Imaging of molecular target modulation in oncology: challenges of early clinical trials

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Abstract The development of molecularly targeted anti-cancer therapies has resulted in a paradigm shift in the clinical drug development process. Phase 1 studies now routinely incorporate pharmacodynamic biomarker endpoints, specifically to evaluate whether a targeted therapy has the desired impact on its target and whether this leads to clinical benefit. Non-invasive molecular imaging using positron emission tomography (PET) has shown promise in this setting, in particular where target modulation directly impacts on glucose metabolism or cell proliferation. In this review, we discuss the challenges in identifying PET biomarkers of target modulation by a novel targeted therapy in the preclinical setting and in their translation into the clinical setting.

Keywords PET imaging · Molecular targeted therapy · Proof of mechanism · Proof of concept

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Introduction

Recent years have seen tremendous advances in understanding of the molecular mechanisms by which cancers develop and progress [1]. The development of drugs targeting these molecular processes is changing the face of modern oncology [2]. The majority of targeted therapies either interferes with key signal transduction pathway proteins, or interacts with cell surface receptors or antigens. The specificity of these drugs for their target generally means that their clinical use is associated with better tolerability than is shown by cytotoxic therapies still in general use.

Although promising, the development of molecularly targeted therapies in oncology has resulted in a paradigm shift in the clinical drug development process due to the significant challenges in obtaining reimbursement for targeted cancer therapeutics [3]. Rather than being drugs used in every patient with a particular cancer, molecular targeted drugs will generally have a smaller market since the relevant target may be present in only a small proportion of patients with a given form of cancer. Because of this and the enormous costs of drug development, pharmaceutical companies are seeking to make early strategic decisions regarding ongoing clinical development of targeted therapies. Therefore, in addition to the traditional safety and pharmacokinetics endpoints, phase I protocols are increasingly incorporating pharmacodynamic analyses to determine whether a drug has the desired impact on its target (proof of mechanism) and whether this leads to clinical benefit (proof of concept) [4].

While proof of mechanism has traditionally involved analysis of a putative biomarker in pre- and post-treatment biopsies, there are significant barriers in obtaining robust and adequately powered tissue biomarker results in clinical

trials. These include the unwillingness of subjects to undergo repeated biopsies for research purposes, the inaccessibility of lesions, biopsy sampling errors due to tumour heterogeneity, and inadequate biopsy samples [5]. The development of imaging biomarkers has the potential to overcome many of these limitations.

Although reduction in tumour dimensions on computed tomography (CT) or magnetic resonance imaging is often used as surrogate for treatment efficacy, many targeted therapies exert their effects through inhibition of tumour cell proliferation, resulting in tumour stabilisation rather than regression. Thus, according to the response evaluation criteria in solid tumours (RECIST) [6], growth arrest indicates stable disease rather than response. In addition, even when regression does occur, it may be a late phenomenon. The use of more appropriate pharmacodynamic biomarkers of tumour response to these drugs has therefore been recognised as essential to guide the clinical evaluation of these agents.

Molecular imaging

Molecular imaging involves the use of agents or techniques that enable visualisation of physiological or biological processes in an intact organism. In contrast to tissue biomarker studies, molecular imaging is non-invasive and easily repeated, overcoming the significant issues associated with tissue collection and quality. Although a wide range of molecular imaging modalities exist, in this review we focus on the use of positron emission tomography (PET) for defining target modulation in the early evaluation of novel targeted therapies in oncology.

PET is a tomographic imaging modality that utilises molecules labelled with positron-emitting radioisotopes to evaluate biological phenomena in cancer. Isotopes such as ^{15}O , ^{11}C , ^{13}N and ^{18}F can be incorporated into various small molecules and drugs. The rapid decay of these agents, ranging from a couple of minutes to a couple of hours, allows sensitive imaging at early time points after administration. Larger biomolecules including peptides and antibodies may take longer to clear from the circulation and therefore require imaging to be performed at later time points. Various radiometals are suitable for labelling such agents and include ^{68}Ga , ^{64}Cu , and ^{89}Zr with a half-life of decay ranging from an hour to several days.

There are many tracers with the potential to make PET the preferred imaging modality in oncology [7]. However, [^{18}F]-fluorodeoxyglucose (FDG) is the most commonly used agent in oncological practice. Its use is based on Warburg's fundamental observation that augmented aerobic glycolysis is a hallmark of cancer cells [8, 9]. FDG uptake reflects the combined effects of vascularity

(delivery of FDG to the cell), glucose transporter expression, the activity of hexokinase as the initial rate-limiting enzyme of glycolysis, and the metabolic rate of the cell. The high sensitivity and specificity of FDG PET imaging have facilitated its widespread use for staging and monitoring of tumour response to therapy in both clinical and research settings [10–12].

Imaging cell proliferation

Tumour growth is characterised by unrestrained cell division and tumour cells therefore have higher rates of proliferation than normal cells. The PET tracer, 3'-deoxy-3'-[^{18}F]fluoro-L-thymidine (FLT) is a thymidine analogue used to assess cell proliferation [13, 14]. It is taken up into the cell by the nucleoside salvage pathway transporters, predominantly by the family of equilibrative nucleoside transporters. Once inside the cell FLT is phosphorylated in the cytosol by thymidine kinase-1 (TK1) generating FLT monophosphate, which is not a substrate for further metabolism and so becomes trapped in the cell. As thymidine kinase expression is cell cycle regulated, increasing from late G1 and reaching maximal levels in the S-phase before being degraded during mitosis [15, 16], an FLT signal depends on cell proliferation and therefore represents an indirect PET tracer of cell proliferation. This is further supported by correlation of FLT uptake with other biomarkers of cell proliferation such as Ki-67 [17–19].

However, recent studies have also shown that modulation of FLT uptake may reflect changes in expression of the nucleoside transporters resulting from alterations in cellular use of salvage and *de novo* pathways for nucleotide production. Indeed, anticancer drugs that inhibit key enzymes in the *de novo* DNA synthesis pathway result in increased cellular FLT uptake due to the cells switching to use the salvage pathway for thymidine production [20, 21].

FLT PET imaging has shown some promise in the assessment of early response to conventional and targeted therapies [22], including docetaxel in breast cancer [23], chemoradiotherapy in head and neck cancer [24, 25], and gefitinib in lung adenocarcinoma [26]. Its use in this setting, however, remains controversial with several studies demonstrating no correlation between changes in FLT uptake and morphologic tumour response [27–29].

Challenges in identifying imaging biomarkers of target modulation in the preclinical setting

While an ideal imaging biomarker would directly demonstrate that a drug modulates its target, there are significant fiscal and regulatory impediments to the development of target-specific imaging biomarkers in oncology [7].

Therefore, in practice, imaging biomarkers rely on identification of common downstream phenotypic changes that occur as a consequence of target modulation. As most anti-tumour drugs ultimately exert their effects by impairing tumour metabolism or cell proliferation, studies to identify imaging biomarkers of response often focus on the evaluation of FDG PET and FLT PET scans. Where proof of mechanism can be demonstrated using PET, the same imaging biomarker may also be useful as a proof-of-concept biomarker, if it can be shown to predict tumour response to the drug. Preclinical studies play an important role in demonstrating that target modulation is indeed associated with changes in imaging biomarkers, and that these are, in turn, predictive of tumour response.

Continual technological advances over the past 15 years have facilitated the development of high-resolution pre-clinical PET systems, which provide a powerful platform for translational research [30]. Preclinical tumour models have become increasingly sophisticated in recent years. Moving beyond xenografts generated from human cell lines grown in culture, there is now a vast array of genetically engineered mouse models (GEMMs) with spontaneous tumour development recapitulating the genomic background of human cancers. Another important advance has been the development of the technique of patient-derived xenografts (PDX) in which patient tumour tissue is directly implanted into and then passaged through mice [31]. PDX models faithfully recapitulate the heterogeneity and biology of the clinical specimen from which they were obtained, thereby overcoming a significant limitation of traditional cell culture-based xenografts [32].

While FDG and FLT are the most commonly used PET tracers for evaluating novel targeted therapies, they present some limitations as regards translation between the pre-clinical and clinical settings that need to be considered when performing biomarker studies to identify imaging surrogates for target modulation. In contrast to patient tumours, many tumour xenografts exhibit low baseline FDG uptake. As a result, identifying a preclinical xenograft model with a signal-to-noise ratio sufficient for assessing drug-induced changes in uptake can be challenging, particularly when a specific tumour genotype is required for drug activity (e.g. expression of a mutant form of a target kinase). In contrast, tumours arising in GEMMs (e.g. *Kras*^{G12D}; *Pten*^{del} mouse model of ovarian cancer [33], and the TH-MYC^N mouse model of neuroblastoma (Wood, Cullinane, Hicks et al., unpublished data) more faithfully reflect FDG uptake in the clinical setting with respect to the imaging phenotype.

The biodistribution of FLT differs significantly between mice and humans (Fig. 1). In humans, FLT accumulates in the highly proliferative bone marrow compartment, is metabolised through the liver, and has relatively low

Fig. 1 Variable biodistribution of FDG and FLT in mouse and human FDG PET (*left*) and FLT PET (*right*) images of a mouse bearing a 2008 ovarian tumour (*top*) and a human ovarian cancer patient (*bottom*). Arrows indicate the position of the 2008 tumour. FDG uptake into preclinical tumour models is generally less robust than FLT uptake (*top panels*). In contrast, a pattern of high FDG and low FLT uptake is frequently observed in human tumours (*lower panels*). The characteristic FLT uptake into liver and bone marrow in humans is not observed in the mouse (*right panels*). Substantial FDG uptake into the harderian gland behind the eye is observed in mice (color figure online)

tumour uptake compared to FDG. Conversely, mice demonstrate significantly higher plasma levels of thymidine than do humans, have little basal bone marrow FLT uptake, and excrete FLT unchanged in the urine without significant hepatic uptake [34, 35]. FLT uptake in tumour xenografts is inversely related to the intrinsic tumour thymidine concentration, which, when coupled with the high rate of proliferation typically associated with human xenografts, results in a high proportion of human xenografts with high basal FLT uptake in mice. In contrast, tumours arising in GEMMs and syngeneic tumour models generally show low FLT uptake, presumably due to their relatively high intratumour thymidine levels.

In preclinical imaging biomarker studies, the higher signal-to-noise ratio associated with FLT may lead to more striking drug-induced changes than are seen with FDG [36, 37] (Fig. 1), but may misinform translation into the clinical environment. Therefore, careful consideration of the differences between these tracers in the preclinical versus clinical settings is required when selecting a PET tracer for early clinical biomarker evaluation.

Challenges in extrapolating findings from preclinical studies to the clinical domain

Preclinical imaging biomarker studies have two key objectives. The first is to define the association between a tissue biomarker that directly measures the effect of the drug on its target and the imaging biomarker. In practice, while a cohort of tumour-bearing mice is subjected to longitudinal PET imaging, tumours are, at the same time, harvested from a parallel cohort for ex vivo biomarker analysis and testing of correlations between the different biomarkers investigated. The second aim is to explore the impact of different drug doses and schedules to define the relationship between drug exposure and the tissue and imaging biomarkers. Achievement of these key goals in preclinical studies will indicate the optimal drug dose and schedule, as well as the optimal PET tracer and imaging time for evaluation in clinical trials.

Where preclinical studies support the association between an imaging biomarker and an appropriate tissue biomarker of target modulation, further investigation of this association in early clinical development of the compound is warranted. These proof-of-mechanism biomarker studies are typically performed in an expanded cohort of patients once the maximum tolerated dose of the drug has been determined in a Phase 1 study. The patient population in which the study is performed must have tumours in which the expression of the drug target is relevant. This usually requires screening of patient tumours for expression of the appropriate target, for example a mutant kinase such as BRAF^{V600E} in melanoma patients for evaluation of type 1 BRAF inhibitors or ALK-positive tumours for drugs such as crizotinib. In rare cases, where a tumour is driven by a single oncogene such as *BCR-ABL* in chronic myeloid leukaemia, selection of patients with this disease is sufficient for evaluation of inhibitors of the oncogenic protein. These studies must also be adequately powered to demonstrate a significant difference between pre- and post-treatment biomarker analyses, where one exists. Typically, these studies involve 15–30 patients [38–40]. However, the existence of a logical link between tumour biology and preclinical proof-of-concept and clinical proof-of-mechanism studies does not guarantee that these imaging biomarkers will be successfully introduced into clinical therapeutic response assessment. Below we describe examples in which the successful identification and evaluation of imaging biomarkers for proof of mechanism of novel targeted therapies in the preclinical and clinical settings have been achieved; however, these biomarkers have not yet entered daily clinical practice.

FDG PET as an imaging biomarker in oncogene-driven tumours

Imatinib

Gastrointestinal stromal tumour (GIST) is a rare tumour characterised by activation of the c-KIT oncogene [41]. In vitro studies using cell lines expressing the activating mutations in c-KIT found in GIST revealed that the small molecule KIT inhibitor, imatinib, causes a rapid reduction in cellular uptake of the glucose analogue, 2-deoxyglucose. Furthermore in vivo, imatinib therapy resulted in a rapid reduction in FDG uptake and membrane expression of GLUT-1 [42]. The direct association between imatinib and, in subsequent studies, nilotinib [43] treatment and inhibition of glucose uptake therefore supported the use of FDG PET in proof-of-mechanism studies of drugs targeting this receptor. In the clinical setting, FDG uptake into GISTs that express imatinib-sensitive mutations in c-KIT is significantly reduced within 48 h of imatinib therapy, consistent with the ability of the drug to modulate its target. Despite this powerful molecular imaging signal of drug effectiveness, CT remains the most common modality for assessing response following modification of RECIST criteria to reduce the misclassification of response in comparison to FDG PET. The so-called Choi criteria were established to reconcile the observation that some patients who demonstrated apparent progression according to RECIST had a complete metabolic response on FDG PET and had a favourable prognosis [44]. This phenomenon has since been called “pseudoprogression”. The ability of these revised CT criteria to approximate the findings on FDG PET, which was used as the validating gold standard, has been considered sufficient to allow CT to remain the preferred modality for response assessment in most clinical situations.

Vemurafenib

Activating mutations in the *BRAF* oncogene are known to occur in approximately 50 % of malignant melanoma cases. As B-Raf signalling promotes cell proliferation and survival through the mitogen-activated protein kinase (MAPK) pathway, several mutant B-Raf kinase inhibitors including vemurafenib and dabrafenib have been developed. In vitro studies suggest that activation of the MAPK signalling pathway by mutant BRAF impacts on several pathways regulating glucose metabolism, including the liver kinase B1–AMP-activated protein kinase (LKB1–AMPK1) energy sensor pathway and GLUT-1 expression [45, 46]. Extracellular signal-related kinase (ERK), which functions downstream of B-Raf, phosphorylates the tumour suppressor LKB1, which leads to uncoupling of the

LKB1–AMPK1 complex such that LKB1 is no longer able to activate AMPK, thereby promoting cell growth, proliferation and survival in conditions of energy stress [45]. ERK also phosphorylates a number of transcription factors that regulate the expression of the glucose transporter GLUT-1 [46, 47]. Together, these findings suggest that FDG PET may be an indirect readout of B-Raf inhibitor-mediated MAPK pathway inhibition.

In the preclinical setting, treatment of BRAF^{V600E} mutant melanoma models with vemurafenib indeed led to a rapid reduction in FDG uptake, supporting the hypothesis that FDG PET is a useful pharmacodynamic marker of BRAF inhibition [48] (Fig. 2). Further, it was shown that development of resistance is associated with restoration of FDG uptake [49].

Fig. 2 Preclinical and clinical PET imaging of early tumour response to therapy FDG PET images of Balb/c nude mice bearing BRAF^{V600E} mutant LOX-IMVI tumours imaged at baseline and 1 day following combination therapy with the BRAF inhibitor PLX-4720 (*top panels*). FDG PET images of a melanoma patient with a BRAF^{V600E} mutant tumour responding to the BRAF inhibitor vemurafenib (*bottom panels*). Baseline uptake of FDG is typically higher in melanoma in patients than preclinical melanoma models but both demonstrate the concept of early metabolic response following modulation of signalling through the involved oncogenic pathway

FDG PET has also been investigated as an imaging biomarker following vemurafenib therapy in two early-phase clinical studies. Bollag et al. [50] demonstrated a major reduction in FDG uptake following 14 days of treatment with vemurafenib. McArthur et al. [40] also performed FDG PET imaging in 31 patients prior to and on day 15 of vemurafenib therapy and all showed a metabolic response according to prospectively defined criteria. As this study was performed in patients at different dose levels, the results also provide insight into the relationship between plasma levels and target inhibition.

Together, these data suggest that FDG PET is a robust clinical imaging marker of inhibition of BRAF^{V600E} mutant melanoma (Fig. 2). Nevertheless, a metabolic response was not necessarily associated with tumour regression on subsequent CT. Thus, it appears that target modulation may be a necessary, but sometimes insufficient, condition for therapeutic response. This leaves in question whether monitoring of therapeutic response to BRAF inhibitors should be performed by early FDG PET, potentially allowing a change in treatment or dose escalation in non-responders, or should simply continue to be evaluated by morphological imaging, using CT later during treatment.

FLT PET as an imaging biomarker for target inhibition by a CDK4/6 inhibitor

FLT has been successfully evaluated as a biomarker of target inhibition and response to a number of targeted therapies, in particular compounds that directly impact on the cell cycle. Progression through the cell cycle requires co-ordinated interaction between the cyclins and their partner proteins, the cyclin dependent kinases (CDKs). Upon binding to cyclin D1, CDK4 drives G1 cell cycle progression through its phosphorylation of Rb, which leads to release of the E2F transcription factor and expression of S-phase genes [51–53]. Deregulation of the CDK4/cyclin D/Rb pathway occurs commonly in cancer through amplification of cyclin D, mutational activation of CDK4 or loss of the negative regulator, p16 [54]. CDK4 has therefore been identified as a target for therapeutic intervention in cancer. Consistent with its mechanism of action, the CDK4/6 inhibitor PD-0332991 induced arrest in the G1 phase of the cell cycle in p16-null MDA-MB-231 cells in vitro [35]. FLT PET was therefore investigated as an imaging biomarker in preclinical proof-of-mechanism studies for this agent. Target modulation by PD-0332991 following treatment of the MDA-MB-231 xenograft model was demonstrated by hypophosphorylation of Rb in tumour tissue and this correlated closely with inhibition of FLT uptake [35]. Drug-induced changes in FLT uptake were also correlated with other tissue markers of cell proliferation, including bromodeoxyuridine, Ki-67 and TK-1

expression. These results strongly support the use of FLT PET in early clinical studies to demonstrate proof of mechanism for this cell cycle-specific targeted agent.

FLT PET has been explored as an imaging biomarker in early clinical studies to evaluate PD-0332991 in the setting of mantle cell lymphoma, which exhibits the t(11;14) (q13;q32) translocation and aberrant B cell expression of cyclin D1 [39]. A substantial reduction in FLT, but not FDG, uptake was observed in tumours at an early time point during therapy in the majority of patients and this correlated well with reduction in Rb phosphorylation and Ki-67-positive cells. Together, these findings provide proof of mechanism of this drug in the clinical setting. Nevertheless, FLT PET has not yet entered clinical practice for the monitoring of any form of therapy.

Conclusion

To date, FDG and FLT have been the key PET tracers evaluated as imaging biomarkers of target modulation in the field of oncology. As discussed, there have been a number of cases in which these tracers have proven to be markers of target inhibition and to be predictive of tumour response. Nevertheless, despite its recognized limitations, morphologic imaging remains the dominant technology for therapeutic response assessment. It needs to be recognized, however, these agents are at best indirect markers of target inhibition by a drug. Although specific PET tracers that directly inform target modulation by novel targeted drugs may provide a means of demonstrating proof of mechanism and thus expedite drug development in the future, unless existing molecular imaging techniques such as FDG and FLT become accepted for clinical therapeutic response assessment of targeted agents, there will continue to be a strong disincentive for the development of such tracers.

Conflict of interest Carleen Cullinane, Benjamin Solomon and Rodney Hicks have nothing to disclose.

Ethical approval Compliance with Ethics Guidelines.

Human and Animal studies All studies involving animal or human subjects were performed with approval from the appropriate Peter MacCallum Cancer Centre ethics committee and therefore have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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