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Short Research Article

Imaging hypoxia *in vivo* by controlling the electrochemistry of copper radionuclide complexes†

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Abstract: Tissue hypoxia is a feature of cancer, heart disease and stroke, and imaging it may become clinically important. Copper-ATSM ($ATSMH_2$ = 2,3-butanedione *bis*(*N*-methyl)thiosemicarb-azone), labelled with ^{60}Cu, ^{62}Cu or ^{64}Cu, is selectively taken up in hypoxic cells *in vitro* and *in vivo* by a bioreductive mechanism, and is a prototype hypoxia imaging agent amenable to improvement. *In vitro* studies with several differently alkylated analogues of CuATSM show that hypoxia selectivity is a general property of complexes with two alkyl groups at the diketone backbone, offering a range of pharmacokinetic properties while retaining hypoxia selectivity. This pharmacokinetic control affords a route to development of second-generation hypoxia imaging agents with optimized properties for different clinical applications. Combinatorial synthesis of these analogues, including asymmetric ones, is possible by combining several diketones with several thiosemicarbazides and separating the products chromatographically. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: copper; hypoxia; PET; imaging

Introduction

Tissue hypoxia is an important feature of many pathologies, especially in cancer, cardiovascular disease and brain pathologies such as stroke. The ability to image hypoxic tissues by radionuclide scanning using gamma or positron emitting isotope labelled tracers would aid patient management in these areas. For example, knowledge of locoregional tumour hypoxia would enable additional radiation dose to be delivered to hypoxic regions to compensate for their reduced sensitivity to radiotherapy. Nitroimidazole derivatives labelled with the positron emitter ^{18}F are the earliest examples of tracers targeted to hypoxia.[1] They enter cells non-specifically and are enzymatically reduced to form radicals, which are quickly re-oxidised in the presence of oxygen, allowing escape from the tissue. In the absence of oxygen, the radicals become covalently bound to intracellular macromolecules and are trapped. The nitroimidazoles suffer from disadvantages such as slow uptake and clearance, leading to late imaging times incompatible with the short half life of ^{18}F.[2]

The copper(II) *bis*(thiosemicarbazone) complex CuPTSM (Figure 1), labelled with positron emitting isotopes of copper (of which Cu-64, Cu-61 and Cu-62 are the most promising[2]) provides a starting point for the development of metal-based hypoxia imaging agents, as it is taken up non-selectively in cells very rapidly and trapped by a bioreductive mechanism: intracellular reduction to Cu(I) followed by dissociation and binding of copper to high affinity sites in the cell.[3] It was suggested that by tuning the redox potential, the process could be controlled so that trapping occurs only in the more reducing environment of hypoxic cells.[4]

Results and discussion

Ligands with different alkylation patterns (Figure 1) were synthesised by condensing various vicinal diketones and *N*-alkylthiosemicarbazides. Their copper

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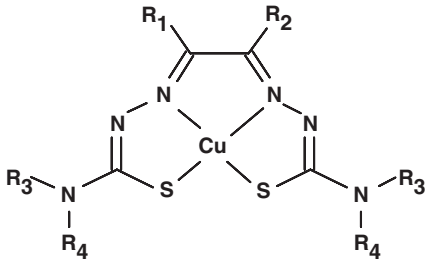

	R1	R2	R3	R4
GTS	H	H	H	H
GTSM	H	H	CH_3	H
PTS	CH_3	H	H	H
PTSM	CH_3	H	CH_3	H
PTSM	CH_3	H	CH_3	CH_3
PTSE	CH_3	H	C_2H_5	H
PTSP	CH_3	H	C_6H_5	H
ATS	CH_3	CH_3	H	H
ATSM	CH_3	CH_3	CH_3	H
CTS	C_2H_5	CH_3	H	H
CTSM	C_2H_5	CH_3	CH_3	H
DTS	C_2H_5	C_2H_5	H	H
ATSE	CH_3	CH_3	C_2H_5	H
DTSM	C_2H_5	C_2H_5	CH_3	H

Figure 1 Structures of complexes.

complexes showed a systematic correlation between the alkylation pattern and the Cu(II/I) redox potential (Figure 2). Complexes with only hydrogens at R1 and R2 were reduced in the range –0.43 to –0.44 V; those with one alkyl group were reduced between −0.48 and –0.53 V, and those with two, between −0.57 and –0.60 V; a shift of ca. −0.08 V per alkyl group. The effect of *N*-terminal alkylation (R3, R4) was much less pronounced (ca. −0.01 V per alkyl group).[5–7]

The effect of shifts in redox potential on hypoxic selective uptake in cells was measured in cultured rat mammary tumour cells. A strong correlation was found between reduction potential and hypoxia selectivity (Figure 3): complexes with redox potentials in the range −0.58 to −0.60 V showed marked selectivity for hypoxic cells compared to normoxic cells.[5,6] CuATSM reached the highest overall selectivity. It was also selected by Fujibayashi and co-workers[4] as the prototype for clinical investigation in cancer[8] and cardiovascular disease[9] and is now undergoing clinical evaluation in many centres.[10] The bioreductive basis of intracellular trapping, involving mitochondrial and cytoplasmic components[11] is supported by EPR measurements on cultured cells. The hypoxic selectivity correlates with the reversibility of the Cu(II/I) redox potential as well as the potential itself. Reduction of the hypoxic-selective complexes is reversible (and indeed a Cu(I) species has been isolated in the case of ATSM[11]), and the reduced species is quickly re-oxidized by O_2, whereas the non-selective complexes undergo rapid dissociation following reduction, precluding re-oxidation.[12] Thus, selectivity may be controlled by balancing rates of reduction, re-oxidation and dissociation of the reduced species, which in turn are controlled by structural[13] and electronic[12,14] properties.

Despite its clinical promise, CuATSM remains a prototype. Factors unrelated to hypoxia, such as the kinetics of blood clearance and excretion, liver and kidney uptake may dictate which will be the best clinical hypoxia-imaging agents for specific applications. Complexes may be required that show selectivity at different levels of oxygen; for example, a complex

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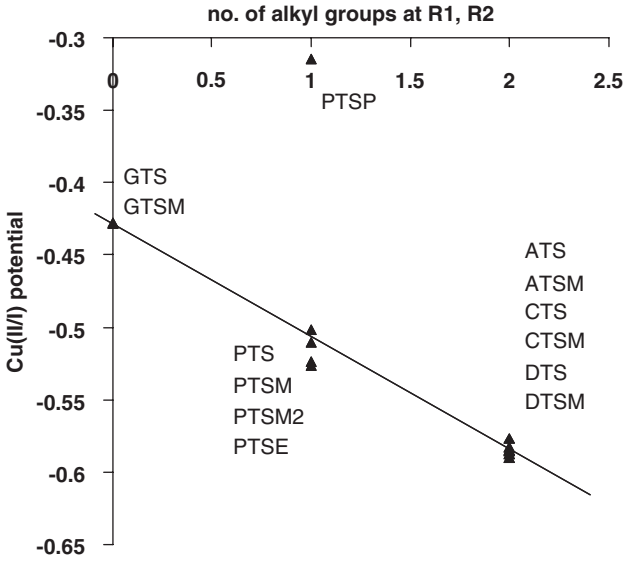

Figure 2 Effect of backbone alkylation on Cu(II/I) redox potential.

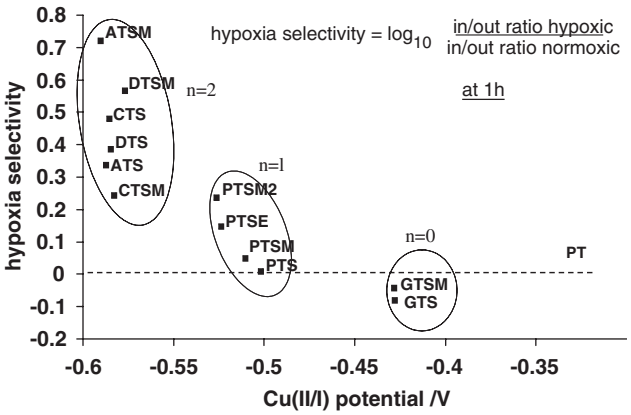

Figure 3 Relationship between redox potential and hypoxic selectivity in cultured cells.

suitable for use in hypoxic tumours may be of no value at the higher oxygen levels present in hypoxic myocardium. Thus different complexes will be preferable in different clinical circumstances.

Observations during the initial studies suggest potential for development of analogues with improved properties. For example, although CuATSM reached the highest plateau hypoxic selectivity of those tested in cell culture systems, several other complexes reached their plateau much more quickly (Figure 4).[6] This might prove advantageous for imaging, yet most of these complexes have yet to be studied *in vivo*. Another example is that CuATS and CuATSM behave very similarly except that CuATS does not cross the blood-brain barrier.[15] The concept that different complexes may detect different levels of hypoxia is supported by comparison of CuATSM and CuATSE. Both exhibit a sigmoid profile of uptake versus pO_2 but the half-wave pO_2 for CuATSE is significantly higher than that for CuATSM (Figure 5).[16] Thus, it is expected that some cells that are insufficiently hypoxic to retain CuATSM will retain CuATSE.

Preliminary *in vivo* biological studies comparing CuATSM and CuATSE show that CuATSE has several potential advantages over the prototype CuATSM, warranting further evaluation and suggesting that more of the variously alkylated complexes should be evaluated *in vitro* and *in vivo*. In rats with EMT6

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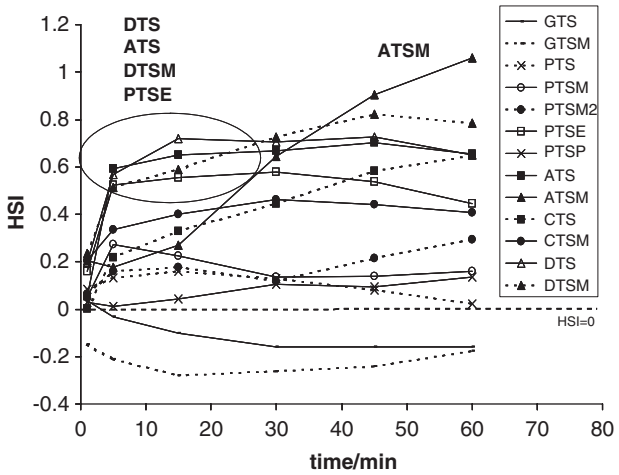

Figure 4 Time dependence of uptake in cultured cells, expressed as hypoxia selectivity index.

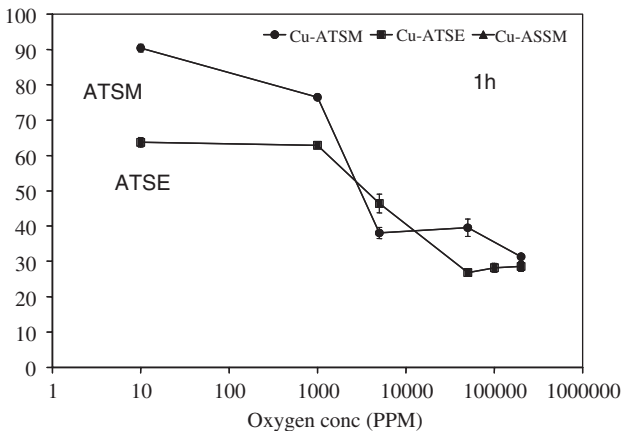

Figure 5 Cellular uptake as a function of ambient oxygen concentration, comparing CuATSE and CuATSM.

tumours, CuATSE shows evidence of hypoxia selectivity, in that uptake in tissues expected to have a hypoxic fraction under normal conditions (i.e. tumour) was significantly reduced in animals breathing 100% O_2, while uptake in normoxic tissues was unaffected. Uptake in liver, which is problematic for CuATSM, is significantly reduced for CuATSE. Uptake in kidneys is also significantly lower, while tumour uptake is increased.[16]

Encouraged by these results, we have continued to broaden the range of *bis*(thiosemicarbazone) ligands for evaluation by including asymmetric ligands with two different side arms. This greatly expands the number of variants even if the range of alkyl groups is limited to methyl and ethyl. A rational synthesis of asymmetric ligands, using an appropriate protection strategy, has been described previously[17] but is relatively long and involved. A more economic and practical approach, at least on a pilot scale, is a quasi-combinatorial one in which mixtures of two or more diketones and two or more *N*-alkylthiosemicarbazides are condensed. The condensation proceeds cleanly to give a roughly statistical mixture of *bis*(thiosemicarbazone) products (Figure 6) which can be separated by HPLC on a scale adequate for conducting biological evaluation. Mixtures of at least 20 ligands have been synthesised and separated in this way. This approach will generate a large number of closely related radiopharmaceuticals with subtly differing properties for evaluation as hypoxia imaging agents. A library ligands made from n symmetrical and m unsymmetrical diketones, and x thiosemicarbazides, contains $mx^2 + 0.5nx(x + 1)$

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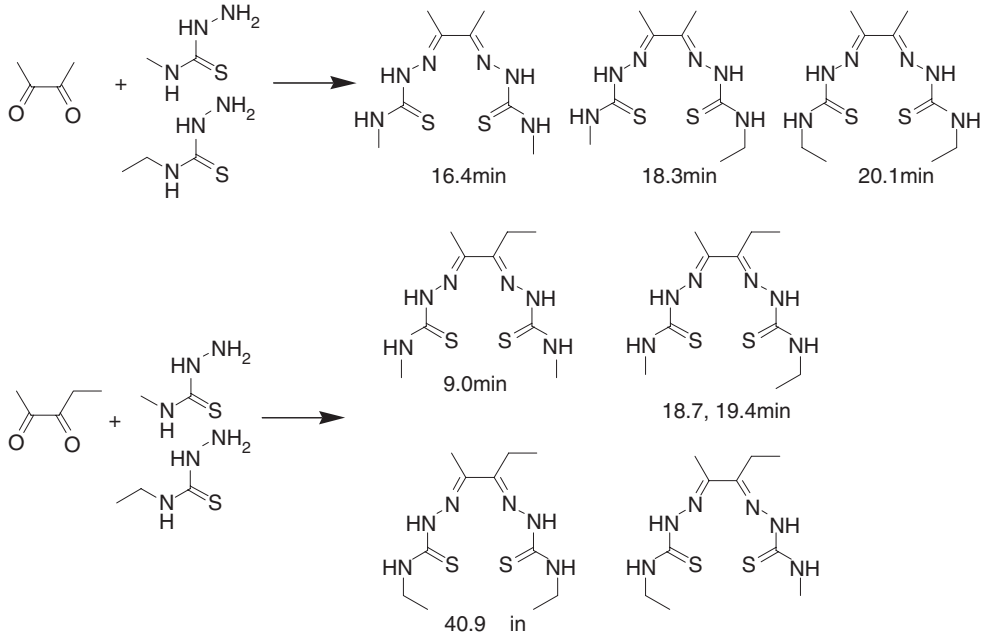

Figure 6 Examples of quasi-combinatorial synthesis of ***bis***(thiosemicarbazone) libraries, showing HPLC elution times (top: C8 reverse phase column, water/acetonitrile gradient; bottom, C8 reverse phase column, isocratic 80% water, 20% acetonitrile).

ligands. For example, if $n = 2$, $m = 3$ and $x = 5$, there are 105 ligands.

Conclusion

CuATSM should be seen as a prototype hypoxia imaging agent. Studies of subtly modified analogues will lead both to replacement of CuATSM with improved tracers, and a broadening of the clinical applications, by identifying complexes with optimal properties for specific uses where the requirements might be significantly different. Despite its promise as a hypoxia -specific agent, interpretations of scans requires caution, as more detailed biological and clinical studies of CuATSM are unearthing a number of anomalies. One is that there may be some specific tumour types in which other uptake and retention mechanisms unrelated to hypoxia may prevail.[18] Another is that circulating radioactivity in patients injected with CuATSM is very quickly (within minutes) converted to hydrophilic forms (Lewis JS, personal communication), casting doubt on the interpretation of later scans. The most effective approach to synthesis of the required ligands, at least during the development stages, may be a quasi-combinatorial one in which a mixture of diketones is treated with a mixture of thiosemicarbazides, and the resulting mixture of *bis*(thiosemicarbazone) ligands is separated by chromatography. This will give easy access to unsymmetrical ligands with two different thiosemicarbazone side arms, which would otherwise require relatively complex multi-step syntheses involving protection strategies.

Acknowledgements

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