NON-INVASIVE MOLECULAR IMAGING FOR PRECLINICAL CANCER THERAPEUTIC DEVELOPMENT

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Abstract

Molecular and non-invasive imaging are rapidly emerging fields in preclinical cancer drug discovery. This is driven by the need to develop more efficacious and safer treatments, the advent of molecular targeted therapeutics, and the requirements to reduce and refine current preclinical *in vivo* models. Such bioimaging strategies include magnetic resonance imaging (MRI), positron emission tomography (PET), single positron emission computed tomography (SPECT), ultrasound, and optical approaches such as bioluminescence and fluorescence imaging. These molecular imaging modalities have several advantages over traditional screening methods, not least the ability to quantitatively monitor pharmacodynamic changes at the cellular and molecular level in living animals non-invasively in real-time. This review aims to provide an overview of non-invasive molecular imaging techniques, highlighting the strengths, limitations and versatility of these approaches in preclinical cancer drug discovery and development.

Keywords: Non-invasive imaging, Cancer Pharmacology, Bioluminescence, Ultrasound, Positron Emission Tomography (PET), Magnetic resonance imaging (MRI)

Abbreviations: BLI, Bioluminescence imaging; BRET, bioluminescence resonance energy transfer; CT, computed tomography; DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; FDG, 18-fluoro-deoxy-glucose; HF-US, high frequency ultrasound; MB, microbubbles; MRI, Magnetic resonance imaging; PD-US, power Doppler ultrasound; PET, positron emission tomography; QD, quantum dot; SPECT, single positron emission computed tomography; US, ultrasound;

Introduction

Despite advances in cancer treatment, response of many tumour types is sub-optimal and in many cases not curative. Therefore new treatments or better targeting of current treatments for cancer therapy are of the upmost importance. As knowledge of molecular systems and pathways expands and improves, development of novel agents that are directed to specific molecular targets has become forefront. This approach aims to increase selective toxicity to cancer cells, reduce the likelihood of therapeutic resistance, and limit patient morbidity commonly associated with chemotherapy. The development and improvement of these 'molecular targeted' therapies is a major goal of current anticancer drug development. Analytical tools that enable the assessment of new therapies targeted specifically to individual pathways/molecules in living mammals are already benefitting researches, with the potential to provide a lot more information in the future.

Limitations of current preclinical in vivo models in cancer drug development.

In vitro studies can be used to give basic information on the toxicity of a drug against cancer cell lines, to evaluate drug target interactions and to define biochemical and gene expression pathways (Massoud *et al.*, 2003). These studies do not however answer questions regarding clinical response in the whole body system (de Jong *et al.*, 2010). Ultimately the value of laboratory and preclinical studies depend on their capacity to accurately predict clinical response as a surrogate to humans, due to the ethical and practical concerns this raises (de Jong *et al.*, 2010). Over the past few decades animal models have played a key part in revealing many biochemical and physiological processes involved in the onset of cancer and its development in living organisms (de Jong *et al.*, 2010). The primary objectives of these animal models are firstly to mimic the human disease as closely as possible and secondly to proficiently test new therapies (Liao *et al.*, 2007). *In vivo* models allow target-orientated drug screening and can yield pharmacokinetic and pharmacodynamic information, both are which are clinically relevant. However, despite animal models giving valuable information regarding drug efficacy, there are several factors that should be considered when extrapolating mouse data to the clinical testing, a topic succinctly reviewed by de Jong and Maina (de Jong *et al.*, 2010). One such example is the use of subcutaneous tumour xenograft models, a tumour environment which does not accurately mimic the clinical situation.

Subcutaneous tumour xenograft models are traditionally used as a frontline screen for assessing therapeutic efficacy and drug-target interactions, the response been determined via calliper-based measurements, and monitoring the lifespan of animals (Suggitt *et al.*, 2005; Zhang *et al.*, 2007). This strategy has proved successful for several agents now in the clinic, including traztuzumab for treatment of HER2-overexpressing breast cancer (Baselga *et al.*, 1998; Vogel *et al.*, 2002), Melphalan in the treatment of rhabdomyosarcoma (Horowitz *et al.*, 1988) and Vorinostat for treatment of cutaneous T-cell lymphoma (Kelly *et al.*, 2003; Marks *et al.*, 2007). Despite these successes and this strategy providing high quality information on therapeutics, several limitations exist which impinge upon the utility of this approach for molecular-targeted therapeutics. Firstly, these models do not fully recapitulate the tumour cellular heterogeneity of the clinical tumour environment. Secondly, significant efforts and advances are being made with molecular-targeted agents against tumour vasculature, including vascular-disrupting and anti-angiogenic agents. Therapeutic strategies such as these may exert their cytostatic effect very efficiently, but since they are not cytotoxic will not necessarily lead to a decrease in tumour size (Cai *et al.*, 2006). Therefore monitoring tumour size using callipers could underestimate the effect of these agents, give little information regarding the internal structure of the tumour and thus provide inconclusive information regarding their efficacy.

A major issue with the use of calliper-measured subcutaneous xenograft tumour models is their lack of applicability for evaluation of metastatic disease (Suggitt *et al.*, 2005). Metastasis is the leading cause of death among cancer patients so the development of robust model systems for front-line drug discovery in this area is an urgent requirement. Although preclinical models exist involving both spontaneous and experimentally induced metastatic tumours, these are often time consuming and labour intensive methodologies being non-amenable to calliper-based measurements and requiring termination of multiple animals for tumour load determination (Grosios *et al.*, 1999). Consequently, there is a need for improved preclinical models or strategies which translate and represent the clinical situation.

Molecular imaging as a tool for preclinical cancer pharmacology studies.

Molecular imaging is a rapidly emerging field with a multitude of characteristics that make it useful for the drug discovery process. Technically it has many advantages over traditional techniques as it is rapid, can be high-throughput, is non-invasive and is less labour intensive than pathology- and chemistry-based assays (Massoud *et al.*, 2007). It allows imaging to be performed at the cellular and molecular level in living animals, in real-time and with a truly

quantitative outcome (Laxman *et al.*, 2002; Massoud *et al.*, 2007; Wessels *et al.*, 2007). With regards to the requirement to replace, refine and reduce (the 3Rs) the use of animals in research, molecular imaging can be used to follow tumour development or therapeutic effect over a period of time in the same animal, thereby reducing the numbers of animals being used whilst simultaneously improving the data set as each animal serves as its own control (Contag *et al.*, 2002; Fomchenko *et al.*, 2006). A further benefit of sequentially studying tumour pathogenesis in one animal is that information regarding the possible behaviour of a tumour in humans, especially regarding metastasis and therapy response, is more achievable.

Earlier detection and characterisation of disease are additional benefits of bioimaging, as is the information that can be obtained which is specific to particular molecular events e.g. evaluation of therapeutic interactions (Contag *et al.*, 2002; Laxman *et al.*, 2002). *In vivo*, bioimaging can be used for target and therapeutic validation in a dynamic environment (Gwyther *et al.*, 2007; Laxman *et al.*, 2002). It also allows quicker assessment of drug/target interactions and identification of therapeutic efficacy prior to any macroscopic or phenotypic changes (Massoud *et al.*, 2007; Morse *et al.*, 2007). Clinically this is important as efficacy of a treatment can be determined earlier and if necessary the treatment regime can be altered (Morse *et al.*, 2007). Some types of bioimaging also have the potential to image specific intracellular pathways, a feature which has previously been illusive (Laxman *et al.*, 2002).

Imaging modalities in preclinical cancer drug discovery: Advantages and limitations.

There are seven main types of bioimaging; magnetic resonance imaging (MRI), positron emission tomography (PET), single positron emission computed tomography (SPECT), computed tomography (CT), ultrasound (US) and optical imaging (including bioluminescence imaging (BLI) and fluorescence imaging). All of these techniques demonstrate merit during drug development, with each of them having advantages and disadvantages (Table 1).

Magnetic resonance imaging (MRI)

MRI is based on the absorption and emission of energy in the radio frequency range of the electromagnetic spectrum, and the fact the body is primarily composed of hydrogen-rich fat and water. When induced by a strong magnetic field the hydrogen nuclei emit a nuclear magnetic resonance signal that is determined by the direction of the spin induced. MRI gives high spatial resolution of about $50\mu m^3$ (Czernin *et al.*, 2006) and good soft-tissue contrast. MRI is a very versatile technique and is widely used in small animal studies to address

tumour physiology and quantification of tumour volume (Fomchenko *et al.*, 2006). However MRI has low sensitivity and a relatively long acquisition time (up to one hour), thereby limiting its utility for preclinical drug development studies (Lyons, 2005). Its ability to amalgamate anatomical and functional information provides great insights into disease processes, including cancer (Hasegawa *et al.*, 2010). Improvements in the methodology for MRI are constant (Schroder *et al.*, 2006), including the use of higher magnetic fields and enhancing contrast agents, which has led to an improvement in the sensitivity of MRI (Hasegawa *et al.*, 2010).

MRI has been used to successfully report efficacy of suicide gene therapy in delaying tumour growth in a mouse model of orthotopic glioma (Breton *et al.*, 2010) and to demonstrate complete infiltration of intraprostatic injected gene therapies in preclinical mouse models of prostate cancer (Kassouf *et al.*, 2007). More recently, MRI has been used with drug-containing liposomes, either gadolinium-labelled or paramagnetic, to facilitate image-supervised therapeutic delivery and subsequent monitoring of efficacy (Grange *et al.*, 2010; Strijkers *et al.*, 2010). Advancements in technology have now led to more sensitive quantitative MRI techniques, such as high-field MRI and dynamic contrast-enhanced MRI (DCE-MRI), which are even more valuable for the research into tumour vasculature and the effects of drugs.

Dynamic contrast-enhanced-magnetic resonance imaging (DCE-MRI). DCE-MRI is a quantitative method of investigating the structure and function of tumour microvasculature and microcirculation (Ali *et al.*, 2010; O'Connor *et al.*, 2007). Advantages of this technique are that in addition to initial anatomical MRI information, data are subsequently acquired every few seconds over a period of 5-10 minutes providing dynamic detail of features such as blood flow (O'Connor *et al.*, 2007). DCE-MRI has been used to characterise tumour angiogenesis (Brix *et al.*, 2010), and has the potential to aid investigation of antiangiogenic and vascular-disrupting therapeutics, although it is not restricted to such agents. Recently DCE-MRI has been used to show co-treatment with a folate-linked liposomal doxorubicin and a TGFβ-Receptor-1 inhibitor (A-83-01) enhanced the therapeutic effect of doxorubicin, indicated by DCE-MRI monitored tumour leakage of the gadolinium-liposome complex (Taniguchi *et al.*, 2010). Furthermore, DCE-MRI demonstrated pancreatic xenograft tumour response to the HIF-1α inhibitor PX-478, before any anatomical changes were recorded (Schwartz *et al.*, 2010).

In general MRI, although constrained by acquisition time and sensitivity, can be used preclinically to help develop new therapies both cytotoxic and target molecule specific, for a broad range of cancers. This strategy does address the requirement for a refinement of usage and reduction in animal numbers according to the 3Rs. In addition, MRI also allows tumour response to be followed in the same animal therefore improving the power of a study despite fewer animals being used. A drawback to the use of MRI in pre-clinical studies is the cost of equipment for smaller labs where *in vivo* work is done.

Positron Emission Tomography (PET)

PET produces a three-dimensional image of functional processes in the body, measuring biochemical function rather than structure, and thus provides a crucial insight into cancer biology and pharmacology (Zaidi *et al.*, 2009). Mechanistically, a probe comprising a metabolically active molecule (such as glucose or water) incorporating a γ-ray emitting radioisotope, is introduced into animal and its uptake and metabolism monitored. The commonest probe is 18-fluoro-deoxy-glucose (FDG), of which uptake indicates glucose metabolism and thus the enhanced glycolysis associated with malignancy, enabling differentiation between malignant and benign tissue (Otsuka *et al.*, 2007; Vansteenkiste, 2002). Unfortunately, due to the requirement of a cyclotron for radio-nucleotide production and dedicated synthetic chemical equipment, PET systems are generally limited to research laboratories associated with a clinical centre. However, where they are used, PET has strong potential for translational research from small animals to humans (Laforest *et al.*, 2007). Additionally, PET has the advantage over other imaging modalities in that it also permits evaluation of changes in tumour metabolism and proliferation, drug biodistribution, and pharmacokinetics, all of which aid assessment of drug efficacy (Avril *et al.*, 2007).

A huge variety of cancer treatments have been investigated using PET, including treatment of malignant gliomas using bevacizumab and irinotecan (Chen *et al.*, 2007), defining optimal does of mTOR inhibitors (Cejka *et al.*, 2009), screening novel HDAC inhibitors (Leyton *et al.*, 2006), evaluating ovarian tumour response to the antivascular agent AVE8062 and taxanes (Kim *et al.*, 2007), and determining the therapeutic effect of the EGFR tyrosine kinase inhibitor lapatinib (Diaz *et al.*, 2010). In addition, radiotracers to indicate changes in receptor expression following therapeutic interventions have also been studied (Kramer-Marek *et al.*, 2009), as was the case with the pharmacodynamics of C75, a fatty acid synthase inhibitor and emerging target for anticancer therapy (Lee *et al.*, 2007a).

The ability to use PET to determine drug and target distribution in cancer cells has been used to monitor whether yttrium-90 spheres, a novel treatment for advanced liver cancer, are preferentially delivered to tumour cells following injection into the hepatic arteries (Tehranipour *et al.*, 2007). Furthermore, this application of PET has been adopted to determine tumour specificity of the JAA-f11 antibody on the survival of mice with metastatic 4T1 breast tumours (Rittenhouse-Olson, 2007) and to confirm that the sigma-2 receptor was a valid target for cancer drug development through preferential expression in tumour tissue but not normal tissues (Kashiwagi *et al.*, 2007).

One problem reported for PET in preclinical studies was the heterogeneity of glucose uptake in different areas of a tumour, resulting in a lack of correlation between PET and standard calliper measurements, evidenced with enzastaurin, a novel protein kinase C-beta II inhibitor (Pollok *et al.*, 2009). Despite this potential issue for experimental reproducibility, the use of PET preclinically is a viable option as it allows treatment optimisation which is one of the main goals of preclinical studies prior to clinical trial. However, as with MRI, the equipment necessary for imaging small animals is expensive and not available to the majority of laboratories.

Single Positron Emission Computed Tomography (SPECT)

SPECT is an imaging technique that detects low energy γ-rays arising from radioisotope decay and has resolution of 1-2mm. An advantage of this technique over PET is the capacity to detect multiple probes simultaneously. Conversely, SPECT has lower sensitivity and therefore requires higher amounts of probe (Lyons, 2005). A detailed comparison of PET versus SPECT methodologies is provided by Rahmin and Zaidi (Rahmim *et al.*, 2008).

In cancer drug development, SPECT has been used to investigate the dosage effects of the human monoclonal antibody hu3S193, targeted to the Lewis-Y (Krug *et al.*, 2007), and using Tc-99m tagged VEGF-C to analyze VEGF-induced signalling pathways and changes in VEGF-Receptor expression in response to the antiangiogenic agent PTK787 (Ali *et al.*, 2010). Similarly, small high-affinity anti-HER2 molecules have been investigated as suitable tracers of SPECT visualisation of HER2-expressing tumours (Ahlgren *et al.*, 2009), with the benefit of also permitting assessment of treatment-induced effects on HER2 expression.

New probes are continually being engineered to optimise current and develop new methods that may help in targeted cancer drug development. For instance, activity of matrix metalloproteinase-14 (MMP-14), a hallmark of cancer metastasis, has been probed using a

technetium-99m SPECT marker developed to be "activatable" by MMP-14 *in vivo*, a strategy which may facilitate development of targeted molecular therapies (Watkins *et al.*, 2009).

Computed Tomography

Computed tomography (CT) is a medical imaging method whereby contrast agents are administered intravenously, and then digital geometry processing is used to generate 3-Dimensional images from a series of 2-Dimensional X-ray scans. CT is the most commonly used tool for clinical assessment of the structural features of cancer and along with MRI is the modality of choice to monitor tumour response to therapy (Torigian *et al.*, 2007). The benefits of CT include its ability to separate anatomical structures at different depths within the body, making it more useful than standard X-rays. It is inherently high contrast therefore even extremely small differences in tissue density can be distinguished. Images created by CT scans can be manipulated so that they can be viewed in different planes.

Conventional CT imaging used in the clinic is not suitable for small animal studies due to differences in species size and modality requirements, therefore dedicated systems were developed for preclinical studies (Paulus *et al.*, 2000; Schambach *et al.*, 2010). This microCT modality offers higher-resolution volumetric imaging of the anatomy of living small animals and has proved useful in monitoring the preclinical response of bone metastatic deposits to radiofrequency ablation (Proschek *et al.*, 2008; Schambach *et al.*, 2010) and small molecule inhibitors of heat-shock proteins (Kang *et al.*, 2010). The greatest utility of microCT is detection of metastases, demonstrated through its ability to detect micrometastatic pheochromocytoma tumours in the livers of a nude mouse model, providing a tool for evaluating treatment strategies for this cancer (Ohta *et al.*, 2006). Similarly, microCT has been employed preclinically to measure bone and tumour volumes in the evaluation of new treatment options for prostate cancer (Kang *et al.*, 2010; Morgan *et al.*, 2008), non-invasive real-time monitoring of lung cancer and treatment response (Fushiki *et al.*, 2009) and to follow colorectal tumorigenesis through the use of micro-CT colongraphy (Durkee *et al.*, 2008).

Several studies have now demonstrated that microCT has significant potential for preclinical anticancer drug development. However, there still remain several issues regarding this technique, including imaging resolution, which often falls short of the intended 100μm objective due to scan quality and reader ability (Durkee *et al.*, 2008), and the inherently poor contrast between different soft tissues (Prajapati *et al.*, 2011). In preclinical studies resolution is vitally important since the commonest use of CT is to monitor micrometastatic deposits,

which may be impossible to detect unless scans are of exceptional quality are achieved (Schambach *et al.*, 2010). Consequently, higher resolution often involves exposure to a higher dose of radiation which in itself results in issues regarding exposure levels, although advances in technology have decreased this somewhat (Prajapati *et al.*, 2011; Schambach *et al.*, 2010). Taking this into account, although CT is a viable imaging technique for preclinical cancer pharmacology studies, it does have pitfalls and limitations and is generally more expensive than other optical imaging techniques.

High-Frequency Ultrasound in Preclinical Cancer Drug Discovery

Ultrasound (US) uses sound waves greater than 20,000Hz generated by pulse/echo transducers and integrated by signal processing software to produce grey scale images. The first high frequency (HF) US instrument specifically designed for micro-imaging of the mouse was described 10 years ago (Foster *et al.*, 2002). Since then, US has developed a clear and growing role in pre-clinical imaging and drug development due to the significant advantages it has over other pre-clinical imaging modalities in that it is relatively inexpensive, fast, portable, easy to use, works in real time and does not involve ionizing radiation. Several systems are currently available for pre-clinical imaging and these have recently been compared for versatility and performance (Moran *et al.*, 2011). In addition to anatomical imaging, US also lends itself to functional imaging with Doppler US or US contrast agents allowing qualitative and quantitative assessment of tumour blood flow and perfusion and tumour angiogenesis.

Anatomical Imaging using high frequency US. Small animal imaging uses high frequency US in the range 25 to 50MHz for anatomical imaging giving spatial resolution of 90 x 30 μm (Turnbull *et al.*, 1995) allowing high definition imaging of mouse organs such as liver, kidney, eyes and heart (Graham *et al.*, 2005; Jolly *et al.*, 2005; Sun *et al.*, 2008). HF-US is applicable for imaging mouse colon and measuring colon wall thickness *in vivo* (Abdelrahman *et al.*, 2012) and for imaging ovarian structures in mice (Jaiswal *et al.*, 2009). HF-US has also been used to study tumour growth and development in different genetically engineered mouse models (GEMM) and in human cancer cell xenografts where grey scale resolution allows for the differentiation of subtle changes in anatomy and monitoring of changes in disease pathology (Figure 1). This is particularly important when considering spontaneously arising tumours in GEMM. Zhao et al. (2010) used HF-US to determine preleukaemic changes and splenomegaly in a transgenic model of acute myeloid leukaemia

(Zhao *et al.*, 2010). In conditional *Kras Trp53* mutant mice, HF-US was the modality of choice for non-invasive detection of pancreatic tumours (Olive *et al.*, 2006). In this context, HF-US is particularly useful in prescreening cohorts for presence of tumours prior to randomisation and drug treatment, thereby reducing the numbers of animals used and refining experimental protocols.

Measurement of tumour size in longitudinal studies is an important readout in anti-cancer drug discovery and 3-dimensional HF-US (3D HF-US) has been shown to be more accurate, precise and reproducible than manual caliper measurement (Ayers *et al.*, 2010). This is particularly important when assessing small irregular shaped tumours (Cheung *et al.*, 2005) and xenograft tumours which are often irregularly shaped. Furthermore, as 3D HF-US can also be used to determine tumour volume and burden within organs, it can facilitate longitudinal studies to assess responses to novel and existing drugs in GEMM and orthotopic models (Singh *et al.*, 2010).

As described earlier, although subcutaneous tumour xenograft are traditionally used as a frontline screen for assessing therapeutic efficacy they do not accurately recapitulate the clinical location or environment (Suggitt *et al.*, 2005). To address the deficiencies of subcutaneous models, orthotopic tumour xenografts are increasingly being explored for increased clinical relevance (Suggitt *et al.*, 2005). Anatomical imaging in real time using HF-US provides the basis for image-guided injection thus facilitating production of orthotopic tumour models. For example, US guided cell injections were used to create clinically relevant models of human pancreatic cancer (Huynh *et al.*, 2011). Similarly, US image guided injection of syngeneic colonic carcinoma cells was used to create a robust mouse model of liver metastasis without the need for invasive surgery (Hawcroft *et al.*, 2012).

A limitation of US is that it cannot be used to image bone, which reflects US waves, making US unsuitable for analysis of brain and bone tumours. It has also been suggested with US is that the imaging and observations from the technique are largely operator dependent with discrepancies observed between studies, a feature which can, however, be controlled by development of robust imaging protocols (Abdelrahman *et al.*, 2012).

HF-US in Functional and Molecular Imaging. In addition to its ease of use and high resolution in anatomical imaging, US can be used to assess tumour angiogenesis by qualitative and quantitative assessment of tumour blood flow using Doppler US, or addition of a US contrast agent or microbubbles (MBs). This is increasingly important given the

development of anti-angiogenic therapies and the consequent need for non-invasive strategies for continued tumour assessment and monitoring of drug pharmacodynamics.

Doppler US detects blood flow by the Doppler shift frequency, with both 2D and 3D power Doppler (PD-US) being capable of monitoring and imaging blood flow velocities in murine tumour models (Goertz *et al.*, 2002). One such example was the use of 3D PD-US to monitor angiogenic therapeutic response in a GEMM of prostate cancer (Xuan *et al.*, 2007). However, a limitation of PD-US in this context is caused by the signal-to-noise ratio and presence of imaging artefacts which make accurate determination of tumour blood flow difficult and slow moving blood undetectable. This may be important when assessing overall tumour blood flow in experimental tumours.

A further type of US with potential for preclinical cancer pharmacology studies is contrast enhanced HF-US which uses gas-filled phospholipd microbubbles (MBs) of 2-7μm in size. The small size of these MBs makes them true vascular tracers as they are restricted to the vascular network and can readily pass through the lungs, unlike contrast agents from other imaging modalities. MBs are hyperechoic, producing bright signals on the grey scale image which can be artificially coloured to produce images of tumour blood flow (Figure 2A). Relative tumour blood flow can be quantitated using time intensity curves (Figure 2B) or destruction replenishment imaging giving relative rates of blood flow and maximum perfusion. Using this methodology, therapeutic response was detected earlier than determination of tumour volume in an orthotopic model of breast cancer, with the area under the time-intensity curve and peak intensity correlating to treatment efficacy (Hoyt *et al.*, 2010). Furthermore, the effect of discontinuation of the anti-angiogenic therapy Bevacizumab was also evaluated by contrast-enhanced US, in an orthotopic model of renal cancer (Guibal *et al.*, 2010). This study identified a quantitative change in tumour perfusion between those continuously treated with Bevacizumab and those in which treatment was interrupted. Moreover, contrast enhanced-US was shown to be more sensitive at detecting therapeutic response than histological assessment of microvessel density (Guibal *et al.*, 2010).

Recently, contrast-enhanced US has been adapted to simultaneously monitor *in vivo* pharmacodynamics, achieved through surface modification of MBs to include targeting moieties to vascular biomarkers. Targeted contrast enhanced HF-US imaging has been used for *in vivo* assessment of avβ3 integrin, endoglin (CD105), and VEGFR2 levels in mouse models of breast, ovarian and pancreatic cancer (Deshpande *et al.*, 2011). This relatively new approach combining the sensitivity of molecular imaging with low cost, easy to use US imaging has the potential to enhance and accelerate pre-clinical drug development.

Optical Imaging in Preclinical Cancer Drug Discovery

The term optical imaging encompasses preclinical approaches using either luminescence or fluorescence detection as a means for evaluating drug activity, molecular events or biological activity of potential drug targets. These techniques typically require less equipment and expertise and are more cost-effective than the methodologies described above, making them more suitable techniques for smaller laboratories. This system relies upon detection of either a fluorescent or luminescent event and provides no results regarding the physical characteristics or pathology of the tissue or animal, requiring the overlay of the detected events on an anatomical image of the animal or tissue.

The use of optical imaging for monitoring biological changes is now a well established methodology within drug discovery and pharmacology (Fomchenko *et al.*, 2006). Early in the drug discovery process *in vitro* investigations are performed on cancer cell lines, and here optical imaging can be used to determine the therapeutic or pharmacological effect on cancer cell lines treated with different compounds (Loo *et al.*, 2007), to investigate transcription factor activity (Weiss *et al.*, 2010) or to study specific protein expression following drug treatment (Sakoguchi-Okada *et al.*, 2007). Furthermore, mechanistic information can be obtained through the use of tagged proteins or promoter-driven reporter expression within these experimental models. In preclinical cancer pharmacology, there are now a wide range of cancer cells available which express optical imaging proteins or probes, representing all the major human solid tumour types.

A downside to the use of optical imaging is that the target or investigated cells require genetic modification to express either luciferase or a fluorescent protein prior to their use, making it unlikely that such an approach will ever translate to the clinic. Nevertheless, the high selectivity, specificity and applicability of this approach remain a valuable tool for preclinical evaluation of cancer therapeutics and their acceleration and progression into the clinic.

Bioluminescence Imaging. The principle of BLI relies upon detection of photons emitted from the oxygen-mediated conversion of luciferin to oxyluciferin by cells genetically-modified to express luciferase (Figures 3 and 4), a process which does not necessitate an external light source (Gould *et al.*, 1988). Several luciferase genes have now been identified and cloned from various natural sources, with the most common ones for imaging purposes being ATP-dependent luciferase isolated from the North American firefly (*Photonis Pyralis*)

and ATP-independent luciferase from the anthozoan sea pansy (*Renilla Reniformis*) (Gould *et al.*, 1988; Snoeks *et al.*, 2010). Firefly luciferase catalyzes D-luciferin to give a flash of green light at 562nm, whereas Renilla luciferase catalyzes coelentrazine to generate blue luminescence with a wavelength centred at 482nm (Bhaumik *et al.*, 2004). This lack of cross-reactivity between firefly and renilla luciferase substrates also means that the BLI system can be utilised for dual-label and target imaging (Figure 4).

Anatomical Bioluminescence Imaging. The most common use of BLI in the preclinical development of drugs is to monitor any change in tumour volume following treatment of xenograft (subcutaneous or orthotopic) tumours in rodents, usually mice (Figures 3 and 4). This system is underpinned by the fact that luciferase-driven photon emission can be detected externally, even when the cells are located several millimetres below the skin (Lyons, 2005; Moriyama *et al.*, 2008). In drug efficacy studies, a decrease in luminescence is attributed to the cytotoxic effects of the drugs as a result of either induction of cell death or a reduction in cell metabolic ability. In terms of this application of BLI, there have been an extensive number of preclinical cancer pharmacology studies across a wide range of tumour cell types, including brain, breast and lung carcinoma (Grozio *et al.*, 2007; Holzmuller *et al.*, 2010; Kemper *et al.*, 2006; Lim *et al.*, 2009; Ozawa *et al.*, 2010; Zeng *et al.*, 2010), sarcomas (Rousseau *et al.*, 2010; Wang *et al.*, 2010) and multiple myeloma (Jia *et al.*, 2010). Importantly in terms of the utility of BLI to cancer pharmacology, it is now established that neither luciferase itself or bioluminescence imaging affect tumour growth *in vitro* or *in vivo* (Tiffen *et al.*, 2010).

Preclinical models of metastasis for assessment of new therapeutic strategies are extremely hard to develop and there are limited systems that adequately represent the human disease. Unlike conventional preclinical tumour models, BLI has the capability to detect micrometastatic disease and has been reliably demonstrated to detect as few as 500 cells *in vivo* at specific anatomical sites (Troy *et al.*, 2004). This thereby permits tumour dissemination and appearance of micrometastatic disease to be tracked temporally and non-invasively. Similarly, using BLI, spontaneous tumour growth can also be monitored using genetically-modified murine models which can provide vital information regarding tumour pathogenesis and treatment strategies (Hawes *et al.*, 2010). In this context, there are a plethora of studies that have used BLI as a means to monitor tumour metastases and others that have utilised these models to evaluate the treatment of metastatic disease, across a range of tumour types (Cordero *et al.*, 2010; Drake *et al.*, 2010; McNally *et al.*, 2010 ; Nogawa *et*

al., 2005; Shelton *et al.*, 2010; Takahashi *et al.*, 2010; Vikis *et al.*, 2010; Wang *et al.*, 2010; Zhang *et al.*, 2010).

Although there is compelling evidence provided by a multitude of studies confirming a strong correlation between photon emission and tumour burden, a number of studies have now suggested that the intensity of this signal can plateau or even decline during tumour progression (Dickson *et al.*, 2007; Jurczok *et al.*, 2007). One explanation for this phenomenon is the increased degree of necrotic tissue in these advanced tumours which despite not being biochemically active and metabolising luciferin still contributes to the tumour mass, providing a disparity between tumour size and bioluminescence output (Jurczok *et al.*, 2007). This increase in the proportion of necrotic tissue and reduced gross luciferase activity in these tumours is supported by histological studies (Jurczok *et al.*, 2007). In addition, this increase in necrotic tissue within the tumour is also related to an increase in subtumoral hypoxia, which impacts upon luciferase activity which is an oxygen dependent process (Gould *et al.*, 1988). Another issue affecting the viability of BLI for cancer pharmacology studies is the location of the tumour within the animal. Although BLI can detect tumours and cells located a distance below the skin (Lyons, 2005; Moriyama *et al.*, 2008), signal attenuation has been noted in deeper tumours (Kang *et al.*, 2006) and dominant signals produced by one organ have been shown to mask a weaker signal produced by another (Nogawa *et al.*, 2005). Taken together, despite appearing minor in terms of the major successes of BLI, these limitations to this strategy must be borne in mind when evaluating advanced or larger tumours, and multifocal metastatic models.

Bioluminescence Imaging of Pharmacodynamics. In light of the evolution of molecular-targeted cancer therapeutics and the need to extract as much information from *in vivo* studies as possible, the most significant advantage and potential for BLI is the assessment of molecular-target interactions and drug pharmacodynamics. This approach has now been demonstrated in several studies with general success. Induction of tumour apoptosis simultaneously with retardation of tumour growth with a panel of chemotherapeutics was observed in a non-invasive preclinical *in vivo* study (Scabini *et al.*, 2011). Induction of apoptosis was demonstrated using a conjugate of luciferin and the caspase 3/7 substrate Z-DEVD, and tumour cells engineered to express luciferase. Upon apoptosis induction, the conjugated substrate is cleaved by the caspases and releases luciferin which is converted by the luciferase-expressing tumour cells to produce the bioluminescent signal (Scabini *et al.*, 2011). Similarly, a caspase-3 activated luciferase-reporter strategy was used to demonstrate

that concomitant therapy with 5-fluoruracil and tumour necrosis factor alpha-related apoptosis-inducing ligand (TRAIL) enhances apoptotic activity *in vivo*, resulting a significantly greater antitumour response (Lee *et al.*, 2007b). In another iteration of this approach, BLI was utilised to show that inhibition of N-linked glycosylation activity reduces receptor tyrosine kinase activity in tumour cells and is a novel therapeutic strategy for targeting tumours resistant to epidermal growth factor inhibitors (Contessa *et al.*, 2010). In this study, the BLI approach was also applied to determine safe and efficacious *in vivo* dosing of tunicamycin, which blocks N-glycan precursor biosynthesis (Contessa *et al.*, 2010). Recently, BLI has also been used to evaluate tumour glycolysis pathways and demonstrate that lactate but not pyruvate concentrations correlate with tumour response to fractionated irradiation, an *in vivo* observation that was not predicted using *in vitro* assays (Sattler *et al.*, 2010).

One area of preclinical cancer pharmacology where BLI has proved highly useful is the assessment of agents targeting angiogenesis or disrupting existing tumour vasculature (Angst *et al.*, 2010; Snoeks *et al.*, 2010; Sun *et al.*, 2010a; Zhao *et al.*, 2008). Unlike the majority of chemotherapeutic approaches which are cytotoxic and induce tumour regression, those strategies which target the tumour blood supply normally induce a cytostatic response and subsequent tumour necrosis. Consequently, palpation of these treated tumours would not indicate tumour shrinkage and may prove misleading in terms of a response. Use of BLI for assessment of tumour response to these agents provides much greater information regarding tumour response. Evidence for this application is succinctly reviewed elsewhere (Snoeks *et al.*, 2010; Zhao *et al.*, 2008).

Despite showing good results *in vitro* and in conventional *in vivo* tumour models, many cancer therapeutics fail to progress into the clinic as a consequence of poor bioavailability, drug resistance and target selectivity (Jones *et al.*, 2006; Wender *et al.*, 2007). Identifying and predicting the potential for these limitations preclinically has often proved difficult, involving *ex vivo* drug and tissue analysis or extensive pharmacokinetic studies. Over recent years BLI strategies have been utilised in preclinical pharmacology assessments to address many of these issues with specific agents. The potential for modulating the multidrug resistance (MDR) gene and subsequently P-glycoprotein expression *in vivo*, and thus chemotherapeutic response, has been demonstrated using BLI with Renilla luciferase (Jeon *et al.*, 2010). Bioavailability and tumour drug delivery have been monitored using BLI (Cirstoiu-Hapca *et al.*, 2010; Peng *et al.*, 2010). Similarly, luciferin-transporter conjugates have been used as tools for real-time determination of drug uptake into cells and tumours *in*

vivo (Jones *et al.*, 2006; Wender *et al.*, 2007). Furthermore, activity of the metabolic enzyme cytochrome P450 3A4 has been monitored *in vivo* using BLI (Weisheng *et al.*, 2005), an important factor when considering pharmacokinetics, metabolism and clearance of a novel anticancer agent.

Fluorescence Imaging. In agreement with BLI, fluorescence optical imaging requires either modification of the target cells to express a specific fluorescent protein or introduction of a fluorescently-tagged reporter construct into the animal. The principle of fluorescence imaging is similar to BLI in that it relies upon detection of emitted photons; but in contrast to BLI this does not require addition of an exogenous substrate and relies upon excitation of the fluorophore using a light source or laser. There are now a vast number of fluorescent proteins, both natural and engineered, with a wide variety of emitted colours across the visible spectrum from blue to far red. The utility of these fluorescent proteins for *in vivo* preclinical cancer pharmacology studies has been shown in many studies representing all areas of the preclinical cancer drug discovery and pharmacology process, including drug efficacy, monitoring of tumour growth and detection and analysis of angiogenesis and metastatic tumour spread (Liu *et al.*, 2007).

Relative to BLI, fluorescence imaging has lower sensitivity and a higher background signal due to the requirement of an external illumination source to facilitate fluorescent emission. This high background being due to biomolecules having intrinsic fluorescence and the fact that cells and tissues can both quench and scatter emitted light. Consequently, this can limit the depth of penetration of light as the emitted light is absorbed and scattered by various tissues, including haemoglobin, (Tung, 2004). As such, important criteria for selection of fluorescent proteins for *in vivo* molecular imaging studies are the required signal intensity, photostability and potential for tumour autofluorescent interference. The use of fluorescent signals with excitation and emission wavelengths within the near infrared (NIR; 650-900 nm) region of the spectrum has now proven highly applicable in this context. Lower background signals and noise are observed with NIR, due principally to the fact that it is poorly absorbed by haemoglobin and lipids (Weissleder *et al.*, 2008). This therefore improves contrast between target and background tissues and allows much greater *in vivo* tissue penetration and detection (Weissleder *et al.*, 2008). Despite these apparent limitations, fluorescence imaging has proved invaluable in extending our understanding of cancer biology, drug efficacy and preclinical cancer pharmacology.

One major benefit of fluorescence imaging over other imaging strategies has been the ability to simultaneously monitor several processes or molecular events within the same cell or preclinical tumour model, facilitated by the wide range of fluorescent proteins with differential emission spectra. For example, the role, involvement and interactions between tumour cells and the host cellular environment was conclusively addressed using green fluorescent protein (GFP) expressing transgenic mice transplanted with tumour cells engineered to express red-fluorescent protein (Hoffman, 2009). Such an approach permitted the distinction between host and tumour cells, the involvement of specific cell types in tumour development such as macrophages, lymphocytes and fibroblasts (Hoffman, 2009). Using this model, the effects of cancer drug upon each of these cell types was also examined (Hoffman, 2009). A similar approach was also utilised for the evaluation of novel anti-angiogenic compounds (Amoh *et al.*, 2006; Dunphy *et al.*, 2009). In the initial study, human pancreatic tumour cells expressing red fluorescent protein were injected intrasplenically into mice expressing GFP under the control of the nestin promoter, a marker of blood vessel formation, with this model then being used to simultaneously visualise and quantify nascent angiogenesis and the effects of gemcitabine (Amoh *et al.*, 2006). In the latter strategy, GFP expression was driven by the Tie2 promoter, an endothelial specific promoter (Dunphy *et al.*, 2009).

Quantum Dots (QDs). One advance made within the area of fluorescence imaging is the development of QDs, small nanocrystals (1-10 nm) made of inorganic semiconductor materials (Bentolila *et al.*, 2009). QDs exhibit several properties that make them suited for preclinical imaging; the emission wavelength can be precisely tuned and can range from ultraviolet to near-infrared, they are very bright and photostable and they have a wide absorption band but a narrow emission band making them ideal for multiplexed analysis. The relatively large surface area of QDs also allows their utilisation with other contrast agents, permitting the use in multimodality imaging strategies (Bentolila *et al.*, 2009). Consequently, because of these beneficial properties and the lack of detrimental effects upon cellular proliferation or tumourigenicity of cancer cells *in vivo*, QDs are becoming popular for *in vivo* monitoring of cancer cell behaviour and growth (Bentolila *et al.*, 2009; Sun *et al.*, 2010b; Sun *et al.*, 2007; Tavares *et al.*, 2011).

The nanoscale structure and versatility of QDs has also provided the potential for the development of multifunctional theragnostics, whereby the QD is utilised as both a tumour imaging marker and an indicator of drug delivery (Choi *et al.*, 2010; Jain, 2011; Nie *et al.*,

2007). The *in vivo* feasibility of this approach was demonstrated preclinically by QDs targeted to prostate-specific membrane antigen (PSMA) and integrin $\alpha_v\beta_3$, which bound to the surface of prostate and melanoma cells, respectively (Choi *et al.*, 2010). The potential for using QDs as 'markers' for tumour selective drug delivery and evaluation of therapeutic response was recently demonstrated preclinically (Savla *et al.*, 2011). Using QDs linked to doxorubicin and bioconjugated to an aptamer for the mucin-1 tumour marker, the targeting and delivery of doxorubicin to ovarian cancer was shown (Savla *et al.*, 2011). This approach whereby QDs are developed to evaluate imaging and delivery of therapeutic agents has the potential to significantly refine and increase the utility of preclinical cancer pharmacology studies and their translation to the clinic.

Multimodal Imaging in Preclinical Cancer Pharmacology

Although all of the techniques discussed above are proficient in their own right, very often these imaging techniques are combined to allow the best aspects of different techniques to be used in parallel and to gain as much information as possible from the same animal. Combination of modalities providing both functional and anatomical information has proved particularly advantageous, such as MRI/BLI, SPECT/CT and PET/CT (Lyons, 2005; McCann *et al.*, 2009; Mulder *et al.*, 2009; Nam *et al.*, 2010; van Dalen *et al.*, 2007). With regards preclinical cancer pharmacology, there are now a large number of studies in which multimodal imaging has been utilised, of which several studies are described below.

Combination of MRI with optical imaging has been used preclinically to produce a three-dimensional brain image, allowing the morphology, physiology and chemotherapeutic response of glioma to be determined non-invasively (Kang *et al.*, 2006; McCann *et al.*, 2009). For instance, efficacy of the hypoxia-inducible factor-1 (HIF-1) inhibitor 2-methoxyestradiol against glioma was determined in an orthotopic glioma model using BLI and MRI to monitor HIF-1 activity and tumour size, respectively (Kang *et al.*, 2006). Several other preclinical approaches monitoring tumour response and molecular interactions following chemotherapy have also been reported (Medarova *et al.*, 2009), including visualisation and monitoring of tumour angiogenesis (Mulder *et al.*, 2009), temporal determination of optimal prodrug administration for enzyme-prodrug therapy (Li *et al.*, 2008), and combination therapy of chemo- and immuno-therapy against pancreatic cancer (Kim *et al.*, 2008). In the current era of molecular targeted personalised therapeutics, MRI has been applied alongside fluorescence imaging to indicate expression and activity of the epidermal growth factor receptor in

orthotopic glioma, thereby improving the potential for evaluating new and existing treatments for this tumour type (Davis *et al.*, 2010).

The combination of MicroSPECT and CT imaging has proved very informative in preclinical pharmacology studies through provision of tumour metabolic capacity within the framework of anatomical structures. In addition, this multimodal strategy demonstrated beneficial utility through its ability to monitor retention, pharmacokinetics, distribution and excretion of therapeutics, including prostate cancer immunotherapy (Chang *et al.*, 2007), distribution of liposome based drug carriers (Bao *et al.*, 2006), and the evaluation of antibodies as putative therapeutics for tumour-lymph angiogenesis (Zehnder-Fjallman *et al.*, 2007). SPECT/CT has also been used to optimise biodistribution of bombesin analogues, with the potential of using them to target gastrin releasing-peptide receptor-positive tumours (Garcia Garayoa *et al.*, 2007; Mendoza-Sanchez *et al.*, 2011). In this sense, information from such studies can be used to demonstrate whether the pharmacokinetic properties of the drug are optimal or whether improvements need to be made.

SPECT and MRI have been applied to evaluate a variety of therapies including the monitoring of changes in vascular permeability and expression of different angiogenic factors following anti-angiogenic treatment in a rat glioma model (Ali *et al.*, 2010), and to demonstrate the capacity of AC133+ progenitor cells as a breast cancer cell targeted gene delivery system (Rad *et al.*, 2009).

As a consequence of its ability to provide information non-invasively regarding tumour viability and metabolic activity, PET imaging has been combined with many of the other imaging modalities. One of the most commonly utilised approaches is the use of PET with CT, which allows anatomical localisation and size to also be monitored (Otsuka *et al.*, 2007). PET/CT imaging has been shown to be a reliable preclinical tool for the early detection of response to molecular targeted therapeutics such as the kinase inhibitor erlotinib in head and neck cancers, and as such a surrogate marker for predicting tumour response (Vergez *et al.*, 2010). PET/CT imaging also has significant utility for the detection and monitoring of tumour development, progression and response in preclinical models (Walter *et al.*, 2010). In this context, PET/CT imaging of a genetically-engineered mouse model of lung carcinoma has proved valuable in determining whether the clinical efficacy of phosphoinositide 3-kinase inhibitors is restricted to malignancies with specific mutations in this signalling pathway (Engelman *et al.*, 2008).

Incorporation of a third or fourth technique into a multimodal imaging strategy (BLI, MRI and PET) has also been suggested to have potential for preclinical cancer pharmacology

studies (Deroose *et al.*, 2007; Hwang do *et al.*, 2009; Mouchess *et al.*, 2006; Xie *et al.*, 2010). Using a trimodality imaging strategy of BLI/Micro-CT/MRI the effects of zoledronic acid upon tumour progression and bone resorption were evaluated in a neuroblastoma xenograft tumour model (Mouchess *et al.*, 2006). In this study, BLI increased concomitantly with detectable osteolytic lesions and also reflected tumour growth inhibition by zoledronic acid. Bone loss was quantified using micro-CT, and MRI allowed assessment of tumour cells both within the bone marrow cavity and as distant metastases (Mouchess *et al.*, 2006). This simultaneous multimodal strategy thereby allowed a detailed analysis of the tumour and host tissue response. In another study, greater quantification of primary and metastatic tumour burden in mice was achieved using PET/BLI combined with CT (Deroose *et al.*, 2007). The use of a trimodality fusion reporter gene, which allows detection by fluorescence, BLI and PET, combined with CT gave improved sensitivity and allowed molecular signals to be analysed in the context of anatomical structures. PET/CT combination was advantageous as it allowed localisation of lesions not observed by CT due to poor contrast resolution and not seen by PET because of high background signal (Deroose *et al.*, 2007).

More recently, greater imaging capability for use in preclinical cancer pharmacology studies has been achieved through combination of molecular imaging with nanoparticles. Nanoparticles capable of concurrent fluorescence, bioluminescence, bioluminescence-resonance-energy-transfer (BRET), PET and MR imaging have been developed and provide further advantages through cellular uptake, the ability to track tumour dissemination and therapeutic response *in vivo*, and their amenability for molecular targeting (Hwang do *et al.*, 2009; Xie *et al.*, 2010).

Conclusion

Non-invasive and molecular imaging strategies are now well established and powerful methodologies used to evaluate drug efficacy and safety in preclinical cancer pharmacological studies. Through advances in this area we have a much greater understanding of tumour development at the molecular level, and have made significant advances in our ability to monitor or predict therapeutic response. Furthermore, molecular imaging in preclinical studies is increasingly more important in light of the clinical progression toward personalised medicine, with treatment being tailored to a specific tumour protein, gene profile, or genetic polymorphism. In this context, preclinical disease models that can give information regarding specific targets will be hugely beneficial.

The increasing availability of new multifunctional imaging probes, BLI reporter systems and more sensitive equipment in combination with more clinically relevant and improved animal models of human cancers is likely to have increasing impact on the development of new therapeutics and subsequent improved clinical response. The advent of molecular biomaging approaches and advances in this area is further improving the impact of preclinical cancer pharmacology studies, not least through the additional ability to dynamically monitor drug pharmacodynamics and drug-target interactions non-invasively. The most promising advances have been made through the combination of several approaches into a single multimodal imaging strategy, with the ability now to utilise tri- and quadruple-modality approaches within a single animal. Finally, detection of metastatic disease and treatment response against these lesions is an essential requirement for preclinical pharmacological studies, requiring sensitive methodology and clinically applicable disease models. The significant improvements in non-invasive methodologies, multimodal imaging capabilities, and greater detection sensitivity coupled with better metastatic disease models and the ability to monitor response in the same animal over time is now permitting the previously elusive evaluation of metastatic tumour therapeutic response to be evaluated in much greater depth and better translation of therapeutic approaches to the clinic.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest.

Table 1: Advantages and Disadvantages of Imaging Modalities for Preclinical Cancer Drug Discovery

Modality	Advantages	Disadvantages
MRI	- High Spatial Resolution - Good Soft Tissue Contrast - Provides both anatomical and functional information	- Low Sensitivity - Relatively long acquisition time - Requires expensive equipment
PET	- Provides biochemical information - High Sensitivity - Three-Dimensional Imaging - Can monitor changes in tumour metabolism and drug biodistribution	- Limited anatomical information - Requires specialised equipment - Requires radio-nucleotide facilities - Requires expensive equipment
SPECT	- Potential to detect multiple probes simultaneously, in contrast to PET	- Lower sensitivity than PET
CT	- High sensitivity anatomical imaging - Provides three-dimensional image	- Lower resolution - Limited functional information - Poor soft tissue contrast - Requires expensive equipment
Ultrasound	- Good Resolution - Provides both anatomical and functional information - Fast and portable technique - Relatively inexpensive - Amenable to smaller research laboratories	- Inability to image through bone
Optical (BLI and fluorescent)	- Wide applicability - Simultaneously monitor several molecular events - Relatively inexpensive - Amenable to smaller research laboratories	- Requires genetic manipulation of investigated cells - Provides limited anatomical information - Reduced sensitivity with increased imaging depth

Abbreviations: BLI: bioluminescent; CT: computerised tomography; MRI: magnetic resonance imaging; PET: positron emission tomography; SPECT: single photon emission computed tomography.

Figure 1: High frequency ultrasound (HFUS) imaging of the proximal colon of an *Apc$^{Min/+}$* mouse.
The VisualSonics Vevo770 system has a resolution of 30µm allowing identification of normal and pathological colon. (A) shows normal colon where an even thickness of colon wall surrounds the faecal pellet. The wall thickness was measured as 0.18mm [measurement not shown]. (B) shows an adjacent section of colon where an adenoma had formed. This was identified *in vivo* as a significant thickening of the colon wall. The maximum wall thickness was measured as 0.87mm [measurement not shown]. Black arrows show the outer colon wall, white circles delineate the border between faecal pellet and the inner colon wall and white stars indicate the faecal pellet.

Figure 2: Contrast-enhanced HFUS imaging.
The use of microbubble contrast agents with HFUS imaging protocols allows the visualisation and relative quantification of tumour blood flow and perfusion. (A) shows a contrast-enhanced HFUS image of an SW480 human colorectal cancer xenograft where microbubbles are coloured green and the region of interest [tumour] is delineated by the blue line. By imaging the contrast agent over time both qualitative and quantitative data on tumour vascularity and tumour blood flow respectively can be obtained. (B) shows a wash-in time intensity curve where the contrast intensity in arbitrary units is plotted against time in seconds. Analysis of these curves provides values for the maximum intensity of the contrast agent or relative perfusion and the maximum relative rate of tumour blood flow.

Figure 3:Bioluminescence imaging of orthotopic tumour growth
Mice were orthotopically implanted on the caecum with DLD1-1 colorectal cancer cells engineered to express firefly luciferase (under control of the Simian Virus-40 [SV40] promoter). At weekly intervals the mice were injected with D-luciferin and imaged using an IVIS-50 system (Caliper Life Sciences). The image shown depicts the growth of the orthotopic tumour over time.

Figure 4: Potential for dual bioluminescence imaging of tumours *in vivo*
A) Mice bearing subcutaneous tumours engineered to express either firefly luciferase (tumour site 1, FLuc), renilla luciferase (tumour site 2, RLuc) or both lucifearse systems (tumour site 3); **B)** Bioluminescence image following injection of coelenterazine (substrate for renilla luciferase), with light detected from tumours 2 and 3; C) The same mouse imaged 4 hours later following injection with D-luciferin (substrate for firefly luciferase), with light detected from tumours 1 and 3. Neither of the substrates showed any cross-reactivity with the alternative luciferase enzyme, and tumour 3 (a mixture of both cell types) emitted a signal when both substrates were administered, supporting the potential for dual BLI imaging in preclinical cancer pharmacology studies. All images were collected using the IVIS-50 system (Caliper Life Sciences)

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