 at 24 h vs % inhib. 73.85 at 72 h).

From the evaluation of normoxic data in Figure 1 and Table 3 compounds **3, 7, 9, 15, 16**, and **20** were selected on the basis of their higher cytotoxic activity (% inhibition $\geqslant$50 at 10 μM and $\geqslant$60 at 100 μM concentration), and were subsequently tested on a range of concentrations from 10 to 100 μM to obtain dose-response curves. The IC_{50} and IC_{90} values are listed in Table 4.

Table 3
Percentage of growth inhibition on HCT-8 cell line determined after 72 h continuous exposure

Compound	% Cell inhibition	
	10 μM	100 μM
2	9.72 ± 0.28	22.35 ± 3.75
3	24.38 ± 1.73	69.72 ± 0.31
6	13.22 ± 0.86	11.36 ± 0.61
7	12.73 ± 3.8	97.44 ± 0.17
8	3.87 ± 0.83	31.54 ± 1.7
9	11.72 ± 1.0	60.4 ± 3.46
10	3.19 ± 1.67	32.41 ± 2.5
12	3.27 ± 1.3	25.79 ± 1.09
13	−2.36 ± 1.25	42.31 ± 2.0
14	−6.17 ± 3.0	38.04 ± 1.31
15	25.06 ± 3.18	93.22 ± 0.48
16	53.02 ± 6.7	69.6 ± 2.98
17	13.22 ± 2.55	35.49 ± 1.75
18	11.02 ± 0.35	21.59 ± 1.06
19	10.86 ± 0.1	48.47 ± 2.43
20	73.85 ± 1.43	98.68 ± 0.018
a43[a]	−1.62 ± 0.6	55.54 ± 2.3
a45[b]	−4.21 ± 0.17	19.68 ± 0.63

[a] See Ref. 29.
[b] See Ref. 30.

Notably, all compounds caused antiproliferative effects with IC_{50} values in the range 5–65 μM. In details, compound **20**, **16**, and **7** showed IC_{50} values of 5.98 ± 0.04 μM, 10 ± 1.20 μM, and 23.8 ± 1.0 μM, respectively; it is also interesting to note that compounds **20** and **7** had IC_{90} values of 18.5 ± 0.08 μM and 49.1 ± 0.33. From these data, compound **20**, 8-(4-methylpiperazin-1-yl)-3,9-dinitropyrazolo[5,1-*c*][1,2,4] benzotriazine, emerges as the most potent and active cytotoxic agent under normoxic conditions in the range of concentrations used in both tests.

Figure 1 indicates that the most hypoxic-selective agent was compound **15**, 8-(4-methylpiperazin-1-yl)-3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide. MMC has similar cytotoxic activity in both conditions on HCT-8 cells as reported in the literature.[37]

3.2. ROS

Compounds **15** and **20** were the most interesting and thus were evaluated on reactive oxygen species (ROS) production in normoxic and hypoxic conditions at 10 μM in HCT-8 cells. The involvement of ROS, in fact, in the bioreductive drugs action and in the DNA damage is well known.[38–41]

Under normoxia and hypoxia conditions (Fig. 2), treatment with **15** and **20** caused similar and significantly greater ROS production than the untreated control ($P < 0.05$). MMC produced a slight enhancement of ROS production in a similar manner in both condition. It can be excluded that the 'differential cytotoxic-activity' shown by **15** and **20** in normoxic and hypoxic conditions depended on ROS production.

3.3. Cell-cycle

To examine the effect of compounds **15**, **20**, and MMC on HCT-8 cell cycle phases, flowcytometric analysis was performed, as described in Section 6. Tables 5 and 6 show the percentages of G_0/G_1, S-, G_2/M cell phases, and DNA fragmentation determined by sub-G_1 peak quantitation under normoxic and hypoxic conditions, respectively.

No significant modifications of the cell cycle phases were observed under the experimental normoxic conditions, except for a slight increase in the S-phase cell fraction with MMC (52.6% vs 35.7% of control T_{24DW}) as reported in Table 5. The hypodiploid peak, representing the cells with DNA fragmentation, was detectable for **15**, **20** (39.0% and 42.0%, respectively) and was even more evident for MMC (71.2%) after 24 h exposure. At a later time point (T_{24DW}) decreased cell death (25%) was observed for compound **15** and this trend was maintained at T_{48DW} (27.0%), while for compound **20** the hypodiploid peak increased from 42% at 24 h to 59% of the total cell population at 48 h-DW with an intermediate degree of DNA fragmentation at T_{24DW} (33%).

After 24 h hypoxic exposure (Table 6) and at later time points (T_{24}-, T_{48DW}) no compound seemed to cause any significant cell-cycle perturbation. Only compound **15** and MMC caused a significant increase in DNA fragmentation at T_{24DW} (58.3% and 71.4%, respectively) and T_{48DW} (60% and 99%, respectively) as compared to untreated control (15.6% and 16%). Conversely, with compound **20** the dead cell fraction was decreased to 21% at T_{48DW}. It is interesting to note that despite the initial death of control (T_{24}, 37%), it recuperated vitality after 24 h of incubation in reoxygenated conditions. According to reported data[42–45] MMC induces cell cycle perturbations and apoptosis in cell lines.

4. Discussion

In previous papers[27,28] the critical role of the nitro group at position 3 on the pyrazolobenzotriazine system, independently on substituents at position 7 and 8, for normoxic cytotoxic activity

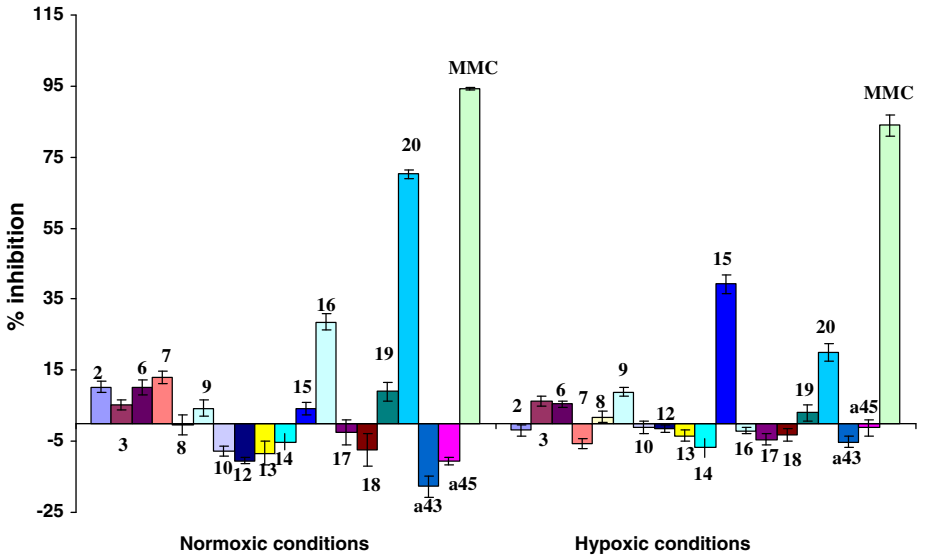


Figure 1. Percentage of growth inhibition in normoxic and hypoxic conditions on HCT-8 cell line, following 24 h incubation of synthesized compounds at 10 μM. The cytotoxic effect was checked by sulforhodamine B (SRB) test. See Section 6. MMC was the reference compound.

Table 4
IC_{50} and IC_{90} values of new compounds determined after 72 h continuous exposure

Compound	IC_{50} (μM)	IC_{90} (μM)
3	58.2 ± 2.45	>100
7	23.8 ± 1.0	49.1 ± 0.33
9	65.2 ± 2.50	>100
15	62.5 ± 2.30	>100
16	10 ± 1.20	>100
20	5.98 ± 0.04	18.5 ± 0.08
MMC	0.083 ± 0.002	5.7 ± 0.75

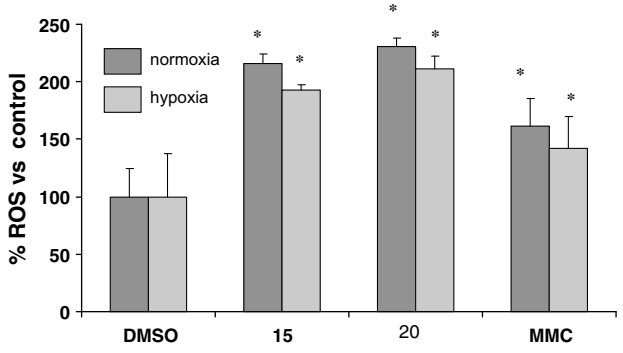


Figure 2. Effects of compounds **15**, **20**, and MMC on reactive oxygen species under normoxic and hypoxic conditions as described in Section 6. Bars are means ± SEM of three separate experiments. $^{*}P < 0.05$ versus untreated control (one-way ANOVA test with Bonferroni post test).

was demonstrated. In the present study the synthesis and preliminary pharmacological evaluation of three chemical series: pyrazolo[5,1-*c*][1,2,4]benzotriazine, pyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazine and their open analogues (**A**, **B**, and **C**, Chart 2) is reported.

With the aim of evaluating the influence of the hydro/lipophilic balance[13,46,47] on pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide system some substituents present in the main categories of cytotoxic agents,[3,6,12,15,17] were introduced.

The mono-substitution at position 8 with groups endowed with lipophilic features such as aromatic- or heteroaromatic rings (**17**, **18**, and **19**), pentyloxy chain (**14**), iodine atom (**a43**)[29], or the introduction in the same position of an dimethylaminoethanolic chain (**13**), methylpiperazinyl- and morpholinyl group (**10** and **12**, respectively), hydroxyl group (**a45**)[30] with hydrophilic features, gave inactive compounds.

While the introduction of a nitro group in position 3 of 8-(4-methylpiperazin-1-yl)derivatives permits identification of a hypoxic agent (**15**), the introduction of a phenyl ring, (**16**), yielded a time-dependent normoxic cytotoxic agent (28.55% at 24 h and 53.02% at 72 h, see Table 1 and Fig. 1) that will be the object of a further study. The synthesis of a dinitro derivative 3,9-dinitro-8-(4-methylpiperazin-1-yl)pyrazolo[5,1,-*c*][1,2,4] benzotriazine **20**, gave a selective cytotoxic normoxic agent.

Derivatives containing pyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazine 5-oxide system, were synthesized since these compounds could be considered 7-aza-analogues of the pyrazolo[5,1-*c*][1,2,4]benzotriazine system, but they were devoid of cytotoxicity in normoxic and hypoxic conditions. The same results occurred for the open analogues (**B** and **C**, Chart 2) **2**, **3**, **8**, **9** synthesized with

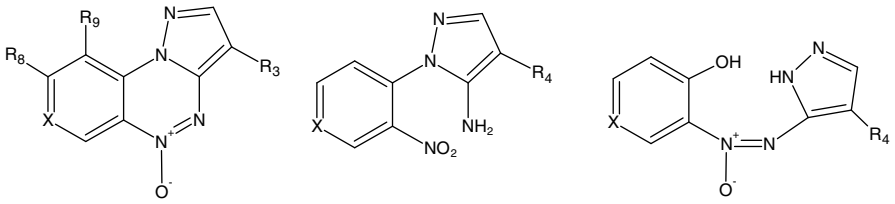
Table 5
Percentage of HCT-8 cells in each phase of the cell cycle and percentage of DNA fragmentation at concentration of 10 μM in normoxic conditions as described in Section 6

Compound	% Cells											
	GoG1			S			G2M			% DNA fragmentation		
	T_{24}	T_{24DW}	T_{48DW}	T_{24}	T_{24DW}	T_{48DW}	T_{24}	T_{24DW}	T_{48DW}	T_{24}	T_{24DW}	T_{48DW}
15	40.3	48	39.8	36.9	39	35.3	22.8	13	24.9	39	25	27
20	40.5	48.6	35.1	37.3	38	35.1	22.2	13.4	35.1	42	33	59
Control	50.6	46.7	38.6	38.6	35.7	28.6	10.8	17.6	32.8	6.8	15	16
Mitomycin	40.3	28.2	nd	36.9	52.6	nd	22.8	19.2	nd	71.2	43.6	44

Table 6
Percentage of HCT-8 cells in each phase of the cell cycle and percentage of DNA fragmentation at concentration of 10 μM in hypoxic conditions as described in Section 6

Compound	% Cells											
	GoG1			S			G2M			% DNA fragmentation		
	T_{24}	T_{24DW}	T_{48DW}	T_{24}	T_{24DW}	T_{48DW}	T_{24}	T_{24DW}	T_{48DW}	T_{24}	T_{24DW}	T_{48DW}
15	34.6	36.8	47	40.7	43	40.4	24.7	20.2	12.6	46.3	58.3	60
20	43.8	51.2	31.1	40.5	38.1	45.9	15.6	10.6	23	42.8	29.3	21
Control	42.8	46	47.5	38.4	33.6	37	18.8	20.4	15.5	37	15.6	16
Mitomycin	36.3	43.2	nd	49.1	42.7	nd	14.6	14.1	nd	42.8	71.44	99

Table 7
ClogP value of tested compounds



Series	Compound	R_4 or R_3	X	R_8	R_9	Clog*P*[a]
B	**2**	$CONH_2$	N			−1.30 ± 1.43
B	**3**	COOEt	N			0.81 ± 1.43
A	**6**	COOH	N	H	H	−0.9 ± 1.52
A	**7**	COOEt	N	H	H	−0.06 ± 1.52
C	**8**	COOEt	N			0.49 ± 1.31
C	**9**	COOEt	C–NO_2			3.30 ± 1.10
A	**10**	H	CH	*N*-Methylpiperazine	H	0.50 ± 1.36
A	**12**	H	CH	Morfoline	H	0.06 ± 1.35
A	**13**	H	CH	$O(CH_2)_2NMe_2$	H	0.69 ± 1.40
A	**14**	H	CH	$O(CH_2)_4CH_3$	H	2.96 ± 1.32
A	**15**	NO_2	CH	*N*-Methylpiperazine	H	0.23 ± 1.53
A	**16**	Ph	CH	*N*-Methylpiperazine	H	2.26 ± 1.53
A	**17**	H	CH	Ph	H	2.68 ± 1.32
A	**18**	H	CH	*p*-OMe-Ph	H	2.49 ± 1.33
A	**19**	H	CH	2-Tienyl	H	2.48 ± 1.34
A	**20**[b]	NO_2	CH	*N*-Methylpiperazine	NO_2	1.63 ± 1.62
A	**a43**[c]	H	CH	I	H	1.95 ± 0.91
A	**a45**[d]	H	CH	OH	H	0.18 ± 1.31

[a] The Clog*P* values were determined using the ACD/log*P* software.
[b] N_5-Deoxide compound.
[c] See Ref. 29.
[d] See Ref. 30.

the aim of clarifying if the planarity of the system was necessary to cytotoxicity.

In this preliminary in vitro study, Clog *P* values of tested compounds were calculated (see Table 7) using the ACD/log*P* software[48] because Clog*P* could be an important parameter predicting a molecule's ability for cellular uptake. From the analysis of Clog*P* data it could be speculated that, for all studied compounds, this parameter is not indicative of final activity. In fact, compound 10, 8-(4-methylpiperazin-1-yl)pyrazolo[5,1,-*c*][1,2,4]benzotriazine *5*-oxide (Clog*P* 0.50 ± 1.36) is inactive and its 3-nitro derivative, **15**, (Clog*P* 0.23 ± 1.53) is hypoxic-selective, despite a similar lipophilicity parameter. On the other hand, compounds a43,[30] inactive and **20**, the normoxic-selective, also have similar Clog*P* value (1.95 ± 0.91 vs 1.63 ± 1.62). Therefore, the presence of suitable substituents, such as nitro-, *N*-methylpiperazinyl-, and *N*-oxide, opportunely combined to exert cytotoxicity, seems to be necessary.

5. Conclusion

From these preliminary results, two potential selective hypoxic and normoxic lead compounds, **15** and **20** can be identified. Probably the N-oxide function is necessary for preferential hypoxic cytotoxicity of **15**; while **20**, a *N*-deoxide dinitroderivative, shows preferential cytotoxicity in normoxic conditions. While overall our experimental results reveal a great killing potential for both compounds **15** and **20**, as determined by certain DNA fragmentation and ROS production, the differential cytotoxicity in normoxic and hypoxic conditions was not clarified. This suggests that other differences in the metabolism or physiology of hypoxic and normoxic cells are responsible for the differential sensitivities of hypoxic and normoxic cells to compound **15** and **20**.

Additional experiments with other methodologies are required to understand the mechanism involved in the cell death induced by our compounds in normoxic and hypoxic conditions.

6. Experimental

6.1. Chemistry

Melting points were determined with a Gallenkamp apparatus and were uncorrected. Silica gel plates (Merk F_{254}) and silica gel 60 (Merk 70–230 mesh) were used for analytical and column

chromatography, respectively. The structures of all compounds were supported by their IR spectra (KBr pellets in nujol mulls, Perkin-Elmer 1420 spectrophotometer) and ^{1}H NMR data (measured with a Bruker 400 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The coupling constant values ($J_{H6\text{-}H7,\ H7\text{-}H6}$; $J_{H7\text{-}H9,\ H9\text{-}H7}$) were in agreement with the assigned structure. The chemical and physical data of new compounds are shown in Tables 1–3; Microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N, and the results were within ±0.4% of the theoretical value.

6.2. General procedure for the synthesis of 1 and 3

A suspension of 3-nitro-4-hydrazinopyridine[31] (1.5 mmol) and 2-ethoxy-1,1-ethenedicarbonitrile (1.5 mmol) or 2-cyano-3-ethoxypropeneate (1.5 mmol) in ethanol (50 ml) were refluxed until the starting material disappeared (4 h). The work up of final solution gave the 1-(3-nitropyridin-4-yl)-5-aminopyrazole-4-carbonitrile or the ethyl-1-(3-nitropyridin-4-yl)-5-aminopyrazole-4-carboxilate **1a** and **1c**, respectively.

6.2.1. 1-(3-Nitropyridin-4-yl)-5-aminopyrazole-4-carbonitrile (1)

From 3-nitro-4-hydrazinopyridine[31] and 2-ethoxy-1,1-ethenedicarbonitrile. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3300, 3200, 2215; ^{1}H NMR (DMSO-d_6) δ 9.27 (s, 1H, H-2′); 8.95 (d, 1H, H-6′); 7.90 (s, 1H, H-3); 7.86 (d, 1H, H-5′); 7.24 (br s, 2H, NH_2, exch.). Anal. C, H, N.

6.2.2. Ethyl 1-(3-nitropyridin-4-yl)-5-aminopyrazol-4-carboxylate (3)

From 3-nitro-4-hydrazinopyridine[31] and 2-cyano-3-ethoxypropeneate. Yellow crystals. TLC eluent: dichloromethane/methanol 10:0.5 v/v; IR ν cm^{-1} 3300, 3200, 1687; ^{1}H NMR ($CDCl_3$) δ 9.20 (s, 1H, H-2′); 8.88 (d, 1H, H-6′); 7.82 (s, 1H, H-3); 7.64 (d, 1H, H-5′); 5.40 (br s, 2H, NH_2, exch.); 4.24 (q, 2H, CH_2); 1.38 (t, 3H, CH_3). Anal. C, H, N.

6.2.3. 1-(3-Nitropyridin-4-yl)-5-aminopyrazole-4-carboxiamide (2)

A solution of compound **1** (1.0 mmol) in 5 ml of concentrated sulfuric acid was heated at 60 °C with stirring for 2 h. After cooling the solution was treated with ice/water and neutralized with concentrated ammonia. The solid was filtered and recrystallized by suitable solvent. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3350, 3200, 1659; ^{1}H NMR (DMSO-d_6) δ 9.20 (s, 1H, H-2′); 8.94 (d, 1H, H-6′); 7.95 (s, 1H, H-3); 7.84 (d, 1H, H-5′); 7.48 (br s, 1H, NH, exch.); 6.98 (br s, 1H, NH, exch.); 6.75 (br s, 2H, NH_2, exch.). Anal. C, H, N.

6.3. General procedure for the synthesis of 5, 8, and 9

A suspension of 1.0 mmol of suitable 5-aminopyrazole **2**, **3**, and **4**[30] in 10% solution of sodium hydroxide (10 ml) was stirred. In case of compound **5** the precipitate was filtered, instead the final solution was made slightly acid in case of compound **8** and acidified with conc hydrochloric acid in case of compound **9**. The raw products were recrystallized by suitable solvent.

6.3.1. 3-Carbamoylpyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazine 5-oxide (5)

From **2** at room temperature for 2 h. Orange crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2.1 v/v/v; IR ν cm^{-1} 3440, 3100, 1550; ^{1}H NMR (DMSO-d_6) δ 9.58 (s, 1H, H-6); 9.15 (d, 1H, H-8); 8.65 (s, 1H, H-2); 8.28 (d, 1H, H-9); 7.65 (br s, 1H, NH, exch.); 7.25 (br s, 1H, NH). Anal. C, H, N.

6.3.2. Ethyl 5-(4-hydroxypyridin-3-yl-*ONN*-azoxy)-1*H*-pyrazole 4-carboxylate (8)

From **3** at 30 °C for 2 h. Yellow crystals. TLC eluent: dichloromethane/methanol 10:0.5 v/v; IR ν cm^{-1} 3340, 1687; ^{1}H NMR (DMSO-d_6) δ 13.60 (br s, 1H, NH, exch.); 11.58 (br s, 1H, OH, exch); 9.20 (s, 1H, H-2′); 8.82 (d, 1H, H-6′); 8.58 (d, 1H, H-5′); 8.40 (s, 1H, H-3); 4.24 (q, 2H, CH_2); 1.38 (t, 3H, CH_3). Anal. C, H, N.

6.3.3. Ethyl 5-(3-nitro-6-hydroxyphenyl-*ONN*-azoxy)-1*H*-pyrazole 4-carboxylate (9)

From **4**[30] at 30 °C for 1 h. Orange crystals. TLC eluent: dichloromethane/methanol 10:0.5 v/v; IR ν cm^{-1} 3440, 3100, 1710; ^{1}H NMR ($CDCl_3$) δ 12.60 (br s, 1H, OH, exch.); 9.20 (d, 1H, H-2′); 8.40 (dd, 1H, H-4′); 8.14 (s, 1H, H-3); 7.35 (d, 1H, H-5′); 4.46 (q, 2H, CH_2); 1.43 (t, 3H, CH_3). Anal. C, H, N.

6.3.4. 3-Carboxypyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazine 5-oxide (6)

A suspension of **5** (2.0 mmol) in 10 ml of concentrated sulfuric acid was rapidly cooled to 0 °C and treated with cooled sodium nitrite solution (3 g in 6 ml of water), with stirring. The final syrup solution was poured into ice/water and the precipitate was filtered; it was pure enough for the next step. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2.1 v/v/v; IR ν cm^{-1} 2700–2600, 1700, 1550; ^{1}H NMR (DMSO-d_6) δ 9.52 (s, 1H, H-6); 9.00 (d, 1H, H-8); 8.38 (s, 1H, H-2); 8.20 (d, 1H, H-9). Anal. C, H, N.

6.3.5. 3-Ethoxycarbonylpyrazolo[5,1-*c*]pyrido[4,3-*e*][1,2,4]triazine 5-oxide (7)

A suspension of 0.43 mmol of acid **6** in 20 ml of anhydrous ethanol, containing 1 ml of concentrated sulfuric acid, was refluxed for 2 h until the starting material disappeared in TLC. After cooling the solution was evaporated and the residue was purified by recrystallization. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1650, 1550; ^{1}H NMR ($CDCl_3$) δ 9.80 (s, 1H, H-6); 9.10 (d, 1H, H-8); 8.60 (s, 1H, H-2); 8.25 (d, 1H, H-9); 4.50 (q, 2H, CH_2); 1.45 (t, 3H, CH_3). Anal. C, H, N.

6.4. General procedure for the synthesis of 10–13

A solution of 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[30] (0.5 mmol) in 15 ml of ethoxyethanol was reacted with an excess of N-methylpiperazine, morpholine or *N*,*N*-dimethylethanolamine and maintained between 60 and 100 °C for 2–12 h. The reaction was monitored by TLC and when the starting material disappeared, the final solution was worked-up.

The crude final product was obtained and purified by suitable solvent by recrystallization.

6.4.1. 8-(4-Methylpiperazin-1-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (10)

From starting material[30] and *N*-methylpiperazine, 2.5 ml, at 80 °C for 2 h. Yellow crystals. TLC eluent: chloroform/methanol 9:2 v/v; ^{1}H NMR ($CDCl_3$) δ 8.38 (d, 1H, H-6); 8.08 (d, 1H, H-2); 7.50 (d, 1H, H-9); 7.08 (dd, 1H, H-7); 6.68 (d, 1H, H-3); 3.70 (m, 4H, –N(CH_2)$_2$–); 2.75 (m, 4H, Me-N(CH_2)$_2$–); 2.50 (s, 3H, N-CH_3). Anal. C, H, N.

6.4.2. 8-(4-Methylpiperazin-1-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine (11)

From starting material[30] and N-methylpiperazine, 8 ml, at 100 °C for 2 hours, under nitrogen flow. Yellow crystals. TLC eluent: chloroform/methanol 9:2 v/v; ^{1}H NMR ($CDCl_3$) δ 8.38 (d, 1H, H-6); 8.18 (d, 1H, H-2); 7.56 (d, 1H, H-9); 7.24 (m, 2H, H-7 and H-3); 3.70 (m, 4H, –N(CH_2)$_2$–); 2.75 (m, 4H, Me-N(CH_2)$_2$–); 2.50 (s, 3H, N-CH_3). Anal. C, H, N.

6.4.3. 8-Morpholinpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (12)

From starting material[30] and morpholine, 3 ml, at 60 °C for 12 h. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.41 (d, 1H, H-6); 8.08 (d, 1H, H-2); 7.52 (d, 1H, H-9); 7.09 (dd, 1H, H-7); 6.68 (d, 1H, H-3); 3.92 (m, 4H, $O(CH_2)_2$–); 3.58 (m, 4H, $N(CH_2)_2$–). Anal. C, H, N.

6.4.4. 8-(*N*,*N*-Dimethylaminoethyloxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (13)

From starting material[30] and *N*,*N*-dimethylaminoethanol, 3 ml, 10% sodium hydroxide solution 1.0 ml at 50 °C for 12 h. Yellow crystals. TLC eluent: chloroform/methanol 10:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.47 (d, 1H, H-6); 8.09 (d, 1H, H-2); 7.74 (d, 1H, H-9); 7.20 (dd, 1H, H-7); 6.72 (d, 1H, H-3); 4.35 (t, 2H, OCH_2); 2.90 (t, 2H, NCH_2); 2.45 (s, 3H, CH_3). Anal. C, H, N.

6.5. 8-*n*-Pentyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (14)

A mixture of starting compound[30] (100 mg, 0.288 mmol), 10 ml of dichloromethane, 5 ml of 40% sodium hydroxide solution, 0.1 mole of *tetra*-butylammonium bromide and *n*-propanol in large excess (5 ml) was vigorously stirred at 30–50 °C for 12 h. The organic layer was then separated and the aqueous layer extracted twice with 10 ml of dichloromethane. The combined organic extracts were evaporated and the residue was recovered with isopropyl ether and recrystallized by the suitable solvent. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v; ^{1}H NMR ($CDCl_3$) δ 8.47 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.71 (d, 1H, H-9); 7.15 (dd, 1H, H-7); 6.73 (d, 1H, H-3); 4.24 (t, 2H, CH_2O); 1.92 (m, 2H, CH_2); 1.49 (m, 4H CH_2CH_2); 0.98 (t, 3H, CH_3). Anal. C, H, N.

6.6. General procedure for the synthesis of 17–19

Tetrakis-(triphenylphpsphine)palladium(0) (30 mg, 0.026 mmol) and 8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (0.32 mmol)[29] were combined in anhydrous tetrahydrofurane (4.0 ml). The suitable boronic acid (0.62 mmol) in absolute ethanol (2.5 ml) and aqueous sodium carbonate (2 M, 4 ml) were added and the reaction was heated to reflux for 12 h and monitored by TLC. The suspension was treated with water, the crude product was filtered, and recrystallized by the suitable solvent.

6.6.1. 8-Phenylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (17)

Starting material[29] and phenylboronic acid. Green crystals. TLC eluent: isopropyl ether/ciclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.62 (m, 2H, H-6 and H-9); 8.12 (s, 1H, H-2); 7.88 (dd, 1H, H-7); 7.80 (m, 2H, Ph); 7.55 (m, 3H, Ph); 6.80 (d, 1H, H-3). Anal. C, H, N.

6.6.2. 8-(4-methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (18)

Starting material[29] and 4-methoxyphenylboronic acid. Yellow crystals. TLC eluent: isopropyl ether/ciclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.58 (d, 1H, H-6); 8.55 (d, 1H, H-9); 8.13 (s, 1H, H-2); 7.84 (dd, 1H, H-7); 7.75 (d, 2H, Ph); 7.08 (d, 2H, Ph); 6.78 (d, 1H, H-3); 3.92 (s, 3H, OCH_3). Anal. C, H, N.

6.6.3. 8-(Thien-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (19)

Starting material[29] and 2-thienylboronic acid. Dark yellow crystals. TLC eluent: isopropyl ether/ciclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.56 (m, 2 H, H-6 and H-9); 8.14 (s, 1H, H-2); 7.86 (dd, 1H, H-7); 7.68 (m, 1H, H-5′ 8-tienyl); 7.54 (m, 1H, H-3′ 8-tienyl); 7.14 (m, 1H, H-4′ 8-tienyl); 6.78 (d, 1H, H-3). Anal. C, H, N.

6.7. General procedure for the synthesis of 15 and 16

A solution of 8-chloro-3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[28] and 8-chloro-3-phenylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[35] (0.5 mmol) in toluene (10 ml), potassium carbonate (60 mg) and *N*-methylpiperazine (0.1 ml) was kept at reflux temperature for 20 h. When the reaction was concluded the suspension was treated with water and the organic layer was separated, dried and evaporated. The final product was purified by suitable solvent.

6.7.1. 8-(4-Methylpiperazin-1-yl)-3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (15)

From 8-chloro-3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[28]. Red crystals. TLC eluent: toluene/ethyl acetate/methanol 8:3:2 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.64 (s, 1H, H-2); 8.37 (d, 1H, H-6); 7.44 (d, 1H, H-9); 7.22 (dd, 1H, H-7); 3.75 (m, 4H, –$N(CH_2)_2$–); 2.68 (m, 4H, Me-$N(CH_2)_2$–); 2.45 (s, 3H, N-CH_3). Anal. C, H, N.

6.7.2. 8-(4-Methylpiperazin-1-yl)-3-phenylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (16)

From 8-chloro-3-phenylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide.[35] Red-orange crystals. TLC eluent: toluene/ethyl acetate/methanol 8:3:2 v/v/v; ^{1}H-NMR ($CDCl_3$) δ 8.37 (m, 3H, H-6, H-2, and H-9); 8.01 (d, 2H, Ph); 7.48 (m, 2H, Ph); 7.32 (t, 1H, Ph); 7.07 (dd, 1H, H-7); 3.68 (m, 4H, –$N(CH_2)_2$–); 2.68 (m, 4H, Me-$N(CH_2)_2$–); 2.45 (s, 3H, N-CH_3). Anal. C, H, N.

6.8. 8-(4-Methylpiperazin-1-yl)-3,9-dinitropyrazolo[5,1-*c*][1,2,4]benzotriazine (20)

To a suspension of **7** (0.3 mmol) in cooled conc. sulfuric acid (6 ml), was added drop wise fuming nitric acid (0.5 ml): the red solution was stirred at 0 °C for 2 h. Addition of ice/water and 10% sodium hydroxide solution yields a precipitate which was filtered and purified by recrystallization. Red crystals. TLC eluent: chloroform/methanol 9:2 v/v; ^{1}H NMR ($CDCl_3$) δ 8.80 (s, 1H, H-2); 8.72 (d, 1H, H-6); 7.60 (d, 1H, H-7); 3.60 (m, 4H, –$N(CH_2)_2$–); 2.68 (m, 4H, Me-$N(CH_2)_2$–); 2.43 (s, 3H, N-CH_3). Anal. C, H, N.

6.9. Pharmacological methods

Puck's EDTA solution without phenol red: 140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM $NaHCO_3$, 0.8 mM EDTA, and 9 mM Hepes buffer (pH 7.3).[49]

Chemicals: 2′,7′-diclorofluoresceinadiacetato (DCFDA) was from the Eastman Kodak Company (Rochester, NY). Stock solutions of DCFDA (50 μM) were prepared in PBS (phosphate saline buffer) and stored at 4 °C.

Compounds were solubilized in dimethyl sulfoxide (DMSO) at 100-times the desired maximum test concentration (maximum final DMSO concentration of 0.1%; this concentration was not toxic) and stored frozen. Compounds were then diluted with complete media to obtain the 10× desired final maximum test concentration. Additional serial dilutions were made to provide a total of five drug concentrations plus control.

6.9.1. Cell cultures

The cell line used in this study was a human colorectal carcinoma, HCT-8 obtained from the American Type Culture Collection (Rockville, MD). The HCT-8 cell line was maintained in RPMI 1640 (Euroclone Ltd, UK) supplemented with 10% fetal calf serum (FCS) (Euroclone Ltd, UK) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). Cells were incubated at 37 °C, 5% CO_2/95% air and subcultured twice weekly. We optimized the growth of tumor

cells under hypoxic conditions by increasing the glucose concentration to 4.5 g/L to the culture medium.

6.9.2. Cytotoxicity assay

Before studying the cytotoxic effects of our compounds in hypoxia we investigated the effect of hypoxia on HCT-8 cells proliferation (data not shown) using sulforhodamine B (SRB) test according to the procedure described by Skehan et al.[36] Cell proliferation was slightly less than normal for the first 24 h of hypoxic treatment, then we have chosen 24 h drug exposure with drug washout and reoxygenation at 24–48 h.

To identify a normoxia/hypoxia-selective cytotoxin, cells were seeded at 5000 cells/well and cultured at 37 °C in normoxia in a humidified incubator containing 5% CO_2 for 24 h to assure complete adherence of the cells to the plates before addition of experimental drugs in complete medium. To evaluate the cytotoxic effects in normoxia at established times (72 or 24 h exposure), the compounds were diluted to desired concentrations in complete medium and in replicates of eight wells/condition were added. To evaluate the cytotoxic effects in hypoxia, cells and drug solutions were allowed to equilibrate for 2 h into a Ruskinn Concept 400 annormoxic incubator flushed with the 0.1% O_2 mixture (95% N_2 and 5% CO_2). Then drug solutions were added to wells for 24 h and after drug washout cells were reoxygenated for 48 h. In every assay 0.1% DMSO (negative control) and MMC (positive control) were tested. At the end of incubation the cells are fixed in situ by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B solution at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM Tris base, and the absorbance read on an automated plate reader at a wavelength of 540 nm. The IC_{50} drug concentration was that causing a 50% reduction in the net protein increase (as measured by SRB staining) in control cells.

6.9.3. Flow cytometric measurement of ROS

The dichlorofluorescein method for determining ROS production of in vitro has been described previously.[50] Briefly HCT-8 cells (in 5 ml of D-MEM high glucose medium with 10% FCS) were plated in 25 cm^2 flasks at a density of $1–2 \times 10^6$ cells, left to adhere overnight, and then treated with 10 μM compounds **15** and **20** and MMC for 24 h under normoxic and hypoxic conditions. Then 5 μM DCFH-DA was added under subdued lighting for 20 min at 37 °C under shaking. Cells were harvested with Puck's EDTA, washed twice with PBS and the fluorescence intensity was measured in a Coulter Epics Elite flow cytometer (Coulter, Miami, FL, USA) using a 535 nm filter. Viable cells can deacetylate DCFH-DA to dichlorofluorescin which is not fluorescent but reacts quantitatively with the oxygen species within the cells to produce the fluorescent dye 2′,7′-dichlorofluorescein (DCF) which remains trapped within the cell and can be measured to provide an index of intracellular oxidation. Fluorescence data were obtained using the WinMDI software and are expressed as mean ± SEM of fluorescent cells from at least three experiments and calculated as the percentage of fluorescence over basal values of DMSO treated cells.

6.9.4. DNA Flow cytometric analysis

HCT-8 cells (in 5 ml of D-MEM high glucose medium with 10% FCS) were plated in 25 cm^2 flasks at a density of $1–2 \times 10^6$ cells, left to adhere overnight then treated with 10 μM compounds **15**, **20** and MMC for 24 h under normoxic or hypoxic conditions. After drug washout, cells were reoxygenated for 48 h.

At experimental times (T_{24}, T_{24DW}, and T_{48hDW}) floating cells were collected, adherent cells were harvested with Puck's EDTA and pooled with the floating ones and fixed in 70% ice-cold ethanol and stored at 4 °C. Cells were then rehydrated in PBS and stained in propidium iodide (PI, 50 μg/ml) solution containing RNase A (5 U/ml) for 30 min.[51] PI-stained cells were analyzed for DNA content using a flow cytometer (Coulter XL, Coulter Cytometry, Hialeah, FL, USA). The red fluorescence emitted by PI was collected by a 620 nm long-pass filter. The percentage of cells in cycle phases and the percentage of those with fragmentated DNA, hypodiploid peak(sub-G_1) was determined using WinMDI2.8 Windows Multiple Document Interface Flow Cytometry Application (Cylchred Windows 95 Version 1.02).

6.9.5. Statistical analysis

The results were represented as mean ± SEM and statistical analysis was performed using one-way Anova test and Bonferroni's multiple comparison test (GraphPad Prism software, Inc., CA, USA).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.055.

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