A Novel Design Strategy for Stable Metal Complexes of Nitrogen Mustards as Bioreductive Prodrugs

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Tumor hypoxia provides a key difference between healthy and cancerous cells. It can be exploited to produce drug selectivity, offering a reductase-rich environment for prodrug activation. Nitrogen mustard drugs are cytotoxic, but usually unselective. Polyamine mustards are candidates for conversion into hypoxia-selective prodrugs via complexation with metals. Reduction to a less stable complex can free the active drug. The novel Cu(II) complexes of N-mustard derivatives of 1,4,7-triazacyclononane (tacn), 1,4,7,10-tetraazacyclododecane (cyclen), and 1,4,8,11-tetraazacyclotetradecane (cyclam) were assessed in vitro as hypoxia-selective cytotoxins. The cyclen mustard complex showed 24-fold selectivity as a hypoxia-selective bioreductive prodrug, with an IC_{50} value of 2 μM against the lung tumor cell line A549. Reversible redox behavior and stability of the cyclen−Cu(II) complex in aqueous solution correlated with good hypoxia selectivity. The two other related complexes showed irreversible redox behavior and low aqueous stability and were not hypoxia-selective. The use of macrocyclic nitrogen mustard complexes represents a promising new strategy in the design of hypoxia-selective cytotoxins.

Introduction

Tumor hypoxia is not dependent on tumor size, stage of development, extent of necrosis, or histological environment. There is a large body of laboratory and clinical evidence confirming the existence of hypoxia in human tumor cells.[1] Tumor hypoxia has been linked to the unsuccessful outcome of therapeutic treatments, especially radiotherapy.[2,3] Chemotherapy is often inhibited in hypoxic cells due to the requirement for molecular oxygen in the toxicity of many anticancer agents.[4] In addition, cell proliferation is greatly reduced as a result of lowered oxygen concentration, so agents that specifically target the cell cycle (particularly S-phase) will be less toxic in hypoxic cells. Aside from contributing to treatment resistance, hypoxia also contributes to adverse malignant effects within the tumor microenvironment by promoting metastasis and angiogenesis.[5,6] Since hypoxia is known to have an adverse effect on the success of treatment it is regarded as a problem in cancer therapy. However, the presence of hypoxia as a major physiological difference between tumors and normal tissue provides an opportunity to achieve tumor selectivity. One way of doing this is to develop bioreductive drugs, which are prodrugs selectively activated by intracellular reductases under hypoxic conditions.

Nitrogen mustard alkylating agents can interact covalently with many cellular components, especially enzymes and DNA. The reactive aziridinium ions formed[7] can alkylate nucleophiles such as histidine and cysteine residues of proteins,[8] or guanine residues of DNA.[9] Binding to enzyme backbone peptides can cause conformational changes of the active site and these can result in deactivation of the enzyme. Bifunctional nitrogen mustards are known to form DNA cross-links.[10,11] We have previously synthesized a range of conformationally restricted nitrogen mustards and shown them to be very efficient at cross-linking DNA, with high selectivity for guanine residues.[12−14] The action of nitrogen mustards is fairly indiscriminate and cytotoxicity is high (IC_{50} values around 10^{-6} M), giving them the ability to kill almost all types of tumor cells, both cycling and noncycling. This can be a disadvantage, as such compounds generally exhibit undesirable cytotoxicity in healthy cells. However, the reactivity of these compounds is almost completely dependent on the electron density on the nitrogen. This allows for versatile and stable deactivation through the manipulation of the electronic properties of the molecule around the central nitrogen.

Previously, this was attempted via coordination with Co(III); however, the insolubility in water and nonspecific release of linear polyamine mustards from their Co(III) complexes created problems with the development of these as bioreductive prodrugs.[15] Their cytotoxicity to aerobic cells was also still quite high.[16] This seemed to be due to rapid hydrolysis of the reduced [Co(II)] complex even under aerobic conditions, but also could have resulted from release of mustard from the oxidized [Co(III)] complex. Strategies involving the complexation of Cu(II) to linear mustards encountered similar problems.[17]

Here we report the synthesis of three macrocyclic nitrogen mustard compounds and their cytotoxicity

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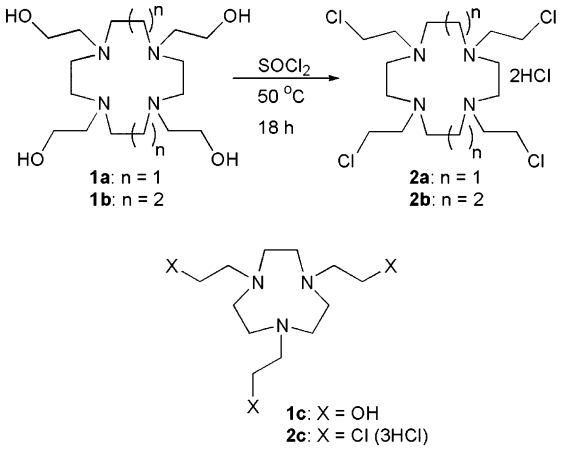
Scheme 1. Synthesis of Mustard Ligands



against the human chronic myeloid leukemia cell line K562. We also report the formation of novel prodrugs of these compounds via complexation with Cu(II) and the selectivity of their in vitro cytotoxicity against the lung-derived human tumor cell line A549 under hypoxic vs oxic conditions. We have found that low kinetic lability of the complex (before and after reduction) is important to the selectivity of the biological activity and can be qualitatively analyzed using UV−vis spectroscopy and cyclic voltammetry. All the Cu(II) complexes discussed here were water-soluble, and our most 'aqueous stable' mustard complex, **3a**, exhibited very promising hypoxia-selective cytotoxicity.

Chemistry

Polyazamacrocyclic nitrogen mustard ligands **2a**−**c** were synthesized in a manner similar to that used for analogous literature compounds, as in Scheme 1. Their Cu(II) complexes **3a**−**c** (Figure 1) were formed. The 'aqueous stability' was assessed using UV−vis spectroscopy to detect changes in the visible absorbance of the complexes due to hydrolytic chemical change of the ligands, giving qualitative approximations of the thermodynamic and kinetic stabilities of the complexes. The corresponding stabilities of the reduced (Cu[I]) complex were qualitatively assessed based on the reversibility of the cyclic voltammagram. Compounds **3a** and **3c** were crystallized and their structures analyzed using X-ray crystallography. **3b** could not be crystallized.

Biological Assays

The free ligands were assessed in vitro for their cytotoxicity against the human chronic myeloid leukemia cell line K562 using the MTT cell proliferation assay. Tumor tissues were exposed to varying concentrations of the drugs for 1 h. The complexes were assessed for hypoxia selectivity by comparing the cytotoxicities against the human lung-derived tumor cell line A549 (measured using the MTT assay, 24 h exposure) under aerobic (oxic) and anaerobic (hypoxic) conditions. The A549 cell line is commonly used for assaying hypoxia selectivity of bioreductive prodrugs and is not known to be sensitized to the MTT assay or hypoxic conditions.[18]

Results and Discussion

The uncomplexed macrocyclic mustards showed good cytotoxicity against the human chronic myeloid leukemia cell line K562, with IC_{50} values in the μM range. The activity was typical of N-mustard drugs, in a similar range to that seen for chlorambucil and melphalan (Table 3). The Cu(II) complexes formed relatively easily and are water-soluble (up to 10 mM, depending on counterion). The low yields obtained for **3b** and **3c** and the difficulties experienced in the attempted crystallization of **3b** are probably due to their instability in aqueous solution. The crystal structures of **3a** and **3c** are shown in Figure 2 (important bond lengths and angles are given in Tables 1 and 2; full crystal data are given in the Supporting Information). The crystal structure for **3a** shows that the Cu(II) ion exhibits square pyramidal geometry. The crystal structure of the tacn compound, **3c** (Figure 2), shows that one of the 2-chloroethyl arms has been hydrolyzed to 2-hydroxyethyl. It is likely that this was the result of an intramolecular hydrolysis facilitated by the coordination of a water molecule to the Cu(II) center. The Cu(II) acts as a Lewis-acid, allowing the water to be deprotonated at neutral pH and activating it for intramolecular hydrolytic attack on a nearby 2-chloroethyl substituent. The resulting 2-hydroxyethyl group then occupies the coordination site, preventing further water complexation and hydrolysis. This type of Lewis-acid activation of water is well-known for triazamacrocyclic metal complexes.[19,20] The resulting complex exhibits distorted octahedral geometry (Figure 2).

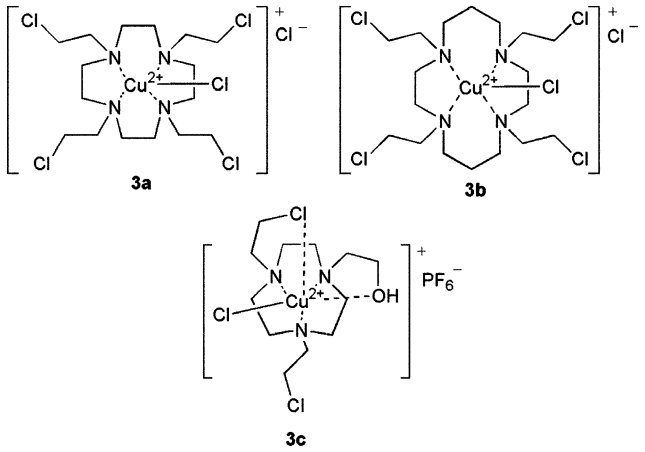


Figure 1. Structures of complexes.

The kinetic labilities of the oxidized forms of the complexes **3a**−**c** in aqueous solution were estimated qualitatively. Due to the facile hydrolysis of these ligands, it was not possible to measure the kinetics or the thermodynamic stability constant K_{stab} quantitatively and directly by typical titration methods.[21] The kinetic lability could, however, be qualitatively estimated by monitoring the hydrolysis of the ligand functionality in aqueous solution (as exemplified in Scheme 2).

These N-mustard ligands can undergo aziridinium ion formation and hydrolysis in water to their 2-hydroxyethyl derivatives, which can also coordinate to Cu(II) ions in solution. The hydrolysis of free mustards is known to happen appreciably and fairly rapidly in vitro even in the presence of biological nucleophiles.[7,22] The differences between the λ_{max} values for the mustard complex and the poly-(2-hydroxyethyl) derivatives are sufficient so that changes in the primary component of

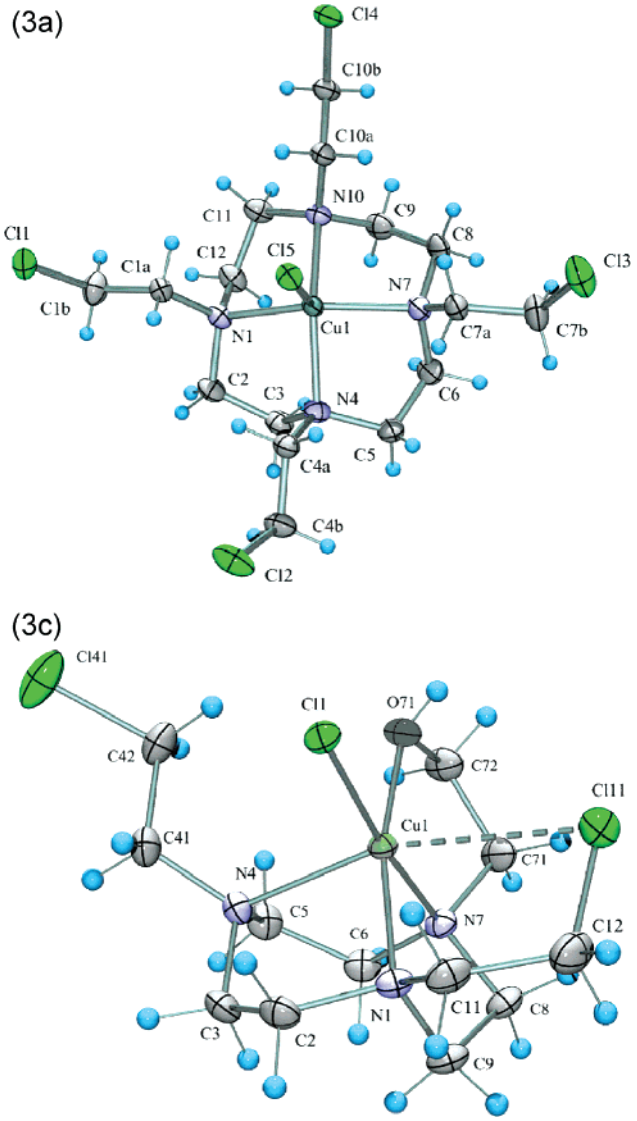


Figure 2. Crystal structures of **3a** and **3c**.

Table 1. Molecular Geometry for **3a**

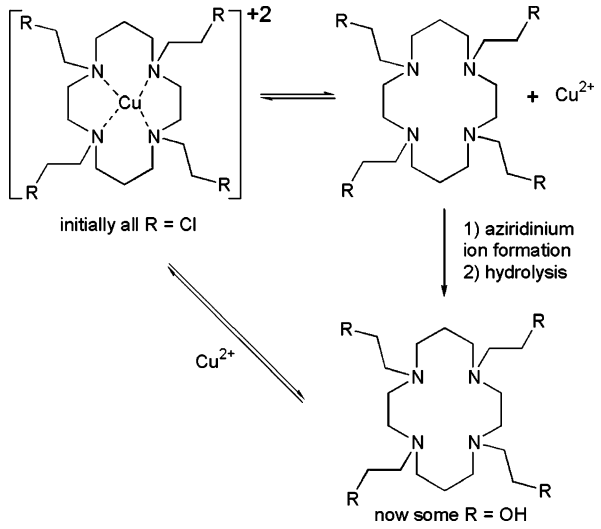
bond lengths, Å, (error)		bond angles (deg)	
Cu1−N1	2.099(3)	N1−Cu1−N4	85.11
Cu1−N4	2.060(4)	N1−Cu1−N7	146.0
Cu1−N7	2.062(3)	N4−Cu1−N7	86.51
Cu1−N10	2.065(2)	N4−Cu1−N10	148.95
Cu1−Cl5	2.3618(5)	N7−Cu1−N10	87.03
		N10−Cu1−N1	85.33

Table 2. Molecular Geometry for **3c**

bond lengths, Å (error)		bond angles (deg)	
Cu1−N1	2.0487(15)	N1−Cu1−N4	85.22
Cu1−N4	2.2490(15)	N1−Cu1−N7	85.38
Cu1−N7	2.0305(15)	N4−Cu1−N7	84.42
Cu1−O(7)1	2.0299(13)	N1−Cu1−Cl1	98.06
Cu1−Cl1	2.2683(4)	N1−Cu1−O(7)1	166.32
Cu1−Cl(1)1	3.086(3)	N4−Cu1−Cl1	99.92
		N4−Cu1−O(7)1	101.62
		N7−Cu1−Cl1	174.64
		N7−Cu1−O(7)1	83.53

an aqueous solution of a mustard complex can be observed over time. **3a** and **3b** were evaluated in this way, and their 'aqueous stability' is defined as $t_{1/2}(\delta)$: the time necessary for a λ_{max} shift of half the difference between those for their mustard and poly-(2-hydroxyethyl) derivatives. **3a** was remarkably kinetically stable, exhibiting no discernible change in its λ_{max} over the course of 14 days [$t_{1/2}(\delta) > 2$ weeks]. **3b**, however, was far less stable, with a $t_{1/2}(\delta)$ value of just 24 h. The data for **3a** and **3b** were consistent with information published for the relative kinetic and thermodynamic properties of similar substituted cyclen and cyclam macrocycles.[23,24]

Scheme 2. Hydrolytic Decomposition of the Ligand in Less Stable Mustard Complexes



Thermodynamic and kinetic stabilities of the reduced [Cu(I)] complexes were also estimated qualitatively using cyclic voltammetry. Reversible redox behavior indicates that the reduced complex is sufficiently stable to remain intact and in contact with the working electrode during the time scale of the voltammetry sweep. Less stable reduced complexes should show irreversible redox behavior, since the concentration of the reduced complex at the electrode will change before it can be reoxidized. Under biological conditions, stable reduced complexes should remain intact long enough to be reoxidized in the presence of cellular oxygen; conversely, reduced complexes that are less stable may be released too quickly even in aerobic tissue.

Cyclic voltammetry and cytotoxicity data are given in Table 3. Representative voltammagrams for **3a**−**c** are shown in Figure 3. The voltammagram for **3a** retained reversibility down to a scan rate of 10 mV/s (the practical limit of the instrumentation and conditions used).

The cytotoxicities of the complexes under aerobic and hypoxic conditions support the prediction that redox reversibility would give hypoxia-selective release of the N-mustard (as suggested by Blower et al.[17]). **3a** was 24 times more cytotoxic under hypoxic conditions, indicating that it targets slow-growing hypoxic cells in vitro, probably through reduction of the complex and release of the mustard (most likely via one e^- reductases),[26] since similar Cu(II) complexes of tetraazamacrocycles used under physiological conditions are usually not very cytotoxic.[27,28] Its aerobic toxicity was approximately 10 times less than that previously reported for the most promising Co(III) complex of a linear mustard.[16] A mechanism is suggested for the activation of this complex in Scheme 3. **3a** is one of the best hypoxia-selective cytotoxins that has been tested on the cell line used in this study, the lung tumor-derived A549.

Table 3. Biological Activities and Physical Characterization of Compounds Tested

parent compound	free mustard	IC_{50}[a] (μM)	Cu(II) complex	$t_{1/2}(\delta)$ (days)	E_{pc}[b] (mV)	cell kill (μM)[c] IC_{50} (air)	cell kill (μM)[c] IC_{50} (N_2)	HCR[d]	ACR[e]
cyclen	**2a**	22	**3a**	>14	−37 (rev)	53.4 ± 10	2.2 ± 0.25	24	–
cyclam	**2b**	7.5	**3b**	1	−140 (irrev)	10.1 ± 1	51.3 ± 10	–	5
tacn	**2c**	10.5	**3c**	n.d.	−240 (irrev)	8.4 ± 0.7	15.9 ± 1	–	2

[a] Against the human chronic myeloid leukaemia cell line K562. [b] vs NHE. [c] Against the human lung tumor-derived cell line A549. [d] HCR is the hypoxic cytotoxicity ratio: IC_{50} (air)/IC_{50} (N_2). [e] ACR is the aerobic cytotoxicity ratio: IC_{50} (N_2)/IC_{50} (air).

3b and **3c** showed no evidence of deactivation via complexation. They exhibited typical characteristics of classical nitrogen mustard drugs. Their IC_{50} values under aerobic conditions were similar to those for their free ligands, and they even showed some degree of aerobic selectivity—a common behavior in drugs which target fast growing oxic cells. This supported the observations from UV−vis analysis that suggested the lability of the oxidized complex was very important for deactivation of the mustard ligand. It is likely that these complexes release the active ligand regardless of the level of oxygenation of the system, making bioreduction less relevant as a mechanism for activation. Given their relatively high reduction potentials, they could be reduced too easily or activated via a different bioreduction pathway, e.g. by DT-diaphorase (which is oxygen-independent). However, it is clear that these compounds (**3b** and **3c**) have no selectivity for hypoxia and are not good prodrugs. Thermodynamic and kinetic stabilities of polyazamacrocycle metal complexes can vary widely depending on the metal ion, parent ring size, and the conformation of the chelate rings formed.[23,29,30] Indeed, cyclam complexes are known to be more kinetically labile than their cyclen analogues.[24] Our evidence shows that these properties are very important to the behavior of metal complexes as prodrugs.

Conclusion

Polyazamacrocyclic N-mustards represent a new class of potent cytotoxins. Their potency is improved when protected from hydrolysis in aqueous media until they are delivered directly to cells via an in situ cellular activation process, e.g. bioreduction. The 'aqueous stabilities' of both the oxidized and reduced forms of metal-complexed prodrugs of these compounds are important to the selectivity of their activation. Cu(II) complexes of N-mustards of three typical azamacrocyclic ring structures were compared, and the cyclen-based mustard complex **3a** showed the best aqueous stability and hypoxia selectivity. This compound provides an attractive lead for further development of this new strategy for bioreductive metal complex design. Previous work with bioreductive ^{64}Cu(II) complexes has shown that a lower (i.e. more negative) reduction potential corresponds with increased selectivity of bioreduction and release of the radiolabeled copper.[31,32] The reduction potentials can be lowered by increasing the electron-donating character of the ligand.[31] Work is currently underway to synthesize analogues of **3a** with electron-donating substituents around the macrocyclic ring, to increase the selectivity of these cytotoxins for hypoxic tissue, as well as to characterize and understand further the mechanism of activation and selectivity of these prodrugs.

Experimental Section

Synthesis of Mustard Ligands. The mustard ligands were synthesized from their 2-hydroxyethyl analogues (as in Figure 2). Poly-*N*-(2-hydroxyethyl) polyazamacrocycles (**1a**−**c**) were synthesized as described previously.[33]

1,4,7,10-Tetra(2-hydroxyethyl)-1,4,7,10-tetraazacyclododecane (1a). The macrocyclic polyamine (1.94 g, 88.8 mmol) was dissolved in EtOH (20 mL) and cooled to 5 °C in with an immersion cooler. Excess ethylene oxide (5.3 mL) was added and the solution stirred overnight at 5 °C. The solvent was evaporated and the residue precipitated from $CHCl_3$/Et_2O to give a white powder (2.71 g, 69% yield); ^{1}H and ^{13}C NMR spectra agreed with literature values;[34] δ_H ($CDCl_3$) 2.16−2.23 (24H, m), 3.27−3.31 (8H, t, J = 5), 4.87 (4H, bs); δ_C ($CDCl_3$) 48.8, 54.1, and 60.1.

1,4,8,11-Tetra(2-hydroxyethyl)-1,4,8,11-tetraazacyclotetradecane (1b) was prepared as above to give a white solid (1.84 g, 98% yield); ^{1}H and ^{13}C NMR spectra agreed with literature values;[34] δ_H ($CDCl_3$) 1.48−1.61 (4H, m), 2.48−2.59 (24H, m), 3.52−3.58 (8H, m); δ_C ($CDCl_3$) 25.1, 49.1, 51.9, 55.6, and 59.3.

1,4,7-Tris(2-hydroxyethyl)-1,4,7-triazacyclononane (1c) was prepared as above (not crystallized) to give a clear oil (0.086 g, 85% yield); ^{1}H and ^{13}C NMR spectra agreed with literature values;[35] δ_H ($CDCl_3$) 2.63 (12H, s), 2.75−2.77 (6H, t, J = 5), 3.58−3.61 (6H, t, J = 5); δ_C 53.3, 59.8, and 60.4.

1,4,7,10-Tetra(2-chloroethyl)-1,4,7,10-tetraazacyclododecane dihydrochloride (2a). The poly-*N*-(2-hydroxyethyl) derivative **1** was stirred with $SOCl_2$ (5 mL/100 mg 1) while heating to 50 °C overnight. The excess thionyl chloride was removed in vacuo, leaving the hydrochloride salt (2.68 g, 70% yield): δ_H (D_2O) 3.17 (bs, 16H), 3.26 (bs, 8H), 3.73 (8H, m)$_C$ (D_2O) 39.3, 49.1, 54.9; Microanalysis: Found: C, 38.65; H, 7.09; N, 11.10%. $C_{16}H_{34}N_4Cl_6$ requires C, 38.81; H, 6.92; N, 11.31%.

1,4,8,11-Tetra(2-chloroethyl)-1,4,8,11-tetraazacyclotetradecane dihydrochloride (2b) was prepared as above to give a cream solid (0.114 g, 90% yield); ^{1}H and ^{13}C NMR spectra agreed with literature values;[36] δ_H (D_2O) 1.92−2.18 (4H, m), 3.13−3.37 (16H, m), 3.72−3.79 (16H, m); δ_C (D_2O) 17.5, 43.5, 47.9, 56.1, and 57.9; Microanalysis: Found: C, 36.42; N, 9.45%. $C_{18}H_{40}N_4Cl_8$ requires C, 36.26; N, 9.40%.

1,4,7-Tris(2-hydroxyethyl)-1,4,7-triazacyclononane trihydrochloride (2c) was prepared as above to give a cream solid (0.138 g, 99% yield); δ_H (DMSO-d_6) 2.94 (6H, broad s), 3.20 (6H, broad s), 3.39 (6H, broad s); δ_C (DMSO-d_6) 40.5, 49.3, 56.5; *m/z* (FAB+, glycerol) 316.1 ([(M - 3HCl) + H]$^+$, 100%), 197.1 (5%), 147.0 (8), 106.3 (20), 70.8 (4), 57.0 (4); found [(M − 3HCl) + H]$^+$ 316.1106, $C_{12}H_{24}N_3{}^{35}Cl_3$ requires 316.1114.

Formation of Cu(II) Complexes (Figure 1). 1,4,7,10-Tetra(2-chloroethyl)-1,4,7,10-tetraazacyclododecane-[Cu(II)]Cl_2 (3a) was formed from the hydrochloride salt of **2a** (754 mg, 1.52 mmol) and anhydrous $CuCl_2$ (205 mg, 1.52 mmol) in methanol/water (30 mL, 5:1). The solution was warmed to ~50 °C for 10 min. The deep blue-colored complex precipitated upon cooling the solution and was filtered and dried with suction to give a blue powder (716 mg, 75% yield, purity: 98% based on reverse-phase HPLC, see Supporting Information). MS: *m/z* (FAB+, glycerol) 520.3 (12%), 485.3 ([(M − 2Cl) + H]$^+$, 100%) 483.3 (57), 421.3 (7), 419.3 (3), 185.1 (14), 147.1 (6), 93.5 (79), 75.7 (37), 57.9 (27); Found [(M − 2Cl) + H]$^+$ 485.0650, $C_{16}H_{32}N_4{}^{35}Cl_3{}^{37}ClCu$ requires 485.0651. The material was crystallized as its tetrafluoroborate salt by adding excess NH_4BF_4 to a hot, saturated solution of the chloride

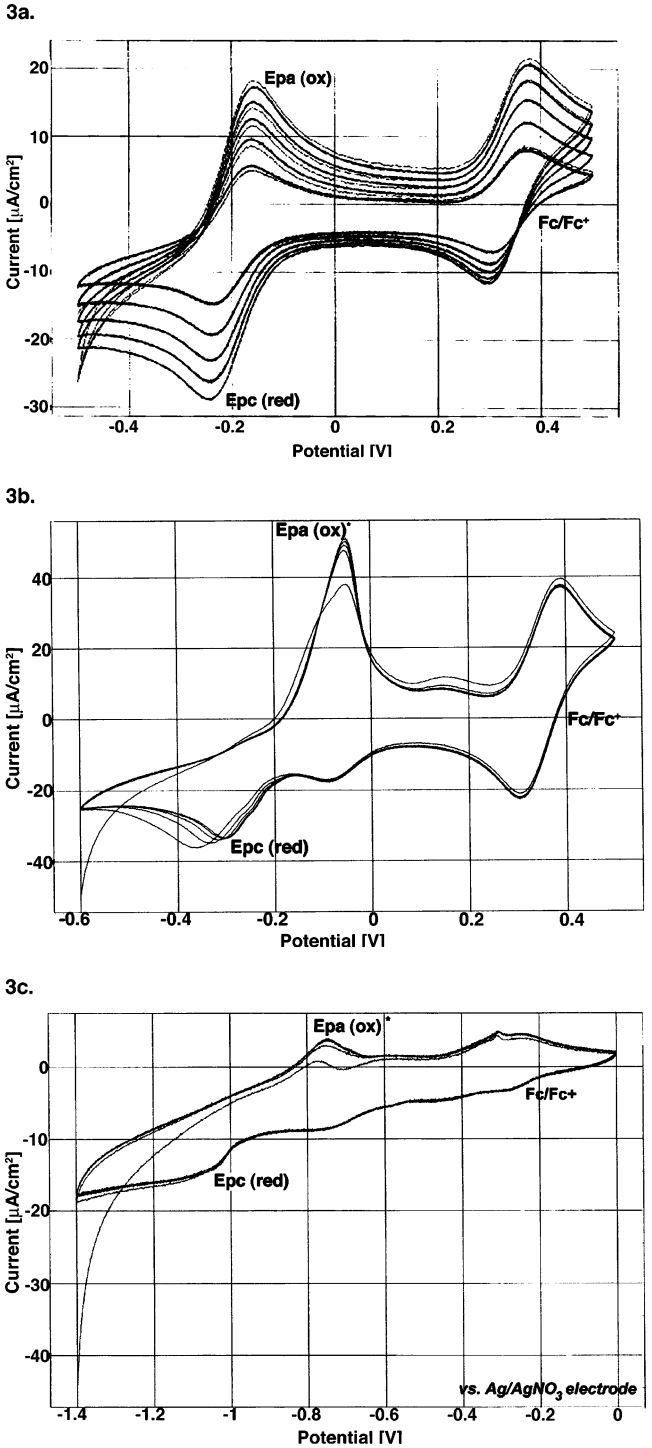


Figure 3. Cyclic voltammagrams for **3a**−**c** using a platinum macrodisc (2 mm) working electrode. **3a**: 1.0 mM in 100 mM aqueous phosphate buffer, pH 7.2; vs sat. Ag/AgCl electrode; scan rates from 50 to 400 mV/s (showing reversibility); **3b**: 1.0 mM in 100 mM aqueous phosphate buffer, pH 7.2; vs sat. Ag/AgCl reference electrode; scan rate 100 mV/s; **3c**: 1.0 mM in 100 mM aqueous phosphate buffer, pH 7.2 (lower concentration of ferrocenecarboxylic acid internal standard); vs 100 mM $Ag/AgNO_3$ (nonaqueous) reference electrode; *ligand has changed before oxidation.

salt in water. The structure of **3a** was confirmed by X-ray crystallography (shown in Figure 2).

1,4,8,11-Tetra(2-chloroethyl)-1,4,8,11-tetraazacyclotetradecane[Cu(II)]Cl_2(2NaCl)(H_2O) (3b) required neutralization in saturated NaCl in order to effect complexation. A solution of **2b** (159 mg, 0.256 mmol) and $CuCl_2$ (36 mg, 0.26 mmol) in sat. NaCl (5 mL) was neutralized with 1 M aq NaOH (15 drops), causing a color change to deep blue-violet. The solvent was removed in vacuo to give a bright green solid, which was washed with H_2O (10 mL) and filtered (23 mg, 14% yield, purity: 94% based on microanalysis). MS: *m/z* (FAB+, glycerol) 513.3 ($[(M - 2Cl) + H]^+$, 11%), 511.3 (6), 451.4 (23), 449.4 (20), 387.4 (9), 369.3 (6), 277.2 (15), 185.1 (100), 106.3 (16), 75.7 (67), 57.9 (77). Found $[(M - 2Cl) + H]^+$ 513.0969. $C_{18}H_{36}N_4{}^{35}Cl_3{}^{37}ClCu$ requires 513.0965. Microanalysis results: 31.86% C, 5.30% H, 8.29% N, 39.47% Cl. Theoretical: $C_{19}H_{39}N_4Cl_6Cu(2NaCl)(H_2O)$ requires 30.04% C, 5.32% H, 7.79% N, 39.41% Cl.

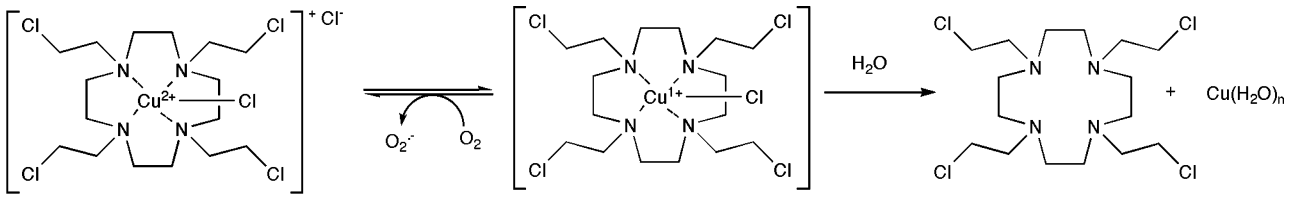
Attempts to crystallize **3b**, regardless of counterion, resulted in hydrolysis of the ligand to its hydroxyethyl analogue, giving the Cu(II) complex of the 2-hydroxyethyl derivative **1b** which crystallized out of solution. The crystal structure (**3d**) is shown in the Supporting Information for this document (Figure 4).

1,4-Bis(2-chloroethyl)-7-(2-hydroxyethyl)-1,4,7-triazacyclononane[Cu(II)]ClPF_6 (3c) was formed from the hydrochloride salt of **2c** (115 mg, 0.270 mmol) and anhydrous $CuCl_2$ (36 mg, 0.270 mmol) in methanol/water (8 mL, 5:1). The solvent was removed in vacuo to give a green glassy solid which was taken up in H_2O (5 mL). Excess NH_4PF_6 was added and the solvent evaporated until dark blue-green crystals formed, which were suitable for X-ray crystallography (15 mg, 10% yield, purity: 94.5% [reverse-phase HPLC, see Supporting Information]). *m/z* (FAB+, glycerol) 362.3 ($[(M - ClPF_6) + H]^+$, 64%), 326.3 (6), 277.4 (13), 262.3 (4), 185.2 (100), 93.5 (74), 75.7 (14), 58.0 (4), 46.1 (3); found $[(M - ClPF_6) + H]^+$ 362.0648, $C_{12}H_{25}N_3O^{35}Cl^{37}ClCu$ requires 362.0647. The crystal structure is given in Figure 2.

Reverse-Phase HPLC for Determination of Purity. Compounds **3a** and **3c** were analyzed using an Agilent 1100 analytical HPLC system. Separation was achieved on a Zorbax Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm particle size) using a gradient of 90/10%−25/75% A/B over 12 min (A: 0.1% aqueous TFA; B: 0.08%TFA/MeCN) with a 2.0 mL/min flow rate and compounds detected with a diode-array UV−vis spectrophotometer. See Supporting Information for spectra. **3b** decomposed in the aqueous solvent system (as seen during attempted crystallizations) and did not produce reproducible data.

X-ray Crystallography. Details of data collection procedures and structure refinement are given in Supporting Information. Single crystals of suitable size were attached to glass fibers using acrylic resin and mounted on a goniometer head in a general position. Data were collected on an Enraf-Nonius KappaCCD diffractometer, running under Nonius Collect software, and using graphite monochromated X-radiation (λ = 0.71073 Å). All data sets were collected at a temperature of 150 K using an Oxford Instruments Cryostream. Typically scan angles of 1−2° were used, with integration times of 50−100 s per image. Precise unit cell dimensions were determined by post-refinement of the setting angles of a large proportion of the data set. The frame images were integrated using Denzo(SMN)[37] and the resultant raw intensity files processed using a locally modified version of DENZOX.[38] Absorption corrections, either by Gaussian quadrature,[39] based on the measured crystal faces, or by a semiempirical correction[40] were applied to all data sets. Data were then sorted and merged using SORTAV.[41] Structures were solved either by Patterson interpretation (DIRDIF-99)[42] or by direct methods (SIR92).[43] All structures except **3c** showed disorder in the macrocyclic ring system, with the major component having ~80−90% occupancy, and all structures showed some disorder in their respective Cl^-, BF_4^-, or PF_6^- anions. For the major componenent only, all non-H atoms were allowed anisotropic thermal motion. Aliphatic C−H hydrogen atoms were included at calculated positions, with C−H = 0.96 Å, and were refined with a riding model and with U_{iso} set to 1.2 times that of the attached C-atom. Refinement was carried out with SHELXL97-2[44] using full-matrix least-squares on F^2 and all the unique data. Neutral atom scattering factors, coefficients of anomalous dispersion, and absorption coef-

Scheme 3. Proposed Mechanism for Bioeduction of Mustard Complex and Release of Cytotoxin



ficients were obtained from published work.[45] Calculations using PLATON[46] indicated that there were no voids in the lattice capable of containing solvent molecules. Thermal ellipsoid plots were obtained using the program ORTEP-3 for Windows.[47] All calculations were carried out using the WinGX package[48] of crystallographic programs.

Aqueous Stability of Complexes. A solution of the complex (1.0 mM) in 100 mM aqueous phosphate buffer, pH 7.2, was allowed to stand for the required period of time. The λ_{max} of the solution was monitored by UV−vis spectroscopy, watching for a shift toward the λ_{max} known for the 2-hydroxyethyl complex. The time required for a λ_{max} shift of half the difference between those for the two complexes was defined as $t_{1/2}(\delta)$. Solutions were monitored for a maximum of two weeks.

Cyclic Voltammetry. The redox potential of each compound was measured with cyclic voltammetry using a PGZ301 Dynamic-EIS VoltaLab potentiostat. The analysis was performed on freshly prepared solutions of the complexes (1.0 mM) in aqueous phosphate buffer (100 mM) at pH 7.2, with ferrocenecarboxylic acid (FCA) as an internal standard (+533 mV vs NHE, or +334 mV vs sat. Ag/AgCl),[49] using a three-electrode cell with a Pt macrodisc working electrode (2.0 mm), Pt wire counter electrode, and the saturated Ag/AgCl reference electrode. The potentials were corrected for the published potential of FCA and reported vs NHE (correction factor: E° [vs NHE] = E° [vs Ag/AgCl] + 199 mV). The solutions were degassed with N_2 for at least 10 min before analysis, to simulate the hypoxic environment.

Cytotoxicity of Free Mustard Ligands. The cytotoxic effects of the free ligands studied were measured against the human chronic myeloid leukaemia cell line K562. Cells were maintained as a suspension in RPMI 1640 medium supplemented with 10% ftal calf serum (FCS) and 2 mM glutamine (Gln) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The IC_{50} values of the series of analogues following a 1 h exposure to drug were determined using the MTT assay[50] as has been previously described.[51] This is based on the ability of viable tumor cells to convert a yellow tetrazolium salt [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2*H*-tetrazolium bromide, MTT] into mauve formazan crystals.

Cytotoxicity and Hypoxia Selectivity of Complexes. The toxicities and hypoxia selectivities of the complexes were determined using the MTT proliferation assay.[18] All media, plates, and other plastic material were placed into the anoxic incubator for at least 24 h prior to the hypoxic experiments. The lung-derived tumor cell line, A549, was exposed to each of the three drugs for 24 h under aerobic or hypoxic conditions. After 24 h exposure, the drug was removed and fresh media instilled into each well. After 96 h incubation at 37 °C, the MTT proliferation assay was performed. The IC_{50} results were expressed as the mean of at least three different experiments (±SEM). HCR is the hypoxic cytotoxicity ratio [IC_{50} (air)/IC_{50} (N_2)], and ACR is the aerobic cytotoxicity ratio [IC_{50} (N_2)/IC_{50} (air)].

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Supporting Information Available: Full crystal data for all structures and HPLC spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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