Review

Radiolabeled antiviral drugs and antibodies as virus-specific imaging probes

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ABSTRACT

A number of small-molecule drugs inhibit viral replication by binding directly to virion structural proteins or to the active site of a viral enzyme, or are chemically modified by a viral enzyme before inhibiting a downstream process. Similarly, antibodies used to prevent or treat viral infections attach to epitopes on virions or on viral proteins expressed on the surface of infected cells. Such drugs and antibodies can therefore be thought of as probes for the detection of viral infections, suggesting that they might be used as radiolabeled tracers to visualize sites of viral replication by single-photon emission computed tomography (SPECT) or positron emission tomography (PET) imaging. A current example of this approach is the PET imaging of herpes simplex virus infections, in which the viral thymidine kinase phosphorylates radiolabeled thymidine analogues, trapping them within infected cells. One of many possible future applications might be the use of a radiolabeled hepatitis C protease inhibitor to image infection in animals or humans and provide a quantitative measure of viral burden. This article reviews the basic features of radionuclide imaging and the characteristics of ideal tracer molecules, and discusses how antiviral drugs and antibodies could be evaluated for their suitability as virus-specific imaging probes. The use of labeled drugs as low-dose tracers would provide an alternative application for compounds that have failed to advance to clinical use because of insufficient *in vivo* potency, an unsuitable pharmacokinetic profile or hepato- or nephrotoxicity.

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1. Introduction

The radionuclide imaging techniques of single-photon emission computed tomography (SPECT) and positron emission tomography (PET) employ short-lived radiolabeled tracers to visualize biochemical processes in animals and humans. The selective retention of an imaging probe at a site of interest is based either on its high-affinity binding to a specific target, such as a hormone receptor, or on the occurrence of a specific chemical modification, such as phosphorylation, that traps it within a cell (Fig. 1). Many different tracer molecules are now employed to study a variety of pathophysiologic processes. When applied to virology research, SPECT and PET have been used almost exclusively to image host responses to infection, rather than to visualize viral replication. The only instance in which a pathogen-specific tracer has been employed to detect and track a virus has been the use of a radiolabeled thymidine analogue to image herpes simplex virus (HSV) infections, based on phosphorylation of the probe by the viral thymidine kinase (TK), which traps it within infected cells (Fig. 2) (Brader et al., 2009; Kuruppu et al., 2007).

This article examines the question of whether viruses other than HSV could be imaged by SPECT or PET, using radiolabeled antiviral drugs or antibodies as probes. In the case of hepatitis C, for example, such tracers might include molecules that block viral replication by binding to specific virus-encoded molecules, such as the NS3 protease (Fig. 3). In contrast, an antiviral compound such as ribavirin, which is phosphorylated and retained within both infected and uninfected cells, would not be a suitable probe for virus-specific imaging.

We begin by providing an overview of the principles of radionuclide imaging, noting the similarities and differences between the mechanisms of action of antiviral drugs and imaging probes. We then describe how SPECT and PET have been used to detect host responses to viral infection and examine how PET has been employed to track the distribution of radiolabeled antiviral drugs in uninfected subjects. Next, we show how the recent development of recombinant oncolytic viruses encoding host reporter molecules provides a proof of concept for virus-specific imaging. We then examine how researchers have taken advantage of the selective phosphorylation of thymidine analogues by the HSV TK to image HSV infections, and how the TK has also been used as a recombinant marker to image tumors, gene therapy and other conditions. Next, we identify a number of processes unique to viral replication that might serve as targets for radiolabeled pathogen-specific tracers. We then review nine different DNA and RNA virus families, identifying approved and experimental antiviral drugs that target virus-encoded molecules, and might have potential as radiolabeled probes. We conclude by explaining how researchers can determine the potential of an antiviral drug or antibody as a radiolabeled imaging probe by using the ^{3}H- or ^{14}C-labeled compound in simple *in vitro* and *ex vivo* experiments.

## 2. Basic concepts of radionuclide imaging

2.1. Antimicrobial drugs and imaging probes

Antiviral therapy and nuclear medicine share a common origin in the efforts of Paul Ehrlich to develop antimicrobial medications in the early 1900s. His concept of the "magic bullet" was based on the observation that certain dyes stained microbes, but not surrounding cells, in tissue sections, suggesting that the selective binding of other small molecules to pathogens could be exploited for therapeutic purposes. To find a substance that specifically inhibited *Treponema pallidum*, Ehrlich tested more than 600 compounds with varied side chains until he found one that was toxic for the spirochete, but not its host. Two decades later, Georges de Hevesy followed the same line of reasoning when he used radioactive compounds as "tracers" to explore mechanisms of drug action. At that time, bismuth was a component of a number of compounds employed for the treatment of syphilis. To determine their distribution within the body, de Hevesy introduced radioactive bismuth into the medications, inoculated them into the bloodstream of rabbits and followed their distribution and clearance from blood and tissues (Hevesy and Paneth, 1938; Lomholt, 1925). The concept of using a low-dose radioactive tracer to study a biochemical process in which it directly participates is still followed today in developing probes that target cell surface and intracellular receptors, enzymes and other molecules (Eckelman et al., 2008; Phelps, 2000; Rudin et al., 2005).

In some respects, the basic mechanisms that underlie radionuclide imaging are similar to those of antiviral therapy, in which a drug produces a beneficial effect within a region of interest (a locus of viral infection), while having a minimal impact on other tissues. The optimal pharmacokinetic properties of an antiviral agent differ, however, from those of an ideal imaging probe: continuous high drug levels in the blood and tissues are beneficial for therapy, but for SPECT or PET imaging, such persistence of the tracer would result in a high background signal and poor image contrast. As discussed below, an ideal imaging probe differs from a drug, in that it should be retained at sites of interest, but cleared rapidly from non-target tissues.

2.2. Image acquisition by SPECT and PET

SPECT and PET imaging are based on the use of radiolabeled probes that are selectively retained at the site of a specific physiological process. Photons emitted from the probe are registered by a detector and the signals are translated by a computer into
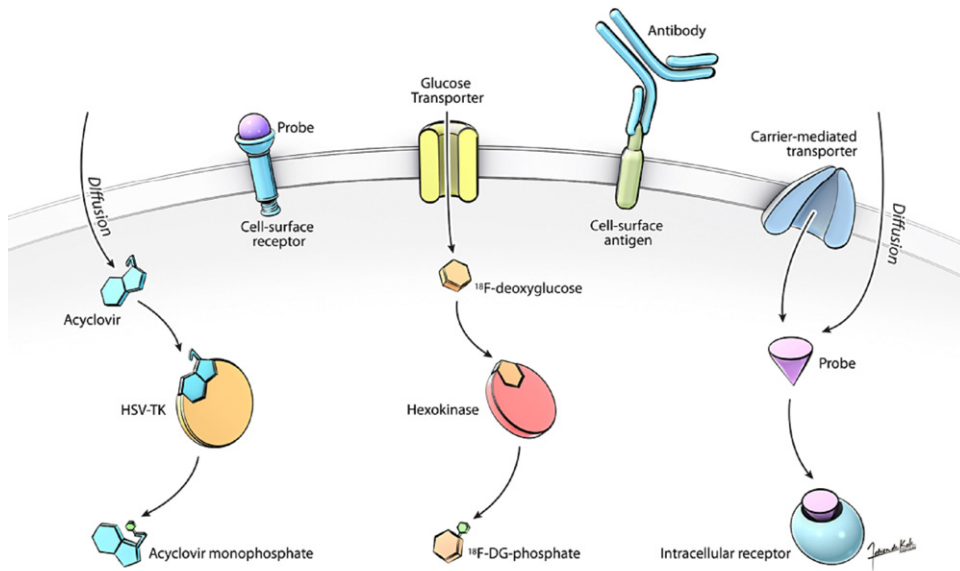
Fig. 1. SPECT and PET imaging are based on the selective retention of radiolabeled tracers at sites of interest. An antibody or peptide that is too large or too highly charged to diffuse through the cell membrane can be used to target an antigen or receptor on the cell surface. A small molecule that is transported or diffuses across the cell membrane may be retained in the cytoplasm or nucleus if it binds to a receptor or other target. Alternatively, the probe may undergo phosphorylation by a cellular (e.g. hexokinase) or viral (HSV-TK) enzyme, so that it cannot diffuse out of the cell. (Figure by Fabian De Kok-Mercado)

a visible image. In SPECT imaging, photons in the energy range of 80–400 kEV impact on sodium iodide crystal detectors within camera heads that revolve around the subject, enabling the assembly of a three-dimensional image (Fig. 4A). Photons from outside the region of interest and nonspecific scatter arising within the sub-ject are blocked by collimators (perforated metal blocks) placed in front of the detectors, increasing the signal-to-noise ratio (SNR). Because the SPECT cameras can determine the direction of a gamma ray source more accurately than its depth within the body, the method's spatial resolution is in the order of 7.5–10 mm.
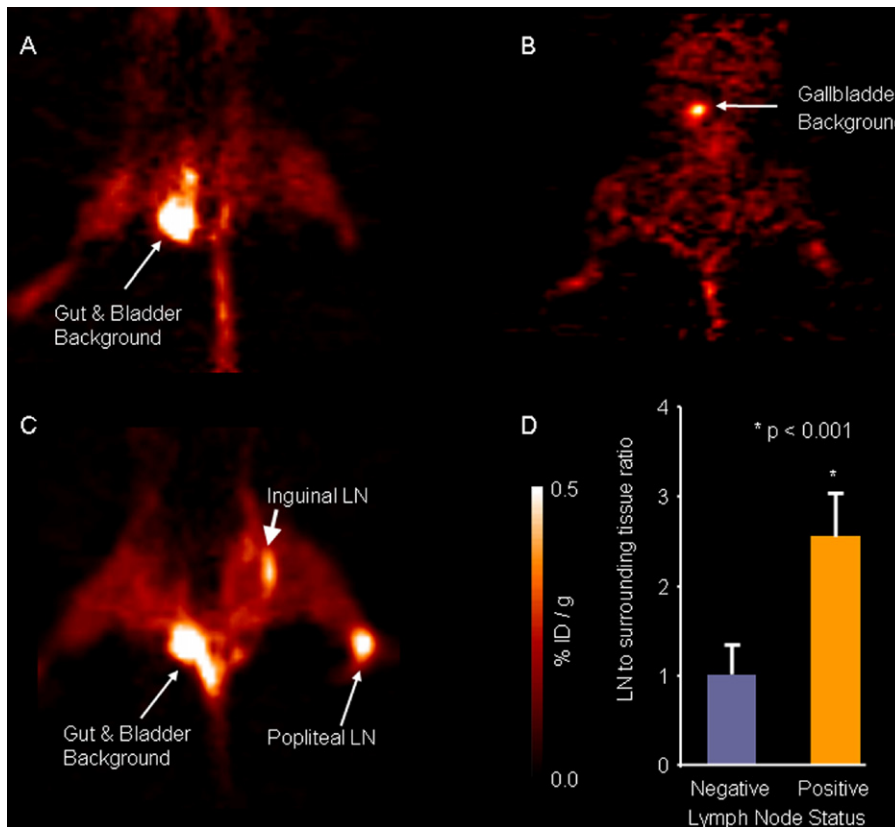
Fig. 2. PET imaging of HSV infection, in which the virus is used as an experimental anti-tumor agent. The probe is $[^{18}F]$-2′-fluoro-2′-deoxy-1-b-D-β-arabinofuranosyl-5-ethyluracil (^{18}F-FEAU), which is phosphorylated by the viral TK, but is not a substrate for the corresponding host cell enzyme. A and B show background uptake of the probe in the gut and bladder of uninfected control mice. C shows additional uptake of probe in virus-infected tumor metastases in inguinal and popliteal lymph nodes. From Brader et al. (2009), with permission.
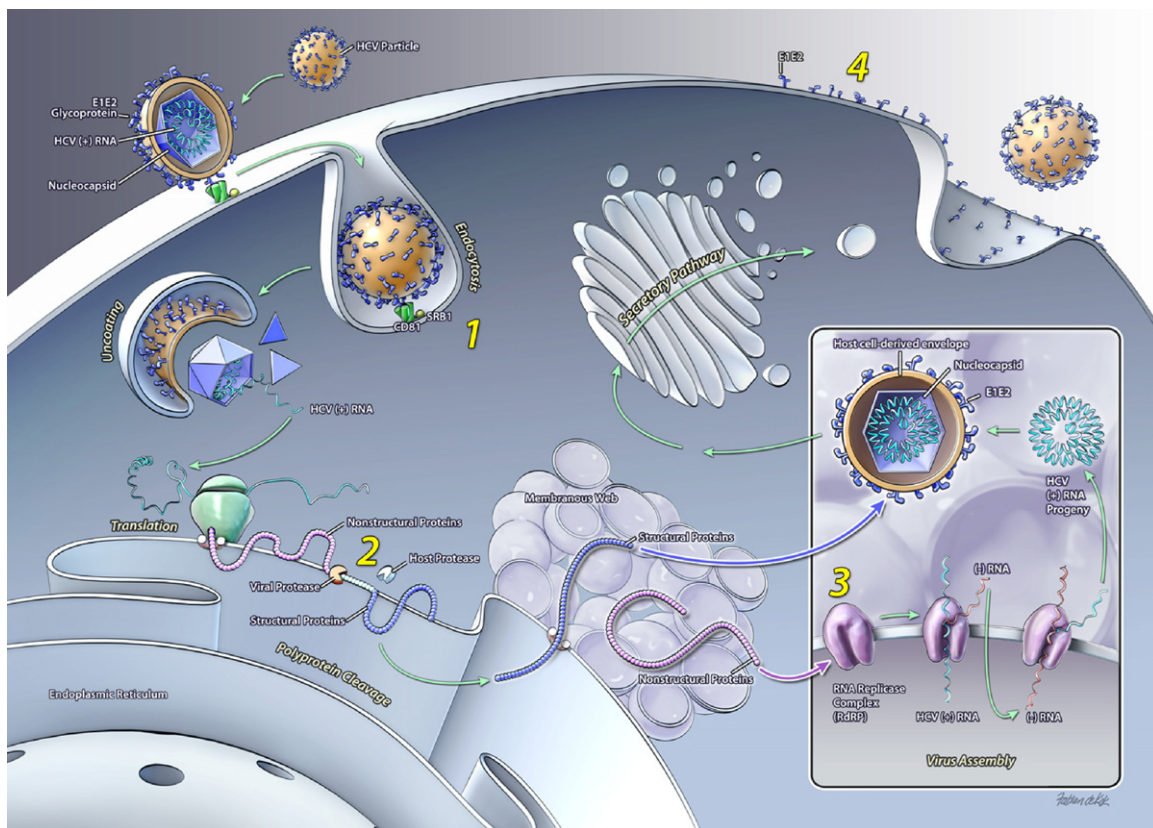
Fig. 3. Potential targets for virus-specific imaging probes in the replication cycle of hepatitis C virus. 1. Binding of a peptide inhibitor to a fusion sequence in the viral envelope glycoprotein. 2. Binding of an inhibitor to the active site of the viral NS3 protease. 3. Binding of a non-nucleoside inhibitor to the RNA replication complex. 4. Binding of an antibody or aptamer to the viral E1–E2 protein on the cell surface. (Figure by Fabian De Kok-Mercado)

In contrast to the registration of single photons over a range of energies by SPECT, PET detects the pair of 511 kEV photons that are released in opposite directions when a positron emitted by the probe within body tissues collides with an electron (Fig. 4B). PET instruments are therefore configured as a ring of detectors surrounding the subject. By registering a signal only when two photons of the correct energy strike opposite sides of the ring simultaneously, the SNR is markedly increased. This intrinsic mechanism allows PET to provide better spatial resolution than SPECT (approximately 4 mm for clinical imaging and 1–2 mm for animal microPET systems), and facilitates the accurate quantitation of radioactive decay within a region of interest. Such quantitative data lend themselves to statistical analysis of pathologic abnormalities and assessment of response to treatment, and are especially useful for monitoring cancer therapy.

2.3. Considerations in the design of SPECT and PET probes

2.3.1. Location of the imaging target: on the cell surface or within the cell?

The first question to consider in designing an imaging probe is the location of its intended target: on the cell surface, where it can be accessed directly from the bloodstream, or within the cell, where it can be reached only by a tracer capable of traversing a lipid bilayer. Cell-surface targets can be imaged using antibodies, antibody fragments and peptides, because these large, charged molecules can reach such targets from the plasma. For example, radiolabeled anti-CD4 antibodies are being used to detect and quantitate CD4+ T cells in lymphoid tissues of retrovirus-infected macaques (Fig. 5) (Di Mascio et al., 2009a). In contrast, when the target is in the cytoplasm or the nucleus, size and charge become

critical considerations, and effective tracers are generally small, nonpolar molecules.

Target location and the chemical properties of the tracer, also affect the timing required to obtain suitable high-contrast images. Because high molecular weight probes are cleared more slowly from non-target tissues than smaller molecules, it may be necessary to label them with a radionuclide with a longer half-life. As discussed in greater detail below, such pharmacokinetic considerations affect image contrast and the SNR, by determining how long it takes for the tracer to be cleared from areas other than the site of interest. In the case of licensed medications that are being considered for use as radiolabeled probes, such clearance data may already have been obtained in pharmacokinetic studies.

2.3.2. Nature of the target: saturable or non-saturable?

The interactions of drugs and probes with their targets can be divided into two different types: those that reach a maximum level of activity ("become saturated") as the concentration of drug or probe increases, and those that continue to display greater activity as the concentration increases (non-saturable targets). Saturable targets in antiviral therapy are generally those in which the therapeutic molecule occupies a target site, and only a limited number of sites are available for binding. Examples include the binding of pleconaril within a cleft in the capsid of some picornaviruses, the blocking of the influenza M2 ion channel by amantadine, and the binding of a non-nucleoside reverse transcriptase (RT) inhibitor to a locus on the HIV RT, distinct from its active site. For radionuclide imaging, examples of saturable targets include beta-adrenergic, muscarinic and estrogen receptors, which bind labeled analogues (Eckelman et al., 1979; Katzenellenbogen et al., 1980).
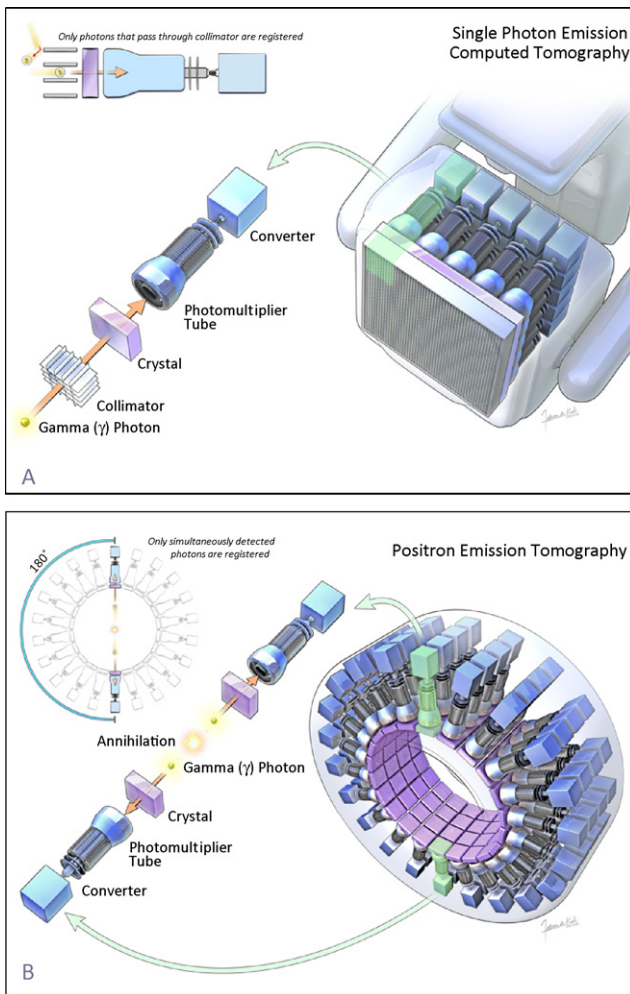
Fig. 4. Detection of signals for SPECT and PET imaging. A. The single gamma rays emitted by the decay of SPECT isotopes trigger a light signal in a crystal that is amplified by a photomultiplier tube, converted to an electical signal and converted to an image by a computer. Gamma rays originating outside the area of interest are blocked by a collimator. B. Decay of a PET isotope releases a positron that collides with an electron in tissues, generating a pair of gamma rays that travel in opposite directions. Only simultaneously detected gamma rays are registered by the detector. (Figure by Fabian De Kok-Mercado)

Several factors determine whether a radiolabeled probe that binds to a saturable target will generate a useful image. First, the probe must have the needed pharmacokinetic properties to reach the target (see below). Second, it should have a high affinity for its binding site, as measured by the K_d (the concentration at which 50% of binding sites are occupied at equilibrium). Third, there must be a sufficient number of targets within the region of interest so that the accumulation of the bound tracer will result in a detectable signal. In practice, this means that it is possible to image low-density targets, as long as the K_d of the tracer–target interaction is in the nanomolar range (or less) and the fraction of labeled probe molecules (the specific activity) is high. When these conditions are met, a minute quantity of tracer, much too small to cause any pharmacologic effect, can produce an image (Jagoda et al., 2004; Eckelman, 1994; Eckelman et al., 2002, 1979). Compounds that are too toxic for therapeutic use might therefore still find application as imaging probes.

In contrast, the target of a non-saturable probe is generally an enzyme that alters the chemical structure of the probe, in a manner that favors its retention at the target site. For antiviral therapy, the classic non-saturable target is the HSV TK, which phosphorylates

acyclovir, causing it to accumulate within HSV-infected cells. As described below, this property of the HSV TK has also permitted the extensive use of the recombinant enzyme as an imaging reporter for SPECT and PET. The principal application of a non-saturable target in radionuclide imaging makes use of 2-deoxyglucose (DG), a glucose molecule lacking a 2-hydroxyl group that is readily phosphorylated by the host cell hexokinase, but cannot be further metabolized, and remains trapped within the cell (Fig. 1). DG can be labeled with fluorine-18 at the 2 position, to form ^{18}F-DG (FDG), without changing its metabolic activity. Because malignant cells tend to have a high rate of glucose metabolism, DG was originally tested as an anti-cancer drug, but it proved too toxic for clinical use (Woodward and Hudson, 1954; Pauwels et al., 2000). At low dose, however, FDG is not toxic, and it can be used as a probe to label cells with high glycolytic activity. FDG is now widely used to identify foci of increased glucose metabolism, such as tumors or sites of inflammation, by PET imaging (Fig. 6) (De Winter et al., 2002; Kumar et al., 2008).

In contrast to imaging a saturable target, in which the signal strength is determined by the number of radiolabeled probe molecules that bind to the desired site, the success of imaging a non-saturable target is not limited by the number of targets within the region of interest. For example, when ^{18}F-FDG is used to visualize a site of high glucose metabolism, such as the influx of neutrophils that occurs in response to endotoxin, each affected cell accumulates a large amount of phosphorylated label, amplifying the signal (Chen et al., 2006; Chen and Schuster, 2004). By bringing about the selective retention of phosphorylated acyclovir and other thymidine analogues within infected cells, the HSV TK provides a similar advantage, both for antiviral therapy and for imaging.

Because of the signal amplification inherent in the use of a non-saturable target, the specific activity of the probe and the number
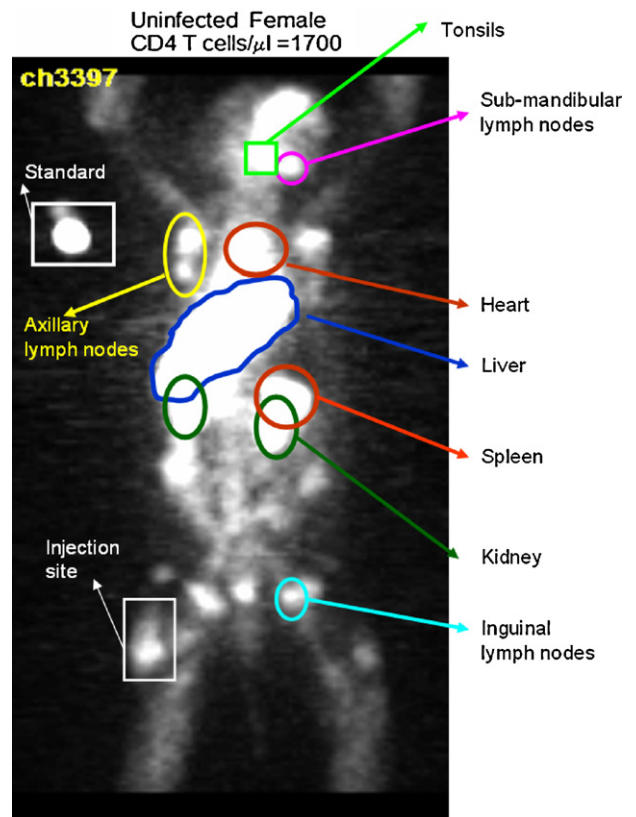
Fig. 5. Total-body SPECT image of a healthy macaque, showing uptake of the ^{111}In-labeled anti-CD4 antibody DTPA-OKT4A/hIgG4. Lymphoid tissues are identified. From Di Mascio et al. (2009a), with permission.
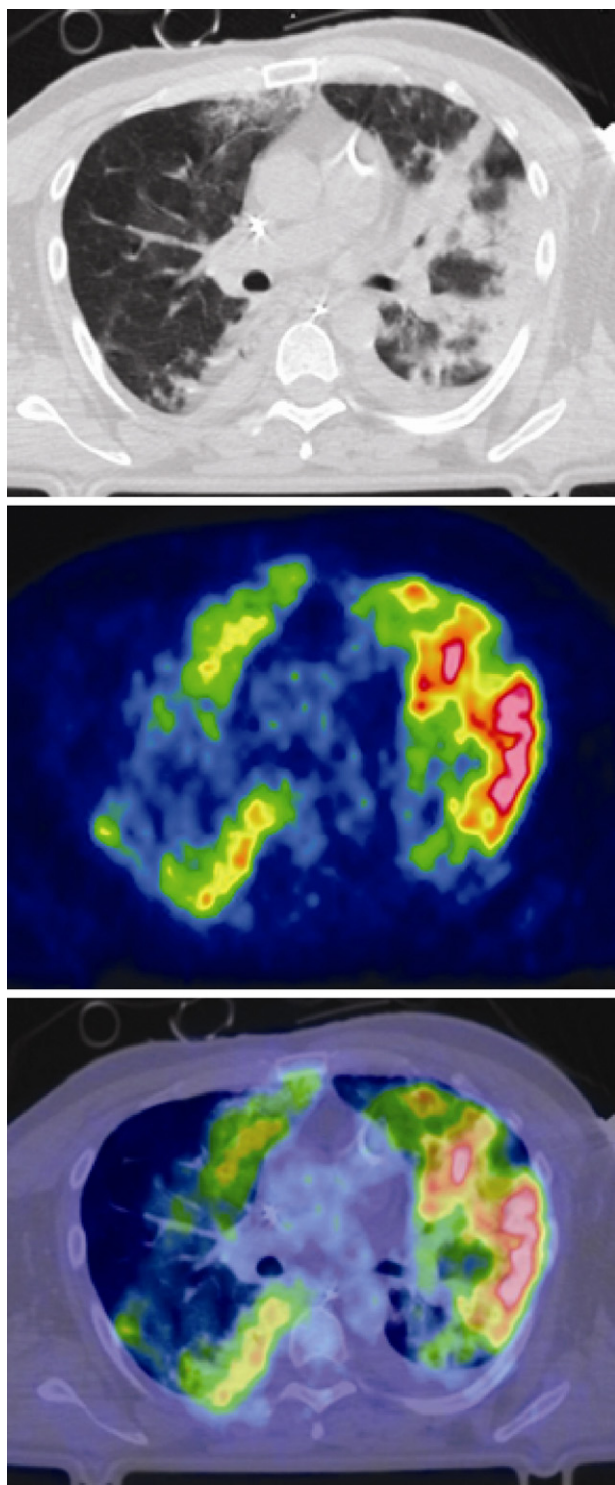
Fig. 6. ^{18}FDG-PET/CT scan of the thorax of a patient with severe swine-origin H1N1 influenza. A. CT scan shows patchy infiltrates and areas of apparently normal aeration. B. The PET image shows elevated tracer retention, principally in areas that contain infiltrates, but also in some apparently well-aerated regions. Red indicates the highest and blue the lowest level of activity. Previously unpublished images from Bellani et al. (2010), courtesy of Giacomo Bellani.

of target sites are less critical considerations in the acquisition of a useful image. In practical terms, this makes it possible for a tracer such as ^{18}F-FDG to be stored longer between synthesis and administration to subjects because one can compensate for the diminished radioactivity of the probe by giving a larger dose. For a saturable target, in contrast, such a delay could impair image acquisition,

because tracer molecules that have undergone radioactive decay will compete with the labeled probe for available binding sites.

2.3.3. Choosing the radionuclide

The radioisotopes with which virologists and medicinal chemists are most familiar, ^{3}H and ^{14}C, are weak beta-emitters with half-lives measured in years. Because hydrogen and carbon are constituent elements of most, if not all-antiviral medications, these isotopes can be introduced into a drug during its synthesis, and the product will not differ in structure or biological properties from the unlabeled compound. The long half-lives also mean that ^{3}H- and ^{14}C-labeled drugs can be stored and used for years without loss of activity. When employed for *in vivo* pharmacokinetic studies, the compound can be detected in blood, urine or tissue samples by scintillation counting, in which beta decay triggers the emission of a photon of light.

There are some key differences between such isotopes and the short-lived radionuclides used for PET and SPECT imaging. PET is based on positron-electron annihilations that yield pairs of 511 kEV photons, which because of their high energy are minimally attenuated or scattered during their passage through soft tissues. SPECT isotopes, on the other hand, emit photons in the lower energy range of 60–400 kEV, which are more likely to be scattered or absorbed. In addition, degradation of image quality is worsened, the further the target is from the detector. An advantage of SPECT, however, is that because the various tracers emit photons with a range of energy peaks, it is possible to image more than one target in the same session, by setting the instrument's computer algorithms to separate signals of different energies. PET isotopes, in contrast, all produce photons of the same energy, making it necessary to perform separate imaging sessions if two different probes are used to study the same subject.

As shown in Tables 1 and 2, many different radioisotopes can be used to label SPECT and PET probes. The choice of which one to use for a particular study will be based on a number of considerations:

- the availability of an imaging instrument;
- access to a cyclotron;
- expertise of the radiochemistry laboratory; and
- the technical challenges of incorporating a given isotope into the probe.

The latter point includes questions such as the chemical stability of the resulting molecule and the retention of its biological activity. The half-life of the selected radionuclide should also be consistent with the expected clearance time of the tracer, to ensure that sufficient radioactivity will be detected in the region of interest to provide a useful image.

The need for chemical stability and a short half-life can both potentially be met by performing PET imaging with a ^{11}C-labeled probe, because the replacement of one carbon atom by another during synthesis leaves the molecule's physiologic activity unchanged, while the 20-min half-life makes it possible to image the same subject repeatedly, at intervals of a few hours. However, the isotope's short half-life means that its use is only practical if one possesses an on-site cyclotron and adequate radiochemistry support. Those lacking such resources would be better off using a ^{18}F-labeled probe, because its 108-min half-life makes it possible for synthesis to take place off-site, but still allows a subject to be imaged twice daily. The limitation of this approach is that the introduction of a fluorine atom into the probe may require a difficult chemical synthesis, and it may also alter the molecule's biochemical characteristics (in this case, its antiviral efficacy). It will therefore be essential to test the activity of the probe *in vitro* to ensure that its biological activity has been retained.

Table 1
The radionuclides most commonly employed in PET imaging, with some of their advantages and disadvantages.

Radionuclide	$T_{1/2}$	Advantages	Disadvantages	Chemistry for radionuclide incorporation
^{15}O	122 s	Probe can be labeled without any change in its molecular structure	Requires on-site cyclotron. Not feasible for commercialization of probes	Water ($H_2{}^{15}O$) production
^{13}N	9.9 min			
^{11}C	20 min	Substitution of ^{12}C with ^{11}C possible so no change in molecular structure	Requires on-site cyclotron. Not feasible for commercialization of probes	Methylation
^{68}Ga	68.3 min	Generator commercially available, affordable	Addition of chelate structure usually requires large molecule	Metal chelation
^{18}F	110 min	Direct labeling of probe; excellent spatial resolution. Low energy (0.635 MeV; 97% β^+). Available and affordable	May not be appropriate for labeling large molecules with slow pharmacokinetics, due to short half-life	Nucleophilic or electrophilic substitution
^{64}Cu	12.7 h	Useful for probes with slow pharmacokinetics	Limited commercial availability and expensive	Metal chelation
^{76}Br	16.2 h	Direct labeling of probe	Limited commercial availability and expensive	Halide addition or substitution
^{89}Zr	78.4 h	Relatively inexpensive	Loss of spatial resolution due to high energy (0.901 MeV; 22.8% β^+)	Metal chelation
^{124}I	100.3 h	Direct labeling of probe; commercially available	Loss of some spatial resolution due to high energy (2.1 MeV; 24% β^+)	Halide addition or substitution

2.3.4. Labeling an imaging probe

The methods by which a radionuclide can be incorporated into an imaging probe fall into two broad categories: those in which the tracer molecule's original chemical structure remains unchanged, and those in which the structure is modified to permit addition of the radioactive moiety. The first approach includes the introduction of the radionuclide into the primary synthetic reaction and its direct exchange for its stable counterpart. The second includes three different techniques: chemical modification of the original molecule to permit the addition of a radiolabel; addition to the original molecule of a structure that already contains the radiolabel; and addition to the original molecule of a chemical structure that can noncovalently bind (chelate) the isotope.

No matter how labeling is performed, the criteria for success are the same. Most importantly, addition of the radionuclide should not interfere with the high-affinity binding of the tracer to its target. Labeling also should not reduce the lipophilicity of a probe that must pass through cell membranes or cross the blood–brain barrier to reach its target, and should not prevent its interaction with a specific transporter system. Finally, attachment of the radionuclide to the probe should be sufficiently stable to ensure that it remains in place while it is carried through the bloodstream to the target.

2.3.5. Tracer pharmacokinetics

Successful *in vivo* imaging depends upon the proper systemic distribution, cellular uptake and elimination of a tracer following its administration. To ensure rapid and uniform delivery throughout the body, most imaging probes are administered by intravenous injection. As noted above, the pharmacokinetics of a virus-specific imaging probe should resemble those of an ideal antiviral drug, in terms of its uptake into infected cells, but should differ from a therapeutic agent in being cleared quickly from uninfected tissues. (Such a pharmacokinetic pattern would provide optimal contrast between sites of viral replication and normal tissues.) Because clearance takes time, the radioisotope must have a long enough half-life to ensure that adequate activity remains in the region of interest once the background signal has reached a low level, so as to maximize the target-to-background ratio. In contrast to antiviral therapy, in which the desired drug concentration in tissues tends to be in the micromolar range, radiotracer concentrations are typically in the pico- to nanomolar range.

3. Imaging host responses to viral infection

Radionuclide imaging has been used to study viral diseases for more than three decades, by employing indirect methods to identify sites of infection. Beginning in the 1970s, it was found that cytomegalovirus pneumonitis and other infections in immunodeficient patients could be detected by administering a small intravenous dose of ^{67}Ga citrate and imaging them with a gamma camera (Hamed et al., 1979; Reinders Folmer et al., 1986). As a result of increased perfusion and enhanced vascular permeability, the radionuclide accumulated at sites of infection. Other approaches include the intravenous infusion of ^{111}In-labeled polyclonal human immunoglobulin or a patient's own ^{111}In-tagged leukocytes (Buscombe et al., 1993; Kumar, 2005). Although these techniques can detect the presence of an infection, they cannot identify its causative agent, so a microbiologic diagnosis must be obtained by collecting appropriate samples and performing laboratory tests.

FDG-PET imaging is increasingly used to study host responses to infection, especially in research settings. As described above, the technique relies on the preferential retention of phosphorylated ^{18}F-FDG in cells with high levels of glucose metabolism, which occur in infectious and inflammatory processes (Love et al., 2005;

Table 2
The radionuclides most commonly employed in SPECT imaging, with some of their advantages and disadvantages.

Radionuclide	$T_{1/2}$	Advantages	Disadvantages	Chemistry for radionuclide incorporation
^{99m}Tc	6 h	Generator is commercially available and affordable		Metal chelation
^{123}I	13.2 h	Direct labeling of probe; commercially available		Halide addition or substitution
^{111}In	67.3 h	Commercially available	When attached to proteins, mAbs and mAb fragments, tends to accumulate in the kidneys and liver	Metal chelation
^{67}Ga	78.3 h	Cyclotron produced, commercially available	When attached to proteins, mAbs and mAb fragments, tends to accumulate in the kidneys and liver	Metal chelation
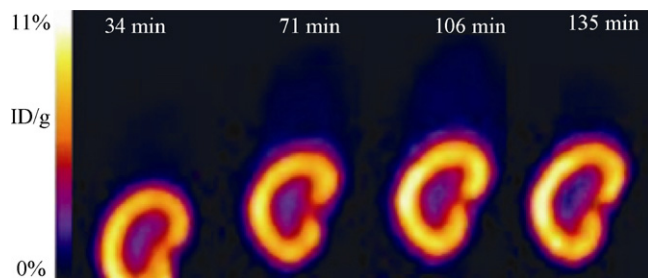
Fig. 7. PET images of the right kidney of a rat at various time points after injection with (S)-^{18}F-FPMPA. Areas of highest tracer retention are shown in orange and red. From Di Mascio et al. (2009b), with permission.

Mackie, 2004). Early in the HIV pandemic, for example, FDG-PET was used to demonstrate that zidovudine therapy had a beneficial effect in patients with HIV-related dementia, as shown by a measurable reduction in cerebral ^{18}F-FDG retention (Brunetti et al., 1989; Yarchoan et al., 1987). The same technique has shown that peripheral lymph nodes have a greater glucose uptake in untreated HIV-infected individuals than in patients on antiretroviral therapy, indicating that lymph nodes are sites of viral replication (Brust et al., 2006). FDG-PET was used recently to characterize a patient with severe swine-origin H1N1 influenza, showing that an intense inflammatory response was present both in areas of dense pulmonary consolidation as seen by CT scan and in regions of aerated lung (Fig. 6) (Bellani et al., 2010, 2009). The value of such studies would obviously be enhanced if the same patient could also be imaged with an influenza-virus-specific probe, to determine the relative chronology and physical distribution of viral replication and host inflammatory responses.

4. PET imaging of drug distribution in uninfected subjects

Whole-body PET imaging has been used to supplement traditional methods of measuring the uptake, distribution and excretion of antiviral drugs in laboratory animals. For example, PET was used to study the pharmacokinetics of the tenofovir analogue ^{18}F-PMPA in rats, revealing increased concentrations in the renal cortex that appear to correspond to the drug's occasional nephrotoxicity for humans (Fig. 7) (Di Mascio et al., 2009b). Similarly, in an effort to understand the cause of the neurologic side effects of the anti-influenza drug oseltamivir, investigators employed PET to examine the uptake and retention of the ^{11}C-labeled drug in the brains of mice (Hatori et al., 2009; Konno et al., 2008). Although a study of the pharmacokinetics of ^{11}C-labeled stavudine in rats was based on measuring radioactivity in excised tissues, the authors noted that the same questions could have been answered by *in vivo* imaging (Livni et al., 2004).

PET has also been used to follow the deposition and retention of an inhaled dose of radiolabeled zanamivir, another inhibitor of the influenza viral neuraminidase, in human subjects (Bergstrom et al., 1999; Cass et al., 1999). These studies only involved uninfected individuals, but it is evident that the same procedure could be used to track the distribution of zanamavir in persons infected with seasonal influenza viruses, either before or after treatment. Such experiments would help to determine the effect of therapy on the course of illness, and would reveal whether the radiolabeled drug could be used to detect and track the spread of virus in the respiratory tract.

5. Proof of concept: recombinant viruses encoding imaging reporters

The nascent field of cancer "virotherapy" exploits the ability of viruses to replicate selectively in malignant cells as a novel
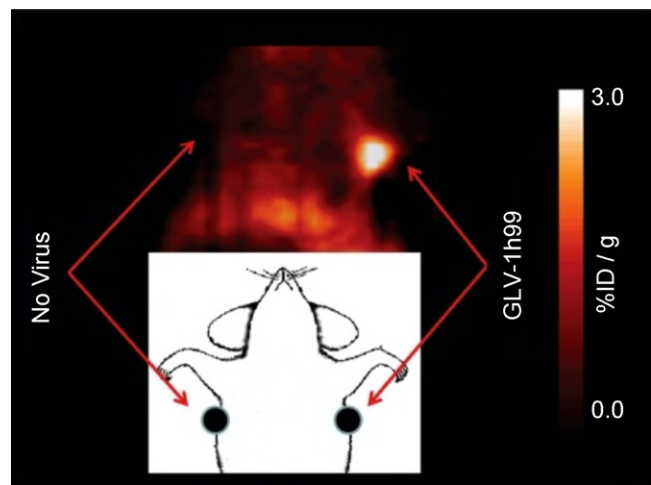
Fig. 8. PET image of a mouse with bilateral tumors, one of which has been inoculated with a recombinant vaccinia virus encoding the human norepinephrine transporter. The probe, [124]I-MIBG, is taken up by the virus-infected, but not by the uninfected tumor. From Chen et al. (2009b), with permission.

approach to killing tumors. To monitor the replication of the oncolytic virus, recombinant agents have been constructed that encode a reporter for radionuclide imaging. Examples include:

- a recombinant vaccinia virus encoding the human norepinephrine transporter, which enables PET imaging by causing [124]I-meta-iodobenzylguanidine (MIBG) to accumulate within infected cells (Fig. 8) (Chen et al., 2009b);
- a recombinant vaccinia virus encoding the type-2 somatostatin receptor, which binds the somatostatin analog ^{111}In-pentetreotide on the cell surface, so that virus-infected tumors can be imaged by gamma scanning (McCart et al., 2004);
- recombinant measles and vesicular stomatitis viruses encoding the thyroidal sodium iodide symporter, which causes infected cells to take up ^{123}I for PET imaging, or ^{131}I for radiotherapy (Dingli et al., 2004; Goel et al., 2007). Herpesviruses are also being used as experimental oncolytic agents; in this case, the virus' own TK provides a reporter for PET or SPECT imaging (see below).

The strategy of introducing a gene encoding an imaging reporter into a viral genome provides proof of concept for the notion of employing a naturally occurring virus-encoded molecule as a target for a radiolabeled imaging probe. The approach described above, however, has a significant limitation that would not be seen using an unmodified virus: the introduction of an additional gene may interfere significantly with viral replication. This problem was observed, for example, when a gene encoding green fluorescent protein was introduced into Ebola Zaire virus; although the modified agent replicated normally in cell culture, it was attenuated for mice (Ebihara et al., 2007). The ideal imaging approach for the study of viral infections in laboratory animals should therefore make use of unmodified pathogens.

6. Virus-specific radionuclide imaging: the herpesviral TK as a reporter

The possibility of using a naturally occurring virus-encoded molecule as an imaging reporter was first explored in the early 1980s, when acyclovir was approved as an antiviral drug for the treatment of HSV infections. The ability of this and other nucleoside analogues to selectively inhibit HSV replication is based on their ability to undergo phosphorylation by the viral thymidine kinase (TK), but not by corresponding host enzymes. TK supports

DNA synthesis in dividing cells or during pathogen replication by phosphorylating the 5′ hydroxyl group of deoxythymidine (dT) to produce dTMP, which is then converted to the triphosphate form by other cellular kinases (Eriksson et al., 2002).

All multicellular organisms and bacteria possess a TK, and the enzyme is also encoded by large DNA viruses (Gentry, 1992). Two families of TK enzymes are recognized, based their chemical structure and substrate specificity (Deville-Bonne et al., 2010). The first group includes the TK1 enzyme found in the cytoplasm of human cells, a tetrameric molecule which phosphorylates only dT and deoxyuridine, using ATP as the phosphate donor (Welin et al., 2004). TK1 is expressed during the S-phase of the cell cycle, and it is therefore present in significant quantity only in dividing cells. Labeled deoxythymidine analogues such as ^{18}F-3′-deoxy-3′-fluorothymidine (FLT) can therefore be used as probes to selectively image tumors by PET (Langen et al., 1969; Shields, 2006). Other members of this enzyme family include the poxviral TK, which has a similarly narrow range of substrate specificity (Gentry, 1992).

The second class of TKs consists of homodimeric enzymes that are able to phosphorylate a range of substrates, including a variety of nucleoside analogues (Eriksson et al., 2002). This group includes a second human enzyme, TK2, that is found at low concentrations in mitochondria. It also includes the TK encoded by the alpha-herpesviruses, of which HSV is the best-known member. The broad substrate specificity of the HSV TK is the basis of antiviral therapy with acyclovir and other nucleoside analogues. Acyclovir enters cells from the bloodstream by diffusion or active transport across the cell membrane. In uninfected cells, the drug is not modified by TK1, and only a minimal amount is phosphorylated by the mitochondrial TK2, so that almost all of it diffuses back into the bloodstream and is eliminated by the kidneys. In HSV-infected cells, however, the viral TK converts acyclovir to its monophosphate, which is then converted by cellular kinases to the triphosphate. Because acycylovir triphosphate has a 20- to 100-fold greater affinity for the HSV DNA polymerase than for the host enzyme, it is preferentially incorporated into replicating viral genomes, causing chain termination (Elion, 1983). The selective retention of acyclovir and related antivirals in HSV-infected cells and their rapid clearance from uninfected tissues enhances their potential utility as imaging probes, by increasing the target-to-background ratio.

Until recently, efforts to image HSV infections have focused on detecting and monitoring viral encephalitis in laboratory animals. Beginning in the early 1980s, investigators succeeded in showing that, even though the blood–brain barrier normally restricts the entry of deoxythymidine analogues into the central nervous system, the increased permeability associated with viral infection permitted the entry of a sufficient amount of probe to label infected cells. In a series of studies, rats were infected intraocularly with HSV-1, and after an appropriate interval were injected intravenously with a small dose of ^{14}C-labeled 2′-fluoro-5-methyl-1-β-D-arabinosyluracil (FMAU) or with ^{131}I- or ^{125}I-labeled E-5-(2-iodovinyl)-2′-deoxyuridine (IVDU) (Gill et al., 1984; Klapper et al., 1988; Price et al., 1983; Saito et al., 1984; Cleator et al., 1988). Foci of encephalitis were identified either by killing animals and performing autoradiography on brain slices, or by imaging live animals using a gamma camera. However, only weak signals were obtained. Similar studies have been performed more recently using ^{11}C-labeled FMAU, but the results of *in vivo* imaging were not shown (de Vries et al., 2000). In general, this experience has shown that the limited transport of probe across the blood–brain barrier significantly restricts the utility of radionuclide imaging for HSV encephalitis.

In contrast to those discouraging results, success in PET imaging of HSV infections has recently been reported in a different area of research: the use of viruses for experimental cancer therapy. Herpesviruses are one of a number of agents that are being explored for

their capacity to infect and kill tumor cells. The fact that they naturally encode a TK means that their replication in laboratory animals can readily be monitored by radionuclide imaging. Viral replication in tumors has been virualized using several different radiolabeled deoxythymidine analogues: [^{18}F]-2′-fluoro-2′-deoxy-1-β-D-β-arabinofuranosyl-5-ethyluracil (FEAU) (Fig. 2) (Brader et al., 2009); ^{124}I-5-iodo-2′-fluoro-1-β-d-arabinofuranosyl-uracil (FIAU) (Jacobs et al., 2001); or 9-(4-[^{18}F]-fluoro-3-[hydroxymethyl]butyl)-guanine (FHBG) (Kuruppu et al., 2007). This work thus represents the first success in imaging viral replication in a living animal by targeting a naturally occurring virus-encoded reporter with a radiolabeled probe.

The beta herpesvirus cytomegalovirus (CMV) does not encode a TK, but employs its UL97 protein kinase to phosphorylate nucleosides. Because UL97 recognizes acyclovir to only a limited extent, the drug cannot be used to treat CMV infections. In 1990, however, a new compound with greater anti-CMV activity, ganciclovir, was introduced into medical practice. An ^{18}F-labeled analogue of ganciclovir, 9-[(3-fluoro-1-hydroxy-2-propoxy)-methyl]guanine (FHPG), has been evaluated as a probe to image CMV infections (Alauddin et al., 1996; de Vries et al., 2000). PET scanning using ^{18}F-FHPG was shown to correctly identify CMV encephalitis in rats, as confirmed by autoradiography, but the utility of the approach was once again limited by the restricted entry of the probe across the blood–brain barrier (Buursma et al., 2005).

Because the HSV-1 TK gene is comparatively small, it should be possible to insert it into the genome of other viruses, to provide a reporter for radionuclide imaging. For example, recombinant HIV and simian immunodeficiency (SIV) viruses encoding the HSV-1 TK has been constructed by using parental viruses lacking the nef gene, which both attenuates them and creates space for additional genomic material (Chakrabarti et al., 1996). Although originally envisioned as live vaccines that would be safe for use, because of their sensitivity to ganciclovir, these recombinant agents could also be employed to study retroviral infections in animals by SPECT or PET.

In addition to these uses, the HSV-1 TK molecule has been extensively exploited as a reporter for SPECT or PET imaging of malignancies and the assessment of gene therapy (Green et al., 2004; Miyagawa et al., 2008; Serganova et al., 2007). In a novel therapeutic approach, HSV itself is now being harnessed to kill malignant cells, and its distribution and replication are being monitored by PET imaging, using ^{124}I-FIAU as the tracer (Bennett et al., 2001; Kuruppu et al., 2007). It has recently been shown that treatment of herpesvirus-induced cancers, such as Burkitt's lymphoma, with the anti-cancer drug bortezomib triggers expression of the viral TK, making it possible not only for the tumors to be visualized by PET, but potentially treated with ganciclovir (Fu et al., 2007).

As noted above, poxviruses also encode their own TK. Because it is a type II enzyme, however, with a substrate specificity similar to that of human cells, poxviruses are not susceptible to acyclovir. Treatment has instead relied on nucleotide analogues such as cidofovir, which enter cells by macropinocytosis, and are then converted to their bi- and triphosphates by cellular kinases. Such drugs therefore inhibit both herpesviruses and poxviruses. Because their initial phosphorylation is performed by a host enzyme, they accumulate in normal cells, making them unsuitable for use as virus-specific imaging agents. Interestingly, recent research has identified some thymidine analogues that inhibit the replication of wild-type vaccinia virus significantly better than a virus lacking a TK gene, suggesting that their mechanism of action resembles that of acyclovir (Prichard et al., 2007, 2006). However, because these compounds can also be processed to some extent by the host TK, they probably have little value for imaging.

In an interesting parallel to viral infections, many genera of bacteria encode a type I TK, making them susceptible to antiher-
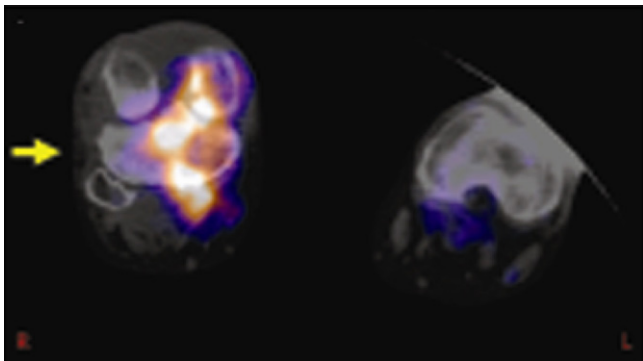
Fig. 9. PET/CT image of septic arthritis in the right knee of a patient, using the bacterial TK as the reporter and [124]I-FIAU as the probe. From Diaz et al. (2007), with permission.

pesviral drugs. Imaging researchers have taken advantage of this property to visualize musculoskeletal infections, both in mice and in human patients. For example, a recent study employed [124]I-FIAU as a PET probe to detect staphylococcal infections following joint surgery (Fig. 9) (Bettegowda et al., 2005; Diaz et al., 2007). The same approach has been used to image *Mycobacterium tuberculosis* in animals, even though the organism lacks its own TK. By introducing the gene for the *Escherichia coli* TK into a mycobacterial strain, researchers were able to use ^{125}I-FIAU to visualize sites of infection by SPECT (Davis et al., 2009).

7. Beyond TK: other imaging targets in the viral replication cycle

The only naturally occurring virus-encoded molecule that has been employed for radionuclide imaging is the HSV TK, but it seems unlikely that this enzyme should be the only possible target for virus-specific imaging. In the following sections, we review basic steps in viral replication that could be exploited for PET or SPECT. A number of these steps are identified in the hepatitis C virus replication cycle (Fig. 3).

7.1. Binding, membrane fusion and entry

To infect a cell, a virus must first bind to its surface (usually to a specific receptor), then undergo a process of fusion with the cell membrane that leads to the disassembly of the capsid and the release of its contents into the cytoplasm. During the fusion process, virion surface proteins undergo specific conformational changes that expose hydrophobic peptide sequences that interact with the lipid bilayer (Fig. 3). While some agents, such as retroviruses, enter directly from the cell surface, for others membrane fusion takes place within endosomes, and is triggered by acidification. In both cases, peptides and other inhibitors have been identified for a wide range of viruses that specifically block the entry process.

7.2. Transcription and genome duplication

Once the viral genome has been delivered into the cytoplasm, gene transcription and genome production depend upon a ready supply of nucleotides and deoxynucleotides for the synthesis of new RNA, for all types of viruses, and DNA (for DNA viruses). A major antiviral strategy is therefore the design of analogues that are accepted as substrates by the viral RNA or DNA polymerase, resulting either in chain termination or their incorporation into polynucleotides, impairing their subsequent function. Because most drugs that inhibit the viral polymerase are converted to their monophosphate by a cellular kinase, and are therefore trapped

within both infected and uninfected cells, they would not be suitable as virus-specific imaging probes. An alternative approach, which has been successful for HIV therapy, and is now being used to target hepatitis C and other viruses, is to identify non-nucleoside molecules that bind to a locus on the surface of the viral polymerase, rather than to its active site, inducing a conformational change that blocks its enzymatic activity (Fig. 3). Because such molecules do not undergo initial processing by a host enzyme, they have the potential to serve as virus-specific probes.

Another potential target for pathogen-specific tracers is the virus-encoded helicase, which separates the strands of double-stranded DNA or RNA molecules (or, in the case of retroviruses, DNA/RNA molecules) during the course of transcription and genome replication. Specific inhibitors of the helicase of a number of viruses are being developed as candidate drugs, and they may also have potential as imaging probes.

While transcription is taking place, viral mRNA must be modified to ensure its efficient translation, through the formation of a methylated 5′ cap. The methylation process is proving to be a fruitful target for antiviral therapy. However, although compounds that inhibit methylation by blocking the host cell enzyme, S-adenosylhomocyteine hydrolase, have potent broad-spectrum antiviral activity, they would not be suitable as virus-specific probes, because they do not bind to a viral target. In contrast, compounds that specifically block a virus-encoded methyltransferase, such as the flavivirus NS5 protein, might make good tracers for radionuclide imaging (Dong et al., 2008).

7.3. Protein cleavage by a virus-encoded enzyme

Translation of viral mRNA by the host cell protein-synthetic machinery is often followed by cleavage of the protein products to their mature forms. In the case of the positive-sense, single-stranded RNA viruses, for example, the entire genome is first translated into one large polyprotein, which is then cleaved into individual components by cellular enzymes and its own endogenous protease (Fig. 3). Because they differ from those of the host cell, the active sites of a number of viral proteases have proven to be rewarding targets for antiviral drugs. A number of compounds have been identified that bind irreversibly and with high specificity to the active sites of the proteases, and could potentially be used as radiolabeled probes.

7.4. Virion assembly and exit

Once nucleocapsids and other virion structural components have been generated, they are carried by endogenous transport pathways to the inner surface of the cell membrane, where assembly and release of viral particles takes place. Efforts to develop specific inhibitors of this process are under way, but no drugs have yet been shown to have protective activity *in vivo* (Harty, 2009). Because the same transport pathways are shared by uninfected cells, it is unlikely that drugs that block such mechanisms could be used as pathogen-specific tracer molecules.

7.5. Targets on the surface of virus-infected cells

Viral envelope proteins accumulate on the cell surface during the course of replication, anchored by lipophilic peptide sequences (Fig. 3). Such surface markers, which make infected cells vulnerable to the immune system through the binding of specific antibodies and subsequent cell-mediated destruction, could also be targets for probes such as radiolabeled antibodies. Interestingly, certain tumors induced by viral infection, such as EBV-associated lymphomas, hepatocellular carcinoma in patients with hepatitis B and C and cervical carcinomas induced by papillomaviruses, express

viral antigens, making them vulnerable to destruction by antibodies tagged with high-energy alpha emitters (Dadachova et al., 2006). It has been suggested that the same strategy could be used to eradicate HIV in individuals with low levels of residual infection (Casadevall et al., 2007). A similar strategy, using antibodies labeled with gamma-ray or positron-emitting isotopes, might be used for SPECT or PET imaging.

8. Some candidate probes for virus-specific imaging

Could drugs other than anti-HSV medications be used to visualize viral infections by SPECT or PET? Most licensed antivirals are nucleoside or nucleotide analogues that undergo initial phosphorylation by cellular kinases, and therefore could not serve as pathogen-specific probes. However, as noted above, the replication cycles of various DNA and RNA viruses offer a variety of targets for drugs and probes that interact specifically with virus-encoded molecules. The following sections identify examples of candidate tracers for nine different virus families.

8.1. Arenaviruses

- The small-molecule drug ST-294 inhibits the replication of Junin and other New World arenaviruses, while the benzimidazole derivative ST-193 targets the Old World agent, Lassa virus (Bolken et al., 2006; Larson et al., 2008). Based on mutagenesis studies of the virion surface GP2 molecules, both compounds interfere with viral entry, but the specific mechanism is not known.

8.2. Coronaviruses

- The extensive requirement for protein cleavage during the coronavirus replication cycle has made the viral papain-like (Plpro) and chymotrypsin-like (3Clpro) proteases major targets for drug development. Highly specific small-molecule inhibitors of both enzymes have been identified that inhibit the SARS coronavirus, and might also be used to image infections (Ghosh et al., 2008; Ratia et al., 2008).

8.3. Flaviviruses (see Fig. 3 for identification of potential targets)

- Inhibitors of flaviviral proteases, such as the dengue virus NS3, block replication without causing cellular toxicity, suggesting that they are specific for the viral target (Lescar et al., 2008). The small-molecule BILN 2061, which binds to the active site of the hepatitis C virus NS3, was effective in reducing viral load in patients (Lamarre et al., 2003). Although its development was halted by evidence of cardiotoxicity, this would not necessarily prevent the compound from being used at low dosage as an imaging probe.
- Drugs that directly inhibit the virus-encoded methyltransferase are currently under development for dengue, West Nile and other flaviviruses (Dong et al., 2008).
- Non-nucleoside molecules have been identified that inhibit the RNA-dependent RNA polymerase of bovine pestivirus (Paeshuyse et al., 2009). Others are now under evaluation for the treatment of hepatitis C.
- The C-terminal portion of the flavivirus NS3 molecules is a helicase, which is essential for replication. Inhibitors have been identified for hepatitis C and other flaviviruses (Leyssen et al., 2008; Lescar et al., 2008).
- The hepatitis C virion surface glycoproteins E1 and E2 are expressed on the surface of infected cells, making them potential targets for antibody-dependent cellular cytotoxicity and for imaging by a radiolabeled antibody fragment or a labeled DNA

aptamer (Drummer et al., 2003; Nattermann et al., 2005; Chen et al., 2009a).

8.4. Herpesviruses

- The UL97 protein kinase of CMV carries out multiple functions, including nucleoside phosphorylation (Prichard, 2009). The recently discovered antiviral mirabavir inhibits this enzyme, suggesting that it could be used as a radiolabeled tracer to image CMV infections.

8.5. Orthomyxoviruses

- The prototypes of drugs that bind directly to virions to inhibit their replication are the adamantanes, amantadine and rimantidine, which block the M2 ion channel, preventing core acidification that is required for virion disassembly in the endosome (Beigel and Bray, 2008). The specific nature of this interaction suggests that small doses of radiolabeled amantadine or rimantadine could be used to localize an influenza virus infection and track it's spread, both in laboratory animals and in humans.
- Two licensed drugs, oseltamivir and zanamivir, inhibit the cell-to-cell spread of influenza A and B viruses by binding to the neuraminidase active site (Beigel and Bray, 2008). This specific interaction might make them effective imaging probes. As noted, the distribution of inhaled ^{11}C-zanamavir has been visualized by PET in healthy human volunteers (Cass et al., 1999; Bergstrom et al., 1999). The same study, performed in influenza-infected patients, could provide useful information on drug distribution and the extent of viral infection.

8.6. Paramyxoviruses

- Two small-molecule compounds, VP-14637 and JNJ-2408068, block the entry of respiratory syncytial virus (RSV) into cells at low nanomolar concentrations by binding to a small hydrophobic pocket in the center of the F protein (Douglas et al., 2005).
- The RSV F protein is also the target of the human mab palivizumab, used to prevent infection in high-risk infants (Georgescu and Chemaly, 2009). A new antibody, motavizumab, with a greater virus-specific binding affinity and a lower degree of nonspecific binding to host tissues, has recently been developed (Wu et al., 2007).
- Conserved heptad repeat regions of the fusion proteins of the highly virulent paramyxoviruses, Hendra and Nipah, are the targets of peptide mimics, which block infection *in vitro* at low nanomolar concentrations (Porotto et al., 2007).
- The recently discovered compound RSV604 blocks the formation of RSV nucleocapsids by binding to a specific site on the N protein at submicromolar concentrations (Chapman et al., 2007). It is now in Phase II clinical trials.

8.7. Picornaviruses

- The entry of picornaviruses into cells requires the disruption of interactions among the four capsid proteins, so as to release the viral genome into the cytoplasm. A number of compounds have been identified that insert into "pockets" in the virion surface, stabilizing the capsid and preventing its disassembly (De Palma et al., 2008). Such compounds have protected mice against lethal poliovirus infection, and pleconaril, which acts by this mechanism, has been extensively tested for antirhinovirus activity in humans (De Palma et al., 2008; McKinlay and Steinberg, 1986). The highly specific interaction of such compounds with the viral

capsid suggests that they could be used to image sites of picor-naviral infection.

- The 3C protease of human rhinovirus is inhibited by a number of compounds, including the small-molecule rupintrivir, which binds irreversibly to the enzyme's active site (De Palma et al., 2008). Because the viral enzyme has no known human homologue, it should be a good target for virus-specific imaging.
- The benzimidazole analogue enviroxime inhibits the replication of rhinoviruses and enteroviruses by blocking viral RNA replication, apparently by directly inhibiting the action of the 3A protein. The compound protected mice against lethal Coxsackie myocarditis and has shown activity in protecting humans against rhinoviruses (Victor et al., 1997; De Palma et al., 2008).

8.8. Poxviruses

- The complex process by which poxviruses exit cells has provided targets for antiviral therapy. The small-molecule ST-246 interacts specifically with the vaccinia virus F13L protein, which is involved in the addition of a membrane layer needed to form extracellular enveloped virions (Yang et al., 2005; Duraffour et al., 2008). ST-246 is nontoxic for mammalian cells, animals and humans, suggesting that it uniquely targets the viral enzyme, and could therefore be used as a radiolabeled probe to image poxvirus infections.

8.9. Retroviruses

- The HIV fusion inhibitor, T-20, is a polypeptide that binds to the hydrophobic fusion region of the viral GP41, preventing it from undergoing the conformational change required for fusion with the cell membrane. A labeled version of this peptide might therefore be an effective tracer to identify sites of HIV replication.
- Non-nucleoside inhibitors of the HIV reverse transcriptase (RT) interfere directly with its activity by binding to loci other than the active site. A number of such compounds are now licensed for the treatment of HIV infection (Sluis-Cremer and Tachedjian, 2008). Because no equivalent of the HIV RT is encoded by the human genome, radiolabeled derivatives of these drugs might prove useful in targeting sites of HIV replication.
- The potential of protease inhibitors as effective antiviral drugs was first proven for the treatment of HIV infection, and a number of different compounds are now approved for clinical use. The most recently licensed, darunavir, has a strong binding affinity for the HIV protease ($K_d = 4.5 \times 10^{-12}$ M), suggesting that it would make an effective probe (Lefebvre and Schiffer, 2008).

9. Getting started: testing a drug or antibody for its potential as an imaging probe

To assess whether a drug or antibody has promise as an imaging probe, it is not necessary to label it with one of the isotopes listed in Tables 1 and 2 and attempt to image an infected animal by PET or SPECT. Instead, a preliminary evaluation can be performed using the ^{14}C- or ^{3}H-labeled compound, which is often synthesized for pharmacokinetic studies and is therefore available for other types of research. Three types of experiments can be performed. The first is an *in vitro* assay, in which the labeled drug is added to the liquid medium of a number of flasks or wells of virus-infected and uninfected cells. At various time points, the supernatant is removed, the cells are rinsed and harvested, scintillation counting is performed, and the amount of radioactivity retained by infected and uninfected cells is compared. The accumulation of a significantly larger quantity of radiolabeled drug in virus-infected than uninfected cells indicates that it may also be preferentially retained at sites of infec-

tion *in vivo*. Additional studies would include testing the specificity of binding, by adding a large excess of unlabeled drug; determination of the K_d of the labeled drug, the concentration of binding sites (B_{max}) and the kinetics of binding; and competition studies with other unlabeled analogues (Cheng and Prusoff, 1973; DeBlasi et al., 1989; Jeffries et al., 1997).

Investigators could then move forward to an *ex vivo* experiment, in which virus-infected and uninfected animals are killed at various time points, necropsies are performed and relevant organs (for example, the lungs in influenza or the brain in encephalitis) are collected and frozen. Thin sections are cut and placed onto microscope slides, which are then immersed in a solution of labeled ^{14}C- or ^{3}H-labeled drug, rinsed to remove unbound tracer and placed against film or phosphor imaging plates for autoradiography (Saito et al., 1984; Frey and Albin, 2001; Cagnin et al., 2007; Patel and Gibson, 2008; Stumpf, 2005). Once a sufficient exposure time has elapsed, the films are examined to determine if radiolabeled drug has preferentially been retained at sites of viral infection. To check for specificity, various amounts of unlabeled ("cold") drug can be added to the solution of labeled compound, to determine if it will compete for binding to sites of infection, reducing the detected signal. Additional thin sections would be used for immunohistologic studies, to confirm the presence of virus-infected cells.

At the same time that *ex vivo* studies are being performed, investigators can carry out *in vivo* experiments, in which ^{14}C- or ^{3}H-labeled drug is administered to infected and uninfected animals (preferably small rodents). After an appropriate delay, the animals are killed, organs of interest are removed, samples are collected for determination of viral titer by plaque titration and of probe concentration by scintillation counting or autoradiography, and sections are prepared for immunohistologic study. If sites of maximum viral replication also show the highest levels of radioactivity, and much lower signals are detected in uninfected tissues, this would suggest that the drug has potential as a virus-specific probe. As in *ex vivo* studies, the specificity of binding can be further evaluated by administering various amounts of unlabeled drug along with the labeled probe, to see if competition takes place.

If initial testing of the ^{14}C- or ^{3}H-labeled drug confirms its potential as an imaging probe, the next step will be to work with a radiochemist to determine the necessary precursors and methods for synthesis of a SPECT or PET tracer. Once that synthesis has been carried out, evaluation of the new radiolabeled probe should include *in vitro* and/or *ex vivo* studies to ensure that it has retained its biological activity. At the same time, imaging specialists should be consulted to design initial studies in an appropriate animal model of viral infection.

In addition to offering a second application for certain licensed antivirals that directly target viral replication, radionuclide imaging may also provide a use for some compounds that are active *in vitro* and in animal models, but for various reasons have not moved forward to licensure. As in the case of deoxyglucose, which failed in trials as a cancer drug, but is now the most widely used tracer for PET imaging, this might include candidate antivirals that are nephro- or hepatotoxic at therapeutic doses. Rather than discarding such drugs, researchers should consider evaluating them for use as virus-specific imaging probes.

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