REVIEW ARTICLE

The future of imaging: developing the tools for monitoring response to therapy in oncology: the 2009 Sir James MacKenzie Davidson Memorial lecture

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ABSTRACT. Since the days of Sir James MacKenzie Davidson, radiology discoveries have been shaping the way patients are managed. The lecture on which this review is based focused not on anatomical imaging, which already has a great impact on patient management, but on the molecular imaging of cancer targets and pathways. In this post-genomic era, we have several tools at our disposal to enable the discovery of new probes for stratifying patients for therapy and for monitoring response to therapy sooner than is possible using conventional cross-sectional imaging methods. I describe a chemical library approach to discovering new imaging agents, as well as novel methods for improving the metabolic stability of existing probes. Finally, I describe the evaluation of these probes for clinical use in both pre-clinical and clinical validation. The chemical library approach is exemplified by the discovery of isatin sulfonamide probes for imaging apoptosis in tumours. This approach allowed important desirable features of radiopharmaceuticals to be incorporated into the design strategy. A lead compound, [^{18}F]ICMT11, is selectively taken up *in vitro* in cancer cells and *in vivo* in tumours undergoing apoptosis. Improvement of the metabolic stability of a probe is exemplified by work on [^{18}F]fluoro-[1,2- $^2\text{H}_2$]choline ("[^{18}F]-D4-choline"), a novel probe for imaging choline metabolism. Deuterium substitution significantly reduced the systemic metabolism of this compound relative to that of non-deuteriated analogues, supporting its future clinical use. In order for probes to be useful for monitoring response a number of validation and/or qualification studies need to be performed, including assessments of whether the probe measures the target or pathway of interest in a specific and reproducible manner, whether the probe is stable to metabolism *in vivo*, what is the best time to assess response with these probes and finally whether changes in radiotracer uptake are associated with clinical outcome. [^{18}F]Fluorothymidine, a probe for proliferation imaging has been validated and qualified for use in breast cancer. In summary, the ability to create new molecules that can report on specific targets and pathways provides a strategy for studying response to treatment in cancer earlier than it is currently possible. This could fundamentally change the way medicine is practised in the next 5–10 years.

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Radiological discoveries, particularly in the areas of magnetic resonance and X-ray computed tomography, have been shaping the way cancer patients are managed since the days of Sir James MacKenzie Davidson. In addition to these cross-sectional imaging approaches, radiologists and nuclear medicine physicians increasingly have at their disposal more functional and molecular imaging technologies for making evidence-based decisions. These include methods for patient stratification for therapy, as well as methods for early assessment of

response. These methods also have a role in oncology drug development [1]. The evolution of discoveries within this field has largely resulted from a clearer understanding of the molecular pathology of cancer, including cancer-causing genes and the cellular pathways that their encoded proteins control, and of tumour–host interactions (Figure 1). Knowledge of cancer types, which are addicted to specific gene products, and the increasing availability of new drugs, which target subsets of patients that express these proteins, have also spurred a need to develop biomarkers for identifying such patients and monitoring their response to therapy [2, 3]. A recent review by Contractor and Aboagye [4] showed that imaging biomarkers are being used to assess the target activity and response of patients to novel mechanism-based therapeutics.

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Figure 1. A better understanding of the molecular pathology of cancer involving tumour–host interactions provides a mechanism for probe discovery in imaging science.

Particularly attractive for biomarker discovery are targets and pathways that are associated with the six hallmarks of cancer. These are essential alterations that collectively define malignant growth and are common to probably all human cancers [5]. The six hallmarks are: i) self-sufficiency in growth signals, ii) insensitivity to growth-inhibitory (antigrowth) signals, iii) evasion of programmed cell death (apoptosis), iv) limitless replicative potential, v) sustained angiogenesis, and vi) tissue invasion and metastasis [5]. Pathways that represent the congruence of many protein activities are probably more attractive and cost-effective targets for the development of imaging biomarkers than those that measure specific molecular targets. For example, the Vogelstein and Kinzler group recently reported that the many genetic alterations that are involved in pancreatic cancer correspond to just 12 alterations in core signalling pathways, including those responsible for the regulation of apoptosis, DNA damage control, control of G1/S phase transition, invasion, homophilic cell adhesion, and signalling involving TGF β , K-Ras, JNK, integrin, small GTPase, Wnt/Notch or hedgehog [6]. Several of these pathways can be exploited for imaging.

The discovery of novel imaging biomarkers is not a simple task and many complementary combinatorial chemistry approaches (*i.e.*, approaches using the rapid synthesis of a large number of different but structurally related molecules or materials) have been used [7–11]. Two approaches for positron-emitting radiotracer biomarker discovery described below exemplify this strategy. Another important milestone for getting new biomarkers into the clinic is their validation and qualification. Validation involves testing whether the biomarker measures what it is supposed to and that the endpoint is

reproducible. Qualification involves confirming that the endpoint is related to the clinical outcome.

Discovery of new radiotracers — the chemical library approach

The chemical library approach for the discovery of new radiotracers is analogous to that used in drug discovery. The process starts with identifying a target – the crystal structure of a protein, the cleavage of a protein or another mechanism of action of a target or pathway – that could be exploited. A series of design goals are then set that are used to inform the synthesis of a library of compounds. There are, however, deviations from the drug development pathway. The lipophilicity of compounds selected for imaging is often less than that for drugs because enhanced elimination of radiotracers from the body is sought after as a means of reducing radiation dose. *Log P* values (the logarithm of the partition coefficient or lipid/aqueous solubility) in the range 1–2 are sufficient for radiotracers used for imaging visceral tumours; higher values (>1.5–2) are more appropriate for cerebral tumours [12]. Because radiotracers have faster clearance rates than drugs at pharmacological doses, it is essential to retain the highest degree of metabolic stability.

Facile radiosynthesis is another important design goal. With the increasing availability of many elegant end-stage radiolabelling methodologies, the desired radiolabelling approach can be included in the design of a combinatorial chemical library such that once a lead candidate is selected, it is obvious which radiolabelling approach will be employed. Two types of the

combinatorial chemical library approach have been described. The first approach, typified by [^{11}C]carbon monoxide-based radiolabelling, has been described by Långström and co-workers [9]. This approach permits many radiolabelled analogues to be synthesised for testing. The second approach, typified by fluorine-18 radiolabelling in the field of apoptosis imaging, has been described by the groups led by Mach [11], Kopka [13] and Aboagye [16]. This approach allows both the cold unlabelled analogues to be tested more extensively and the labelling of the lead compound derived from the library screening. Both combinatorial approaches offer a rapid and thorough way of getting to the optimal imaging biomarker for the target.

Development of apoptosis imaging biomarkers using the chemical library approach

Deregulation of apoptosis (*i.e.*, deregulation of programmed cell death) is a hallmark of cancer, and most anti-cancer agents kill tumour cells by inducing apoptosis [5, 17, 18]. It is envisaged that quantification of apoptosis in patients will enable early prediction of response to therapy (within 1–2 days of therapy).

Outside the field of cancer, quantification of apoptosis has a role in evaluating the pathophysiology of acute myocardial infarction and neurodegeneration. A number of cellular biochemical processes have been exploited for developing imaging biomarkers to assess apoptosis. [$^{99\text{m}}\text{Tc}$]-HYNIC-Annexin-V, a radiopharmaceutical agent for SPECT imaging is the most advanced of the radiotracers used in a clinical context [19]. This compound, and related radiotracers used for positron emission tomography (PET) [20, 21], are ~36 KDa proteins that bind to phosphatidylserine, a phospholipid which flips from the inside to the outside of the cell during apoptosis. Other imaging biomarkers that interact with the cell membrane consequent to changes in membrane potential during apoptosis include a series of *N,N*-didansyl cysteine, dansyl ethylfluoroalanine and malonic acid derivatives [22].

In the past few years, several groups have made attempts to image caspase-3/7 biochemistry, an intracellular enzymatic process that is involved in the apoptosis cascade, as a more specific readout for apoptosis. Two approaches – assessment of enzymatic activity and assessment of the binding of compounds to reactive surfaces on the cleaved enzyme – have been exploited for imaging. Probes based on the caspase-3-specific Asp-Glu-Gly-Asp (DEVD) peptide sequence have been found to be highly polar in nature, a character that influences their cellular uptake. The importance of the unidirectional uptake of probes designed for intracellular targets is exemplified in the use of the HIV-Tat sequence to increase cellular uptake, which is independent of caspase-3 enzymatic activity [23].

More recently, the laboratories of Kopka, Mach and Aboagye have developed isatin sulfonamides for imaging apoptosis by PET [11, 13–16]. These compounds covalently bind the active site of the activated caspase-3/7 enzyme to form an intracellular enzyme-inhibitor complex. Specifically, the dicarbonyl functionality of the isatin sulfonamide (Figure 2) binds to a cysteine residue

at the active site to form a thiohemiketal that involves the electrophilic C-3 carbonyl carbon of the isatin sulfonamide and the nucleophilic cysteine thiolate functionality [24, 25].

All three laboratories (Kopka, Macha and Aboagye) have used a focused chemical library approach for selecting the lead compound. In our case, the design goals for structural optimisation of the series included: i) rapid bi-directional uptake and retention only in cells with activated caspase-3/7, ii) high selective affinity for caspase-3/7, iii) relatively low lipophilicity, iv) low systemic metabolism, v) facile radiosynthesis and vi) high specific radioactivity as this was a binding phenomenon. To permit facile radiosynthesis, we incorporated 2'-fluoroethyl-1,2,3-triazole both in the N-1 position and on the left side ether position of the isatin 5-sulfonamide scaffold; this permits "click chemistry" labelling [26] of the selected lead compound. Initial studies in predictive models *in vitro* demonstrated that the (S)-2-phenoxyethyl moiety was a major site of metabolism. Incorporation of heterocycles and fluorine-substituted aromatic groups reduced metabolic instability; fluorine substitution in itself increased the potency of the compounds [11, 16].

A selected number of analogues from the focused library designed in this example are shown in Figure 2. The lead compound derived from the screen was ICMT11 (highlighted in blue), which showed promise as a marker of apoptosis in living subjects [11, 27]. Apoptosis in 38C13 lymphoma tumours showed increased radiotracer uptake together with increased (~4–5%) cleaved caspase-3 immunostaining at 24 h after chemotherapy (Figure 2). These early studies have highlighted a number of issues that should be addressed in the clinical testing of these new radiotracers. The methods will benefit from simultaneous cross-sectional imaging of tumours as their uptake at baseline is very low. The early response, detectable 24 h after initiating treatment, is also evident with annexin-V-based radiotracers. Whether the peak for apoptosis differs for different anti-cancer drugs and drug-combinations remains to be seen.

Discovery of new radiotracers – methods for improving biomarker performance

The key advantages of using radiotracers like [^{18}F]fluorodeoxyglucose (FDG) for cancer imaging include their high cellular flux in many common tumours, together with their high metabolic stability. The latter property is, however, not present in many of the other oncology radiotracers designed to date. Another radiotracer with high cellular flux in malignant tumours is [^{11}C]choline. This radiotracer and its fluoromethyl analogue suffer from metabolic instability – conversion of the parent compounds to their respective betaine analogues – rendering late PET images (>50 min) difficult to interpret. As described above for the isatin sulfonamide series, there are a number of ways to reduce the metabolic instability of novel compounds. By contrast, there are only a few ways to modify the stability of existing compounds without significantly modifying their affinity or uptake/flux properties. Examples of

Figure 2. Discovery of imaging agents. The combinatorial library approach, exemplified by the discovery of isatin sulphonamide, [¹⁸F]ICMT-11. A selected number of analogues from the focused chemical library are shown. The lead compound derived from the screen was ICMT11 (highlighted in blue). Apoptosis in 38C13 lymphoma tumours showed increased radiotracer uptake together with increased (~4–5%) cleaved caspase-3 immunostaining at 24 h after chemotherapy.

chemical modifications that can improve systemic metabolic stability include the replacement of hydrogen or hydroxyl with fluorine, which has been exemplified in radiochemistry [28–30]. Other chemical strategies, such as the replacement of carbonyl with silanediol isostere, could also prove useful in the future [31]. A number of groups have examined the use of substituting deuterium for hydrogen as a means of improving the stability of radiotracers without changing the structure of the compound [32, 33]. We have exploited the deuterium isotope effect to improve the stability of [¹⁸F]fluorocholine [34]. We developed [¹⁸F]fluoromethyl-[1,2-²H₄]-choline, which has significantly higher *in vivo* metabolic stability than [¹⁸F]fluorocholine, and results in images with high signal-to-noise contrast (Figure 3). Owing to its improved stability, this radiotracer should better enable late imaging of tumours after sufficient clearance of the radiotracer from systemic circulation [34]. Furthermore, the diagnostic sensitivity should be improved through increased availability of substrate for tumour uptake. Future studies

in humans will determine the clinical value of this new radiotracer in imaging prostate cancer, where [¹¹C]choline has been shown to have value [35–37], and for other indications where [¹¹C]choline uptake or choline kinase expression is altered, including breast [38, 39], ovarian [40] and lung cancer [41].

Validation and qualification of new imaging biomarkers

All newly discovered imaging probes must undergo validation and qualification in multiple centres to provide confidence for their use in drug development and for patient management. The level of validation/qualification required increases from non-pivotal phase I/II studies to pivotal phase III/regulatory therapeutic trials or for licensed use in patient management. Our research group has contributed significantly to the validation/qualification of [¹⁸F]fluorothymidine-PET (FLT-PET).

Figure 3. Chemical structures of [^{18}F]fluoromethyl-[1,2- $^2\text{H}_4$]-choline and its non-deuterated analogue. The deuterated form has greater metabolic stability and improved imaging properties.

Since the first major report of the use of FLT-PET for cancer imaging in 1998 [42], there have been more than 50 reported human studies with this radiotracer. Hence this is not meant to be a comprehensive review of studies involving FLT-PET, but rather an account of key studies that have biologically validated/qualified its use in monitoring therapy response. FLT is a safe radiopharmaceutical [43]. Several laboratories have contributed to addressing the important question of whether FLT-PET measures what it is supposed to, *i.e.*, cell proliferation.

As illustrated in Figure 4a, FLT is phosphorylated by thymidine kinase 1 (TK1) at the 5'-OH position to give FLT-phosphate, which is charged and therefore retained within cells to give the PET signal. Cellular uptake of FLT depends on TK1 and ENT1 transporter activity [44–46]. The relationship between FLT (unlike thymidine) and cell proliferation is not by virtue of its incorporation into DNA (there is very little incorporation of FLT into DNA) but rather the regulation of TK1 within the cell cycle. TK1 is transcriptionally regulated in the

cell cycle (Figure 4b). Peak levels of the enzyme are seen in S-phase (the DNA synthesis phase) of the cell cycle and the enzyme is targeted for degradation in late M-phase (mitosis phase), such that there is low TK1 expression in newly divided cells [47, 48]. There is also evidence to support a post-translational signal attenuation mechanism involving increased phosphorylation of TK1 to produce a less active enzyme, which has reduced ability to form the more active tetrameric complex [49–51]. Initial studies in patients with untreated lung and breast cancer showed that simple measurements of uptake, *e.g.*, standardised uptake value (SUV), correlated with Ki67 immunohistology of biopsy material [52, 53]. Kenny and co-workers [53] further demonstrated that the FLT flux constant, *i.e.*, the net irreversible trapping of FLT at steady state, was more strongly associated with cell proliferation as assessed by histology. Modelling of FLT kinetics improves accuracy in visceral tumours, but it was found to be essential for brain tumours [53–57]. Simplified methods of blood sampling have been

Figure 4. (a) Mechanism of cellular retention of FLT involving phosphorylation of the radiotracer by thymidine kinase 1. Phosphorylation increases the charge on the molecule which is then trapped within the cell. A putative deoxynucleotidase could dephosphorylate FLT. (b) A generic model constructed for defining drug types that will inhibit FLT uptake based on transcriptional control of thymidine kinase 1 (TK1). Currently, this model requires empirical data to define both the timing of imaging post-therapy and the relationship between imaging endpoint and cell proliferation in disease models of cancer. In this model hyperphosphorylation of Rb protein by cyclin D/cdk4/6 and cyclin E/cdk2 during G1-S transition leads to release of E2F, which activates transcription of cell cycle genes including TK1. Mitogenic signaling inhibitors, e.g. MEK inhibitor, may act by ultimately inhibiting cyclin D expression and inhibitors of aurora kinase may act through activation of p21/WAF1. R-point, restriction point.

proposed to support this approach [58, 59]. Although encouraging, these studies did not validate FLT-PET as a marker of response to chemotherapy. With the exception of drugs that inhibit thymidylate synthase [45, 60, 61], most drugs tested in pre-clinical trials cause a predictable time- and dose-dependent reduction in FLT-PET signal [44, 62–69]. This decrease in FLT-PET signal is associated with reduced expression of Ki67, cyclin D, hyperphosphorylated Rb and TK1 (Figure 4b).

The information obtained from these pre-clinical studies has formed the basis of FLT clinical trial design in therapeutic studies. Future clinical imaging studies conducted with matched tumour biopsies (neoadjuvant, window-studies, etc) will further validate the clinical utility of FLT-PET in the context of therapeutic response. Interestingly, at least two studies involving breast and lung cancer have demonstrated that FLT-PET can be used to assess response early — at 1 week after treatment [70, 71]. Other studies have used ≥ 2 weeks to assess therapeutic response [72–74]. Another aspect of biological validation is reproducibility: the precision of the

technique in drug-naïve patients. Single-centre studies in breast and lung cancer have shown that the SUV value derived from PET studies (ranging from 30 to 90 min) is reproducible to $\sim 10\%$ [70, 75, 76]. This is not dissimilar to that of [¹⁸F]fluorodeoxyglucose-PET [77].

Do FLT-PET signal changes predict clinical outcome? In other words, in the context of drug efficacy, is the technique qualified for use in human cancers? Only a few studies have been done to support qualification, but the data are very encouraging. For instance, Kenny and co-workers [70] showed that stage III–IV breast cancer patients who responded (tumour size reduction at 6 weeks) to combination fluorouracil-epirubicin-cyclophosphamide treatment had significant reductions in the FLT-PET signal (Figure 5a). The PET endpoint (SUV or flux constant) preceded changes in tumour size. The objective response criterion was derived prospectively from test-retest reproducibility studies (Figure 5b). Furthermore, Sohn and co-workers [71] showed that in non-smokers with advanced or recurrent lung cancer receiving an epidermal growth factor receptor antagonist,

Figure 5. The use of FLT-PET to assess early response (at 1 week post-treatment) to treatment in individual breast cancer patients. (a) Response is observable as a reduction of FLT uptake in the right breast (arrowed). (b) A prospective objective response cut-off, obtained from test-retest reproducibility studies, is used to identify lesions that have responded to treatment.

gefitinib, the change in SUVmax for FLT discriminated responders from non-responders with a sensitivity and specificity of 93%; a retrospective cut-off was used for objective FLT response. Importantly, FLT-PET predicted progression-free survival. The endpoint did not predict overall survival, presumably because patients whose cancers were progressing after gefitinib treatment were offered salvage therapy [71]. Multicentre qualification studies with prospectively defined imaging response criteria will provide further information on the utility of FLT-PET for early response assessment.

Conclusion

In summary, the ability to create new molecules that can report on specific targets and pathways provides a strategy for studying therapeutic response in cancer patients earlier than is possible at present. Better understanding of the molecular pathology of cancer is providing knowledge of key cellular processes on which to focus probe development efforts. The new imaging probes can be made to be target-specific

and metabolically stable. We are already at the stage where pharmacological response in the context of cellular proliferation signals can be measured reliably at 1 week after treatment. New methods for imaging apoptosis could enable us to predict response within hours of treatment initiation. These developments would fundamentally change the way medicine is practised in the next 5–10 years.

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