Letters

Phenazine 5,10-Dioxide Derivatives as Hypoxic Selective Cytotoxins

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Received September 28, 2004

Abstract: The synthesis and evaluation as hypoxic selective cytotoxins of 2-amino- or 2-hydroxyphenazine 5,10-dioxide derivatives and reduced analogues are reported. In vitro cytotoxicities on V79 cells under hypoxic and aerobic conditions were determined. Some derivatives, such as 7(8)-bromo-2-hydroxyphenazine 5,10-dioxide, showed selective toxicity toward hypoxic cells and along with derivatives 7(8)-bromo-2-aminophenazine 5,10-dioxide and 7(8)-chloro-2-aminophenazine 5,10-dioxide behave as hypoxic trigger cytotoxins. These compounds represent interesting leads for further chemical modifications and biological studies.

It is well-known that the imperfect neovascularization seen in growing solid tumors results in limited and an inefficient blood vessel network and restricted and often chaotic blood flow. These and the variable interstitial pressures caused by the growing tumor lead to the presence of hypoxic cells in the solid tumor.[1] Anticancer drugs in clinical use are antiproliferative agents that kill dividing cells, by attacking DNA (synthesis, replication, or processing). These agents are not selective for tumoral cells and their efficacy is limited by the damage that they also cause to normal tissues. This is particularly true in the treatment of solid tumors, where the majority of the cells are not dividing rapidly.[2] Hypoxia appears to be a common and distinct property of cells in solid tumors that promotes an important mechanism for the specific activation of antitumoral prodrugs, namely bioreduction. On the basis that bioreduction is irreversible under hypoxic conditions,[3] bioreductive agents, quinones, nitro derivatives, and *N*-oxides[3b] (Figure 1), have been developed and are either in clinical use or entering in clinical trials. For some *N*-oxide-containing heterocycles, the mechanism of cytotoxicity has been suggested to involve one-electron reductive activation, a enzymatic or nonenzymatic process, or both, which could result in the production of •OH (Figure 1b).[3c] This radical would produce oxidative DNA cleavage, unlike quinones and nitro compounds, without covalent binding to DNA and proteins.[3c] In the case of

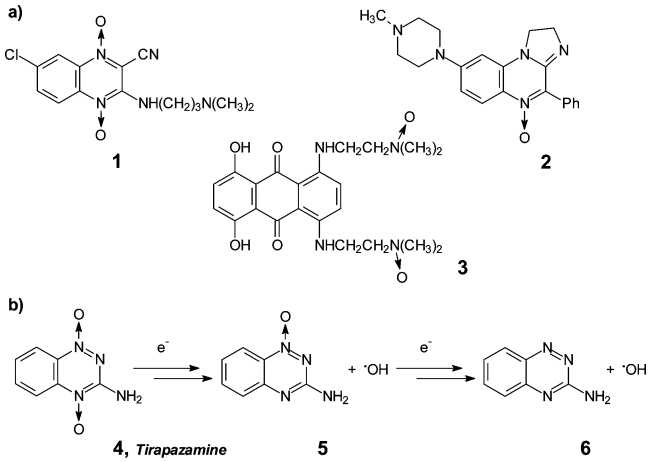

Figure 1. a. Examples of bioreductive *N*-oxides. b. Tirapazamine and its proposed bioreductive mechanism.

tirapazamine (**4**, Figure 1b) the reduction products, **5** and **6**, are not toxic to either hypoxic or aerobic cells.[4] Besides, the strategy to transport a diffusible cytotoxin generated in the bioreduction process into the hypoxic tissue has been used as a tool for triggering antitumoral agents that spread to neighbor aerobic cells.[2a]

On the other hand, it was hypothesized that hybrid compounds (compound **3**, Figure 1a) that possess an *N*-oxide and a π DNA-stacking moiety would be a new generation of bioreductive compounds. These could damage hypoxic cells by generating •OH, like that for tirapazamine, and after bioreduction, damage the hypoxic cells by direct DNA interaction or DNA-related biomolecules.[5] This idea together with our interest in the development of new hypoxic selective cytotoxic agents[3b,6] encourages us to synthesize phenazine 5,10-dioxide derivatives, structurally related to other bioreductive compounds and possessing potential π DNA-stacking moieties. Otherwise, it is well-known that naturally occurring phenazine 5,10-dioxide derivatives, isolated from bacteria and fungus, possess antibiotic activity.[7c] Besides, some studies related to phenazine 5,10-dioxide-DNA damage in oxia and hypoxia have been described,[7] but none of these probe them as cytotoxins under hypoxia in whole cells. In this paper we present a systematic study on the hypoxic cytotoxicity against V79 cells of phenazine derivatives with different substituents covering a wide range of physicochemical properties.

Phenazine 5,10-dioxide derivatives **7**−**19** were obtained by a heterocycle expansion process by reaction of the corresponding benzofuroxan with different phenol derivatives (Scheme 1).[8] As nucleophilic agent, two different phenols were used, *p*-aminophenol and *p*-hydroquinone. The reaction of benzofuroxans with phenols produced the corresponding 7- and 8-substituted-2-aminophenazine 5,10-dioxide (**7**, **8**, and **10**−**13**) and 7- and 8-substituted-2-hydroxyphenazine 5,10-dioxide (**14**, **15**, **17**−**19**) with moderate yields (Table 1). The 7-

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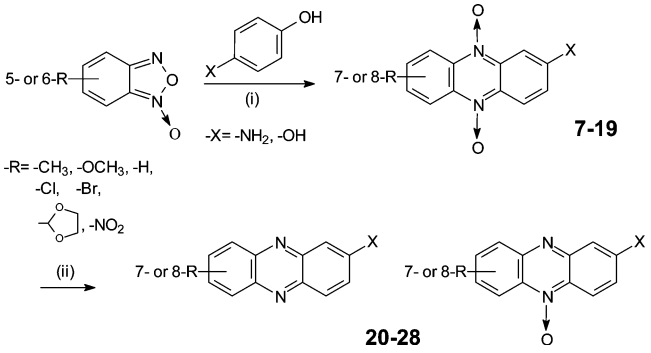
10.1021/jm0492150 CCC: $30.25
Published on Web 12/17/2004

Table 1. Phenazine 5,10-Dioxide (**7**−**19**) and Deoxygenated Analogues (**20**−**28**) and Cytotoxic Effects in Oxia and Hypoxia on V-79 Cells

dioxide derivatives				SF[a,b,c]		dioxide derivatives				SF[a,b,c]		deoxygenated derivatives					SF[a,b,c]	
ref	yield (%)[d]	X[e]	R[e]	air	hypox	ref	yield (%)[d]	X[e]	R[e]	air	hypox	ref	yield (%)[f]	X[e]	R[e]	n[g]	air	hypox
7	46	NH_2	CH_3	61	11	13	7	NH_2	NO_2	1	2	20	25	NH_2	CH_3	1	100	100
8	45	NH_2	OCH_3	87	23					58[h]	65[h]	21	25	NH_2	CH_3	0	98	100
9	45	NH_2	H	2	0	14	42	OH	CH_3	93	100	22	40	NH_2	Cl	0	0	100
				64[h]	19[h]	15	40	OH	OCH_3	100	87	23	40	NH_2	Br	0	0	0
10	40	NH_2	Cl	0	0	16	40	OH	H	100	73						6[h]	33[h]
				26[h]	3[h]	17	37	OH	Cl	100	100						33[i]	78[i]
11	40	NH_2	Br	12	0	18	40	OH	Br	80	0	24	41	NH_2	j	0	100	100
				64[h]	3[h]	19	47	OH	j	100	100	25	42	OH	CH_3	0	100	100
12	85	NH_2	j	14	0							26	40	OH	Cl	0	100	100
				86[h]	1[h]							27	41	OH	Br	0	100	100
				100[i]	36[i]	1[k]	-	-	-	100[i]	5[i]	28	35	OH	CHO	0	98	100

[a] SF air = survival fraction in air at 20 μM. bSF hypox = survival fraction under 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] Yield of benzofuroxans to phenazines transformations; the yields were not optimized. [e] X and R according to Scheme 1. [f] Yield for the reduction with sodium dithionite; the yields were not optimized. [g] n = number of N-oxide. [h] At 10 μM. [i] At 5 μM. [j] CH(1,3-dioxol-2-yl). [k] Positive control.

Scheme 1. Preparation of Phenazine Derivatives[a]



and 8-isomers formation is the result of the well-known tautomerism of benzofuroxan reactant at room temperature.[9] Although, the carbanion could react unselectively and with similar probability with each tautomeric form, some isomeric preferences were observed according to the proportion of each tautomer at the temperature of reaction.[10] In all cases the products were characterized and evaluated as a nonseparable mixture of 7- and 8-isomers because they could not be separated by crystallization or chromatographic methods. Previous results for quinoxaline dioxide derivatives demonstrated that both positional isomers have the same selective hypoxic cytotoxicities against V79 cells.[6a]

On the other hand, deoxygenated derivatives were developed (**20**−**28**). Chemical reductions were performed with sodium dithionite,[11] as a reductive enzyme analogue, to simulate the bioreductive process.[1b] These deoxygenated derivatives could act as π-DNA stacking compounds so they could kill both hypoxic and oxic cells, but these derivatives could also act like tirapazamine, where its bioreductive metabolites **5** and **6** (Figure 1b) are nontoxic to hypoxic or aerobic cells. Attempts to obtain mono- and dideoxygenated derivatives were performed with parent phenazine **7**, varying the molar ratio of reductive agent, yielding derivatives **20** and **21** respectively (Table 1).[10] Derivative **20**, N^5-oxide isomer, was obtained as the unique monoreduced compound when 1 equiv of dithionite was used. The electron donor CH_3 substituent together with the 2-amino group increases the negative charge density on the 4a- and 5a-carbon, not allowing the nucleophilic attack of the reductive reagent at these positions. On the other hand, under the conditions of reduction of derivative **19**, the corresponding acetal moiety was hydrolyzed, yielding dideoxygenated aldehyde **28** as product.

In vitro selective cytotoxicity was evaluated by a clonogenic assay after 2 h of treatment of V79 suspension cultures gassed with air or nitrogen at 20 μM.[6,10] The cytotoxic effects are expressed as cell survival fractions (SF), with respect to the control, under both conditions. For each of the tested derivatives, the average SF value of two different experiments is reported in Table 1. When the products demonstrated cytotoxicity, the assay concentrations were lowered. The collected data for the 2-aminophenazine derivatives show that the 7(8)-substituent affected the displayed activities. Derivatives with electron-withdrawing substituents, **10**−**13** and derivative **9** (with R = H) were very cytotoxic but not selective under hypoxic conditions at 20 μM. Furthermore, when lower concentrations were tested, 10 μM derivative **10** maintained similar cytotoxicity without selectivity while derivative **13** resulted in less cytotoxicity and was not selective. Besides, derivatives **9** and especially **11** and **12** showed cytotoxic effects with some degree of hypoxic selectivity at the lower doses assayed, 10 and 5 μM. On the other hand, the electron-donor derivatives, **7** and **8**, presented less cytotoxic effects and some degree of hypoxic selectivity at 20 μM. In the 2-hydroxy series, it is possible to observe that these derivatives resulted in less cytotoxicity, under both conditions, than the 2-amino analogues (compare activities of derivatives **9** and **16**, **10** and **17**, or **12** and **19**). Only bromo derivative **18** displayed a good hypoxia selective cytotoxicity at 20 μM. Therefore, derivative **18** was tested at different doses to obtain a dose−response curve in air and hypoxia.[10] From this curve it is possible to obtain potency (P) and hypoxic cytotoxicity ratio (HCR) for compound **18**. P is defined as the dose (μM), which gives 1% of control cell survival under hypoxia, and HCR is defined as the dose in air divided by the dose under hypoxia giving 1% of control cell survival. Derivative **18** shows a high potency (P = 10 μM) and an HCR on V79 cells greater than 10, this value being in the same order to other hypoxic selective cytotoxins (i.e., mitomycin C, misonidazole, and the N-oxides **2** and **4**, Figure 1, $HCR_{V79-379A}$ **2** = 10.2, HCR_{V79} **4** = 75.0).[3b,6a] On the other hand, the deoxy-

genated derivatives, **20**−**28**, resulted, in general, in noncytotoxicity under hypoxia. So, this biological behavior indicates that the corresponding parent compounds, phenazine dioxide, could be acting like that of hypoxic selective bioreductive compounds, such as tirapazamine. The unique reduced derivative that showed cytotoxic effects under hypoxia and under oxia, at 20 μM, is derivative **23**. When **23** was evaluated at lower doses, it was possible to observe that cytotoxicity is dose-dependent, finding that cytotoxicity under hypoxia was near zero at 5 μM. Through observation of the oxic cytotoxicity for the metabolic products **22** and **23**, some conclusions about derivatives **10** and **11** could be drawn. Assuming that these compounds could be irreversibly reduced under hypoxic conditions, toxic damage is generated, as the corresponding SF hypox indicates (see Table 1). Besides, the products of these reactions, **22** and **23**, being nontoxic per se, as the corresponding SF hypox indicates (see Table 1), could migrate to the surrounding oxic cells producing damage in these, as the corresponding SF air indicates (see Table 1). These results indicate that the parent compounds, derivatives **10** and **11**, could act as selective hypoxic cytotoxic agents and the bioreductive process could act as a trigger of the oxic-antitumoral agent, compounds **22** and **23**.

In conclusion, our results indicate that phenazine 5,-10-dioxide system could be acting as a bioreductive pharmacophore. Notably, derivatives **7**−**12** and **18** represent excellent starting points for further structural modifications as bioreductive agents or as hypoxic trigger cytotoxins. Compound **18** is a selective hypoxic cytotoxin and compounds **10** and **11** are hypoxic trigger agents. Chemical modifications to improve the activity (DNA damage) and QSAR studies are currently in progress.

Acknowledgment. Collaborative work was performed under the auspices of the Iberoamerican Program for Science and Technology (CYTED), Subprogram X.

Supporting Information Available: Experimental details of the preparation and spectroscopic characterization of **7**, **8**, **10**, **11**, **13**, **14**, **18**−**28**. Experimental procedures for the biological tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0492150