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PET imaging of cardiac hypoxia: Opportunities and challenges

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Abstract

Myocardial hypoxia is a major factor in the pathology of cardiac ischemia and myocardial infarction. Hypoxia also occurs in microvascular disease and cardiac hypertrophy, and is thought to be a prime determinant of the progression to heart failure, as well as the driving force for compensatory angiogenesis. The non-invasive delineation and quantification of hypoxia in cardiac tissue therefore has the potential to be an invaluable experimental, diagnostic and prognostic biomarker for applications in cardiology. However, at this time there are no validated methodologies sufficiently sensitive or reliable for clinical use. PET imaging provides real-time spatial information on the biodistribution of injected radiolabeled tracer molecules. Its inherent high sensitivity allows quantitative imaging of these tracers, even when injected at sub-pharmacological (pM) concentrations, allowing the non-invasive investigation of biological systems without perturbing them. PET is therefore an attractive approach for the delineation and quantification of cardiac hypoxia and ischemia. In this review we discuss the key concepts which must be considered when imaging hypoxia in the heart. We summarize the PET tracers which are currently available, and we look forward to the next generation of hypoxia-specific PET imaging agents currently being developed. We describe their potential advantages and shortcomings compared to existing imaging approaches, and what is needed in terms of validation and characterization before these agents can be exploited clinically.

Keywords

Positron Emission Tomography; ¹⁸FMISO; Fluoromisonidazole; Copper bis(thiosemicarbazones); ⁶⁴Cu-ATSM; Hypoxia

1. Introduction

Ischemic heart disease is the leading cause of mortality worldwide and its incidence is expected to continue to rise [1]. There is an ever-increasing need for new diagnostic and prognostic techniques able to detect cardiac ischemia earlier, provide greater information on the vulnerability of the tissue at risk, and better evaluate the effectiveness of interventions designed to reperfuse ischemic tissue. Imaging is currently one of the fastest growing medical technologies with the potential to satisfy these requirements [2]. While imaging approaches have historically been limited to providing structural or contractile information on cardiac function, new molecular imaging techniques now make it possible to obtain non-invasive real-time images of the regional biochemistry of the heart [3]. Imaging with

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specific markers of hypoxia may provide new insights regarding the assessment of myocardial ischemia, the pathophysiology of myocardial hibernation and non-compensated cardiac hypertrophy, the development of cardiomyopathies, natural collateral development, and response to revascularization and angiogenic therapies [4].

2. Supply versus demand: hypoxia and ischemia

We would formally define the terms hypoxia and ischemia as disparities between supply and demand for either oxygen or blood flow, respectively, *that have a pathophysiological consequence*. This definition aids the conceptualization of these processes as dynamic, variable, and potentially reversible. In neither case should ischemia or hypoxia be defined as merely “low blood flow” or “low oxygen saturation”; if the flow and oxygen demand in that tissue were both low, supply and demand could essentially be in balance [5,6]. This highlights a potential limitation of assessing the status of a tissue based on perfusion imaging data alone.

Ischemia causes not only hypoxia, but also the loss of hydrostatic pressure, decreased energy substrate delivery, and the lack of washout of waste and cellular injury products. The terms hypoxia and ischemia should therefore not be used interchangeably (as some authors have done in the past), despite empirical distinction of their relative pathologies being difficult to achieve outside of experimental models. When we consider the potential of a hypoxia-specific imaging approach for cardiology, we are most often describing an imaging agent that characterizes ischemic myocardium by the degree of hypoxia it is experiencing. By focusing on one representative parameter (in the present case, hypoxia) in the chaos of an ischemic event, we hope to obtain a reliable index of tissue vulnerability with reproducible diagnostic or prognostic accuracy. We also hope to gain some insight into the importance of hypoxia alone among all of the other factors which may contribute to ischemic injury.

3. Potential applications of cardiac hypoxia imaging

The majority of current cardiac imaging techniques used clinically measure blood flow and/or perfusion, wall motion, or cellular energy metabolism at rest and stress. Even though these data show a correlation between degree of ischemia and outcome [7], and provide a threshold for the appropriateness of revascularization, it is impossible to determine the clinical significance of reduced blood flow for an individual patient [8]. While generating fully quantitative data (i.e. flow in ml/g muscle mass/minute) might be a step toward this goal, a more attractive approach would be to move from measuring blood flow or perfusion to measuring oxygen deficit specifically. Not only would this give novel insight into the pathophysiology of heart disease, but it would also provide an objective and personalized measurement of the significance of any supply–demand imbalance observed. While hypoxia-specific molecular imaging may become a useful additional technique for identifying and characterizing acute and relatively severe ischemia or hypoxia (and evaluating its treatment), it also has unique potential in identifying myocardium jeopardized by the more subtle effects of chronic low level ischemia or hypoxia.

Myocardial hibernation, for example, is an ischemic syndrome characterized by chronic ventricular hypocontractility that is reversible after revascularization [9]. It is thought to be mediated by repetitive short bouts of ischemia/reperfusion caused by coronary stenosis, which may not necessarily be limiting at rest, but cause intermittent demand ischemia by restricting perfusion reserve [10,11]. Chronically, these cycles of repetitive stunning cause myofilament degeneration, changes in proteomic profile and mitochondrial morphology, and a switch in myocardial substrate selection from fatty acids to glucose [12]. Effectively, this produces a phenotype that is resistant to ischemic injury, but limited in terms of contractile performance. Identification of hibernating myocardium is key because the prognosis after its

surgical correction is good; regional contractile function can be restored within days or up to a year, depending on the duration and severity of the perfusion deficit [13]. The general approach for identifying hibernating myocardium is the demonstration of myocyte viability in the setting of decreased flow. The gold standard is to use [^{18}F]-2-deoxy-2-fluoro-D-glucose (^{18}FDG) to identify regions of myocardium with increased glycolytic activity by PET, co-registered with regions of poor perfusion demonstrated by $^{13}\text{NH}_3$ -PET, for example [14–16]. However, ^{18}FDG is a relatively non-specific tracer with many practical limitations, the worst being that in ischemic/reperfused myocardium, it severely underestimates glucose uptake [17–21] which can potentially lead to underestimation of viability. Furthermore, few, if any, radiotracers of “perfusion” represent perfusion alone; most, like $^{99\text{m}}\text{Tc}$ sestamibi, ^{201}Tl , and $^{13}\text{NH}_3$ are trapped by energy-dependent processes [22–24], which makes them vulnerable to under-estimating the true level of perfusion in energy depleted tissue. There is, therefore, room for improvement. While hypoxia imaging agents are unlikely to replace this longstanding “flow-metabolism mismatch” approach in the short term, the use of a single imaging agent which can specifically define viable tissue experiencing oxygenation mismatch is an attractive additional technique. Since the pathophysiology of hibernation remains poorly understood [25], the addition of a complementary hypoxia biomarker may also provide further insight into its mechanism.

Pressure overload, cardiac hypertrophy and ischemia are the main pathophysiological mechanisms underlying heart failure [26]. Pathologic cardiac hypertrophy is central to this pathophysiology because the lack of proportionate microvascular growth leaves the myocardium vulnerable to ischemia [27]. In this context, ischemia and hypoxia contribute to the pathophysiology of heart failure, but since its clinical presentation is typically less dramatic and more diffuse than acute myocardial infarction, and can occur in the absence of coronary artery disease, our inability to detect it means that we are missing an important opportunity to intervene. This decreased vascularity in hypertrophic myocardium is exacerbated by increases in myocyte size and tissue fibrosis, which increase diffusion distances and decrease oxygen availability to myocytes (and perhaps more importantly, their mitochondria) [27,28]. While up-regulation of hypoxia inducible factor HIF-1 α is increasingly being recognized as an important instigator of cardiac hypertrophy [29–31] and mediator of decline into heart failure [32–34], we have found no reports that successfully demonstrate non-invasive measurement of hypoxia itself in hypertrophic or failing myocardium. As we shall discuss, this is most probably because most existing approaches designed to assess tissue hypoxia have insufficient sensitivity to detect the less extreme levels of hypoxia that are likely to be involved. Quantitative, non-invasive imaging of hypoxia in this context would be highly desirable for determining its importance in the pathogenic process, assessing disease progression and risk of heart failure, and gauging response to treatment with novel angiogenic therapies, for example [35].

4. Requirements of an ideal cardiac hypoxia imaging agent

The ideal hypoxia imaging agent must exhibit several key characteristics. It must rapidly localize in the tissue of interest with a target to background ratio ideally greater than 3 to 1, and it should clear quickly from the blood pool and normoxic tissues to allow rapid imaging of hypoxic tissue [36]. This requirement is amplified in the case of ischemia because of the potential for poor delivery of tracer to the affected region. Hepatic uptake should be low to minimize interference when quantifying myocardial uptake (this is a typical trade-off—lipophilicity promotes cellular uptake through diffusion, but also results in higher liver uptake). Tracers should be simple and quick to synthesize, bearing in mind the short half lives of the radioisotopes used, and exhibit sufficient stability in blood that they remain intact throughout the imaging protocol. They should also have a sufficiently high photon or

positron yield to produce high quality images in a short time and at an acceptable radiation dose to the patient.

Beyond these practical concerns, the key question in designing a hypoxia imaging agent is “what level of ischemia or hypoxia is pathologically, diagnostically or prognostically relevant?” i.e. “what do we want our images to show us?” Selecting a specific level of hypoxia against which to target a hypoxia imaging agent for diagnostic or prognostic purposes is a particularly thorny issue. In fact, it is even difficult to define how hypoxia should be quantified; much like the problem with measurement of perfusion, quantification of absolute tissue oxygen concentrations provides no insight into whether or not that oxygen level is sufficient for normal metabolism in that tissue at that time. Hypoxia therefore can only really be quantified indirectly as the degree of oxygen deficit required to cause a measurable change in a parallel biomarker in the tissue of interest. Thus, the levels of oxygen which define a tissue as hypoxic are likely to be very different in different organs or disease states; while tumor cells survive in oxygen environments of less than 2 mmHg O₂, we would argue that they are not necessarily any more “hypoxic” than post-ischemic hypertrophic myocardium surviving at 10 mmHg O₂ [37], because the oxygen demands of even the hypocontractile heart are so much higher by comparison. Similarly, care must be taken when extrapolating data from experimental models of hypoxia to the clinical situation. While it is relatively easy to accurately and reproducibly induce any level of hypoxia desired in isolated myocytes or isolated heart models, the energy (and oxygen) demands of the myocyte progressively increase when moving from the culture dish to the beating-but-non-pumping Langendorff heart, to the *in vivo* situation [38]. This is apparent when comparing the degree of flow deficit required to cause ATP depletion in the heart—in open chest dogs, PCr/ATP ratios start to fall when regional coronary flow is reduced by as little as 30% [39], while in isolated perfused (un-paced) hearts, PCr levels do not fall until coronary flow rates have been reduced from 11 to 2 ml/min (an 82% reduction) [40]. However, this does not necessarily mean that because workload is lower in isolated hearts, they are less prone to hypoxia; oxygen delivery in the isolated buffer-perfused heart is significantly worse than is achieved *in vivo*. Recent work by Mik et al. [41] has shown that mitochondrial pO₂ levels are generally lower in isolated hearts than when *in vivo*, and the model can only be considered representative of the *in vivo* situation when they are maximally vasodilated. As such, while monitoring oxygen concentrations in cells or tissues may be a useful means of comparing the hypoxia selectivity of one tracer with another in any given model, using tissue oxygen levels as the key to translate tracer behavior across models or to the *in vivo* situation must be done with caution. As most of the hypoxia imaging agents we describe in this review accumulate in tissues when a specific hypoxia-dependent threshold is crossed, perhaps parallel biomarkers defining these thresholds which are conserved across all experimental models may be the most appropriate approach for describing their tissue retention profiles.

Considering all of these things together, it seems unlikely that there will be a “one size fits all” tracer for imaging tissue hypoxia; a spectrum of hypoxia-defining imaging agents for different applications and for different stages of disease will be necessary. In acute ischemia, the term “ischemic cascade” was originally coined to describe the progression of injury from clinically silent to clinically recognizable (Fig. 1) [42]. It is our aim here to be able to visualize less severe ischemia sooner after its onset, to allow the earliest possible intervention. Molecular imaging approaches, such as quantitative hypoxia measurement, potentially provide biomarkers well in advance of those available to functional or ECG-based characterization. We have expanded this molecular imaging “target zone” in Fig. 2. While the “earliest” approach would perhaps be to quantify reductions in blood flow or perfusion [43,44], imaging a decrease in supply without reference to demand gives little insight into its pathophysiological importance. Several studies describe earlier and more

severe endocardial damage during demand ischemia with arterial stenosis, despite having comparable or greater blood flow than the rest of the heart. Regional oxygen or energy demand is therefore a greater predictor of ischemic injury than flow delivery in this context [45–47]. Furthermore, images of perfusion deficit are “negative contrast”, and more difficult to quantify than the “positive contrast” or “hot spot” images that metabolic-/hypoxia-dependent imaging agents can potentially provide.

^{31}P magnetic resonance spectroscopy (MRS) is able to determine absolute phosphocreatine and ATP levels in the heart, and measure intracellular pH [48], which are all associated with oxygen or blood flow deficit. Significant reductions of phosphocreatine: ATP ratios have been demonstrated in patients with heart failure [49,50] and hypertrophic cardiomyopathy [51] correlating with declining left ventricular ejection fraction [52]. Similar changes have also been recently demonstrated in healthy volunteers in a normobaric hypoxia chamber [53]. Unfortunately, due to the low inherent sensitivity of MR, clinical applications of MRS suffer from long measurement times, poor signal to noise and low spatial resolution. ^{31}P MRS is therefore unlikely to be able to compete with the high sensitivity, relative speed and resolution of PET for quantifying hypoxia clinically (although it does have the advantage of using an endogenous signal rather than a potentially perfusion-limited one). We describe MRS here for another reason, that of validation. Loss of ATP is arguably the pivotal point in governing the fate of a compromised cardiac myocyte. It precedes loss of ionic homeostasis and susceptibility to electrophysiological changes, and follows the loss of any energetic buffering capacity that intracellular glycogen or phosphocreatine may provide (Fig. 2). While MRS may not be the best technique for measuring this critical tipping point clinically, it may provide the best parallel biomarker for the development of a useful hypoxia-/ischemia-dependent imaging agent in preclinical/isolated heart studies. We therefore propose that the ideal acute hypoxia imaging agent should identify the level of intracellular O_2 deficit which corresponds with the onset of loss of intracellular ATP as measured by MRS. Prototype preclinical dual PET/MR systems already exist which would be ideal for this purpose [19,54], and clinical equivalents are currently in development around the world.

In terms of chronic (and probably less severe) hypoxia in hypertrophy or failure, loss of ATP may represent too severe a target at which to aim potential hypoxia imaging agents. Since the main mediator of hypoxic injury in the pathogenesis of cardiac hypertrophy is up-regulated HIF-1 α expression, HIF-1 α up-regulation would seem to be the most appropriate parallel biomarker to target [29–31].

5. Potential non-invasive approaches for quantifying myocardial hypoxia

In the search for hypoxia-specific imaging tracers, two distinct classes of molecules have been most extensively studied; the nitroimidazoles and the copper bis(thiosemicarbazone) complexes. While this brief review is limited to PET applications, numerous nitroimidazole complexes have been evaluated for hypoxia imaging by SPECT. The uptake mechanism is thought to be common across this family of tracers, with various structural modifications being employed to improve upon the basic pharmacokinetic limitations of the parent misonidazole compound. Each has met with varying degrees of success, but so far all experience the same limitations, to a greater or lesser degree, as ^{18}F MISO, which we describe here [55]. For a more detailed account of the history and current state of the art in SPECT-based nitroimidazole derivatives, we direct the reader to the excellent reviews by Sinusas, Strauss et al. and more recently, Krohn et al. [4,56,57].

6. Fluoromisonidazole (^{18}F MISO)

Nitroimidazoles are believed to enter cells by passive diffusion, becoming bioreduced once internalized to form nitro anion radicals (Fig. 3). In the presence of molecular oxygen, these

species are rapidly re-oxidized back to their uncharged form, which is then able to diffuse back out of the cell [58], or become reduced again in a futile cycle. In the absence of oxygen, bioreduction of the nitro ion radical continues, resulting in the stepwise production of nitrosohetero-cycles, hydroxylamines and amines, which become trapped within the cell [56,58]. While the identities of the bioreductants are currently unclear, flavoproteins including cytochrome P450 and xanthine oxidoreductase have been implicated [59]. The eventual binding partners of the $^{18}\text{FMISO}$ metabolites have not yet been identified.

While $^{18}\text{FMISO}$ (for structure, see Table 1) is the most extensively studied 2-nitroimidazole for hypoxia imaging, there has not been any significant research into its potential cardiovascular application for almost 20 years [60–64]. Early work by Martin et al. demonstrated 4- and 8-fold increases in $^3\text{H-FMISO}$ accumulation in isolated cardiomyocytes in hypoxic (~3.5 mmHg) or anoxic culture after 1 h. This increased to 15- and 26-fold respectively after 3 h. They observed no change in either cell morphology or creatine kinase (CK) leakage in hypoxic cells, but anoxia caused significant CK leakage and morphologic changes [61]. This meant that either $^{18}\text{FMISO}$ accumulates in hypoxic dying cells (which make its use more difficult in terms of visualizing compromised but viable myocardium), or that the net elevated uptake in the remaining viable cells exceeded the effect of myocyte loss.

Using isolated perfused hearts, Shelton et al. studied washout of $^{18}\text{FMISO}$ from hypoxic (perfused with buffer containing <40 mmHg O_2) or low flow ischemic hearts after they had been loaded with the tracer for 20 min. They observed 48% and 41% $^{18}\text{FMISO}$ retention after 20 min of reoxygenation/reperfusion respectively, which was double that observed in normoxic or post-ischemic hearts [63]. This approach of loading tracer into the heart over an extended period and studying its washout pharmacokinetics rather than following the fate of a simple bolus perhaps masks its poor first pass extraction. Using PET to quantify $^{18}\text{FMISO}$ uptake in an LAD-occluded canine model, this group did observe increased accumulation of the tracer after a bolus injection within 60 min (Fig. 4). However, this was achieved by subtracting the high residual blood pool signal using previously acquired H_2^{15}O perfusion data. Ischemic changes were confirmed by ST segment elevation by ECG, although the functional consequence of this degree of ischemia in terms of tissue viability or the extent of recovery on reperfusion in these animals was not specifically addressed.

Martin et al. reported ischemic zone:blood ratios of only 1.8:1 when LAD flow was completely occluded, and 1.6:1 when partially occluded, in open chest dogs 4 h after the injection of tracer [65]. They noted that $^{18}\text{FMISO}$ blood plasma clearance was biphasic, with a rapid distribution phase followed by a first order elimination phase with a half life of 275 min. They suggested that if $^{18}\text{FMISO}$ was to be used clinically, significant delays between administration and imaging would be necessary to achieve sufficient contrast between blood pool and target tissue. While they did demonstrate viability in tissue positively labeled by $^{18}\text{FMISO}$, and recovery of function when they briefly reperfused it, they also observed significant $^{18}\text{FMISO}$ uptake into tissue identified as infarcted by histological staining. This conflicts with the contention that $^{18}\text{FMISO}$ accumulates in hypoxic but viable tissue. However, as tracer accumulation was studied over a 4 hour imaging protocol, tracer in necrotic regions may have accumulated during early ischemia before the infarct evolved.

Shelton et al. went on to investigate the relationship between ischemic duration (after 3, 6 or 24 h LAD occlusion) and $^{18}\text{FMISO}$ accumulation [64]. They found that after 3 h of occlusion, $^{18}\text{FMISO}$ accumulation in myocardium experiencing 40% of normal flow or less was increased compared to that in myocardium with normal flow, although they did not investigate the biochemical or physiological consequence of this degree of flow reduction.

Post-mortem tissue counting demonstrated that tracer trapping was greatest 3 h after occlusion, progressively halving at subsequent ischemic time points, as tissue viability declined. However, this investigation could only be accurately performed by calculating residual fractions from the myocardium post-mortem. As with previous studies, they confirmed that occluded:normal tissue contrast was less than 1.5:1 after 45 min, at a time point when blood pool concentrations were still as high as tissue concentrations (images of tissue uptake in this study were also derived by subtraction of blood pool data). This last paper seems representative of its predecessors. ^{18}F MISO does seem to accumulate in viable but ischemic tissue (although possibly in necrotic tissue too). However, ischemic:normal tissue contrast is poor, rarely exceeding 2:1, even in the most extreme experimental models. In conjunction with a blood clearance half life of 275 min (clearly not ideal with ^{18}F 's half life of 110 min), it is perhaps unsurprising that ^{18}F MISO has failed to be widely adopted as a cardiac hypoxia imaging agent [66].

7. Copper bis(thiosemicarbazone) complexes (BTSCs)

Copper radionuclides represent a versatile set of diagnostic and therapeutic tools offering a range of long and short half life β^+ , β^- , Auger, and γ emissions for a variety of applications [67]. The copper bis(thiosemicarbazonato) complex ^{64}Cu -PTSM (for structure see Table 1) is a perfusion tracer which rapidly diffuses into cells, whereupon it is non-selectively bio-reduced, resulting in the release of its radiocopper, which is scavenged by intracellular copper binding proteins and trapped within the cell [68–71] (Fig. 6). By modifying the ligand backbone of these complexes, it is possible to change their physiochemical properties, such as reduction potential, giving the capacity to ‘tune’ them to release radiocopper within specific intracellular environments [72–73]. For example, since the cell becomes increasingly reductive as hypoxia or ischemia progresses (due to loss of mitochondrial NAD(P)H recycling and glycolytic lactic acid production [74–76]), these agents exhibit potential as hypoxia/ischemia imaging agents. Hypoxia selectivity, quantified by the “hypoxia selectivity index”, HSI [72,77], can be controlled via alkylation of the diketone backbone, while other properties important to optimizing imaging quality or cell uptake (lipophilicity, serum protein binding, membrane permeability, detailed structural parameters etc. [78]) can be controlled by alkylation of the terminal amino groups (Table 1). There is also a possibility to alter the donor atoms to control redox potential [79].

Cu-ATSM (ATSMH_2 =diacetyl-bis(N^4 -methylthiosemicarbazone), first described in 1996, with a reduction potential of -0.59 V (compared to -0.51 V for Cu-PTSM , as measured against a Ag/AgCl reference electrode [80]), was the first BTSC complex demonstrated to exhibit hypoxia selectivity [67,81–82]. While it has since been extensively investigated for characterizing hypoxic tumors [83–87] [88], relatively few studies have addressed its potential application for imaging ischemic or hypoxic myocardium [89–90]. Fujibayashi et al. were the first group to report selective trapping of ^{62}Cu -ATSM in isolated perfused rat hearts. Under normoxia, they demonstrated rapid washout of a tracer bolus within 10 min to a stable baseline representing retention of 20% of injected radiocopper. This retention increased to 80% when perfusion was switched to a hypoxic buffer, representing a 4-fold increase in radiocopper accumulation due to hypoxic perfusion. Unfortunately cardiac function was not monitored during their protocol, and no other biomarkers were obtained, so it is difficult to interpret the potential biological relevance of their findings, but it was nonetheless a promising first study. Advancing to an *in vivo* rat model of coronary artery occlusion, they then demonstrated that ^{62}Cu -ATSM accumulation increased as uptake of the perfusion tracer ^{201}Tl decreased (except in regions with very low ^{201}Tl accumulation, where it was presumed that the tissue was necrotic). In a further study, quantitative autoradiography revealed a reciprocal relationship between regions of high flow/metabolism by ^{11}C -acetate distribution, and regions of hypoxia delineated by ^{64}Cu -ATSM, Fig. 5 [91].

These images were obtained from tissues frozen within 10 min of ^{64}Cu -ATSM injection, highlighting its rapid washout from normoxic tissue.

In recognition of the distinct pathophysiological states of hypoxia and ischemia, Lewis et al. employed three models to assess $^{60,61,64}\text{Cu}$ -ATSM accumulation in canine myocardium: systemic global hypoxia achieved by inhalation of hypoxic gas; acute supply ischemia induced by LAD occlusion (~50% flow reduction), and acute demand ischemia achieved by stenosis and dobutamine administration. Systemic hypoxia led to a quadrupling in cardiac tracer accumulation, associated with a 7-fold increase in myocardial blood flow. While it could be argued that this increase in flow confers greater tracer delivery to the myocardium, it also confers faster washout. Tracer accumulation in each experiment correlated well with decreasing arterial pO_2 levels, although how this translates to intracellular pO_2 is unclear. During both acute supply and demand ischemia, tracer retention was only increased in myocardium which was hypoxic but not necrotic, a promising result in the context of the potential utility of ^{64}Cu -ATSM for identifying salvageable myocardium. Pharmacokinetic modeling of tracer uptake confirmed that tracer retention was independent of blood flow in all experimental groups [89]. In these studies, the rapid blood pool clearance of Cu-ATSM allowed PET imaging within 20 min of tracer injection; a significant advance over what had been previously possible with nitroimidazole-based imaging agents.

In 2001, Takahashi et al. described a preliminary trial of ^{62}Cu -ATSM PET in seven patients with coronary artery disease [90]. While four patients exhibited increased ^{18}F FDG uptake in eighteen segments, increased ^{62}Cu -ATSM accumulation was only observed in one segment from one patient with unstable angina (which co-localized with ^{18}F FDG), albeit with a good degree of contrast between the blood pool and occluded region within 20 min of injection (1:3.1). *In vitro*, Cu-ATSM releases radiocopper in only extremely hypoxic cells (the threshold in isolated cells is exceeded when the medium is gassed with a pO_2 of 0.8 mmHg or even lower [91,92]); such low oxygen tensions typically occur within the core of a poorly perfused tumor, but are less likely to occur in ischemic but salvageable myocardium. Although interstitial myocardial O_2 partial pressures have been recorded falling to as low as 0.1 mmHg in patients undergoing coronary artery bypass grafting using polarographic microelectrodes [93], this is an extremely acute artificial situation under cardioplegic protection. In more pathophysiologically relevant conditions, coronary stenoses of around 55% only cause endocardial O_2 levels to fall to around 7 mmHg *in vivo* (also by polarographic electrode) [94,95]. Considering that the intended use of these imaging agents is to delineate vulnerable *but salvageable* myocardium, we suggest that the redox potential for Cu-ATSM may be too low for useful application in cardiology. This may explain the disparity between the relatively low tracer uptake Takahashi et al. observe in their stable cardiac patients, but promising results (higher tracer uptake) obtained under experimental conditions where it is possible to induce more severe (but less pathophysiologically relevant) levels of hypoxia or ischemia.

8. What needs to be done to develop and validate the BTSCs?

8.1. Screening of the BTSC library

Cu-ATSM is only one of an almost limitless array of potential BTSC compounds. The strength of this family is their inherent and subtle “tuneability”, such that different complexes could potentially be used to characterize different levels of hypoxia associated with different disease processes. However, to date, Cu-ATSM is the only hypoxia-selective BTSC that has ever been tested in the heart, despite seemingly being better suited to imaging extreme hypoxia in a hypo-perfused tumor. While its pharmacokinetic properties are extremely promising, and bode well for the general approach, it is perhaps targeting levels of hypoxia more severe than regularly occur in patients with heart disease.

Development and screening of more members of this library of tracers is therefore essential. Not only do we need to identify new tracers which display selectivity for lower levels of hypoxia (and optimize them in terms of pharmacokinetics, liver clearance etc.), but we need to define the levels of hypoxia that they identify, in line with our previously described ischemic cascade. We also need to understand what pathologies are associated with these levels of hypoxia, and to use parallel biomarkers wherever possible to validate the appropriateness of each hypoxia-selective tracer identified. In the investigations that we have described in this review, if the accumulation of potential hypoxia tracers has been correlated with anything at all, it is usually either decreased perfusion, oxygenation, or contractile function. Since these agents are intended to replace perfusion imaging agents, and the basic tenet of their application is to relate supply to demand, the relevance of comparing their uptake to perfusion agents alone is questionable. In scant few investigations of these tracers (including the misonidazoles) have biochemical correlates of hypoxia or ischemia been confirmed. For any of these tracers to be validated as truly hypoxia-specific, or even hypoxia sensitive, parallel demonstration of hypoxia or ischemia as defined at the beginning of this review *must* be performed. This is particularly important for *in vivo* studies, where it is more difficult to reliably induce and confirm the existence of ischemia or hypoxia.

Despite their increasing use, and recent and ongoing clinical trials in applications for hypoxia imaging in oncology, the details of the uptake and trapping mechanisms of these complexes remain a matter of hypothesis rather than accepted fact. Indeed, as Dearling and Packard discuss in their recent review [96], even their specificity to hypoxia remains to be validated. A number of other important issues must also be resolved before data obtained from this class of compounds can be fully exploited.

8.2. What is the effect of perfusion?

Tracer delivery and washout are potential sources of variation with respect to the final amount of radiocopper trapped in a tissue, independent of its hypoxia selectivity. Wood et al. observed that after 10 min of tracer injection, Cu-ATSM images in rats bearing carcinosarcoma allografts correlated strongly with flow, while after 60 min, this correlation was lost, revealing a hypoxia-dependent biodistribution [97]. Similarly, Fujibayashi et al. showed that regions of acute myocardial ischemia exhibited sub-regions lacking uptake of both ^{64}Cu -ATSM (10 min post-injection) and ^{11}C -acetate (1 min post-injection) surrounded by regions of high Cu-ATSM uptake in the hypoxic border zone [98], delivery of both tracers presumably being limited by flow. Conversely, the *in vivo* pharmacokinetic data obtained by Lewis et al. showed no relationship between flow and tracer uptake [99]. The importance of blood flow in BTSC hypoxia imaging remains unresolved, and requires careful dissection.

8.3. What is the intracellular site of complex reduction?

The subcellular site of reduction of these complexes is likely to provide insight into their trapping mechanisms, and help identify the bioreductants involved. In brain, 70–80% of Cu-PTSM and Cu-ATSM was reduced by mitochondria, while in a variety of cancer cell types, mitochondrial retention is reported to represent approximately 10% of the total [82,100–102]. Thus far, reduction of these complexes has only been investigated in individual subcellular fractions [100,103–105], rather than intact tissues which have been exposed to copper complexes and then fractionated. Errors in accounting for relative differences in volumes and concentrations of each fraction potentially skew the measured contribution of each fraction to total cellular bioreduction, but some tissue-specific differences have been identified nonetheless. No studies have yet been performed to determine the subcellular site of tracer reduction in cardiac tissue.

8.4. What is fate of the radiocopper?

The identification of the binding partners of radiocopper once released from its ligand may provide some insight into its mode of reduction and trapping, and aid image interpretation and pharmacokinetic modeling. This has only been addressed in one study so far, where the fate of radiocopper released from Cu-PTSM appeared to be similar to that of Cu-citrate in brain and liver, suggesting that it enters normal cellular pools for copper ions [106]. It is not yet known whether this is also true in cardiac tissue, or whether the fate of radiocopper is ischemia- or hypoxia-dependent, or varies between different BTSC complexes. Burgman et al. have suggested a further selectivity mechanism, that of transporter-mediated efflux of radiocopper from the cell, where the cell-dependent differences that they observe in radiocopper retention may be governed by differences in expression of the copper exporters ATP7A and ATP7B [107]. This has not yet been investigated further.

8.5. What is the identity of the bioreductants?

In 1993, Fujibayashi et al. employed EPR spectroscopy to demonstrate the reduction of Cu(II) in Cu-PTSM to a Cu(I) species by murine brain homogenates, supporting previous work by Warren et al. [108] and Minkel and Petering on Cu-KTS [109]. This single-electron reduction was temperature-dependent, raising the possibility of it being enzyme mediated, potentially by the thiol-containing enzymes of the mitochondria, although they provide no evidence for this [103]. Using electron transport chain inhibitors to identify the enzymes responsible for Cu-PTSM reduction in brain sub-mitochondrial particles, Taniuchi et al. observed increases in tracer reduction with rotenone and antimycin A, but not TTFA. This suggests that while bioreduction of the complex may be NADH-dependent, (as originally proposed by Petering [110]), NADH alone did not reduce the complex, inferring that NADH-dependent redox-active proteins may be responsible [111]. It is unclear whether data obtained from sub-mitochondrial particles can be extrapolated to intact cells, or even intact mitochondria in this approach.

In tumor cells, where tracer reduction is reported to be primarily cytosolic rather than mitochondrial, Obata et al. showed that Cu-ATSM reduction by cytosolic fractions was also heat sensitive and NAD(P)H sensitive. They demonstrated that this reduction was inhibited in a dose-dependent manner by the flavin enzyme inhibitor DPIC, and pHMB, a thiol-enzyme inhibitor, suggesting that cytosolic electron transport chain enzymes were responsible. Further evidence for this was provided by treatment of microsomal fractions with inhibitors of cytochrome b5 reductase and cytochrome P450 reductase, resulting in inhibition of tracer reduction [101].

The importance of non-enzymatic reductants is also the subject of debate. Glutathione, as a highly abundant thiol (~1 $\mu\text{mol/g}$ wet weight in heart), with a redox potential of -0.23 V at pH 7, has long been implicated as a potential bioreductive agent for these complexes [112,113]. While the measured rates of thiol-mediated complex reduction obtained *in vitro* seem prohibitively slow, they were originally derived from spectrophotometric studies with high concentrations of tracer, such that thiol concentrations were only 20 times greater than those of the copper complex [113]. This vastly underestimates the thiol: complex ratios existing when these complexes are injected in sub-femtomolar concentrations *in vivo* [96], and may therefore misrepresent their actual rate of reduction. This issue is a fundamental problem with all of the *in vitro* mechanistic studies performed thus far because much higher concentrations of complex are required for EPR and HPLC analysis than would be required if radiometric methods were employed. It is very difficult to assess the relative importance of each of these bioreductive mechanisms when each is likely to have a different saturation threshold which may affect the observed rates of reaction. When the complexes are present in tracer concentration *in vivo*, it is unlikely that any of them will be saturated.

In the only study to evaluate the effect of glutathione modification on radiocopper uptake from a tracer *in vivo*, Barhart-Bott and Green demonstrated only small decreases in tissue Cu-PTSM retention in rats treated with the glutathione depletion agents diethylmaleate and buthionine sulfoximine [114]; they did not, however, actually measure glutathione levels in these animals, so it is difficult to interpret their findings. The other question with respect to the importance of thiol status in this process is that of time frame. Fujibayashi et al. demonstrate dramatic changes in tracer retention within 5 min of the induction of hypoxia in their isolated hearts [82]. While intracellular glutathione concentrations are known to change during ischemia [74], it is currently unclear whether their rate of change is sufficiently rapid to implicate them as responsive mediators of radiocopper reduction (during acute ischemia/hypoxia at least). However, chronic changes in glutathione status may be important in determining the baseline intracellular redox status to which these complexes are thought to respond. Further investigation of the importance of cardiac thiol status on tracer reduction and trapping in relevant *in/ex vivo* experimental models is therefore essential.

8.6. What is the role of oxygen?

There are at least two non-mutually exclusive mechanisms by which the lack of oxygen may cause radiocopper reduction and loss from these complexes (Fig. 6). When intracellular oxygen is not available to allow NADH recycling through the electron transport chain, there is an increase in intracellular NADH/NAD⁺ and NADPH/NADP⁺ ratios [75]. While NAD(P)H is not capable of reducing copper BTSC complexes directly, its increasing concentration during hypoxia may up-regulate either cytosolic or mitochondrial enzymes capable of using it as a cofactor in radiocopper reduction [96]. In this model hypoxia therefore determines the initial rate of radiocopper reduction. The second mechanism of oxygen selectivity is that of re-oxidation of the unstable reduced complex by molecular oxygen. If the lifetime of the stable complex inside the cell is thought of as a cycling between reduction and oxidation, re-oxidation of the unstable complex from Cu(I) to Cu(II) by molecular oxygen is likely to be a key regulator of whether these tracers dissociate and deposit their radiocopper, or diffuse back out of the cell unchanged. This has been shown to be possible chemically in a non-biological system, but as yet, this has not been demonstrated in live cells [115]. The relative importance of these two mechanisms is currently unknown.

8.7. What is the effect of acidosis?

One further potential modifier of BTSC complex dissociation is protonation, which may be more prevalent during acidosis [96,115,116]. While it has been shown *in vitro* that Cu-BTSC complexes are stable under acidic but oxygenated conditions [113,115], and at this time there is no experimental data to directly support cellular retention of radiocopper by protonation alone, density functional theory calculations by Holland et al. suggest that a decrease in cellular pH may protonate the unstable Cu(I) complex, and increase its rate of dissociation [116,117]. If it occurred, double protonation would also decrease the complexes' lipophilicity, tending to decrease their washout rates, increase their retention times within the cell, and hence increase their chances of interacting with intracellular reducing species [96].

In tumors, where low flow coincides with an increasingly reductive environment, low oxygen, and low pH, these likely prime determinants of radiocopper trapping co-exist in the same location. This is also likely to be true in the severely flow-limited ischemic heart, but identifying such moribund cardiac tissue can be performed much more cheaply and quickly with other imaging techniques. In the more subtle pathophysiological conditions where these imaging agents may find unique application, however, the prime determinants of tracer trapping may not all coincide. During demand ischemia, for example, while the tissue may be compromised with respect to oxygen, residual flow may limit acidosis, meaning that one

parameter which may be important in tracer retention is absent. The impact of this on tracer retention is currently unknown. The relationship between each potential trapping parameter, such as intracellular pH, tracer reduction, and retention in normoxia and hypoxia therefore needs to be empirically characterized in an intact tissue model, or *in vivo*, with translationally relevant concentrations of tracer.

9. Summary

The non-invasive imaging of tissue hypoxia promises novel and unique insight into the biochemistry of the ischemic myocardium, with the potential to provide new prognostic and diagnostic information on cardiac dysfunction, ranging from identification of areas of acute risk to propensity to heart failure. Unfortunately, to date, the misonidazole imaging agents originally designed for this purpose have been plagued by significant pharmacokinetic problems, which have meant that this approach has failed to deliver upon this promise. Preliminary trials of the copper bis(thiosemicarbazonato) complexes indicate that this new class of imaging agents has significantly better pharmacokinetic properties, coupled with the distinct advantage that they may be tuneable to specifically identify different hypoxia thresholds for different applications. To date, only one agent, Cu-ATSM, has been evaluated in the heart, with mixed results. However, this particular agent is targeted at levels of hypoxia far more extreme than would be routinely encountered in the myocardium. A library of these compounds exists, which is likely to contain agents more appropriate for cardiac application. Not only is it essential to screen these new compounds, including asymmetric ones [118], for hypoxia selectivity at levels relevant to cardiac pathophysiology, but much more work is needed to fully understand how this class of agents work, and to validate them appropriately, before they can be fully exploited in the clinic.

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References

- [1]. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med.* 2006; 3:e442. [PubMed: 17132052]
- [2]. Fraser AG, Buser PT, Bax JJ, Dassen WR, Nihoyannopoulos P, Schwitter J, et al. The future of cardiovascular imaging and non-invasive diagnosis. *Eur Heart J.* 2006; 27:1750–3. [PubMed: 16820369]
- [3]. Chen IY, Wu JC. Cardiovascular molecular imaging: focus on clinical translation. *Circulation.* 2011; 123:425–43. [PubMed: 21282520]
- [4]. Sinusas AJ. The potential of myocardial imaging with hypoxia markers. *Semin Nucl Med.* 1999; 29:330–8. [PubMed: 10534235]
- [5]. Hearse DJ. Myocardial ischaemia: can we agree on a definition for the 21st century? *Cardiovasc Res.* 1994; 28:1737–44. [PubMed: 7867024]
- [6]. de Boer RA, Pinto YM, van Veldhuisen DJ. The imbalance between oxygen demand and supply as a potential mechanism in the pathophysiology of heart failure: the role of microvascular growth and abnormalities. *Microcirculation.* 2003; 10:113–26. [PubMed: 12700580]
- [7]. Nagel E, Klein C, Paetsch I, Hettwer S, Schnackenburg B, Wegscheider K, et al. Magnetic resonance perfusion measurements for the noninvasive detection of coronary artery disease. *Circulation.* 2003; 108:432–7. [PubMed: 12860910]
- [8]. Hachamovitch R, Hayes SW, Friedman JD, Cohen I, Berman DS. Comparison of the short-term survival benefit associated with revascularization compared with medical therapy in patients with

- no prior coronary artery disease undergoing stress myocardial perfusion single photon emission computed tomography. *Circulation*. 2003; 107:2900–7. [PubMed: 12771008]
- [9]. Rahimtoola SH. A perspective on the three large multicenter randomised clinical trials of coronary bypass surgery for chronic stable angina. *Circulation*. 1985; 72:V123–35. [PubMed: 3905053]
- [10]. Heusch G, Schulz R, Rahimtoola SH. Myocardial hibernation: a delicate balance. *Am J Physiol*. 2005; 288:H984–99.
- [11]. Camici PG, Rimoldi O. Myocardial hibernation vs. repetitive stunning in patients. *Cardiol Rev*. 1999; 7:39–43. [PubMed: 10348965]
- [12]. Feldhaus LM, Liedtke AJ. mRNA expression of glycolytic enzymes and glucose transporter proteins in ischemic myocardium with and without reperfusion. *J Mol Cell Cardiol*. 1998; 30:2475–85. [PubMed: 9925382]
- [13]. Depre C, Vanoverschelde JL, Melin JA, Borgers M, Bol A, Ausma J, et al. Structural and metabolic correlates of the reversibility of chronic left ventricular ischemic dysfunction in humans. *Am J Physiol*. 1995; 268:H1265–75. [PubMed: 7900881]
- [14]. Michelena HI, Vecker WA. Radionuclide-based insights into the pathophysiology of ischemic heart disease: beyond diagnosis. *J Investig Med*. 2005; 53:176–91. doi: 10.2310/6650.2005.00401.
- [15]. Uren NG, Camici PG. Hibernation and myocardial ischemia: clinical detection by positron emission tomography. *Cardiovasc Drugs Ther*. 1992; 6:273–9. [PubMed: 1637733]
- [16]. Camici PG, Prasad SK, Rimoldi OE. Stunning, hibernation, and assessment of myocardial viability. *Circulation*. 2008; 117:103–14. [PubMed: 18172050]
- [17]. Southworth R. Hexokinase-mitochondrial interaction in cardiac tissue: implications for cardiac glucose uptake, the 18FDG lumped constant and cardiac protection. *J Bioenerg Biomembr*. 2009; 41:187–93. [PubMed: 19415474]
- [18]. Garlick PB, Medina RA, Southworth R, Marsden PK. Differential uptake of FDG and DG during post-ischaemic reperfusion in the isolated, perfused rat heart. *Eur J Nucl Med*. 1999; 26:1353–8. [PubMed: 10541837]
- [19]. Southworth R, Dearling JL, Medina RA, Flynn AA, Pedley RB, Garlick PB. Dissociation of glucose tracer uptake and glucose transporter distribution in the regionally ischaemic isolated rat heart: application of a new autoradiographic technique. *Eur J Nucl Med Mol Imaging*. 2002; 29:1334–41. [PubMed: 12271416]
- [20]. Hariharan R, Bray M, Ganim R, Doenst T, Goodwin GW, Taegtmeyer H. Fundamental limitations of [18F]2-deoxy-2-fluoro-D-glucose for assessing myocardial glucose uptake. *Circulation*. 1995; 91:2435–44. [PubMed: 7729031]
- [21]. Doenst T, Taegtmeyer H. Profound underestimation of glucose uptake by [18F]2-deoxy-2-fluoroglucose in perfused rat heart muscle. *Circulation*. 1998; 97:2454–62. [PubMed: 9641698]
- [22]. Barrington SF, Chambers J, Hallett WA, O'Doherty MJ, Roxburgh JC, Nunan TO. Comparison of sestamibi, thallium, echocardiography and PET for the detection of hibernating myocardium. *Eur J Nucl Med Mol Imaging*. 2004; 31:355–61. [PubMed: 14647986]
- [23]. Piwnica-Worms D, Kronauge JF, LeFurgey A, Backus M, Hockett D, Ingram P, et al. Mitochondrial localization and characterization of 99Tc-SESTAMIBI in heart cells by electron probe x-ray microanalysis and 99Tc-NMR spectroscopy. *Magn Reson Imaging*. 1994; 12:641–52. [PubMed: 8057769]
- [24]. Schwaiger M, Muzik O. Assessment of myocardial perfusion by positron emission tomography. *Am J Cardiol*. 1991; 67:35D–43D.
- [25]. Depre C, Vatner SF. Mechanisms of cell survival in myocardial hibernation. *Trends Cardiovasc Med*. 2005; 15:101–10. [PubMed: 16039970]
- [26]. Bonow RO, Udelson JE. Left ventricular diastolic dysfunction as a cause of congestive heart failure. *Ann Intern Med*. 1992; 117:502–10. [PubMed: 1503353]
- [27]. Hoenig MBC, Rosenswieg A, Sellke FW. The cardiac microvasculature in hypertension, cardiac hypertrophy and diastolic heart failure. *Curr Vasc Pharmacol*. 2008; 6:292–300. [PubMed: 18855717]

- [28]. Des Tombe AL, Van Beek-Harmsen BJ, Lee-De Groot MBE, Van Der Laarse WJ. Calibrated histochemistry applied to oxygen supply and demand in hypertrophied rat myocardium. *Microsc Res Tech.* 2002; 58:412–20. [PubMed: 12226811]
- [29]. Shyu KGLJY, Wang BW, Fang WJ, Chang H. Carvedilol prevents cardiac hypertrophy and overexpression of hypoxia-inducible factor-1 α and vascular endothelial growth factor in pressure-overloaded rat heart. *J Biomed Sci.* 2005; 12:409–20. [PubMed: 15942707]
- [30]. Krishnan J, Suter M, Windak R, Krebs T, Felley A, Montessuit C, et al. Activation of a HIF1[alpha]-PPAR[gamma] axis underlies the integration of glycolytic and lipid anabolic pathways in pathologic cardiac hypertrophy. *Cell Metab.* 2009; 9:512–24. [PubMed: 19490906]
- [31]. El Jamali A, Freund C, Rechner C, Scheidereit C, Dietz R, Bergmann MW. Reoxygenation after severe hypoxia induces cardiomyocyte hypertrophy in vitro: activation of CREB downstream of GSK3 β . *FASEB J.* 2004; 18(10):1096–8. [PubMed: 15155564]
- [32]. Anversa P, Capasso JM. Loss of intermediate-sized coronary arteries and capillary proliferation after left ventricular failure in rats. *Am J Physiol.* 1991; 260:H1552–60. [PubMed: 2035675]
- [33]. Biolo A, Greferath R, Siwik DA, Qin F, Valsky E, Fylaktakidou KC, et al. Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of hemoglobin, myo-inositol trispyrophosphate. *PNAS.* 2009; 106:1926–9. [PubMed: 19204295]
- [34]. Bekereditian R, Walton CB, MacCannell KA, Ecker J, Kruse F, Outten JT, et al. Conditional HIF-1 α expression produces a reversible cardiomyopathy. *PLoS One.* 2010; 5:e11693. [PubMed: 20657781]
- [35]. Dobrucki LWMD, Hu J, Sadeghi MM, Sinusas AJ. Regional hypoxia correlates with the uptake of a radiolabelled targeted marker of angiogenesis in rat model of myocardial hypertrophy and ischemic injury. *J Physiol Pharmacol.* 2009; 60:117–23. [PubMed: 20083860]
- [36]. Nunn A, Linder K, Strauss HW. Nitroimidazoles and imaging hypoxia. *Eur J Nucl Med Mol Imaging.* 1995; 22:265–80.
- [37]. Chung Y. Oxygen reperfusion is limited in the postischemic hypertrophic myocardium. *Am J Physiol.* 2006; 290:H2075–84.
- [38]. Williamson JR, Kobayashi K. Use of the perfused rat heart to study cardiac metabolism: retrospective and prospective views. *Basic Res Cardiol.* 1984; 79:283–91. [PubMed: 6477381]
- [39]. Zhang J, Ugurbil K, From AHL, Bache RJ. Myocardial oxygenation and high-energy phosphate levels during graded coronary hypoperfusion. *Am J Physiol.* 2001; 280:H318–26.
- [40]. Kreutzer U, Mekhamer Y, Chung Y, Jue T. Oxygen supply and oxidative phosphorylation limitation in rat myocardium in situ. *Am J Physiol.* 2001; 280:H2030–7.
- [41]. Mik EG, Ince C, Eerbeek O, Heinen A, Stap J, Hooibrink B, et al. Mitochondrial oxygen tension within the heart. *J Mol Cell Cardiol.* 2009; 46:943–51. [PubMed: 19232352]
- [42]. Nesto RW, Kowalchuk GJ. The ischemic cascade: temporal sequence of hemodynamic, electrocardiographic and symptomatic expressions of ischemia. *Am J Cardiol.* 1987; 59:C23–30.
- [43]. Camici PG, Rimoldi OE. The clinical value of myocardial blood flow measurement. *J Nucl Med.* 2009; 50:1076–87. [PubMed: 19525470]
- [44]. Jerosch-Herold M. Quantification of myocardial perfusion by cardiovascular magnetic resonance. *J Cardiovasc Magn Reson.* 2010; 12:57. [PubMed: 20932314]
- [45]. Lowe J, Cummings R, Adams D, Hull-Ryde E. Evidence that ischemic cell death begins in the subendocardium independent of variations in collateral flow or wall tension. *Circulation.* 1983; 68:190–202. [PubMed: 6851046]
- [46]. Jameel MN, Wang X, Eijgelshoven MHJ, Mansoor A, Zhang J. Transmural distribution of metabolic abnormalities and glycolytic activity during dobutamine-induced demand ischemia. *Am J Physiol.* 2008; 294:H2680–6.
- [47]. Fujiwara H, Ashraf M, Sato S, Millard R. Transmural cellular damage and blood flow distribution in early ischemia in pig hearts. *Circ Res.* 1982; 51:683–93. [PubMed: 6754127]
- [48]. Garlick PB, Radda GK, Seeley PJ, Chance B. Phosphorus NMR studies on perfused heart. *Biochem Biophys Res Commun.* 1977; 74:1256–62. [PubMed: 843355]
- [49]. Hardy CJ, Weiss RG, Bottomley PA, Gerstenblith G. Altered myocardial high-energy phosphate metabolites in patients with dilated cardiomyopathy. *Am Heart J.* 1991; 122:795–801. [PubMed: 1877457]

- [50]. Neubauer S, Krahe T, Schindler R, Horn M, Hillenbrand H, Entzeroth C, et al. 31P magnetic resonance spectroscopy in dilated cardiomyopathy and coronary artery disease. Altered cardiac high-energy phosphate metabolism in heart failure. *Circulation*. 1992; 86:1810–8. [PubMed: 1451253]
- [51]. Hansch A, Rzanny R, Heyne JP, Leder U, Reichenbach JR, Kaiser WA. Noninvasive measurements of cardiac high-energy phosphate metabolites in dilated cardiomyopathy by using 31P spectroscopic chemical shift imaging. *Eur Radiol*. 2005; 15:319–23. [PubMed: 15630572]
- [52]. Neubauer S, Horn M, Cramer M, Harre K, Newell JB, Peters W, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation*. 1997; 96:2190–6. [PubMed: 9337189]
- [53]. Holloway C, Cochlin L, Codreanu I, Bloch E, Fatemian M, Szmigielski C, et al. Normobaric hypoxia impairs human cardiac energetics. *FASEB J*. 2011; 25(2):792–6. [PubMed: 20978235]
- [54]. Garlick PB, Marsden PK, Cave AC, Parkes HG, Slates R, Shao Y, Silverman RW, Cherry SRPET. NMR dual acquisition (PANDA): applications to isolated, perfused rat hearts. *NMR Biomed*. 1997; 10:138–42. [PubMed: 9408923]
- [55]. Hoffend J, Linke G, Mohammed A, Tiefenbacher CP, Eisenhut M, Haberkorn U. 99mTcO(BAT-NI), a novel nitroimidazole tracer: in vivo uptake studies in ischaemic myocardium. *Eur J Nucl Med Mol Imaging*. 2003; 30:494–501. [PubMed: 12574972]
- [56]. Strauss HW, Nunn A, Linder K. Nitroimidazoles for imaging hypoxic myocardium. *J Nucl Cardiol*. 2:437–45. [PubMed: 9420823]
- [57]. Krohn KA, Link JM, Mason RP. Molecular imaging of hypoxia. *J Nucl Med*. 2008; 49:129S–48S. [PubMed: 18523070]
- [58]. Whitmore GF, Varghese AJ. The biological properties of reduced nitroheterocyclics and possible underlying biochemical mechanisms. *Biochem Pharmacol*. 1986; 35:97–103. [PubMed: 3510061]
- [59]. Kedderis GL, Miwa GT. The metabolic activation of nitroheterocyclic therapeutic agents. *Drug Metab Rev*. 1988; 19:33–62. [PubMed: 3293954]
- [60]. Martin G, Cerqueira M, Caldwell J, Rasey J, Embree L, Krohn K. Fluoromisonidazole. A metabolic marker of myocyte hypoxia. *Circ Res*. 1990; 67:240–4. [PubMed: 2364494]
- [61]. Martin GV, Caldwell JH, Graham MM, Grierson JR, Kroll K, Cowan MJ, et al. Noninvasive detection of hypoxic myocardium using fluorine-18-fluoromisonidazole and positron emission tomography. *J Nucl Med*. 1992; 33:2202–8. [PubMed: 1460516]
- [62]. Martin GV, Biskupiak JE, Caldwell JH, Rasey JS, Krohn KA. Characterization of iodovinylmisonidazole as a marker for myocardial hypoxia. *J Nucl Med*. 1993; 34:918–24. [PubMed: 8509858]
- [63]. Shelton ME, Dence CS, Hwang D-R, Welch MJ, Bergmann SR. Myocardial kinetics of fluorine-18 misonidazole: a marker of hypoxic myocardium. *J Nucl Med*. 1989; 30:351–8. [PubMed: 2738664]
- [64]. Shelton ME, Dence CS, Hwang D-R, Herrero P, Welch MJ, Bergmann SR. In vivo delineation of myocardial hypoxia during coronary occlusion using fluorine-18 fluoromisonidazole and positron emission tomography: a potential approach for identification of jeopardized myocardium. *J Am Coll Cardiol*. 1990; 16:477–85. [PubMed: 2373827]
- [65]. Martin GV, Caldwell JH, Rasey JS, Grunbaum Z, Cerqueira M, Krohn KA. Enhanced binding of the hypoxic cell marker [3H]fluoromisonidazole in ischemic myocardium. *J Nucl Med*. 1989; 30:194–201. [PubMed: 2738648]
- [66]. Strauss HW, Nunn A, Linder K. Nitroimidazoles for imaging hypoxic myocardium. *J Nucl Cardiol*. 1995; 2:437–45. [PubMed: 9420823]
- [67]. Blower PJ, Lewis JS, Zweit J. Copper radionuclides and radiopharmaceuticals in nuclear medicine. *Nucl Med Biol*. 1996; 23:957–80. [PubMed: 9004284]
- [68]. Green MA. A potential copper radiopharmaceutical for imaging the heart and brain: copper-labeled pyruvaldehyde bis(N4-methylthiosemicarbazone). *Int J Rad Appl Instrum B*. 1987; 14:59–61. [PubMed: 3583756]

- [69]. Green MA, Klippenstein DL, Tennison JR. Copper(II) bis(thiosemicarbazone) complexes as potential tracers for evaluation of cerebral and myocardial blood flow with PET. *J Nucl Med.* 1988; 29:1549–57. [PubMed: 3261785]
- [70]. Shelton ME, Green MA, Mathias CJ, Welch MJ, Bergmann SR. Kinetics of copper-PTSM in isolated hearts: a novel tracer for measuring blood flow with positron emission tomography. *J Nucl Med.* 1989; 30:1843–7. [PubMed: 2809749]
- [71]. Shelton ME, Green MA, Mathias CJ, Welch MJ, Bergmann SR. Assessment of regional myocardial and renal blood flow with copper-PTSM and positron emission tomography. *Circulation.* 1990; 82:990–7. [PubMed: 2394015]
- [72]. Dearling JLJ, Lewis JS, Mullen GED, Rae MT, Zweit J, Blower PJ. Design of hypoxia-targeting radiopharmaceuticals: selective uptake of copper-64 complexes in hypoxic cells in vitro. *Eur J Nucl Med Mol Imaging.* 1998; 25:788–92.
- [73]. McQuade P, Martin KE, Castle TC, Went MJ, Blower PJ, Welch MJ, et al. Investigation into ⁶⁴Cu-labeled bis(selenosemicarbazone) and bis(thiosemicarbazone) complexes as hypoxia imaging agents. *Nucl Med Biol.* 2005; 32:147–56. [PubMed: 15721760]
- [74]. Zhu X, Zuo L, Cardounel AJ, Zweier JL, He G. Characterization of in vivo tissue redox status, oxygenation, and formation of reactive oxygen species in postischemic myocardium. *Antioxid Redox Signal.* 2007; 9:447–55. [PubMed: 17280486]
- [75]. Katz A, Edlund A, Sahlin K. NADH content and lactate production in the perfused rabbit heart. *Acta Physiol Scand.* 1987; 130:193–200. [PubMed: 3604709]
- [76]. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev.* 2005; 85:1093–129. [PubMed: 15987803]
- [77]. LJ Dearling J, J Blower P. Redox-active metal complexes for imaging hypoxic tissues: structure–activity relationships in copper(II) bis(thiosemicarbazone) complexes. *Chem Commun.* 1998:2531–2.
- [78]. Blower PJ, Castle TC, Cowley AR, Dilworth JR, Donnelly PS, Labisbal E, et al. Structural trends in copper(ii) bis(thiosemicarbazone) radiopharmaceuticals. *Dalton Trans.* 2003:4416–25.
- [79]. Castle TC, Maurer RI, Sowrey FE, Went MJ, Reynolds CA, McInnes EJJ, et al. Hypoxia-targeting copper bis(selenosemicarbazone) complexes: comparison with their sulfur analogues. *J Am Chem Soc.* 2003; 125:10040–9. [PubMed: 12914467]
- [80]. Dearling JL, Lewis JS, Mullen GE, Welch MJ, Blower PJ. Copper bis(thiosemicarbazone) complexes as hypoxia imaging agents: structure–activity relationships. *J Biol Inorg Chem.* 2002; 7:249–59. [PubMed: 11935349]
- [81]. Wada K, Fujibayashi Y, Tajima N, Yokoyama A. Cu-ATSM, an intracellular-accessible superoxide dismutase (SOD)-like copper complex: evaluation in an ischemia–reperfusion injury model. *Biol Pharm Bull.* 1994; 17:701–4. [PubMed: 7920437]
- [82]. Fujibayashi Y, Taniuchi H, Yonekura Y, Ohtani H, Konishi J, Yokoyama A. Copper-62-ATSM: a new hypoxia imaging agent with high membrane permeability and low redox potential. *J Nucl Med.* 1997; 38:1155–60. [PubMed: 9225812]
- [83]. Dehdashti F, Mintun MA, Lewis JS, Bradley J, Govindan R, Laforest R, et al. In vivo assessment of tumor hypoxia in lung cancer with ⁶⁰Cu-ATSM. *Eur J Nucl Med Mol Imaging.* 2003; 30:844–50. [PubMed: 12692685]
- [84]. Takahashi N, Fujibayashi Y, Yonekura Y, Welch MJ, Waki A, Tsuchida T, et al. Evaluation of ⁶²Cu labeled diacetyl-bis(N4-methylthiosemicarbazone) as a hypoxic tissue tracer in patients with lung cancer. *Ann Nucl Med.* 2000; 14:323–8. [PubMed: 11108159]
- [85]. Dehdashti F, Grigsby PW, Lewis JS, Laforest R, Siegel BA, Welch MJ. Assessing tumor hypoxia in cervical cancer by PET with ⁶⁰Cu-labeled diacetyl-bis(N4-methylthiosemicarbazone). *J Nucl Med.* 2008; 49:201–5. [PubMed: 18199612]
- [86]. Dehdashti F, Grigsby PW, Mintun MA, Lewis JS, Siegel BA, Welch MJ. Assessing tumor hypoxia in cervical cancer by positron emission tomography with ⁶⁰Cu-ATSM: relationship to therapeutic response—a preliminary report. *Int J Radiat Oncol Biol Phys.* 2003; 55:1233–8. [PubMed: 12654432]

- [87]. Lewis JS, Laforest R, Dehdashti F, Grigsby PW, Welch MJ, Siegel BA. An imaging comparison of ^{64}Cu -ATSM and ^{60}Cu -ATSM in cancer of the uterine cervix. *J Nucl Med*. 2008; 49:1177–82. [PubMed: 18552145]
- [88]. Dietz DW, Dehdashti F, Grigsby PW, Malyapa RS, Myerson RJ, Picus J, et al. Tumor hypoxia detected by positron emission tomography with (^{60}Cu) -ATSM as a predictor of response and survival in patients undergoing neoadjuvant chemoradiotherapy for rectal carcinoma: a pilot study. *Dis Colon Rectum*. 2008; 51(11):1641–8. [PubMed: 18682881]
- [89]. Lewis JS, Herrero P, Sharp TL, Engelbach JA, Fujibayashi Y, Laforest R, et al. Delineation of hypoxia in canine myocardium using PET and copper(II)-diacetyl-bis (N(4)-methylthiosemicarbazone). *J Nucl Med*. 2002; 43:1557–69. [PubMed: 12411560]
- [90]. Takahashi N, Fujibayashi Y, Yonekura Y, Welch MJ, Waki A, Tsuchida T, et al. Copper-62 ATSM as a hypoxic tissue tracer in myocardial ischemia. *Ann Nucl Med*. 2001; 15:293–6. [PubMed: 11545205]
- [91]. Dearling JL, Lewis JS, Mullen GE, Rae MT, Zweit J, Blower PJ. Design of hypoxia-targeting radiopharmaceuticals: selective uptake of copper-64 complexes in hypoxic cells in vitro. *Eur J Nucl Med*. 1998; 25:788–92. [PubMed: 9662602]
- [92]. Lewis JS, McCarthy DW, McCarthy TJ, Fujibayashi Y, Welch MJ. Evaluation of ^{64}Cu -ATSM in vitro and in vivo in a hypoxic tumor model. *J Nucl Med*. 1999; 40:177–83. [PubMed: 9935074]
- [93]. Bjerrum JT, Perko MJ, Beck B. Myocardial oxygen tension during surgical revascularization: a clinical comparison between blood cardioplegia and crystalloid cardioplegia. *Eur J Cardiothorac Surg*. 2006; 29:181–5. [PubMed: 16387508]
- [94]. Barrett JA, Lynch VD, Balkon J, Wolf PS. Measurement of oxygen tension in the ischemic myocardium using encased polarographic oxygen electrodes. *J Pharmacol Meth*. 1986; 15:235–43.
- [95]. Shibano T, Abiko Y. Effects of nitroglycerin, dipyridamole and propranolol on myocardial pH and pO₂ during regional ischemia in the dog heart. *Arch Int Pharmacodyn Ther*. 1983; 264:274–89. [PubMed: 6416195]
- [96]. Dearling JL, Packard AB. Some thoughts on the mechanism of cellular trapping of Cu(II)-ATSM. *Nucl Med Biol*. 37:237–43. [PubMed: 20346863]
- [97]. Wood KA, Honess DJ, Maxwell RJ, Wilson J, Paul RL, O'Doherty MJ, et al. Evaluation of the effects of blood flow on ^{64}Cu -ATSM uptake in a rodent tumour model. *Clin Oncol*. 2007; 19:S35–6.
- [98]. Fujibayashi Y, Cutler CS, Anderson CJ, McCarthy DW, Jones LA, Sharp T, et al. Comparative studies of Cu-64-ATSM and C-11-acetate in an acute myocardial infarction model: ex vivo imaging of hypoxia in rats. *Nucl Med Biol*. 1999; 26:117–21. [PubMed: 10096511]
- [99]. Lewis JS, Herrero P, Sharp TL, Engelbach JA, Fujibayashi Y, Laforest R, et al. Delineation of hypoxia in canine myocardium using PET and copper(II)-diacetyl-bis(N4-methylthiosemicarbazone). *J Nucl Med*. 2002; 43:1557–69. [PubMed: 12411560]
- [100]. Fujibayashi Y, Taniuchi H, Wada K, Yonekura Y, Konishi J, Yokoyama A. Differential mechanism of retention of Cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) (Cu-PTSM) by brain and tumor: a novel radiopharmaceutical for positron emission tomography imaging. *Ann Nucl Med*. 1995; 9:1–5. [PubMed: 7779524]
- [101]. Obata A, Yoshimi E, Waki A, Lewis JS, Oyama N, Welch MJ, et al. Retention mechanism of hypoxia selective nuclear imaging/radiotherapeutic agent cu-diacetyl-bis(N4-methylthiosemicarbazone) (Cu-ATSM) in tumor cells. *Ann Nucl Med*. 2001; 15:499–504. [PubMed: 11831397]
- [102]. Shibuya KFY, Yoshimi E, Sasai K, Hiraoka M, Welch MJ. Cytosolic/microsomal redox pathway: a reductive retention mechanism of a PET-oncology tracer, Cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) (PTSM). *Ann Nucl Med*. 1999; 13:287–92. [PubMed: 10582796]
- [103]. Fujibayashi Y, Wada K, Taniuchi H, Yonekura Y, Konishi J, Yokoyama A. Mitochondria-selective reduction of ^{62}Cu -pyruvaldehyde bis(N4-methylthiosemicarbazone) (^{62}Cu -PTSM) in the murine brain; a novel radiopharmaceutical for brain positron emission tomography (PET) imaging. *Biol Pharm Bull*. 1993; 16:146–9. [PubMed: 8395929]
- [104]. Obata A, Yoshimi E, Waki A, Lewis JS, Oyama N, Welch MJ, et al. Retention mechanism of hypoxia selective nuclear imaging/radiotherapeutic agent cu-diacetyl-bis(N4-

- methylthiosemicarbazone) (Cu-ATSM) in tumor cells. *Ann Nucl Med*. 2001; 15:499–504. [PubMed: 11831397]
- [105]. Shibuya K, Fujibayashi Y, Yoshimi E, Sasai K, Hiraoka M, Welch MJ. Cytosolic/microsomal redox pathway: a reductive retention mechanism of a PET-oncology tracer, cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) (cu-PTSM). *Ann Nucl Med*. 1999; 13:287–92. [PubMed: 10582796]
- [106]. Baerga ID, Maickel RP, Green MA. Subcellular distribution of tissue radiocopper following intravenous administration of 67Cu-labeled Cu-PTSM. *Int J Radiol Appl Instrum B Nucl Med Biol*. 1992; 19:697–701.
- [107]. Burgman P, O'Donoghue JA, Lewis JS, Welch MJ, Humm JL, Ling CC. Cell line-dependent differences in uptake and retention of the hypoxia-selective nuclear imaging agent Cu-ATSM. *Nucl Med Biol*. 2005; 32:623–30. [PubMed: 16026709]
- [108]. Warren LE, Horner SM, Hatfield WE. Chemistry of.alpha.-diketone-bis(thiosemicarbazone)copper(II) complexes. *J Am Chem Soc*. 1972; 94:6392–6.
- [109]. Minkel DT, Petering DH. Initial reaction of 3-ethoxy-2-oxobutylaldehyde bis (thiosemicarbazono) copper(II) with ehrlich ascites tumor cells. *Cancer Res*. 1978; 38:117–23. [PubMed: 201367]
- [110]. Petering DH. Physico-chemical properties of the antitumor agent, 3-ethoxy-2-oxobutylaldehyde bis(thiosemicarbazono) copper(II). *Bioinorg Chem*. 1972; 1:225–71.
- [111]. Taniuchi H, Fujibayashi Y, Okazawa H, Yonekura Y, Konishi J, Yokoyama A. Cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) (Cu-PTSM), a metal complex with selective NADH-dependent reduction by complex I in brain mitochondria: a potential radiopharmaceutical for mitochondria-functional imaging with positron emission tomography (PET). *Biol Pharm Bull*. 1995; 18:1126–9. [PubMed: 8535408]
- [112]. John EK, Green MA. Structure–activity relationships for metal-labeled blood flow tracers: comparison of keto aldehyde bis(thiosemicarbazono)copper(II) derivatives. *J Med Chem*. 1990; 33:1764–70. [PubMed: 2342070]
- [113]. Petering DH. The reaction of 3-ethoxy-2-oxobutylaldehyde bis (thiosemicarbazono) copper(II) with thiols. *Bioinorg Chem*. 1972; 1:273–88.
- [114]. Barnhart AJ, Voorhees WD, Green MA. Correlation of Cu(PTSM) localization with regional blood flow in the heart and kidney. *Int J Radiol Appl Instrum: B Nucl Med Biol*. 1989; 16:747–8.
- [115]. Maurer RI, Blower PJ, Dilworth JR, Reynolds CA, Zheng Y, Mullen GE, et al. Studies on the mechanism of hypoxic selectivity in copper bis(thiosemicarbazone) radiopharmaceuticals. *J Med Chem*. 2002; 45:1420–31. [PubMed: 11906283]
- [116]. Holland JP, Green JC, Dilworth JR, Holland JP, Green JC, Dilworth JR. Probing the mechanism of hypoxia selectivity of copper bis(thiosemicarbazono) complexes: DFT calculation of redox potentials and absolute acidities in solution. *Dalton Trans*. 2006:783–94. [PubMed: 16437173]
- [117]. Holland JP, et al. In vitro kinetic studies on the mechanism of oxygen-dependent cellular uptake of copper radiopharmaceuticals. *Phys Med Biol*. 2009; 54:2103. [PubMed: 19287086]
- [118]. Blower PJ, Went MJ, Martin KE, Smith GE. Imaging hypoxia *in vivo* by controlling the electrochemistry of copper radionuclide complexes. *J Labelled Comp Radiopharm*. 2007; 50:354–9.

Fig. 1. The ischemic cascade, modified with permission from Nesto and Kowalchuk [42] describing the gross events of ischemia, the point at which they are traditionally determined in the clinic by ECG abnormalities (dotted line), and where molecular imaging approaches could move that line to (arrow).

Fig. 2.

Rationale for tracer selection with respect to the ischemic/hypoxic cascade showing ischemic events in their approximate order, and the biophysical means we have available for quantifying them either in isolated perfused hearts, or *in vivo*. Regions in red denote biomarkers too far downstream for useful prognostic use while those in green denote those which may not be sufficiently extreme or specific to accurately delineate the most vulnerable myocardial tissue. Activation of HIF and loss of ATP may be the most appropriate parallel biomarkers against which to target hypoxia imaging agents for acute and chronic ischemic event, respectively.

Fig. 3.

Proposed trapping mechanism of nitroimidazoles in hypoxic tissue. Lipophilic nitroimidazoles diffuse into cells; they can then either diffuse out again, or become reduced to anionic radical species. In the presence of oxygen, they are re-oxidized and regenerated, whereupon they can again leave the cell, or enter a further round of reduction and re-oxidation. In hypoxic cells, oxygen is no longer available to re-oxidize them, and their reduction continues until they form species which covalently bind to intracellular macromolecules, and they become trapped. The green asterisk denotes the radiolabeled species.

Fig. 4. (Reproduced with permission from Shelton et al. [63]). Mid-ventricular PET images from a dog subjected to LAD occlusion. Left image: H_2^{15}O perfusion scan showing perfusion deficit in the anterior myocardium (septum is to the right); right image: ^{18}F MISO accumulation in anterior myocardium. ^{18}F MISO image reconstructed 60 min after tracer injection (10 mCi, i.v.) with blood pool correction performed from perfusion image.

Fig. 5. (Reproduced with permission from Fujibayashi et al. [98]). Autoradiographic images showing tracer accumulation in heart sections from an LAD-occluded rat heart. Left column: morphology photographs; middle column: distribution of ^{11}C -acetate 1 min after injection; right column: distribution of ^{64}Cu 10 min after ^{64}Cu -ATSM administration.

Fig. 6.

Proposed trapping mechanisms of radiocopper from BTSC complexes in hypoxic tissue. Lipophilic BTSC complexes are thought to diffuse into cells (although this is as yet unproven); they can then either diffuse out again, or become reduced to an unstable charged Cu(I) complex. This charged species is unable to leave the cell through diffusion. In the presence of oxygen, this Cu(I) complex is rapidly re-oxidized back to Cu(II), whereupon the complex can leave the cell, or become reduced again. In hypoxic cells, intracellular NADPH, bioreductive enzyme activities and thiol concentrations increase, promoting reduction of Cu(II) to Cu(I). There is also less oxygen available to re-oxidize the unstable complex. The reduced complex dissociates (most likely assisted by protonation, which may be augmented by acidosis), and the Cu(I) becomes sequestered by copper chelating proteins and trapped inside the cell. Protonation/deprotonation may occur in normoxic cells too, but we have shown the dominant mechanisms in this figure for the sake of simplicity. The green asterisk denotes the radiolabeled species.

Table 1

A selection of misonidazole and copper bis(thiosemicarbazonato) complexes, and their physicochemical properties. HSI = hypoxia selectivity. This list is by no means exhaustive, but shows the most fully characterized tracers tested to date. Abbreviations: Cu-PTSM pyruvaldehyde bis(*N*⁴-methylthiosemicarbazone), Cu-ATS 2,3-butanedione bis (thiosemicarbazone), Cu-ATSE: diacetyl-bis(*N*⁴-ethylthiosemicarbazone), Cu-ATSM 2,3-butanedione bis(*N*⁴-methylthiosemicarbazone), Cu-CTS 2,3-pentanedione bis(thiosemicarbazone), Cu-DTS 3,4-hexanedione bis(thiosemicarbazone), Cu-DTSM 3,4-hexanedione bis(*N*⁴-methylthiosemicarbazone), n.a. = not available.

Tracer [reference]	Partition ratio (log P)	Redox potential (V)	HSI	Structure
Misonidazole [110,111]	-0.39	-0.230	n.a.
Fluoromisonidazole [112]	-0.40	n.a.	n.a.
Cu-PTSM [71,82]	1.82	-0.51	0.161
Cu-ATS [71,82]	0.35	-0.59	0.657
Cu-ATSE [113]	2.34	n.a.	n.a.
Cu-ATSM [71,82]	1.61	-0.59	1.058
Cu-CTS [71,82]	1.01	-0.59	0.648

Tracer [reference]	Partition ratio (log P)	Redox potential (V)	HSI	Structure
Cu-DTS [71,82]	1.34	-0.59	0.652
Cu-DTSM [71,82]	1.57	-0.58	0.785