Design of hypoxia-targeting radiopharmaceuticals: selective uptake of copper-64 complexes in hypoxic cells in vitro

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Abstract The well-known perfusion tracer CuPTSM, labelled with ^{62}Cu or ^{64}Cu, is believed to be trapped in cells non-selectively by a bioreductive mechanism. It is proposed that by modifying the ligand to increase its electron donor strength (for example by adding alkyl functionality or replacing sulphur ligands with oxygen ligands), the copper complexes will become less easily reduced and tracers with selectivity for hypoxic tissues could thus be developed. The aim of this work was to prepare ^{64}Cu-labelled complexes of two series of ligands, based on the bis(thiosemicarbazone) (13 ligands) and bis(salicylaldimine) (3 ligands) skeletons, and to evaluate the hypoxia dependence of their uptake in cells. The complexes were incubated with Chinese hamster ovary cells under normoxic and hypoxic conditions, and the cells isolated by centrifugation to determine radioactivity uptake at various time points up to 90 min. Several members of both series demonstrated significant (P<0.05) or highly significant (P<0.01) hypoxia selectivity, indicating that both series of complexes offer a basis for development of hypoxia-targeting radiopharmaceuticals for positron emission tomography (^{60}Cu, ^{61}Cu, ^{62}Cu, ^{64}Cu) and targeted radiotherapy (^{64}Cu, ^{67}Cu).

Key words: Hypoxia – Copper-64 – Positron emission tomography

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Introduction

Radiopharmaceuticals capable of selective uptake in hypoxic tissue could have applications in stroke, heart disease and especially cancer [1]. The ability to measure hypoxia non-invasively in tumours would help predict the outcome of radiotherapy, while selective delivery of cytotoxic radioactivity to hypoxic tumours would offer an alternative treatment: the cytotoxicity of high-LET radionuclides is less dependent on oxygenation than that of X-rays if the high-LET emissions occur within the cell nucleus [2].

Most efforts to develop hypoxia-targeting radiopharmaceuticals have used 2-nitroimidazole derivatives, labelled with fluorine-18 or iodine-123. These undergo intracellular reduction to radicals, which in the absence of oxygen become irreversibly attached to cellular macromolecules before they can be re-oxidised [1]. Nitroimidazoles have also been coupled to chelates of technetium-99m [1]. Recently the simple technetium complex, ^{99m}TcBnAO [3], was discovered to be selectively taken up in hypoxic cells, although the mechanism of this selectivity is unknown.

Copper radionuclides offer an attractive basis for the development of hypoxia-targeting radiopharmaceuticals. Several radionuclides are available for both PET (^{60}Cu, ^{61}Cu, ^{62}Cu and ^{64}Cu) and targeted radiotherapy (^{64}Cu and ^{67}Cu) [4]. Copper has an amenable coordination chemistry, and electrochemistry that could lend itself to redox-mediated trapping in cells. There is evidence [4] that the known perfusion imaging agent ^{64}CuPTSM [PTSM=pyruvaldehyde bis(N^4-methyl-thiosemicarbazone)] is extracted into tissues by a bioreductive mechanism. It is believed to diffuse readily into cells, where it is reduced to a labile copper(I) complex from which the ligand dissociates, liberating the copper which is thus trapped. It is suggested [4,5] that by modifying the ligand to lower the redox potential of the complex (i.e. to make it harder to reduce) it might be possible to prepare complexes that are trapped only in hypoxic tissues.

We have approached this possibility in two ways: by adding electron-donating alkyl substituents to the ligand backbone, and by replacing sulphur atoms with oxygen, a stronger π-electron donor. In this paper we describe an in vitro method of measuring the oxygen dependence of

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radiotracer uptake in cells, and the behaviour in this system of two series of ^{64}Cu complexes designed according to these principles.

Materials and methods

Synthesis and characterisation of ligands and their non-radioactive copper(II) complexes will be described elsewhere. Some of the complexes have been described previously [4, 6]. A direct analogue of PTS was synthesised in which both sulphur atoms were replaced with oxygen, but attempts to complex copper with this ligand failed and it was abandoned in favour of salicylaldimine ligands EDS, EDSM and PDS first used for ^{64}Cu by Green et al. [6] For ^{64}Cu labelling, the ligands were dissolved in dimethylsulphoxide (1 mg ml^{-1}) and 10 µl of this mixed with $^{64}CuCl_2$ in hydrochloric acid (carrier free [7]) buffered to pH 4.5 with aqueous 3 *M* sodium acetate to a total volume not exceeding 120 µl, at room temperature. Salicylaldimine ligands (EDS, EDSM, PDS) were labelled in the same way but optimal yields were obtained at pH 8–11. Quality control was performed using thin-layer chromatography (silica-gel stationary phase, ethyl acetate mobile phase) and logP measurement (by octanol extraction). Complexes were not used for cell uptake experiments unless radiochemical purity exceeded 95%.

Chinese hamster ovary cells (CHO320), chosen because they are adapted to suspension culture, were grown semicontinuously in a defined RPMI 1640 medium. Cell concentration and viability were determined using a haemocytometer and trypan blue staining. Cell suspensions (2×10^5 to 4×10^5 cells/ml, 50 ml) in a 500-ml glass conical flask were equilibrated at 37°C with 95% dinitrogen/5% carbon dioxide (hypoxic) or 95% air/5% carbon dioxide (normoxic) (except in the case of PTSM, where 100% dinitrogen and 100% air were used) with a gentle stream of the water-saturated gas directed over the surface of the suspension while agitating on an orbital shaker at 80 rpm, for 1 h (Fig. 1). Oxygen concentration in the hypoxic suspension was monitored continuously with a Mettler Toledo model 4300 oxygen probe. ^{64}Cu complexes (<60 µl, 0.4–0.2 MBq) were added to each culture while gassing and agitation continued. Samples (200 µl, in triplicate) were transferred to microcentrifuge tubes at intervals up to 90 or 120 min and immediately centrifuged at 2000 rpm for 20 s (no significant loss of viability resulted from the centrifugation). A 180-µl sample of each supernatant was removed for counting, and each tube containing cells and 20 µl medium was also counted. Percent cell uptake was calculated as [residue counts–(supernatant counts/9)]/(residue counts + supernatant counts)×100%. For selected complexes exhibiting hypoxia-dependent uptake, additional controls were performed to determine the fraction of the residue radioactivity that was due to binding to the centrifuge tube, under both hypoxia and normoxia, using cell-free medium.

For statistical classification of uptake behaviour, normal distribution of % uptake under hypoxia and normoxia was assumed, and z-scores were determined both over all time points from 30 min onwards, and separately at each time point from 30 min onwards, and used in a two-tailed test to determine the significance of the % uptake difference between normoxic and hypoxic conditions. Three levels of significance were defined: not significant ($P>0.05$), significant ($0.01<P<0.05$) and highly significant ($P<0.01$).

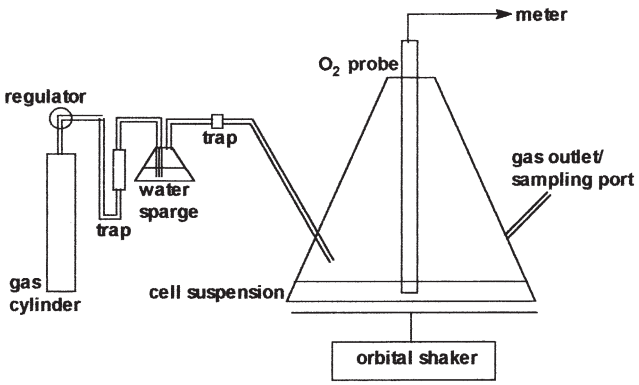

Fig. 1. Schematic representation of apparatus used to measure tracer uptake in hypoxic and normoxic cells

Table 1. Summary of ligands, and logP values, Rf values and hypoxia selectivity of their ^{64}Cu complexes

Ligand	Group	Log P of complex	Rf of complex	Selectivity
GTS	1	0.84	0.2	Normoxia (significant)
GTSM	1	1.98	0.4	Normoxia (significant)
PTS	1	0.53	0.2	Not significant
PTSE	1	1.96	0.5	Normoxia (significant)
PTSP	1	1.96	0.8	Not significant
PTSM2	1	2.39	0.5	Not significant
ATS	1	0.35	0.3	Not significant
CTSM	1	1.85	0.6	Not significant
PTSM	2	1.82	0.5	Hypoxia (significant/highly significant)
ATSM	2	1.61	0.6	Hypoxia (highly significant, $P<0.0001$)
CTS	2	1.01	0.6	Hypoxia (highy significant, $P=0.00095$)
DTS	2	1.34	0.5	Hypoxia (highly significant, $P<0.0001$)
DTSM	2	1.57	0.7	Hypoxia (highly significant, $P=0.0026$)
EDS	3	1.98	0.2	Hypoxia (significant)
EDSM	3	1.59	0.4	Not significant
PDS	3	1.65	0.2	Hypoxia (highly significant, $P<0.0001$)
$CuCl_2$	Control	–1.50	0.0	Not significant

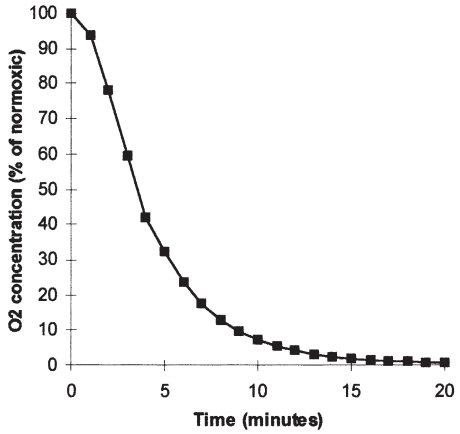

Fig. 2. Clearance of oxygen from cell suspension under the conditions used for deoxygenating prior to addition of tracer (100%=O_2 concentration under normal air atmosphere)

Results

Table 1 shows lipophilicity (log P) and Rf values of the complexes. Figure 2 shows the effect of the gassing conditions on oxygen concentration in the cell suspension. After 20 min purging, O_2 concentration was below 0.2% (cf. the concentration under air is defined as 20%). Radiobiological hypoxia is defined as 0.66% (5 mmHg) [8]. At the beginning of gassing 95% (SD 4.7) of cells were viable, and at the end of the experiment 90% (SD 4.5) were viable under normoxia and 88% (SD 4.3) under hypoxia.

The radioactivity uptake of the cells under normoxic and hypoxic conditions is shown in Figs. 3–5, together with the structures of the complexes. Three groups are defined: group 1 (Fig. 3) comprising thiosemicarbazone complexes showing either no significant selectivity or significant selectivity for normoxic cells; group 2 (Fig. 4) comprising thiosemicarbazone complexes show-

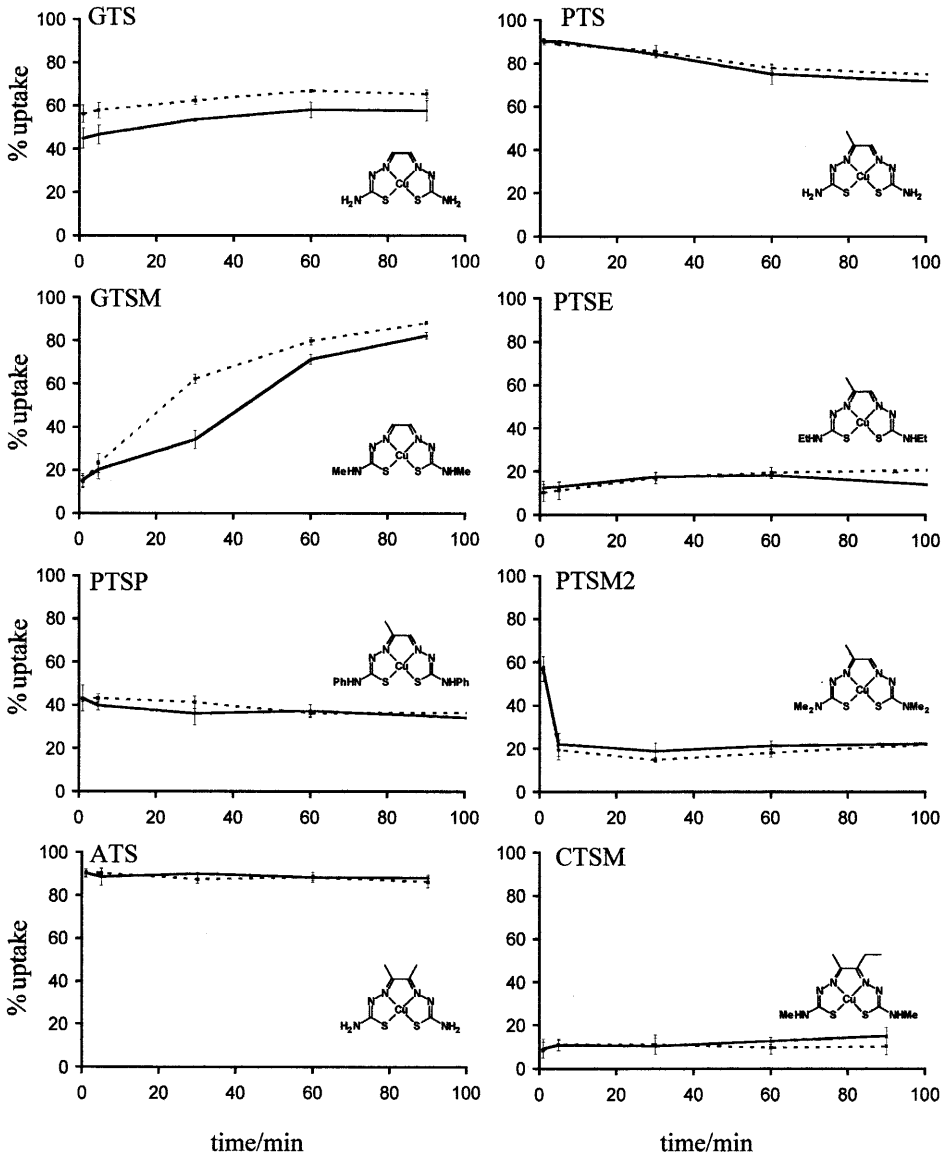

Fig. 3. Cell uptake behaviour of thiosemicarbazone complexes in group 1, showing no selectivity or significant selectivity for normoxic cells (—, hypoxic; - - -, normoxic). *Error bars* represent 1 standard deviation

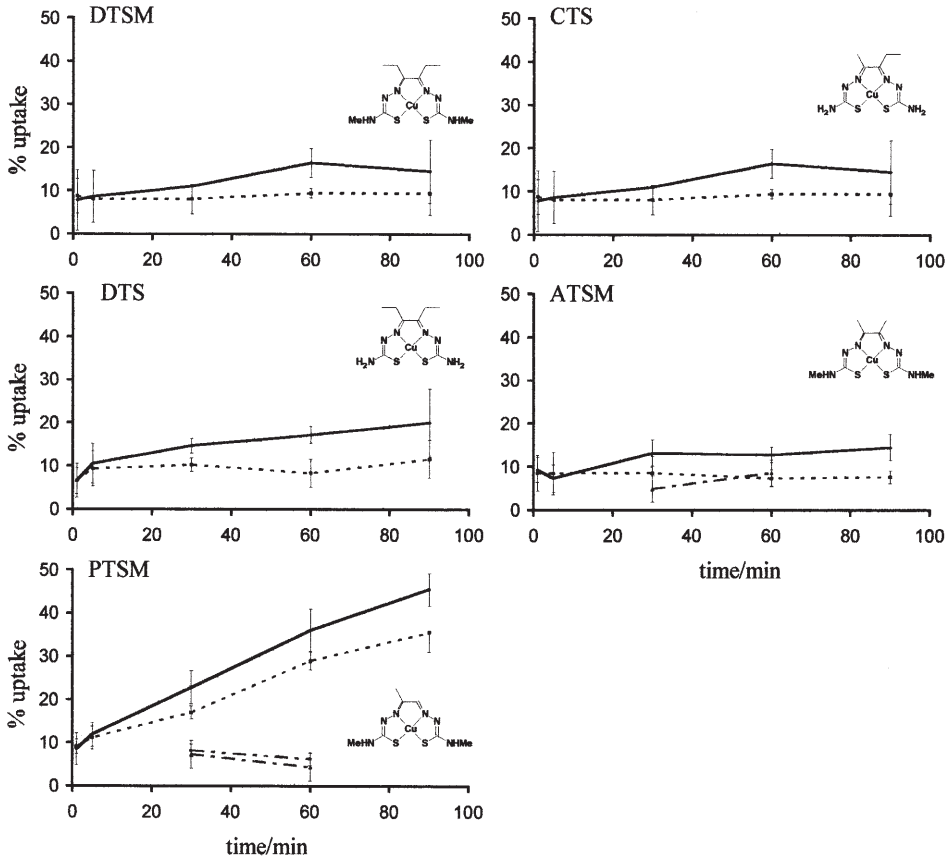

Fig. 4. Cell uptake behaviour of thiosemicarbazone complexes in group 2, showing significant or highly significant selectivity for hypoxic cells (—, hypoxic cells; - - -, normoxic cells, — - —, hypoxic cell-free medium; — - -, normoxic cell-free medium). *Error bars* represent 1 standard deviation

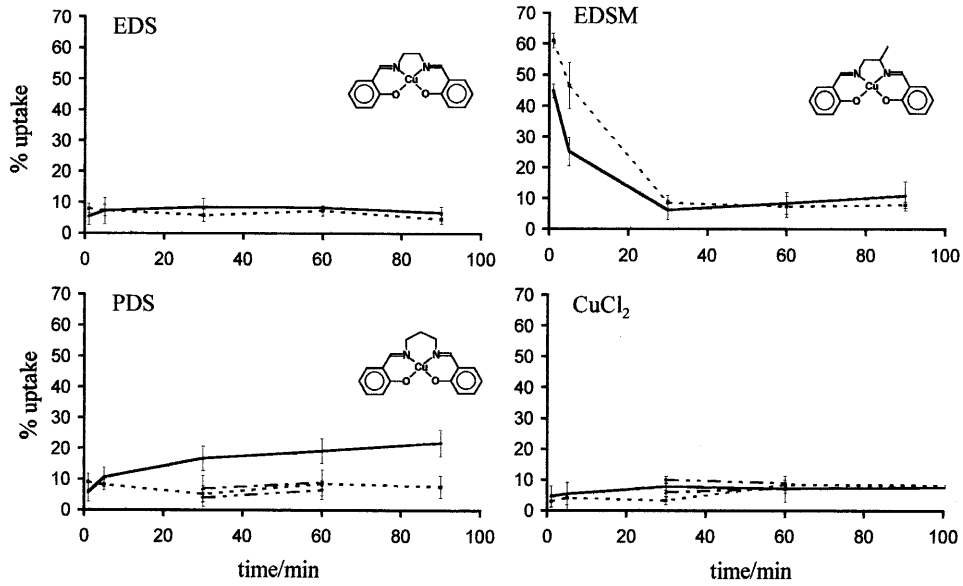

Fig. 5. Cell uptake behaviour of salicylaldimine complexes and copper chloride (control) (—, hypoxic cells; - - -, normoxic cells, — - —, hypoxic cell-free medium; — - -, normoxic cell-free medium). *Error bars* represent 1 standard deviation

ing significant or highly significant selectivity for hypoxic cells (either over all time points from 30 min onwards or at every individual time point from 30 min onwards); and group 3 (Fig. 5) comprising the salicylaldimine ligand complexes and the copper chloride control. The selectivity is summarised in Table 1. $^{64}CuCl_2$ showed low uptake and no significant oxygen dependence. Three complexes showed significant selectivity for normoxic cells, and in none of these was the selectivity highly significant. In group 2 three complexes showed highly significant hypoxia selectivity across all time points from 30 min: ATSM (P<0.0001), CTS (P<0.00095) and DTS (P<0.0001). In addition, PTSM showed highly significant hypoxia selectivity when individual time points from 30 min were compared. In group 3, PDS was highly hypoxia selective (P<0.0001), EDS

was barely significantly hypoxia selective while EDSM showed no significant selectivity. Cell-free controls, where performed, showed that the majority of the normoxic "uptake" was due to oxygen-independent binding to the tubes.

Discussion

The uptake behaviour confirms that hypoxia selectivity can be achieved by manipulation of ligand. The series are remarkable both for the frequency (five out of thirteen thiosemicarbazone complexes and two out of three salicylaldimine complexes) and for the strength (five complexes highly significant) of hypoxia selectivity. In some cases the ratio of hypoxic to normoxic uptake directly observed on the graphs grossly underestimates the true extent of selectivity because a major fraction of normoxic uptake is due to binding to the centrifuge tubes. It is difficult to produce a reliable hypoxic-to-normoxic uptake ratio corrected for this because large percentage errors are introduced by subtracting two almost equal values (i.e. cell-free control and normoxic uptakes).

It is of interest to compare these results with the known biological behaviour of CuPTSM and CuATSM. The hypoxia selectivity of CuPTSM has not been investigated explicitly, and it has been assumed that it is non-selective. However, it has been observed incidentally that brain uptake increases after transient brain ischaemia in rats [9] and that clearance from hypoxic rabbit heart tissue is slower than from normal tissue [10]. The in vitro behaviour described here provides a possible explanation of these in vivo observations. CuATSM has been observed in an isolated rat heart model to be highly hypoxia-selective and is under investigation clinically as a hypoxia tracer [5].

Given the characteristic redox chemistry of copper, the natural cellular (redox-based) mechanisms for trapping, storing and utilising copper, and the frequency with which hypoxia selectivity is observed in the present series of complexes, bioreductive trapping of lipophilic copper complexes could be a property of many classes of copper (II) complexes other than those investigated. There is therefore potential for "tuning" to achieve hypoxia selectivity combined with the necessary uptake/clearance kinetics, stability and whole body distribution required of an ideal hypoxia-targeting radiopharmaceutical.

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