Redox-active metal complexes for imaging hypoxic tissues: structure–activity relationships in copper(II) bis(thiosemicarbazone) complexes

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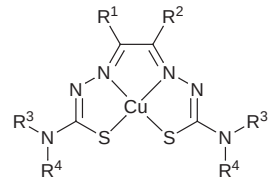
Received (in Cambridge, UK), 29th July 1998, Accepted 12th October 1998

Reduction potential and lipophilicity of the copper(II) bis(thiosemicarbazone) complexes can be independently controlled by alkylation in the diketone backbone and the N-termini of the ligand, allowing optimisation of radiopharmaceuticals strongly selective for hypoxic tissues.

Hypoxia in tumours can affect the outcome of anti-cancer treatments.[1] Hypoxic malignant tissue is relatively resistant to chemotherapy, and to irradiative therapy because of the lack of oxygen, a potent radiosensitiser. Hypoxia is also associated with other important health problems such as heart disease and stroke. Radiopharmaceuticals for imaging hypoxia have therefore been widely sought in recent years.[2] The current lead compound, ^{18}F-fluoromisonidazole,[3] shows imageable differences between normal and hypoxic tissue, but suffers from slow blood clearance and low tumour-to-muscle ratios.[4]

Copper radionuclides have attracted considerable attention in nuclear medicine because they include isotopes with both diagnostic (Cu-60, Cu-61, Cu-62, Cu-64) and therapeutic (Cu-64, Cu-67) potential. They are becoming increasingly available to the medical community through the use of generator systems and improvements in small cyclotron production.[5] The bis(thiosemicarbazone)s chelate copper(II) to form stable mononuclear, square-planar complexes. These have been investigated for use in anti-cancer chemotherapy,[6] and, in radiolabelled form, as a non-tissue-selective blood perfusion tracer $^{62/64}$Cu(PTSM).[7] The latter complex is capable of rapid entry into cells by passive diffusion as a consequence of its low molecular weight, lipophilicity and planarity.[8] It then becomes trapped intracellularly, regardless of tissue type, probably as a consequence of intracellular reduction to a copper(I) complex.[9] This redox-dependent trapping mechanism may allow, through control of redox potential, synthesis of an analogue that is trapped only in cells that provide a more reducing environment than normal, resulting from the absence of molecular oxygen). This approach could lead to design of imaging agents for hypoxia. Indeed, Cu(ATSM) has demonstrated significant selectivity for hypoxic and ischaemic tissue both *in vitro*[10] and *in vivo*,[10,11] while Cu(PTSM) has little[12] or no[13] selectivity. It has been suggested that the difference in selectivity is due to differences in redox potential.[11] Here we show that both lipophilicity and redox potential can be independently controlled through alkyl substitution at the terminal nitrogen atoms and the diketone backbone, respectively, to give complexes with and without selectivity for hypoxic cells.

We have synthesised a series of thirteen such complexes, with a variety of alkylation patterns. All gave satisfactory elemental analysis and FAB-MS results. The Cu-64-labelled complexes were identified with their non-radioactive analogues by thin-layer radiochromatography. The electrochemistry of the complexes was investigated by cyclic voltammetry using a glassy carbon working electrode in dimethyl sulfoxide containing tetrabutylammonium tetrafluoroborate as support electrolyte. The lipophilicity (log *P*) was determined by octanol extraction of the Cu-64 labelled complexes from water. The labelled complexes were screened for hypoxia selectivity using mammalian cancer cells (EMT6) in a suspension. The hypoxic cell suspension was equilibrated for 1 h with an atmosphere of



	R^1	R^2	R^3	R^4
GTS	H	H	H	H
GTSM	H	H	CH_3	H
PTS	CH_3	H	H	H
PTSM	CH_3	H	CH_3	H
$PTSM_2$	CH_3	H	CH_3	CH_3
PTSE	CH_3	H	C_2H_5	H
PTSP	CH_3	H	C_6H_5	H
ATS	CH_3	CH_3	H	H
ATSM	CH_3	CH_3	CH_3	H
CTS	C_2H_5	CH_3	H	H
CTSM	C_2H_5	CH_3	CH_3	H
DTS	C_2H_5	C_2H_5	H	H
DTSM	C_2H_5	C_2H_5	CH_3	H

95% N_2–5% CO_2 while the control (normoxic) suspension was similarly equilibrated with 95% air–5% CO_2. The oxygen concentration, measured with a Mettler Toledo 4300 oxygen electrode, in the hypoxic suspension was then below 0.2% (where 100% is the equilibrium concentration under an atmosphere of air). For comparison, the upper limit defining 'radiobiological hypoxia' is 0.66%.[14] Cu-64 complexes were introduced into the suspension at tracer levels, and samples taken at time points over 1 h and centrifuged to isolate the cells. The 'hypoxia selectivity' was determined from the cell uptake ratios at 1 h incubation, and expressed as $\log_{10}$[(% uptake in hypoxic cells)/(% uptake in normoxic cells)]. Thus, hypoxia-selective complexes have positive hypoxia selectivity values while normoxia-selective complexes have negative values.[15]

Fig. 1 shows typical cell uptake *versus* time profiles for two complexes selected to represent hypoxia-selective and normoxia-selective behaviour. The cyclic voltammograms of all the complexes showed a reversible one-electron Cu(II/I) pro-

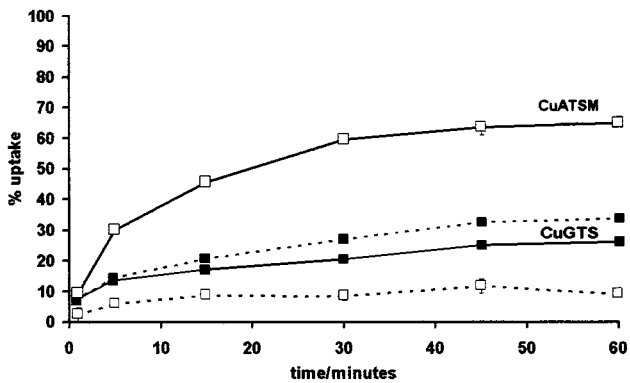


Fig. 1 EMT6 tumour cell uptake profiles selected to typify normoxia-selective (filled squares), and hypoxia-selective (open squares) copper complexes under hypoxic (solid line) and normoxic (broken line) conditions.

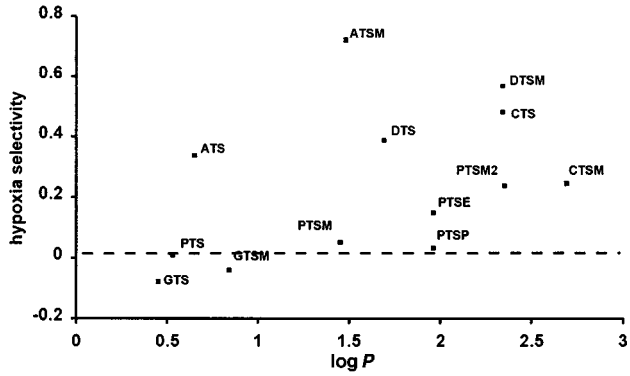


Fig. 2 Plot of hypoxia selectivity (see text for definition) for copper complexes in relation to their lipophilicity values (log *P* octanol/water).

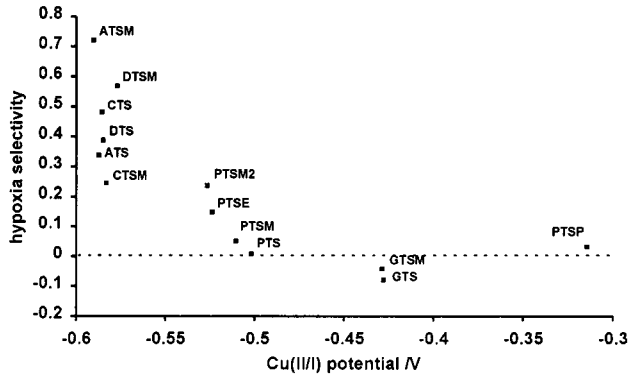


Fig. 3 Plot of hypoxia selectivity (see text for definition) for copper complexes in relation to their Cu(II/I) redox potentials *vs.* Ag/AgCl.

cess. Fig. 2 shows the relationship between lipophilicity (log *P*) and hypoxia selectivity, and Fig. 3 shows the relationship between Cu(II/I) redox potential and hypoxia selectivity.

The inter-relationships between structure, lipophilicity, redox potential and hypoxia selectivity may be summarised as follows. (1) From Fig. 3 it is evident that hypoxia selectivity is strongly dependent on redox potential. (2) On the other hand lipophilic character (Fig. 2), while presumably necessary to allow cell membrane penetration (all of the compounds have log *P* greater than zero), is not an indicator of hypoxia selectivity. (3) The overall number of alkyl groups at positions R^1–R^4 is a poor predictor of redox potential. However, the number in the diketone backbone (R^1 and R^2) is an excellent predictor: complexes with no alkylation at these sites have potentials in the range −0.42 to −0.44 V; those with one alkyl group have potentials in the range −0.50 to −0.53 V; and those with two have potentials in the range −0.57 to −0.59 V. This rule is adhered to by all except the phenyl-substituted complex Cu(PTSP). Alkyl substitution at the N-terminus, on the other hand, does not influence redox potential significantly. (4) Alkyl substitution, either in the complex as a whole or separately in the diketone backbone and N-terminus, is only a very crude predictor of lipophilicity.

These trends support the notion that the redox behaviour of transition metal complexes can be exploited to achieve hypoxia-selective targeting, and that hypoxia selectivity is a function of redox potential in these complexes. Moreover, they provide a basis for designing hypoxia-selective complexes of this type according to redox potential: it appears to be a requirement that the Cu(I/II) redox potential in dimethyl sulfoxide is more negative than −0.57 V *vs.* Ag/AgCl. It might be expected that on shifting the redox potential to much more negative values there will come a point at which the selectivity will diminish again because even hypoxic cells will be incapable of reducing the complexes. This potential is not reached in the present series.

The relationship shown in Fig. 3 suggests that redox potential is not the only factor controlling hypoxia selectivity: although complexes with potentials in the range −0.57 to −0.59 V are all hypoxia selective, they differ in degree of selectivity. Indeed it is to be expected that alkylation pattern would influence selectivity in a complex way through factors such as membrane solubility and steric effects on reaction rates as well as through redox potential. Nevertheless, an appropriate redox potential is the primary requirement for selectivity. The relationship between alkylation pattern and redox potential provides a means of controlling redox potential and lipophilicity separately: R^1 and R^2 can be varied to control redox potential, while R^3 and R^4 can be varied to control lipophilicity and other relevant pharmacokinetic parameters, to produce an ideal radiopharmaceutical for PET imaging of hypoxia.

Many facets of the mechanism of hypoxia-selectivity of these complexes remain to be investigated, including the serum stability[16] of the complexes, whether a single specific intracellular reducing agent is involved, and the reversibility of the trapping in hypoxic cells. Of the complexes investigated here, Cu(ATSM) remains the best candidate in terms of absolute selectivity *in vitro*, and ^{60}Cu(ATSM) is being investigated at the Washington University School of Medicine and Fukai Medical University[17] as an agent for the delineation of hypoxia in humans.

We thank Mr T. A. Perkins for help in producing copper-64 and Mrs E. L. C. Sherman for help with animal cell culture. J. L. J. D. is supported by a studentship and fieldwork grant from the Medical Research Council. We acknowledge the US Dept. of Energy for funding (Grant no. DE-FG02-87ER60512) work undertaken at St. Louis.

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Communication 8/05957H