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Review Article

Nuclear molecular imaging: a novel means of drug discovery in gene therapy?

E.K.J. Pauwels

Department of Radiology, Division of Nuclear Medicine, Leiden University Medical Centre, P.O. Box 9600, 2300 RC Leiden, The Netherlands. e-mail: e.k.j.pauwels@lumc.nl

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Abstract

The next step for medical imaging is molecular imaging. Nuclear imaging with its extremely high sensitivity is well suited to target molecular structures. Nuclear imaging techniques such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) can measure transgene expression in living animals using appropriate reporter gene-reporter probe systems. One such system uses the herpes simplex virus (HSV) thymidine kinase (HSV-1-tk) gene as reporter gene and radiolabeled guanosine analogues such as aciclovir, ganciclovir and penciclovir as substrates. Another system uses receptor genes as reporter gene and receptor-targeting radiolabeled agents such as spiperone and octreotide as probe. In addition, sodium/iodide symporter gene expression can be monitored by iodide uptake and may well serve as a suitable system to obtain quantitative information on gene expression. This new methodology provides temporal quantitative data on biodistribution and pharmacological action, without the need to sacrifice numerous animals. In this way, nuclear molecular imaging may reduce the time of development and testing of new drugs involved in gene therapy and may therefore represent a cost-saving strategy.

Introduction

Genetics is one of the most rapidly evolving areas in medicine. It has an enormous impact on understanding disease, and has provoked a shift from making the diagnosis on the basis of manifestations to the identification of an abnormal molecular process. The mapping of the human genome started a revolution in understanding the molecular basis of disease and the conception that altered biology is the result of changes in gene expression. In its ultimate form, the specific pattern of expression of genes would represent normal or diseased tissues with their typical molecular structure. This genetic profile may be used for diagnostic purposes (*e.g.*, cystic fibrosis, Huntington's disease) or for prediction of drug response (*e.g.*, cancer treatment).

In the past, various analytical techniques have proven successful for probing genetic factors related to disease. These biological assays include blotting to locate a sequence in DNA and RNA expression, as well as the use of proteins, enzymes and peptides as probes for cellular processes such as apoptosis and necrosis. Parallel to these developments, increasing efforts have been directed to the development of noninvasive imaging techniques using targeted and activatable imaging agents in order to exploit the pathways in normal and altered cellular processes. Whereas the aforementioned assays require tissue samples, molecular genetic imaging is basically noninvasive and provides pictorial and quantitative information on cellular processes in space and time.

The basic concept underlying such imaging techniques as positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) is that an expressed protein (enzyme) can be probed with specific agents. Such imaging techniques are particularly helpful for monitoring gene therapy in animal models and human disease. It is well understood that the success of gene therapy is highly dependent on the location and the duration of protein expression, and noninvasive imaging of gene expression is an essential part of the experiment or treatment. Thus, molecular imaging is one of the most promising modalities to facilitate continuous detection of gene expression. It is not the purpose of this article to discuss

these fields in detail, but rather discussion has been limited to agents which may promote the development and testing of drugs involved in gene therapy.

Expert fields in molecular imaging

In a molecular imaging center, knowledge of molecular biology, chemistry and imaging technology converges. Typically, molecular imaging requires the expertise of an interdisciplinary field including biologists, chemists, physicists, veterinarians, geneticists, clinicians and biomedical technicians. In addition, there should be collaboration with industry and government agencies to facilitate the transition from bench to bedside. In the ideal situation, the activities of many experts overlap to increase the sum of the possibilities.

Imaging devices

In recent years, enormous developments have taken place which include the implementation of high-resolution PET devices for animal research and patient studies, the development and introduction of new pulse sequences for MRI, as well as the use of ultra-high-field-strength machines. In general, radiopharmaceuticals developed for nuclear imaging techniques such as PET and SPECT have a high specificity and require minimal amounts of radiolabeled material, on the order of 0.10-10 nmol per dose (usually not more than a few ml). For MRI and computed tomography (CT), higher concentrations on the order of 10-100 μM are necessary to produce sufficient image contrast. The high sensitivity of PET and SPECT (it can detect picomolar concentrations of substances) and the high anatomic resolution of MRI and CT have been combined in the current generation of PET/CT and SPECT/CT scanners in which “image fusion” has been realized. This concept makes it possible to determine specific molecular signals created by the activity of a gene and visualize function and anatomy in one image. For the future, image fusion between MRI and PET can be expected, reducing the radiation burden due to ionizing radiation. An underlying premise of molecular medicine imaging is that the selection of the imaging technology is primarily driven by the clinical question. In this way, the advantages of a specific imaging modality or combination of modalities can be used. In practice, the choice of the imaging device is mainly determined by the probe that is available for the biological question and often comes down to high-sensitivity nuclear imaging.

Radiopharmaceutical chemistry

The design of the imaging probe forms the heart of the developments in molecular imaging. Properly designed, molecular probes are biological tools to reveal the basis of normal and abnormal biochemical processes in disease. Scientists involved in biochemistry, radiopharmaceutical design and synthesis play a key role in the development of these agents. Cooperation with molecular biologists adds in solving important issues for imaging probes, including target specificity, membrane permeability, turnover rate, blood pool clearance and affinity for the target.

Molecular imaging techniques and gene expression

One of the most compelling challenges for molecular imaging is the visualization of gene expression. *In vivo* assessment of gene expression requires an imaging target. Direct visualization of DNA would entail specific targeting of 2 molecules per cell. Besides the fact that the 2 DNA molecules may not be identical, unavoidable nonspecific binding of the imaging agent would obscure the signal arising from the DNA molecule. Similar obstacles are encountered if visualization of mRNA is considered. Also, it would be practically impossible for bulky and remote imaging devices such as PET, SPECT and MRI to pick up the signal from a few molecules in a tiny piece of tissue. A better approach would be the direct imaging of proteins. Proteins are the workhorses of the cell and play a key role in many cellular processes, including enzymatic reactions, receptor function, signaling and neurotransmission. Since proteins may be present as millions of copies per cell, protein imaging offers the advantage of massive amplification of the target molecule. In this way, the expression of specific DNA sequences and the translation of mRNA can be monitored, and enzymatic amplification of protein function may further facilitate molecular imaging research.

Gene transfer

For diagnostic or therapeutic purposes, genetic material may be introduced in diseased tissue in order to encode a protein for therapeutic effect. The transfer of genetic material into the host cell is an important area of study. As genes do not penetrate inside cells by themselves, they must be introduced to host cells using vectors of viral or nonviral nature. Viral vectors are the preferred method, as nonviral vectors such as naked DNA injected by a DNA gun or DNA complexes in liposome formulations have a low transduction efficiency. Viral vectors allow easy incorporation of the transgene into the host DNA using the viral replication machinery. This is achieved by replacing portions of the wild-type viral genome by the genetic material to be introduced into the host cell. Although many of these vectors are immunogenic or oncogenic, they are the most widely used vehicles for gene transfer in the clinical setting.

Gene therapy is still a far from routine procedure. It is only a dream that new genes would be introduced exclusively to target cells. Many other cells are also subjected to gene transfer and the clinical effect is unpredictable

(1). Therefore, many problems remain to be solved and molecular imaging will undoubtedly be of use.

With regard to gene therapy, imaging techniques may clarify various important issues which hamper optimal application in the clinical setting. These include:

- Did the vector used for gene transfer arrive at the target, *e.g.,* the organ of interest?
- How is the distribution in the target?
- Was the transferred gene expressed?
- What is the biodistribution and are there specific areas of undesired accumulation?
- Would the degree of gene expression be sufficient for the desired effect?
- Are the pharmacokinetics optimal for therapy?

Nuclear molecular imaging is a noninvasive methodology and would be the ideal method for *in vivo* mapping of gene expression. Moreover, it would provide quantitative data for assessing transgene delivery and gene expression. As such, molecular imaging with nuclear (scintigraphic) techniques is a unique tool to measure the efficacy of the proposed cure with gene therapy.

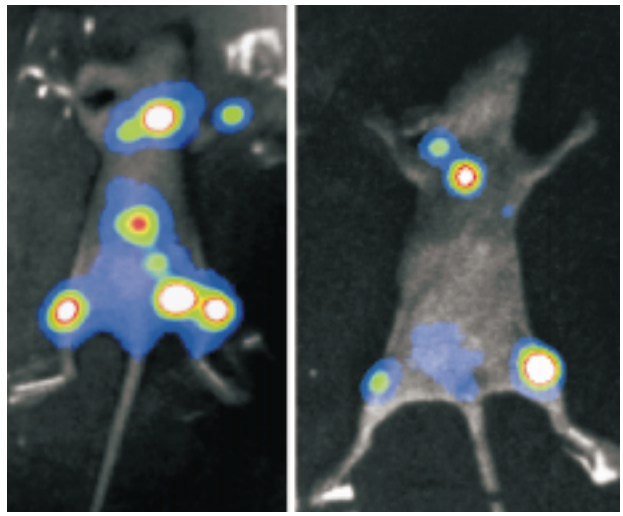

Fig. 1. Experiment in which luciferase-expressing human breast cancer cells (MDA-MB-231) were administered to the left cardiac ventricle of immunodeficient mice (BALB/c *nu/nu*). Nests of cancer cells (micrometastases) are visible in various parts of the body. The metastases were detected about 2 weeks prior to visualization by conventional X-ray. Courtesy of Dr. Gabri van der Pluym, Department of Endocrinology, LUMC, The Netherlands.

Reporter genes and reporter probes

As mentioned, genes may be introduced into the tissue of interest for diagnostic or therapeutic purposes. At present, efforts are being undertaken to use transgene expression to treat malignancies. In this setting, monitoring of gene expression "can be used as a step toward the validation of gene therapy" (2). Thus, a critical step in these experimental techniques is the ability to monitor the temporal changes in the magnitude and location of gene expression. Such an assay would allow researchers and clinicians to modify vectors to facilitate and improve DNA delivery, to evaluate the level of gene expression and the duration of expression in relation to the effect of treatment.

Imaging of transferred genes involves the use of a reporter gene and a reporter probe. The reporter gene encodes for a protein that is easily detected by the reporter probe. This gene-probe system is often referred to as indirect molecular imaging and has been used by molecular biologists for many years to measure gene expression. The technique involves the linking of a reporter gene to a selected promotor construct, its introduction into the target cell by an adequate vector and translation into a reporter protein via mRNA transcription and translation. According to this principle, the reporter protein metabolizes or binds a reporter probe, thereby generating a signal.

The most widely used reporter genes include those that encode for luciferase, β-galactosidase and green fluorescent protein. These optical approaches rely on fluorescence and bioluminescence as a source of contrast. However, due to the attenuation of the emitted visible light photons, this gene-probe system cannot be applied to humans. This optical system finds wide application in basic research, in which it is a very cost-effective method to study gene expression in tissues and rodents. An example is presented in Figure 1.

Consequently, the measurement of gene expression *in vivo* in humans requires other methods, and thus far, scintigraphic imaging with SPECT and PET is the only technology that can be used clinically. Scintigraphic molecular imaging reveals tomographic information with high sensitivity. In addition, when corrected for photon attenuation, scatter and other physical factors, these imaging modalities provide a reliable estimate of the local concentration of the radiotracer.

The three main types of PET/SPECT reporter genes are enzymes, receptors and transporters (symporters) (Table I; Fig. 2), as discussed below.

Enzymes

The HSV-1 thymidine kinase (HSV-1-tk) gene can be considered an enzymatic reporter gene and is often used as a killing gene. For this purpose, the gene is transferred into a malignant tumor cell by infection with recombinant viruses. The protein product of this gene is the HSV-1-TK enzyme. This enzyme phosphorylates thymidine and thymidine analogues, including acycloguanoside derivatives such as aciclovir, ganciclovir and penciclovir, often referred to as prodrugs (Fig. 3). Enzymes such as HSV-1-TK convert acycloguanosine monophosphates to

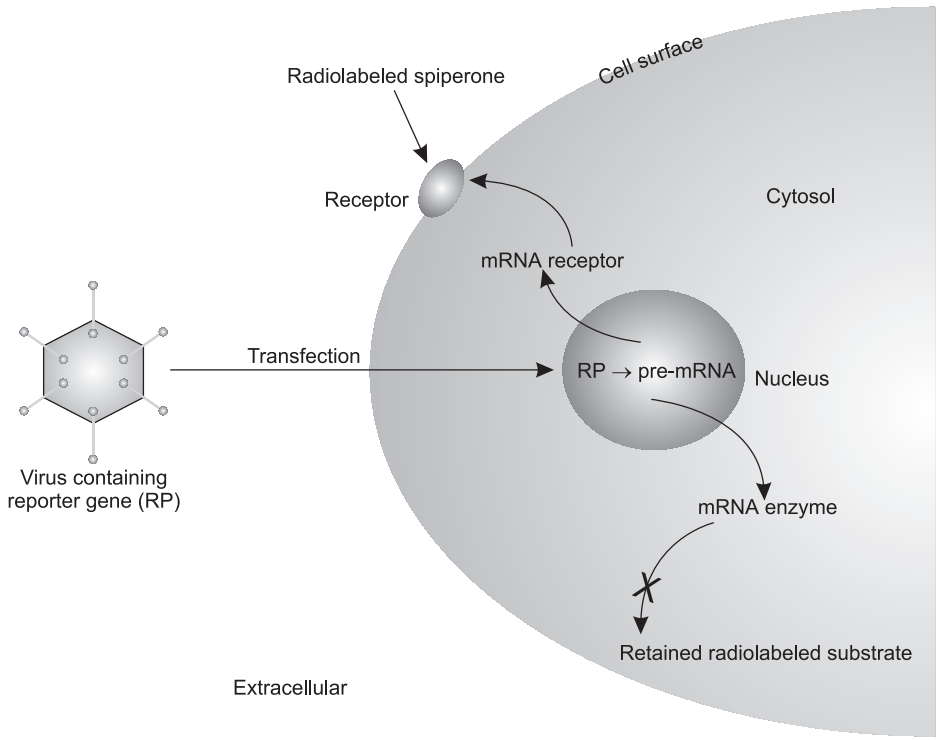

Fig. 2. Schematic representation of the reporter gene-reporter probe system for the assessment of gene expression. Two systems are illustrated: 1. The enzyme reporter gene is transfected into the target cell, where it is transcribed to mRNA and translated into the enzyme. The radiolabeled reporter probe is modified by the enzyme and retained in the cell. After washout of unbound radiolabaled compound, the (quantitative) measurement of reporter probe accumulation in the cell reflects the enzymatic activity of reporter gene expression.2. After transfection of the reporter gene and transcription to mRNA, translation to a ligand-binding protein (receptor) takes place. The expression level of the reporter gene is reflected by the accumulation of the radiolabeled reporter probe usually located on the cell surface.

Table I: Examples of reporter-probe systems for nuclear imaging (more extensive information can be found in Ref. 29).

Reporter gene	Mechanism	Radiolabeled agent (Ref.)	Target
Herpes simplex virus type 1 thymidine kinase (HSV-1-tk)	Phosphorylation	[^{124}I]-FIAU (26)	Intracellular
		[^{18}F]-FHBG (27)	Intracellular
Dopamine D2 receptor	Receptor targeting	[^{18}F]-Spiperone (4)	Cell surface
Somatostatin receptor	Receptor targeting	[^{111}In]-Octreotide (28)	Cell surface
Na/I symporter	Active symport	^{123}I, ^{124}I, ^{131}I (20)	Cell surface

di- and triphosphates. These triphosphates have the ability to kill cells by interacting with cellular DNA (incorporation of chain-terminating derivatives and/or inhibition of DNA polymerase).

Besides being used as a therapeutic gene, HSV-1-tk can be used as part of a scintigraphic reporter gene-reporter probe system. Ideally, a probe is a good substrate for the encoded enzyme and a poor substrate for mammalian enzymatic activity. In this respect, two radiolabeled compounds have adequate characteristics as substrates for HSV-1-TK and are used as scintigraphic reporter probes: the radioiodine-labeled uracil derivative 5-[^{124}I]-iodo-2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil ([^{124}I]-FIAU) and the fluorine-18-labeled penciclovir derivative 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine ([^{18}F]-FHBG). Of these two compounds, the latter is used more often due to the limited availability of ^{124}I, although FIAU appears to be a more efficient probe (3). Figure 4 shows the structural formula of FIAU, which appears to be labeled more easily with radioiodine than with fluorine-18.

Receptor imaging

In a different approach, a reporter gene encodes for a receptor. After delivery of the reporter gene to the cell, transcription to mRNA and translation to a ligand-binding

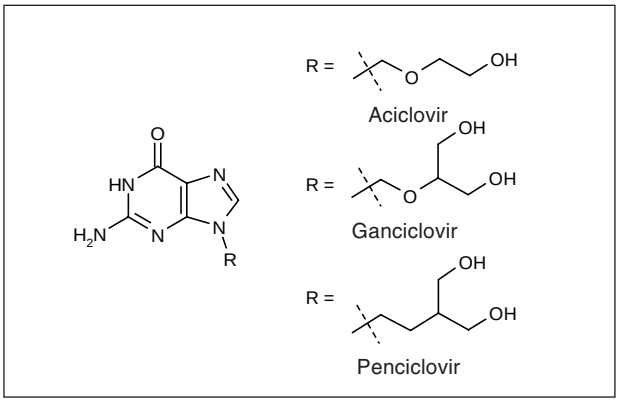

Fig. 3. Structures of acycloguanosine derivatives aciclovir, ganciclovir and penciclovir. The [^{18}F]-fluorinated or radioiodinated versions are potential substrates for HSV-1-TK and suitable for *in vivo* assessment of HSV-1-tk gene expression.

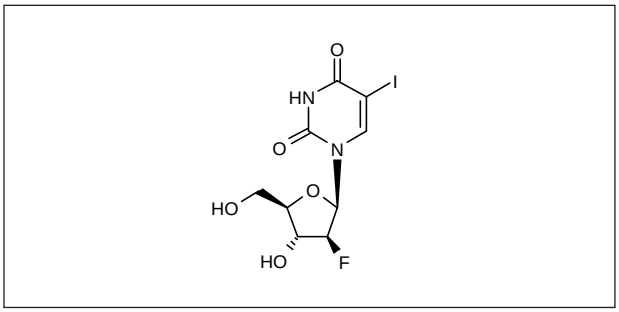

Fig. 4. Structure of FIAU (5-iodo-2'-deoxy-1-β-D-arabinofuranosyluracil). As a high-affinity substrate for HSV-1-TK, radiolabeled (iodine) FIAU is a potential tracer.

protein or receptor take place. The accumulation of the radiolabeled probe by the receptor reflects the expression level of the reporter gene. A clear advantage of this system is that the receptor proteins are usually located on the cell surface, which facilitates imaging, as the targeting agent does not need to cross the cell membrane. Examples of this reporter gene-reporter probe approach are given below.

1. Dopamine D2 receptor

For the more specific diagnosis of brain disease, various radiotracers have been developed that have specific affinity for receptor binding sites. These agents allow the accurate measurement of neuronal function by monitoring receptor activity at the synapse.

The role of the postsynaptic dopamine D2 receptor in the central nervous system has been studied extensively. Various disease states are characterized by focal decreases in D2 receptor binding. This receptor can be part of the reporter gene-reporter probe approach, wherein the reporter gene can encode for this receptor. The human receptor has limited expression in the body, which reduces nontarget accumulation. Once introduced into the target cell by an adequate vector, the receptor-based system will be located on the cell surface. The PET reporter probes [^{18}F]-fluoroethylspiperone and [^{11}C]-raclopride are available for clinical imaging and are useful for demonstrating the ectopically expressed D2 receptor (4).

2. Somatostatin receptor

Somatostatin plays a role in cancer and acts as a tumor growth inhibitor. Somatostatin (sst) receptors are found in neuroendocrine tumors and in several other malignancies, including lung carcinoma, breast carcinoma and lymphoma. Five different sst receptors have been described, but sst_2 receptor is expressed in the majority of cancers, although to varying degrees. On the other hand, the human receptor shows limited expression in normal tissues. The sst_2 receptor gene has been used successfully as a reporter gene, both in DNA constructs using a viral vector (5) and in nonviral delivery vehicles (6). Scintigraphic imaging, especially using SPECT, allows the visualization of primary and metastatic tumors with sst receptor-targeting agents such as [^{111}In]-D-Phe-DTPA-octreotide (Octreoscan®; Mallinckrodt Medical) or [^{99m}Tc]-depretide (Neotect; Amersham Health) as reporter probes.

Sodium/iodide symporter

Iodine is an essential element for the synthesis of thyroxine and triiodothyronine. The production of these thyroid hormones is dependent on the ability of the thyroid gland to concentrate and process this element. The mechanism that transports and concentrates iodide in the thyroid gland is usually referred to as the iodide pump, or the iodide trap. Uptake actually occurs across the membrane of the follicular cells and is mediated by the sodium/iodide symporter (NIS), which belongs to the sodium/glucose cotransporter family. NIS is a glycoprotein located on the cellular membrane and uses the transmembrane sodium ion concentration gradient maintained by sodium/potassium adenosinetriphosphatase. Radioactive iodide (*e.g.*, ^{123}I and ^{131}I) is a well-known agent for assessing functional thyroid disorders and for treating thyroid dysfunction or malignancy. The latter is possible as NIS transport is maintained in thyroid cancer.

Soon after the discovery of NIS, its potential as a reporter gene was suggested and demonstrated in transplanted thyroid cancer cells (7). This transport protein was further tested in animal models and SPECT studies have demonstrated its suitability as a reporter gene, allowing quantification of the magnitude and volume of gene expression (8-10). Radioiodine transport through the NIS provides the possibility of radionuclide-targeted radiotherapy (11). Recently, Dingli *et al.* (12) enhanced

the oncolytic potency of a measles virus strain with NIS. They demonstrated the uptake of ^{123}I with scintigraphy and the killing ability of ^{131}I in myeloma xenografts. Similar results have been obtained by Schipper *et al*. (13) in neuroendocrine tumor cells with NIS expression after gene transfer. Recent research has provided further means for the therapy of colon carcinoma and hepatocellular carcinoma with radioisotopes (14, 15). Unfortunately, rapid efflux of radioiodine has been observed in various NIS-expressing tumors (16-18). In view of the relatively short biological retention time, further research with other radioisotopes, such as rhenium-188 and astatium-211, is warranted (19). An overview of diagnostic and therapeutic applications was presented by Dadachova and Carrasco in 2004 (20) and illustrates the utility of this novel approach emanating from developments in molecular biology.

Gene therapy development

Molecular probes used for imaging often originate from pharmaceutical and pharmacological research in the nonimaging field. Likewise, reporter genes (and their downstream proteins) often emerge from research in molecular biology. The design of a gene-probe system is not an easy proposition and *in vivo* validation is expensive and time-consuming. Nevertheless, studies on the efficacy of gene therapy mostly rely on molecular imaging, as presently only this technique can throw light on crucial questions such as: Did cell targeting succeed? Did the gene transfer affect other tissues? What are the degree and duration of transgene expression?

PET and SPECT offer the possibility of monitoring transgene expression in gene therapy. In this respect, HSV-1-tk is an interesting gene as it is both a reporter gene and a therapeutic (suicide) gene and therapeutic activity can therefore be monitored by using the gene as a reporter gene. However, few genes exert therapeutic activity while also offering *in vivo* validation by direct imaging based on prodrug activation (like the phosphorylation of radiolabeled guanosine derivatives). Monitoring other therapeutic genes therefore requires a different approach and it has been suggested that coupling therapeutic and reporter genes could be the solution (21, 22). Linked genes permit the synthesis of two different proteins from a single mRNA chain, although the expression and, more particularly, the expression rate of both genes may differ. The proof of this principle has been provided by Yu *et al*. (23), who demonstrated co-expression of a gene construct in living animals.

A problem in gene therapy is the use of vectors, usually of adenoviral origin. Before application in humans, a new vector requires extensive safety testing and its biodistribution should be investigated. The radiolabeling of viral vectors could be of help and *in vivo* scintigraphy is a welcome aid in the study and development of gene delivery vehicles (24).

Considerations for the future

The profound impact of molecular imaging on both basic research and clinical management is evident from numerous reports. In view of the clear anatomic depiction with the aid of MRI, the development of specialized probes for molecular imaging is of extreme interest. Such MR probes include so-called “smart” imaging agents, which generate signals under certain physiological and pathological conditions. One example of such a new microenvironment-sensitive MR agent is a probe in which a gadolinium atom remains in a chemical cage until the molecule is “opened” by an enzyme (often β-galactosidase). A number of enzyme applications can be envisaged, especially those in which this microenvironment determines the expression of a gene of interest. Such MR probes are, however, not easy to develop and nuclear reporter-probe systems are more practical and closer to clinical practice. Moreover, nuclear imaging techniques offer the ability to study biodistribution and biokinetics *in vivo* using harmless tracer amounts, the hallmark of nuclear medicine. This allows the visualization of a multitude of molecular targets, depending on the availability of ligands and radionuclides. For targeted radiotherapy, radionuclides with favorable physical characteristics, such as yttrium-90 and lutetium-177 (high- and medium-energy β-emitter, respectively), are available and are used in combination with somatostatin analogues (25).

Along with the increased understanding of abnormal molecular processes as the basis of disease, the use of quantitative visualization of molecular targets is increasing and will take the place of conventional animal studies, including dissection, organ inspection and histology. In addition, temporal changes in biodistribution can be investigated in a single animal. As such, this new approach adds considerably to the ethical aspects of drug testing in animals.

From the clinical point of view, this noninvasive imaging method will provide the basis for optimal timing and balancing the application of drugs. In the past decade, both nonprofit pharmaceutical research institutions and the pharmaceutical industry have embraced these novel imaging techniques for application in drug design and the evaluation of drug efficacy, dosing aspects and safety. Various features of this research, *i.e*., ligand design, receptor studies and genetic intervention, are well covered with nuclear molecular imaging. The outcomes, both quantitative and qualitative, will increase our knowledge of the molecular basis of disease, which will lead to better drug discovery and more adequate drug use, and reduce the cost of medicine as a whole. One thing is for certain: molecular imaging and drug research will be drawn together.

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