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PATHWAYS AND KINETICS OF AQUEOUS DECOMPOSITION AND CARBAMOYLATING ACTIVITY OF NEW ANTICANCER NITROIMIDAZOLE-LINKED 2-CHLOROETHYLNITROSOUREAS*

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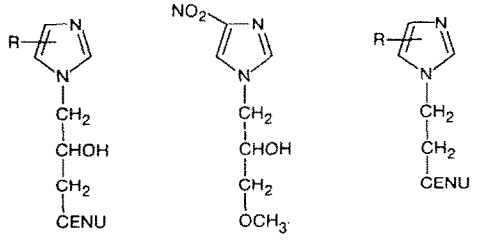
Abstract—The products of decomposition in anaerobic aqueous solution at pH 7.1 and 37° were determined for two series of novel anticancer agents incorporating both nitroimidazole and 2-chloroethylnitrosourea moieties (NI-CENUs) and examples of which exhibit preferential hypoxic toxicity against HeLa-MR cells. The decomposition products identified were vinyl chloride, acetaldehyde, 2-chloroethanol, ethylene glycol and imidazole-bearing compounds of the type including oxazolidinone, ethylamine or urea moieties. Series A NI-CENUs, which contain a 2-hydroxypropyl unit, gave rise to the oxazolidinone intramolecularly compared with the series B agents which gave rise to the imidazole-ethylamine and ureas. The half-lives of the B series agents were comparable with those of 1,3-bis(2-chloroethyl)nitrosourea (BCNU), 2-cyclohexyl-1-(2-chloroethyl)-1-nitrosourea (CCNU) and streptozotocin. The carbamoylation activity of the series B agents was approximately ten times that of series A compounds. This latter property may be related to the greater potency of series B than series A NI-CENUs against Mer$^+$ HeLa-S3 cells via inhibition of relevant repair enzymes.

Hypoxic cells represent one of the main challenges in cancer chemotherapy since they are quite resistant to drug treatments [1–3]. Therefore, the development of drugs which act preferentially on hypoxic cells is currently an important goal in cancer therapy. Such drugs have been considered within the framework of the HSA‖ (hypoxic selective agent) concept [3].

One of the best known HSA drugs is the 2-nitroimidazole derivative, misonidazole (Fig. 1), which has been studied extensively as a hypoxia-selective radiosensitizer [4]. Furthermore, this compound has been shown to be selectively cytotoxic to hypoxic cells *in vitro* and more recently to chemopotentiate alkylating agents at subtoxic sensitizer doses. This evidence supports the suggestion that nitroheterocycles may have a role as adjuvants in cancer chemotherapy [5, 6]. Such considerations have led us to design mixed drugs associating a radiosensitizing unit and an alkylating moiety, ultimately in order to chemopotentiate, *in situ*, the action of the alkylating part of the molecule. Therefore, we recently described [7] a new class of drugs: the NI-CENU (Fig. 1) which combine two complementary fragments: a nitroimidazole moiety structurally related to misonidazole and a 2-chloroethylnitrosourea (CENU) moiety, which types of drugs have an established place in the clinical treatment of human malignancies [8–11]. Another related drug, RSU-1069, in which the imidazole moiety is linked to an aziridine ring, has already been described [12].

Two series of NI-CENUs were synthesized (Fig.



Compound	R	Compound	R
1A	4-NO_2	1B	4-NO_2
2A	5-NO_2	2B	5-NO_2
3A	2-NO_2	3B	2-NO_2

Fig. 1. Structural formulae of series A and B NI-CENUs and misonidazole.

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‖ Abbreviations: HSA, hypoxic selective agent; CENU, 2-chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)nitrosourea; CCNU, 3-cyclohexyl-1-(2-chloroethyl)-1-nitrosourea; CNCC(C), *N,N'*-bis[*N*(2-chloroethyl)-*N*-carbamoyl]cystamine; NI-CENU, 2-nitroimidazole-2-chloroethylnitrosourea; STRTZ, streptozotocin; 5'-AdThd, 5'-amino-5'-deoxythymidine; and GC–MS, gas chromatographic analysis linked to mass spectrometry.

1). The series A compounds differed from those of series B by having a 2-hydroxypropyl in place of an ethyl group, linking the imidazole moiety to the nitroso function.

It was considered that such derivatives would produce, upon decomposition under physiological conditions, an alkylating intermediate and a nitroimidazole-derived chemosensitizing species able to potentiate the toxicity of the alkylating moiety.

Such mixed-function drugs have been found to be highly active *in vivo* against leukemia L1210 [7], and it was also demonstrated [13] that all these compounds exhibit significant activity against nitrosourea-sensitive HeLa-MR and nitrosourea-resistant HeLa-S3 cell lines when compared under either aerobic or hypoxic conditions. However, all of the series A derivatives were less cytotoxic to the HeLa-S3 cells than were their series B counterparts. In HeLa-S3 cells, the 2-nitroimidazole compounds 3A and 3B (Fig. 1) demonstrated enhanced toxicity under hypoxic exposure conditions. This demonstrates that the 2-nitroimidazole ring provides chemosensitizing properties and potentiates the action of the 2-CENU moiety. It was considered that this differential toxicity between series A and B in HeLa-S3 cells may be related to the formation of a carbamoylating isocyanate upon decomposition, under conditions that approximate physiological, of the series B compounds, as is found for other CENUs [14–16]. In contrast, decomposition of the series A derivatives could result in the generation of an oxazolidinone moiety which lacks carbamoylating activity.

To confirm this hypothesis we now report a study of the aqueous decomposition products and pathways, the kinetics of decomposition of each NI-CENU [7], as well as their carbamoylating activities.

MATERIALS AND METHODS

Materials

The samples of the NI-CENUs were prepared and purified by methods previously described [7]. Prior to each experiment, drug purity and concentration were monitored by HPLC. Authentic samples used in the gas chromatographic analysis, 2-chloroethanol, vinyl chloride, ethylene glycol and acetaldehyde, were obtained from the Aldrich Chemical Co. (Milwaukee, WI).

The water used for the buffers was distilled and purified through a Milli-Q system to give a resistivity of 18 MΩ/cm (Millipore). Methanol was of far UV HPLC grade (Fisons). Analytical grade sodium dihydrogen phosphate "Rectapur grade" was purchased from Prolabo. The pH of solution (a) (phosphate buffer, pH 3, 0.1 M, MeOH 1%) was adjusted with phosphoric acid. Solution (b) was methanol.

All the solvents were degassed in an ultrasonic bath and three different linear gradients were used:

(I) (100%a) to 20% (a)/80% (b), v/v, in 7 min (2 ml/min) for analytical studies;
(II) H_2O to 80% MeOH/20% H_2O, v/v, in 10 min for preparative HPLC;
(III) (100% a) to 20% (a)/80% (b), v/v, in 22 min (2 ml/min) for carbamoylation studies.

General procedure for analysis of products of aqueous decomposition of nitrosoureas

Solutions of the nitrosoureas (0.05 mmol) in 100 mM potassium phosphate buffer (0.6 ml) (pH 7.1) in 1 ml air-tight deoxygenated Reacti-vials equipped with Teflon septums were incubated at 37°. The reaction contents were analyzed for (i) volatile and gaseous products, (ii) extractable organic products, and (iii) water-soluble organic products.

Gaseous and volatile components

Samples of the gaseous and volatile products were withdrawn with a hypodermic syringe from the pressurized space in the Reacti-vial and analyzed in a Hewlett–Packard 5840A analytical gas chromatograph. Samples were analyzed on a 4 ft 10% Carbowax 20 m WHP 80/100 column with a helium flow at 20 ml/min. The column was heated at 60° for 10 min [17, 18].

Extractable organic components

A volume of 0.25 ml of methylene chloride was injected into the aqueous layer in the Reacti-vial and shaken vigorously for a few minutes. The methylene chloride layer was withdrawn with a syringe and dried (Na_2SO_4); then the contents were analyzed on the analytical GC using a temperature program in the range 60–100°.

GC–Mass spectral analytical procedure

The gaseous, volatile and methylene chloride extracted components were analyzed by GC–MS. Individual components were identified by GC retention times and characteristic MS fragmentation patterns by comparison with authentic samples. Additional analyses were carried out with a direct inlet system. For example, the methylene chloride extract was introduced by direct inlet and, after removing the solvent by vacuum, the temperature was slowly raised and MS scans were recorded at 10° intervals.

Products retained in aqueous layer

Decomposition products bearing the 2-nitroimidazole chromophore were detected on the basis of their absorption at 295 nm. In series A only one such product was obtained and was isolated by semi-preparative HPLC using gradient III:

5-*[(2-Nitroimidazolyl)-1-methyl]oxazolidinone* (**4**). ^{1}H-NMR (DMSO-d_6): δ3.62, 3.29 (t, 2H, J = 9 Hz, oxazolidinone-4 CH_2); 4.7 (m, 2H, imidazolyl CH_2), 4.94 (m, 1H, oxazolidinone 5H), 7.68 (m, 1H, exchange, NH), 7.65, 7.21 (dd, J = 1 Hz, 1H each, imidazole CH); FAB-MS (thioglycerol), *m/z* 213 (M + H).

In series B, three decomposition products bearing the 2-nitroimidazole chromophore were successively eluted and isolated by semi-preparative HPLC using gradient III. One was an intermediate, **7**, and the following compounds, **5** and **6** were isolated and characterized.

1,3-*Bis[2-nitro-1-imidazolyl)ethyl]urea* (**5**). ^{1}H-NMR (DMSO-d_6): δ3.40 (t, J = 5 Hz, 4H, —NH—CH_2), 4.40 (t, J = 5 Hz, 4H, —NH—CH_2—CH_2), 6.10 (t, J = 5 Hz, 2H, exchange, NH), 7.20, 7.50

Table 1. Products from controlled decomposition of NI-CENUs in potassium phosphate buffer (pH 7.1) at 37°

Source	Decomposition products	GC retention time (min·sec)	Theoretical yield (%)	*m/z* (rel. intensities, fragments)
1A	Vinyl chloride*	1.9	4–6	64(M + 1, 1.4), 62(M^+, 40), 27(M^+ − Cl, 100)
	Acetaldehyde*†	2.20	8–10	44(M^+, 59.3), 43(M + 1, 33.7), 29(M^+ − CH_3, 100)
	1,2-Dichloroethane†	6.14	4–8	102(M + 4, 1.0), 100(M + 2), 7.5), 98(M^+, 11.5), 63(M^+ − Cl, 15.3), 62(M^+ − HCl, 100), 49(31.0)
	2-Chloroethanol†	33.4	6–8	82(M + 2, 0.4), 80(M^+, 2.1), 45(M^+ − Cl, 1.0), 44(M^+ − HCl, 12.1), 31(M^+ − CH_2Cl, 100)
	Ethylene glycol†	48.2	2–3	62(M^+, 3.1), 45(M^+ − OH, 15), 31(CH_2OH, 100)
1B	Vinyl chloride*	1.75	2–4	64(M + 2, 1.3), 62(M^+, 38), 27(M† − Cl, 100)
	Acetaldehyde*†	2.13	6–8	44(M^+, 70.4), 43(M − 1, 43.6), 29(M^+ − CH_3, 100)
	1,2-Dichloroethane†	6.11	7–10	102(M + 4, 1.1), 100(M + 2, 7.2), 98(M^+, 11.3), 63(M^+ − Cl, 13.4), 62(M^+ − HCl, 100), 49(25.5)
	2-Chloroethanol†	35.56	9–11	82(M + 2, 0.6), 80(M^+, 2.3), 45(M^+ − Cl, 1.3), 44(M† − HCl, 10.5), 31(M^+ − CH_2Cl, 100)
	Ethylene glycol†	45.56	Trace	62(M^+, 2.2), 45(M^+ − OH, 12), 31(CH_2OH, 100)
3A	Vinyl chloride*	1.0	3–7	64(M + 2, 1.4), 62(M^+, 37), 27(M^+ − Cl, 100)
	Acetaldehyde*†	1.37	7–10	44(M^+, 70.9), 43(41.2), 29(M^+ − CH_3, 100)
	1,2-Dichloroethane†	7.41	6–10	102(M + 4, 1.5), 100(M + 2. 9.7), 98(M^+, 14.9), 63(M^+ − Cl, 16.1), 62(M^+ − HCl, 100), 49(31.2)
	2-Chloroethanol†	36.20	7–9	82(M + 2, 0.7), 80(M^+, 2.3), 45(M^+ − Cl, 2.5), 44(M^+ − HCl, 9.2), 31(M^+ − CH_2Cl, 100)
	Ethylene glycol†	45.43	1–2	62(M^+, 1.6), 45(M^+ − OH, 14.9, 31(CH_2OH, 100)
3B	Vinyl chloride*	1.02	3–4	64(M + 2, 1.0), 62(M^+, 40), 27(M^+ − Cl, 100)
	Acetaldehyde*†	1.18	7–10	44(M^+, 96.5), 43(54.6), 29(M^+ − CH_3, 100)
	1,2-Dichloroethane†	5.20	6–10	102(M + 4, 1.5), 100(M + 2, 8.9), 98(M^+, 13.6), 63(M^+ − Cl, 14.2), 62(M^+ − HCl, 100), 49(27.4)
	2-Chloroethanol†	32.48	7–11	82(M + 2, 0.8), 80(M^+, 3.3), 45(M^+ − Cl, 2.2), 44(M^+ − HCl, 18.1, 31(M^+ − CH_2Cl, 100)
	Ethylene glycol†	47.2	Trace	62(M^+, 1.75), 45(M^+ − OH, 14), 31(CH_2OH, 100)

* Detected in the gas phase of the sample.
† Detected in dichloromethane solution.

(dd, J = 1 Hz, 4H, imidazole, CH); FAB-MS (thioglycerol) *m/z* 339 (M + H).

2-(2-nitro-1-imidazolyl)ethylamine (6) [7]. ^{1}H-NMR (DMSO-d_6): δ3.30 (m, 2H, NH_2—CH_2), 4.71 (t, J = 5 Hz, 2H, NH_2—CH_2CH_2), 7.18, 7.81 (s, 2H, imidazole CH), 8.40 (m, 2H, exchange, NH_2).

Kinetics of decomposition

To compare our results with those obtained previously with other nitrosoureas such as CCNU [14] the following standard experimental conditions were used. Each compound was dissolved in the minimum of acetone and then diluted with the 0.1 M phosphate buffer (pH 7.4) to a final concentration of 5 mM in the incubation medium and maintained at 37°. The kinetics of decomposition were followed using a Waters Assoc. instrument equipped with two model 6.000A solvent delivery systems, a model 680 solvent programmer, a model U6K sample injector (Waters Assoc.) and a PU 4021 multichannel detector

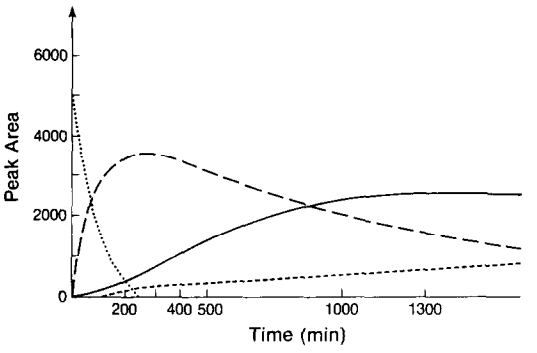

Fig. 2. Kinetics of formation and disappearance of nitroimidazole-bearing products of decomposition of NI-CENU 3B followed spectroscopically. Key: (. . . .) **3**; (———) **5**; (----) **6**; (— —) **7**.

(Philips). Data were computed by means of a PU 4850 video chromatography center equipped with a PU 4900/20 printer/plotter (Philips). Protected by prefilters and a precolumn C_{18} "Guard Pak", the analytical reversed-phase column was a C_{18} "Radial Pak" (100 × 8 mm i.d. 5 μm particle size) in a Waters Assoc. radial compression module RCM 100. The preparative reversed phase column was a μBondapak C_{18} columm (19 mm × 150 mm, Waters Millipore).

The instruments were placed in a thermostated chamber (Frigor–Kulmobel) which was controlled at 18° with a precision of 0.25°.

Carbamoylating activity

For the carbamoylation activity determination, a stock assay solution (11 mM) of 5′-amino-5′-deoxythymidine (5′-AdThd) was prepared. Periodic analysis of an aliquot by HPLC indicated no degradation or concentration change during storage at 5° throughout the course of these experiments. NI-CENU samples were dissolved in acetone at a concentration of 90 mM. The assay procedure was performed as follows: a 0.5-ml glass vial was charged with 200 μl of the stock assay solution and 25 μl of the NI-CENU solution. The mixture was vortexed for 10 sec. The reaction mixture was incubated at 37°, and the aliquot was removed and injected for analysis.

RESULTS

Aqueous decomposition products of NI-CENUs at pH 7.0 and 37°

The volatile and extractable products of the decomposition of NI-CENUs at pH 7.0 and 37° were vinyl chloride, acetaldehyde, 1,2-dichloroethane, 2-chloroethanol and ethylene glycol which were identified by their GC retention times by comparison with authentic samples and by their characteristic fragmentation patterns (Table 1).

In addition, products were isolated in each series bearing the 2-nitroimidazole chromophore (3A, 3B) which was detected on the basis of its absorption at 295 nm. In series A we observed the formation of only one such decomposition product which was stable even after 3 days at 37°. This compound was isolated by semi-preparative HPLC with the gradient III. The UV spectra of both decomposition product and 3A were similar, indicating the presence of a 2-nitroimidazole chromophore. The ^{1}H-NMR and mass spectral data of this product were consistent with the oxazolidinone structure **4**.

In series B, three decomposition products bearing the 2-nitroimidazole chromophore were detected, namely **5**, **6** and **7**. After 4 hr of incubation (at which time there was a maximum concentration of **7**), the gradual disappearance of **7** and the concomitant increase in the concentration of products **5** and **6** were observed (Fig. 2). After 3 days at 37° only two decomposition products containing the imidazole moiety, **5** and **6** were present on the chromatogram. The latter compounds were isolated using semi-preparative HPLC, and their spectroscopic data were consistent with the amino structure **5** and the urea structure **6**. For other NI-CENUs (**1** and **2**), the same decomposition scheme applies.

Kinetics of aqueous decomposition

The kinetics of decomposition of the NI-CENUs under aqueous conditions were determined by following the disappearance of the UV absorbance of the starting drug and the formation of those decomposition products which bear nitroimidazole chromophores. By plotting the peak area of each NI-CENU versus time, exponential curves were obtained which were linearized by logarithmic plots (data not shown). These plots were fitted using least squares

Table 2. Kinetics of aqueous decomposition and carbamoylating activity of NI-CENUs

Compound	K (min^{-1})	$T_{1/2}$ (min)	Correlation coefficient	Carbamoylating activity (%)
1A	0.0238	29	0.999	5.6
2A	0.0201	34.5	0.998	4.4
3A	0.00109	63	0.999	4.5
1B	0.0162	43	0.997	54.0
2B	0.0125	55	0.997	59.4
3B	0.0125	55.5	0.991	50.5
BCNU	0.0133	52 [17]		78.1 [17]
CCNU	0.0144	48 [19]		5.8 [15]
STRPZ		48 [14]		7.00 [17]

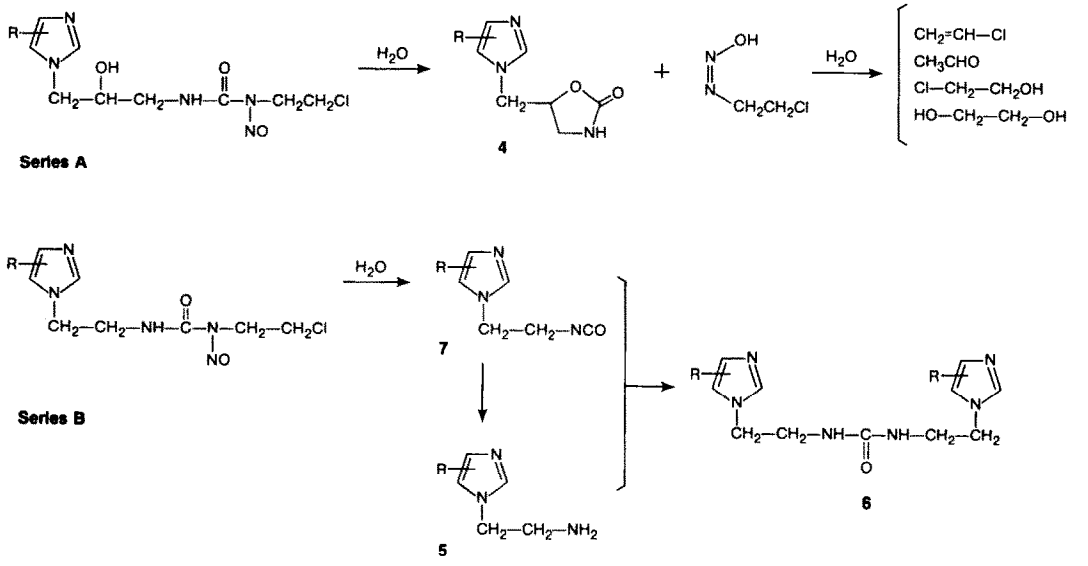

Fig. 3. Scheme summarising aqueous decomposition pathways of series A and series B NI-CENUs.

linear regression analysis and gave correlation coefficients >0.991. The rates of disappearance of NI-CENUs in both series A and series B were shown to be first order. The rate constants determined from the slopes of lines and the half-lives are presented in Table 2. All of the NI-CENUs studied exhibited half-lives comparable with those of BCNU or CCNU [20, 21]; however, the 2-nitroimidazoles were somewhat more stable in both series.

In vitro *carbamoylating activity*

The relative extents of carbamoylating activity of the NI-CENUs were determined using the assay of Brubaker *et al.* [22]. The latter is based on the carbamoylation of the amino group of 5′-AdThd, thereby yielding the corresponding urea (Fig. 3). The ultraviolet absorption of the generated nucleoside permits quantitation of the extent of carbamoylation by measurement of the decrease in the area of the 5′-AdThd peak. Using this assay it was possible to follow the time course of carbamoylation and thus to evaluate the carbamoylating activity of each compound. The extent of carbamoylation after 6 hr is presented in Table 2. The series B NI-CENUs showed carbamoylating activity between 50 and 59%, whereas in the series A, as expected, this value (4.4 to 5.6%) was considerably lower and can be compared with those of streptozotocin [14] or CNCC(C) [17].

DISCUSSION

The products of decomposition of the NI-CENUs under anaerobic aqueous conditions are in accord with the reactions given in Fig. 3. Reversible addition of water to the amide carbonyl bond and the stereoelectronically controlled collapse of the resulting tetrahedral intermediate [23–25] afford, initially, isocyanates bearing the 2-nitroimidazole moiety in addition to *Z*-2-chloroethyldiazohydroxide. The latter gives rise to the products characteristic of CENUs namely vinyl chloride, acetaldehyde and 2-chloroethanol [19, 25, 26]. In the case of the series A NI-CENUs, the isocyanate was trapped intramolecularly, as in the case of streptozotocin or CNCC(C) and structurally related CENUs [14–18, 22] to form the imidazole oxazolidinone **4**.

In the B series compounds, the nucleophilic amine, **5**, reacted with the isocyanate group of **7** to afford the symmetrical urea, **6**. The NI-CENUs examined exhibited rates of decomposition that were comparable with those of BCNU and CCNU [20, 21]; however, the 2-nitroimidazole agents were somewhat more stable.

These results are in accord with recent findings on NI-CENUs [13]. The NI-CENUs decompose under physiological conditions in a similar manner and with a comparable rate to CENUs, and we have demonstrated that NI-CENU derivatives show significant activity against both Mer^+ and Mer^- cell lines. However, as expected, these agents are more toxic towards nitrosourea-sensitive Mer^- HeLa-MR cells, and while NI-CENUs in both series A and B are equally toxic in the Mer^- cells (HeLa-MR), representative compounds from series B are more toxic than their series A counterparts against the Mer^+ cells (HeLa-S3 cells) [13].

This differential toxicity towards Mer^+ and Mer^- cells may be attributable to the generation of the carbamoylating isocyanate upon decomposition of the series B agents which could conceivably inactivate the relevant repair enzymes [15, 16, 27]. In contrast, decomposition of the series A compounds results in the generation of an oxazolidinone moiety lacking carbamoylating activity and which has been shown to be non-cytotoxic [13].

Furthermore, the anaerobic decomposition products of the 2-nitroimidazoles (i.e. **5**, **6**, **7** for 3B and **4** for 3A) could be involved in the chemopotentiation

observed under anaerobic conditions for 3A and 3B. We have already demonstrated that **4** enhances, at sub-lethal concentrations, the hypoxic toxicity of the 4-nitroimidazole 1B [13]. The chemosensitization observed with the NI-CENUs occurs at much lower concentrations (μM) than those required for misonidazole (mM) to produce a comparable dose-enhancement factor with CCNU [13]. These and related aspects are under active investigation in our laboratories.

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