A comprehensive review on controls in molecular imaging: lessons from MMP-2 imaging

Réjean Lebel and Martin Lepage*

Metalloproteinases (MMPs), including MMP-2, play critical roles in tissue remodeling and are involved in a large array of pathologies, including cancer, arthritis and atherosclerosis. Their prognostic value warranted a large investment or resources in the development of noninvasive detection methods, based on probes for many current clinical and pre-clinical imaging modalities. However, the potential of imaging techniques is only matched by the complexity of the data they generate. This complexity must be properly assessed and accounted for in the early steps of probe design and testing in order to accurately determine the efficacy and efficiency of an imaging strategy. This review proposes basic rules for the evaluation of novel probes by addressing the specific case of MMP targeted probes. Copyright © 2014 John Wiley & Sons, Ltd.

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1. INTRODUCTION

Molecular imaging (MI) can evaluate physiological determinants as well as cellular and molecular processes of pathologies and may be key in tailoring treatments – and monitoring the response – for individual patients. Examples of physiological determinants include blood vessel permeability, perfusion, blood flow and tissue pH. Cellular determinants include migration, apoptosis and differentiation. Finally, molecular determinants include the level of production and activity of enzymes along with metabolic changes. Numerous limitations have unfortunately been found on the way to this idealized view of personalized medicine. These include limits in spatial resolution, time resolution, detection sensitivity, imaging depth and ambiguous images from current state-of-the-art imaging modalities, including positron emission tomography (PET), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT) and optical imaging (OI).

Inherent to molecular imaging is the design of novel imaging probes. This process shares numerous similarities with the development of novel drugs. The probe must reach its target, interact with or bind it with sufficient affinity, be sufficiently stable and specific for a long enough time span to play its role without secondary effects, and be eliminated within a reasonable delay. The language used here is fairly loose; this is because different targets have different pharmacological properties that must be included in the design of specific probes. A pre-requisite is of course that the probe has blood stability overlasting most of its pharmacokinetics. The pharmaceutical industry is spending astronomical amounts in order to develop, optimize and test candidate molecules. Specifically, the pharmacokinetics (i.e. effect of an organism on a compound), the pharmacodynamics (i.e. effect of a compound on an organism) and biochemical properties of a compound must all be optimized, but sometimes are conflicting with one another. There is, however, a fundamental difference between drug and probe design. The outcome of a drug can be measured by its efficacy such that unbound or otherwise ‘unused’ drug at the site of interest is not detrimental – except in the case of unacceptable side effects. On the other hand, the presence of free probe in the tissue of interest is a source of background signal that may obscure or overcome the signal of bound or activated probe. In addition, inflammation, blood vessel permeability or activation of a probe in a different tissue followed by transport of the activated probe to the tissue of interest (TOI) can account for a nonzero and time-dependent background signal in the TOI. All of these must be accounted for in molecular imaging.

In this review, we describe a comprehensive experimental approach for the development, in vitro assessment and in vivo evaluation of novel imaging probes. The review is focused on matrix metalloproteinase-2 (MMP-2) and highlights the crucial and essential requirement for appropriate control experiments during every step of probe development and during evaluation of their performance. Detailed attention is given to the issue of nonspecific and specific probe accumulation as a function of time in a tissue of interest.

Metalloproteinases (MMPs) are a family of Zn-dependent proteases which together can degrade most tissue matrix components. Their structure, regulation and substrates have been extensively reviewed elsewhere (1–3). This particular review assumes that the reader is already familiar with most of their
general aspects and will only refer to details that are of importance in the imaging context. Interest in MMP imaging was initially propelled by their suspected role in the progression of cancer. MMPs, and particularly MMP-2 and MMP-9, were thought to be responsible for the digestion of the basal membrane, and thus for the migration of metastatic cells outside their initial niche and into proximal and distal tissues (4). These ideas have been revised or further evolved in the second half of the last decade (5,6), while additional roles and locations were being discovered (7) – yet these findings did not reduce interest or intensity of research dedicated to imaging MMPs. This can be easily explained since MMPs, including MMP-2, are deemed very important progression/intensity biomarkers in several pathologies, including cancer (8), auto-immune diseases (9) and myocardial infarction (10).

In the scope of this review, imaging agents were classified based on their contrast-generating mechanism (e.g. MMP binding, signal induction, cell penetration). An honest attempt was made to include as many MMP agents as possible, but the vast number of papers prohibited us from detailing each of them. Instead, examples are used to illustrate the discussion elements and concerns that are voiced within. This review will be divided into three sections. The first section describes MMPs as imaging targets and what properties must be taken into account when designing a new imaging agent. The second section covers many imaging strategies and associated imaging modalities that have been used for MMP imaging. The last section will discuss specificity and distribution controls in depth – and how to avoid hidden pitfalls in data analysis.

2. TARGETING MMPS IN VIVO: WHAT ARE WE LOOKING AT?

In several MMP imaging studies, the characteristics (activation, role, location, etc.) of MMPs are oversimplified. Fig. 1A shows the classic MMP-2 paradigm which dates from the beginning of the last decade. In order to correctly discuss results obtained...
with a MMP probe, it is critical not to underestimate the complex mechanisms leading to MMP-2 activity regulation. Fig. 1B reflects our current understanding of MMP-2.

2.1. Activity, concentration and activation
MMPs are characterized by several domains, including a catalytic domain that is initially blocked by a pro-domain. This pro-peptide can be removed by another protease (often another MMP) to activate the enzyme (11). From there, endogenous inhibitors, integrin/substrate binding and degradation regulate MMP activity (2,11). In the case of MMP-2, the inhibitor TIMP-2 will regulate the activity of MMP-2 by binding its hemopexin domain. Hence, MMP-2 can either be unactivated (proMMP-2), activated and active (MMP-2) or activated and inhibited (MMP-2-Timp-2 complex). Probe design and targeting strategies must be adapted to the quantity of interest – either MMP-2 concentration or MMP-2 activity. A classic targeting strategy resides on using specific antibodies, which can bind to its active site, thus discriminating between proMMP-2 and MMP-2, or elsewhere on the enzyme (e.g. to the HPX domain (12–15)). On the other hand, no information on the in situ activity of the enzyme can be acquired. This particularity of the regulation of MMP-2 should be kept in mind when reading papers discussing potential MMP involvement in pathologies: qPCR may supply a first-hand approximation of the MMP-2 concentration, and western blotting may even provide insight as to its activation state, but neither will provide information concerning its activity. While in vitro zymography is often preferred for these reasons, this method separates TIMP-2 and MMP-2, and may not properly reflect the in situ MMP-2 activity (16). On the other hand, it allows for the differentiation of MMP-2 and -9 by their molecular weight. In situ zymography is preferred when spatial information is required, but it will only be as specific as the substrate used in the assay – which means that MMP-2 and -9 will probably be co-detected (see Redundancy below) (16). As will be seen later, these concepts can be directly translated to the context of MMP imaging.

2.2. Location and role
Most MMPs are water-soluble enzymes, with a few exceptions, such as MT1-MMP, which is membrane-anchored. Soluble enzymes will diffuse around their source and into the vasculature. For this reason, anchored targets are likely to allow for more focused signal, while soluble enzyme will result in increased background (12). In fact, MMP levels in plasma and urine were reported to be elevated in breast cancer (8,17). Serum levels of MMP-2, and -9 by their molecular weight. In situ zymography is preferred when spatial information is required, but it will only be as specific as the substrate used in the assay – which means that MMP-2 and -9 will probably be co-detected (see Redundancy below) (16). As will be seen later, these concepts can be directly translated to the context of MMP imaging.

2.3. Redundancy
The various MMP genes probably evolved from a single-domain protein which was duplicated and modified to create the current MMP family (3). For this reason, the proteins they encode are highly similar in structure, and an important specificity overlay is present (3). MMP-2, -3, -7, -8 and -9 possess homologous active site features (21), and MMP-2 and MMP-9 are even more closely related and process similar substrates. For this reason, it is difficult to properly evaluate their individual role. Even in situ zymography will detect MMP-2 and MMP-9 since it is based on the degradation of a substrate that is sensitive to both enzymes. While this particular point might not seem alarming, it has been hypothesized, based on pre-clinical evidence and mouse models, that MMP-2 and MMP-9 may well have opposite roles in cancer development (22). Additionally, MMP-2−/− or MMP-9−/− mice reveal distinct, nonoverlapping phenotypes [(7) and references within]. Thus, individual MMP quantification is paramount to understanding their unique role in pathologies.

2.4. Source
Many cancer cells have been shown to produce high levels of MMP-2, and this enzyme was dubbed as a reliable biomarker for cancer. Using these cell lines in a xenograft model seemed a reliable control. On the other hand, our results with MC7-L1 cells (mammary carcinoma) have taught us that, while knocking down MMP-2 expression worked properly in vitro (90% decrease), we obtained only a mere 50% reduction upon inoculation of KD cells in vivo. It has to be noted that endogenous cells (stroma, immune system) cohabit with xenografted cells – and secrete their own enzymes. Most immune cells attempting to repair damage in diseased tissues induce the production or themselves produce a variety of MMPs (23). Macrophages spontaneously secrete MMP-2, whereas T cells require stimulation to do so (24). Neutrophils secrete a factor that can activate MMP-2 production by endothelial cells. The consequence for imaging is that MMP-2 may be secreted by a tissue of interest or by cells transiting in this tissue effecting a different role (e.g. tissue repair around a growing tumor).

3. IMAGING MODALITIES AND PROBE DESIGNS
The major difficulty of molecular imaging is that probes may not be totally specific to their target and, by definition, they are used in a complex biological system. Probes (a) may interact with secondary targets, (b) generate signal or contrast before activation, or their signal/contrast is influenced by their immediate environment, (c) have properties that prevent or help them reach their target in vivo (this can depend on the tissue), and (d) are metabolized. Targets (a) may be scattered through the organism, not only in the tissue of interest, (b) are in finite concentration, which may be insufficient, and (c) may be mobile or inaccessible to the probe (e.g. secreted in blood, located inside cells or in a poorly perfused region). Finally, the subject physiology (a) may prevent probe accumulation through excretion by liver and kidneys (indirectly producing high background in these organs), (b) may prevent access of a probe to a tissue if blood vessel permeability is not sufficient (e.g. blood-brain barrier), or (c) may not
enable sufficient transport of a probe to a targeted site if blood flow is too low.

The observed signal or contrast is a result of all these considerations, and others, making image interpretation difficult. Without appropriate control experiments, interpretation is hazardous, at best. Despite this complexity and elevated costs for maintenance of the required infrastructure, biomedical imaging can claim significant advantages in comparison to classical biochemical and pharmacological methods: it allows for longitudinal studies (i.e. the same animal may be monitored over time), and is noninvasive (or minimally invasive) whereby a target may be studied in its usual environment.

3.1. Imaging modalities

After considering the target and its environment, imaging strategies depend heavily on the imaging modality in which they are to be used. Most studies use SPECT, TEP, MRI or OI, which will be briefly described below. For more information, a thorough and up to date comparative and integrative review of these modalities (and others) was recently published by others (25).

SPECT and PET are based on radioactive isotopes which emit one or two gamma photons, respectively. Nuclear imaging probes (radiotracers) incorporate such an isotope and gamma photons are measured by detectors placed around the subject. The main strength of nuclear imaging is high sensitivity (i.e. subpharmacological doses of radiotracers are required). On the other hand, these modalities require heavier infrastructure for isotope manipulation and radiotracer production. The spatial resolution is also limited to ≥1 mm.

MRI is based on the manipulation and detection of naturally-occurring and nuclear spins of atoms including 1H and 31P (26). No radioactivity or ionizing radiation is present. The vast majority of MRI contrast agents are detected via their magnetic interaction with neighbouring water molecules, which represent an abundant source of 1H. Sensitivity for the detection of contrast agent is low compared with nuclear and optical techniques, and must be addressed during the preparation of the imaging agent. The greatest advantage of MRI is the high-resolution anatomical information it provides, but this tends to become less of an edge as co-modality gains in popularity.

Optical imaging includes techniques based on the detection of chemiluminescence, bioluminescence and fluorescence. A fluorescent probe can absorb a photon at a characteristic energy (wavelength) and re-emit a photon with a slightly lower energy. Excitation may be via an incandescent lamp with appropriate filters or lasers; detection is via a sensitive camera. Most of the research in OI is performed using planar (2D) imagers. While tomographic fluorescence reconstruction systems are available, they are still not the mainstream scanners in terms of MMP OI.

3.2. Contrast modulation

3.2.1. Signal induction

Signal modulation provides efficient contrast in imaging, but can only be achieved for OI fluorescence probes and MRI contrast agents – since PET and SPECT radiotracers cannot be ‘shut down’. Modulating the relaxivity of contrast agents can yield a relaxivity increase by a small factor (i.e., 3) (27) but the relaxivity of the parent compound is not zero. Thus, it may be difficult to separate activation from larger concentration. A ratiometric approach as recently proposed would address this issue (28) – and in vivo adaptation and validation of this method is of the utmost interest. Activatable fluorescent probes have been around for several years, and are the most frequent MMP OI strategy.

Quenched fluorescent probes are either based on fluorophore/fluorophore (FF) or fluorophore/quencher (FQ) FRET ( Förster Resonance Energy Transfer). An FQ pair is composed of a fluorophore (donor) and a spectrally matched quencher. Excitation of the fluorophore is followed by FRET to the quencher whereby the energy is ultimately dissipated by heat (29). Many applications exploit this phenomenon by placing a quencher near a fluorophore on the other side of a MMP sensitive peptide. MMP activity can remove the quencher by cleaving the peptide, giving rise to fluorescence. Auto-attenuation is a decrease in quantum yield caused by a nonradiative transfer between an excited fluorophore and an identical fluorophore (FF). Note that quenching is not perfect and that efficiencies from 99.5 to 80% have been reported (Table 1). In the scope of MMP imaging, OI is the only modality for which signal increasing agents have been reported. Theoretically, contrast in MRI using protease activated self-aggregating particles could be used (30) to increase negative contrast.

The first group (C. Bremer, R. Weissleder, U. Mahmood and C.-H. Tung) to perform MMP-2 imaging in vivo using this method developed an FF FITC-based probe bound to a poly-lysine scaffold (31). This structure was used in other agents by the same group, such as a MMP-2/MMP-9 targeted probe (32). McIntyre et al. introduced a poly/amidoamine (PAMAM)-based MMP-7 targeting agent functionalized with FITC and TMR (PB-M7vis) (33). This agent was later followed by an improved version using near IR/IR fluorophores AF750 and Cy5.5 (34). Commercial probes MMPSense 680 and 750 are also based on similar principles (35). Gold nanorods were also used as a scaffold and functionalized with Cy5.5 bearing MMP-sensitive peptides (36). The large molecular weight of these agents probably improves the contrast obtained – as these are large bulky molecules which will greatly benefit from the enhanced permeability and retention (EPR) effect and accumulate in tumors and inflamed tissues, and where high levels of MMP are likely to be present. Note that high molecular weight agents are likely to be unsuitable for translational imaging approaches owing to their increased blood half-life (37), and slow tissue clearance rate. Also, some FF probes suffer from low quenching efficiency (i.e. 80%), such that a high proportion of the measured signal is from unactivated probe (Table 1). It is important to note that when a fluorophore is removed from the scaffold, its size is greatly reduced, and an important change in pharmacokinetics is to be expected.

FQ agents are usually synthesized as smaller molecules. Pham et al. developed a Cy5.5/NIRQ820 based agent (38), and an MMP-13 targeting agent using Cy5.5/BHQ was prepared by Lee et al. (39). Our group also recently published a set of Cy5/QS21 MMP-2 targeting agents (40). These smaller molecules are likely to penetrate more easily into tissues, but will be eliminated more quickly than most FF probes. We also reported on a potential problem of QS21 – its hydrophobic properties would result in probe aggregation and increased quenching. While this is beneficial for in vitro studies (cleavage of the probe would result in an even greater fluorescence increase), assays in plasma showed that immediate fluorescence increase was detected in plasma – indicating that disaggregation could result in an unspecific signal increase. Quencher-based molecules should be thoroughly examined for such behaviors, as some preliminary data in our
<table>
<thead>
<tr>
<th>Name</th>
<th>Signal moiety and modality</th>
<th>Mode</th>
<th>Theoretical targeting capabilities</th>
<th>In vivo targeting capabilities</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>-</td>
<td>ACPP</td>
<td>MMP-2 &gt; 9</td>
<td>No significantly higher tumor uptake (Fig. 3D of 88)</td>
<td>Assumed from (81)</td>
<td>(82)</td>
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<tr>
<td>NA</td>
<td>-</td>
<td>GdDPThy (Gd)</td>
<td>MMP-2 &gt; 9</td>
<td>In vivo nude mice HT-1080 xPAM-4.9</td>
<td>Assumed from (81)</td>
<td>(83)</td>
</tr>
<tr>
<td>NA</td>
<td>-</td>
<td>ACPP</td>
<td>MMP-2 &gt; 9</td>
<td>In vivo nude mice HT-1080 xPAM-4.9</td>
<td>Not enough data</td>
<td>(19)</td>
</tr>
<tr>
<td>NA</td>
<td>-</td>
<td>Inh.</td>
<td>MMP-2 &gt; 9</td>
<td>Intestine, rectum, and bladder extracts</td>
<td>Not enough data</td>
<td>(19)</td>
</tr>
<tr>
<td>NA</td>
<td>-</td>
<td>FF</td>
<td>MMP-2 &gt; 9</td>
<td>Intestine, rectum, and bladder extracts</td>
<td>Not enough data</td>
<td>(19)</td>
</tr>
</tbody>
</table>

**Table 1.** Metalloproteinase (MMP) targeting probes
<table>
<thead>
<tr>
<th>Name</th>
<th>Signal moiety and modality</th>
<th>Probe type</th>
<th>Structure or sequence</th>
<th>Theoretical targeting capabilities</th>
<th>In vivo targeting capabilities vs ctrl</th>
<th>Specificity</th>
<th>Models</th>
<th>Negative controls</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P947</td>
<td>Gd (MRI)</td>
<td>Inh.</td>
<td>Gd-DOTA-peptide</td>
<td>( r = 5.6 \text{ mW} \text{s}^{-1} )</td>
<td>162% signal inc. vs scrambled probe [Fig. 3 of (73) @ 5 min]</td>
<td>Broadband (incl. MMP-1, -2, -3, -8, -9, -13)</td>
<td>In vivo: AAA in male Lewis rats caused by elastase perfusion</td>
<td>Scrambled (P1135)</td>
<td>Gd-DOTA Sham-operated animals KO animals</td>
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<td></td>
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<td>IC_{50} \text{MMP}: 8 (0.1 \mu m) &lt; MMP-1, 2, 13 (1 \mu m) &lt; MMP-3, 9 (10 \mu m) &lt; MMP-14 (100 \mu m)</td>
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<td>214% inc. sig. vs scrambled ctrl [Fig. 3A of (74)]</td>
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<td>NA</td>
<td>FITC (FOI)</td>
<td>FF</td>
<td>Poly-Lys–[GPGVNGL–FITC]_{n}</td>
<td>( k_{\text{cat}}k_{\text{m}}: 14 \text{ K} \text{m}^{-1} \text{s}^{-1} \text{QE: 88%} )</td>
<td>296% sig. vs scrambled 232% sig. vs ctrl tumor [Fig. 4 of (31)]</td>
<td>MMP-2 (n.c.)</td>
<td>In cellulo: HT1080 (+) vs BT20 (–) In vivo: athymic mice HT1080 (+), BT20 (–) xngft</td>
<td>Cells with ( \Delta \text{[MMP-2]} )</td>
<td>Scrambled probe</td>
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<tr>
<td>NA</td>
<td>Cy5.5 (FOI)</td>
<td>FF</td>
<td>Poly-Lys–[SGFGPRQITA–Cy5.5]_{n}</td>
<td>( k_{\text{cat}}k_{\text{m}}: 188 \text{ K} \text{m}^{-1} \text{s}^{-1} \text{QE: 99%} )</td>
<td>353% sig. vs healthy tissues [Fig. 3 of (32) @ 1 week]</td>
<td>MMP-2, 9 &gt; &gt; 13.7</td>
<td>Myocardial infarct in male C57BL6/6 mice induced by ligation</td>
<td>Remote cardiac region (healthy tissues)</td>
<td>(31)</td>
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<td>Visible sig. inc. (not quantified)</td>
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<tr>
<td>NA</td>
<td>(^{18}) F (PET) and TMRh (FOI)</td>
<td>SS</td>
<td>PEG$_{350a}$–CRSGPLG–VYX((^{18}) F)–K–TMRh</td>
<td>In cellulo (FACS): cells have &gt;10% sig. vs ctrl w/ inh. [Fig. 3 of (88)]</td>
<td>~625% sig. in HT-1080 (+) tumor vs inh. ctrl [Fig. 5 of (88)]</td>
<td>MMP-2 (n.c.)</td>
<td>In cellulo: HT-1080 (+) vs MCF-7 (–) In vivo: nude Balb/C mice HT-1080 (+) and MCF-7 (–) xenografts</td>
<td>Cells with ( \Delta \text{[MMP-2]} ) inh. (1,10-phenanthroline)</td>
<td>(88)</td>
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<tr>
<td>NA</td>
<td>Cy5.5 (FOI)</td>
<td>Inh.</td>
<td>Nonhydroxamate inhibitor–[PEG]$_{3}$–Cy5.5</td>
<td>IC_{50} \text{MMP}: 2 (48 nm)</td>
<td>NA</td>
<td>MMP-2/9 (n.c.)</td>
<td>In cellulo: MCF-7 (–), HT-1080(+), and A673 (+)</td>
<td>Cells with ( \Delta \text{[MMP-2]} )</td>
<td>(69)</td>
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<tr>
<td>NA</td>
<td>Cy5.5 (FOI)</td>
<td>Inh.</td>
<td>Based on CGS27023A/ CGS25966 (hydroxamate acids)</td>
<td>IC_{50} \text{MMP}: 9 (10 \mu m) MMP-2 (16 \mu m)</td>
<td>NA</td>
<td>Broadband</td>
<td>In cellulo: MCF-7 (–), HT-1080(+), and A673 (+)</td>
<td>Cells with ( \Delta \text{[MMP-2]} )</td>
<td>(37)</td>
</tr>
<tr>
<td>Compound</td>
<td>Isotope</td>
<td>Inh.</td>
<td>Type of Compound</td>
<td>K, for MMP-2 (10.5 nM)</td>
<td>K, for MMP-3 (14 nM)</td>
<td>K, for MMP-7 (&lt;6.4 nM)</td>
<td>K, for MMP-9 (7.4 nM)</td>
<td>K, for MMP-12 (&lt;6.4 nM)</td>
<td>K, for MMP-13 (7.3 nM)</td>
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<tr>
<td>RP782</td>
<td>$^{111}$In</td>
<td>Inh.</td>
<td>Macrocyclic compound [Fig. 1 of (61) for structure]</td>
<td>NA</td>
<td>Broadband</td>
<td>In vivo: myocardial infarct in C57BL/6 mice</td>
<td>Enantiomeric ctrl (RP788), healthy animals</td>
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<tr>
<td>RP805</td>
<td>$^{99m}$Tc (SPECT)</td>
<td>Inh.</td>
<td>Macrocyclic compound [Fig. 1 of (61) for structure]</td>
<td>K, for MMP-2 (10.5 nM)</td>
<td>K, for MMP-3 (14 nM)</td>
<td>K, for MMP-7 (&lt;6.4 nM)</td>
<td>K, for MMP-9 (7.4 nM)</td>
<td>K, for MMP-12 (&lt;6.4 nM)</td>
<td>K, for MMP-13 (7.3 nM)</td>
</tr>
</tbody>
</table>

For RP805:

- 639% inc. in %ID g$^{-1}$ patho. vs healthy animals [Fig. 38 of (65)]
- 884% inc. in %ID g$^{-1}$ patho. vs healthy animals [Fig. 28 of (66)]
- In vivo: atheros. induced in NZR with high-cholesterol diet Treated animals/ healthy animals
- In vivo: atheros. induced in NZR with balloon deendothelialization and high-cholesterol diet Treated animals/ healthy animals
- Ex vivo: 363% uptake in aorta of ApoE$^{-/-}$ animals vs healthy animals [Fig. 28 of (67)]
- In vivo: apoE$^{-/-}$ mice under high-cholesterol diet Treated animals/ healthy animals
- 307% uptake in injured artery vs noninjured artery [Fig. 1C of (63)]
- In vivo: injury of the left common carotid artery of apoE$^{-/-}$ mice under high-cholesterol diet Sham operated animals Treated animals (diet withdrawal)

For SAV03(M):

<table>
<thead>
<tr>
<th>Compound</th>
<th>Isotope</th>
<th>Inh.</th>
<th>Type of Compound</th>
<th>IC$_{50}$: MMP-2 (1.9 nM)</th>
<th>TMR 8.42 [Table 1 of (51) @ 120 min]</th>
<th>MMP-2 (n.c.)</th>
<th>In vivo: ddy mice Ehrlich tumor xsgift</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAV03(M)</td>
<td>$^{18}$F (autoradiography)</td>
<td>Valine-based sulfonamide</td>
<td>Inh.</td>
<td>IC$_{50}$: MMP-2 (1.9 nM)</td>
<td>TMR 8.42 [Table 1 of (51) @ 120 min]</td>
<td>MMP-2 (n.c.)</td>
<td>In vivo: ddy mice Ehrlich tumor xsgift</td>
<td>None</td>
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</tbody>
</table>

(Continues)
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<tr>
<th>Name</th>
<th>Signal moity and modality</th>
<th>Probe type</th>
<th>Structure or sequence</th>
<th>Theoretical targeting capabilities</th>
<th>In vivo targeting capabilities vs ctrl</th>
<th>Specificity</th>
<th>Models</th>
<th>Negative controls</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-K11</td>
<td>Gd (MRI)</td>
<td>SS</td>
<td>Gd-DOTA-(INK)-PLGLWAR-(CH4)10-NH2</td>
<td>( r = 8.5 \text{ mm}^{-1}\text{s}^{-1} ) ( r &gt; 5.4 \text{ mm}^{-1}\text{s}^{-1} )</td>
<td>325% sig. vs inh. control [Fig. 3 of (87)]</td>
<td>Broadband</td>
<td>In vivo: CS7BL/6 mice B16.F10 xangrt</td>
<td>Inh. (GM6001)</td>
<td>(87)</td>
</tr>
<tr>
<td>NA</td>
<td>SPIO (MRI)</td>
<td>Misc.</td>
<td>SPIO-C6 or neutravidin-GPLGRGC-PEG</td>
<td>In cellulo: T2 reduced by 40% in conditions used</td>
<td>Not assessed</td>
<td>MMP-2 (n.c.)</td>
<td>In vivo: HT-1080 (+)</td>
<td>Inh. (galdarin)</td>
<td>(30)</td>
</tr>
<tr>
<td>NA</td>
<td>⁶⁴Cu (PET) and Cy5.5 (FOI)</td>
<td>FQ</td>
<td>BBQ650-PLGVR-K(Cy5.5)-EK(DOTA-⁶⁴Cu)</td>
<td>QE: 88%</td>
<td>602% sig. in tumor vs inh. ctrl [Fig. 4 of (103) @ 120 min]</td>
<td>MMP-13 (n.c.)</td>
<td>In vivo: Nude mice U87MG xangrt</td>
<td>Inh. (MMP inhibitor III – broadband)</td>
<td>(103)</td>
</tr>
<tr>
<td>PCA2-switch</td>
<td>Gd-DTPA (MRI)</td>
<td>SS</td>
<td>Gd-DTPA-K(16) SPAAYTA-PEG8</td>
<td>( k_{\text{ea}}K_{\text{mc}} = 120 \text{ Km}^{-1}\text{s}^{-1} )</td>
<td>216% ( R_1 ) variation vs MMP-2 knockdown cells [Fig. 6 of (86) @ 60 min]</td>
<td>Not cleaved by MMP-3/-7, cleaved more slowly by MMP-9</td>
<td>In vivo: Balb/c mice MC7-L1 xangrt</td>
<td>Scrambled peptide Gd-DTPA (perfusion) Cells with ( \Delta [\text{MMP-2}] )</td>
<td>(86)</td>
</tr>
<tr>
<td>PCA7-switch</td>
<td>Gd-DTPA (MRI)</td>
<td>SS</td>
<td>Gd-DTPA-K(36) PLALKRD-PEG12</td>
<td>Assumed from McIntyre (2004)</td>
<td>Qualitative change in the pharmacokinetics</td>
<td>MMP-7 [assumed from Ref. McIntyre (2004)]</td>
<td>In vivo: nude mice SW480 xangrt</td>
<td>Inh. (GM6001) Scrambled peptide Cells with ( \Delta [\text{MMP-7}] )</td>
<td>(84)</td>
</tr>
<tr>
<td>¹¹¹I</td>
<td>¹²³I (SPECT)</td>
<td>Inh.</td>
<td>OH-¹²⁹I-CGS27023A</td>
<td>IC₅₀: MMP-2 (57 nm), MMP-9 (257 nm)</td>
<td>Not assessed</td>
<td>MMP-2/9 (n.c.)</td>
<td>In vivo: CS7BL6 mice</td>
<td>None</td>
<td>(52)</td>
</tr>
<tr>
<td>NA</td>
<td>⁹⁹mTc (SPECT)</td>
<td>Ab</td>
<td>⁹⁹mTc-HYNIC-LgG₂-(tricine)₂</td>
<td>Affinity equal to parent Ab</td>
<td>1.3x aorta to muscle ratio vs ctrl [Table 1 of (15)]</td>
<td>MMP-14 (n.c.)</td>
<td>In vivo: WHHLMI rabbits (atherosclerosis model)</td>
<td>Unspecific Ab Healthy rabbits</td>
<td>(15)</td>
</tr>
<tr>
<td>I, II, III</td>
<td>Cy5 (FOI)</td>
<td>FQ</td>
<td>Cy5-P-QYSV21 (P: SPAAYTA, SLAYYTA, PSWLTTA, respectively)</td>
<td>( k_{\text{eq}}K_{\text{mc}} &gt; 3.9 \text{ Km}^{-1} \text{s}^{-1} ) QE: &gt;96%</td>
<td>Not significant</td>
<td>MMP-2 &gt; MMP-3, 7 and 9</td>
<td>In vivo: Balb/C mice MC7-L1 (+) xangrt</td>
<td>Caa or scrambled peptide Cells with ( \Delta [\text{MMP-2}] ) preactivated probe</td>
<td>(40)</td>
</tr>
<tr>
<td>NA</td>
<td>Cy5.5 (FOI)</td>
<td>FQ</td>
<td>Cy5.5-GLGMNGLGK-BHQ3</td>
<td>QE: 95%</td>
<td>243% sig. vs inhibitor co-injection [Fig. 4 of (39)]</td>
<td>MMP-13 &gt; MMP-7</td>
<td>In vivo: osteoarthritis induction in rat</td>
<td>Inh. (unspecific) Contralateral leg</td>
<td>(39)</td>
</tr>
<tr>
<td>Compound</td>
<td>Conditions</td>
<td>Imaging Modality</td>
<td>Methodology</td>
<td>Characteristics</td>
<td>Notes</td>
<td></td>
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</tr>
<tr>
<td>PB-M7VIS</td>
<td>FITC (activatable)</td>
<td>FF</td>
<td>[FITC–RPLALWRSC] OR [FITC–RPLALWRSC]</td>
<td>$k_{cat}/K_m$: 19 K m$^{-1}$ s$^{-1}$ QE: 80%</td>
<td>$50%$ sig. in MMP-7+ tumors vs MMP-7 – tumor (Fig. 8 of 33)</td>
<td>MMP-7 &gt; 3 &gt; 2</td>
<td>In vivo: nude mice SW480 (±) xengraft</td>
<td>Inh. (BB-94) Cells with Δ[MMP-7] (33)</td>
<td></td>
</tr>
<tr>
<td>CTT</td>
<td>$^{125}$I or $^{99m}$Tc (SPECT)</td>
<td>Inh.</td>
<td>AAX(125)-CTTHWGFTLC OR $^{99m}$Tc-CTTHWGFTLC</td>
<td>CTT variants show in vitro inhibition of MMP-2 (50–75% inh. @ 25 mg ml$^{-1}$ or 100 μM)</td>
<td>TMR and TBR &lt; 0.5 @ 24 h (Fig. 5B of 57)</td>
<td>MMP-2/9 (n.c.)</td>
<td>In vivo: Balb/c mice K51767</td>
<td>&gt;-aa and retroinversion (57)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>$^{123}$I (SPECT)</td>
<td>Inh.</td>
<td>Valine-based</td>
<td>$IC_{50}$: MMP-2 (23–48 nm)</td>
<td>Not suitable</td>
<td>MMP-2 &gt; $\sim$/cMT1/cMT3</td>
<td>In vivo: athymic mice A549 xenografts</td>
<td>None (54)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>Cy5.5 (Plan FOI)</td>
<td>FQ</td>
<td>NIRQ820–GVPLSTMGCC–Cy5.5</td>
<td>$K_{cat}/K_m$: 120 K m$^{-1}$ s$^{-1}$ QE: 86%</td>
<td>Not assessed</td>
<td>MMP-7 &gt; 1, 2, 3, 9</td>
<td>In cellulo: incubation with HT-1080 tumor extracts</td>
<td>None (38)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>$^{125}$I (SPECT)</td>
<td>Ab</td>
<td>125I-bt + Strep.–Ab</td>
<td>NA</td>
<td>160% inc. in %ID g$^{-1}$ vs negative ctrl Ab</td>
<td>MMP-14 (n.c.)</td>
<td>In vivo: C3H/He mice FM3A xengraft</td>
<td>Pre-targeting with negative ctrl Ab (IgG) (14)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>Gd (MRI)</td>
<td>Ab</td>
<td>[Bt$_{10}$-PAMAM–DTPA–Gd + pretarget: Strep.–Ab</td>
<td>Affinity to Strep. similar to unmodified Bt$_{10}$</td>
<td>r = 15.5 mm$^{-1}$ s$^{-1}$, 436 DTPA/PAMAM</td>
<td>202% inc. signal vs control Ab (unspecific Ab) (Fig. 4C of 131)</td>
<td>MMP-14 (n.c.)</td>
<td>In vivo: C3H/He mice FM3A xengraft</td>
<td>Pre-targeting with negative ctrl Ab (IgG) (13)</td>
</tr>
<tr>
<td>PB-M7NI</td>
<td>Cy5.5 (Activ.)</td>
<td>FF</td>
<td>(Cy5.5–M7H)–PAMAM–(PEGS$_{400}$)(AF750)$_6$</td>
<td>$k_{cat}/K_m$: assumed from PB-M7VIS (33) QE: 80%</td>
<td>Signal/Reference ratio of 6.87 between MMP-7 (+) and MMP-7 (−) tumors (Table 1 of 34)</td>
<td>MMP-7 [assumed from Ref. McIntyr (2004)]</td>
<td>In vivo: interstitial neoplasia (APC&lt;sup&gt;Min&lt;/sup&gt;) mice nude mice SW480 (±) xengraft</td>
<td>MMP-2 KO APC&lt;sup&gt;Min&lt;/sup&gt; mice SW480 cells with Δ[MMP-7] (34)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>$^{99m}$Tc (PET)</td>
<td>Ab</td>
<td>$^{99m}$Tc–Ab</td>
<td>Agent has similar MT1–MMP affinity to parent Ab</td>
<td>154% TBR vs negative ctrl (unspecified Ab) in MRMT-1 tumor bearing rats @ 48 h (Fig. 3 of 12)</td>
<td>MMP-14 (n.c.)</td>
<td>In vivo: rats MRMT-1/Walker-256 xengraft C3H/He mice FM3A xengraft</td>
<td>Negative ctrl agent (nonspecific Ab) (12)</td>
<td></td>
</tr>
</tbody>
</table>

(Continues)
<table>
<thead>
<tr>
<th>Name</th>
<th>Signal moiety and modality</th>
<th>Probe type</th>
<th>Structure or sequence</th>
<th>Theoretical targeting capabilities</th>
<th>In vivo targeting capabilities vs ctrl</th>
<th>Specificity</th>
<th>Models</th>
<th>Negative controls</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>68Ga-DOTA-TCP-1</td>
<td>68Ga (PET)</td>
<td>Inh.</td>
<td>GaCl\textsubscript{3}NSGNGC–PEG3–DOTA–68Ga (cyclic: the C form a bridge)</td>
<td>Assumed from parent molecule</td>
<td>TBR: 1.3; TMR: 5.5 \figure{} of (58)</td>
<td>MMP-9 (assumed from parent molecule)</td>
<td>In vivo: RH-1nu/nu rats C8161T/M1 xngfrt</td>
<td>None</td>
<td>(58)</td>
</tr>
<tr>
<td>1f</td>
<td>18F (PET)</td>
<td>Inh.</td>
<td>CGS 25966/27023A derivative</td>
<td>Not assessed</td>
<td>Broadband</td>
<td>In vivo: healthy C57BL/6 mice</td>
<td>None</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td>Cy5.5–C6</td>
<td>Cy5.5 (plan FOI)</td>
<td>Inh.</td>
<td>Cy5.5–KAHWGFTLD (cyclicized by indicated residues)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;: MMP-2 (4 nM), MMP-8 (2 nM), MMP-9 (50 nM), MMP-13 (11 nM)</td>
<td>Not quantified – visible sig. increase vs background</td>
<td>MMP-2/9 (n.c.)</td>
<td>In vivo: athymic nude mice PC-3 xngfrt</td>
<td>Saline injection</td>
<td>(72)</td>
</tr>
<tr>
<td>4egg</td>
<td>99mTc (PET)</td>
<td>ACPP</td>
<td>e\textsuperscript{egg}–SGRIGFLRTA–r\textsubscript{8}</td>
<td>Cell entry 2x vs experiment in presence of inhibitor</td>
<td>Not assessed</td>
<td>MMP-14 (n.c.)</td>
<td>In cellulo: MDA-MB-231 (+) cells penetration assay</td>
<td>Inhibitor</td>
<td>(79)</td>
</tr>
<tr>
<td>MMP AuNR</td>
<td>Cy5.5 (FOI)</td>
<td>FF</td>
<td>Cy5.5–GGLVRGC–Au</td>
<td>QE: 87%</td>
<td>Not quantified – visible sig. increase vs inhibitor-treated tumor</td>
<td>MMP-9 &gt; MMP-13/3 &gt; MMP-8</td>
<td>In vivo: SCC7 (+) xngfrt</td>
<td>Inhibitor</td>
<td>(36)</td>
</tr>
<tr>
<td>NA</td>
<td>Quantum dots (FOI)</td>
<td>ACPP</td>
<td>Fmoc–E&lt;sub&gt;3&lt;/sub&gt;GPLGVR&lt;sub&gt;K&lt;/sub&gt;/GK(bt)</td>
<td>In cellulo: 827x sig. in cells incubated with MMP-2 vs cells without MMP-2</td>
<td>Not assessed</td>
<td>MMP-2 (n.c.)</td>
<td>In cellulo: HT-1080 incubated with MMP-2</td>
<td>None</td>
<td>(78)</td>
</tr>
<tr>
<td>NA</td>
<td>DsRed2 (FOI)</td>
<td>Other</td>
<td>Inhibitory–peptide–transmembrane–fluorescence domains</td>
<td>In cellulo: 23.3x fluorescence in MMP-14+ cells vs cells + inh.</td>
<td>8x sig. inc. in MMP-14 (+) cells vs (−) cells</td>
<td>MMP-14 (n.c.)</td>
<td>In cellulo: HT-1080 (+) in vivo: Balb/c nude mice MCF-7 (±) and HeLa (+) xngfrt</td>
<td>Inh. (GM6001) Scrambled peptide Cells with ΔMMP-14</td>
<td>(92)</td>
</tr>
<tr>
<td>[11C]MSMA</td>
<td>11C (PET)</td>
<td>Inh.</td>
<td>Sulfonamide/valine-based</td>
<td>MSMA: IC&lt;sub&gt;50&lt;/sub&gt;: MMP-1 (1.5 μM), –2 (3 nM), –3 (8 nM), –7 (2.2 μM), –9 (22 μM), –13 (6 nM)</td>
<td>MCF-7 and MDA-MB-435 tumors not visible</td>
<td>Broadband</td>
<td>In vivo: athymic mice MCF-7 or MDA-MB-435 xngfrt</td>
<td>None</td>
<td>(47)</td>
</tr>
<tr>
<td><strong>[11]C-FMAME</strong></td>
<td><strong>[11]C (PET)</strong></td>
<td><strong>Inh.</strong></td>
<td><strong>Sulfonamide/Valine based</strong></td>
<td><strong>Assumed from parent molecule (FMA, IC50 MMP-2: 1.9 μM)</strong></td>
<td><strong>Low TMR and TBR</strong></td>
<td><strong>Broadband</strong></td>
<td><strong>In vivo: athymic mice MCF-7 or MDA-MB-435 xenograft</strong></td>
<td><strong>None</strong> (49)</td>
<td></td>
</tr>
<tr>
<td><strong>NA</strong></td>
<td><strong>177Lu and 125I (SPECT)</strong></td>
<td><strong>ACPP</strong></td>
<td><strong>K1(177Lu)-X-eGALGLPX-eG-y125I</strong></td>
<td><strong>In cellulo: 31x cellular entry increase vs negative ctrl</strong></td>
<td><strong>TMR similar between ACPP and positive control (CPP)</strong></td>
<td><strong>MMP-2/9</strong></td>
<td><strong>In vivo: athymic mice HT-1080 xenograft</strong></td>
<td><strong>Scrambled peptide Positive ctrl (CPP)</strong> (59)</td>
<td></td>
</tr>
<tr>
<td><strong>1a, 7 and 1c</strong></td>
<td><strong>18F</strong></td>
<td><strong>Inh.</strong></td>
<td><strong>Arylsulfone carboxylate</strong></td>
<td><strong>IC50: see Table 1 from reference</strong></td>
<td><strong>Interaction with BSA, 18% specific uptake</strong></td>
<td><strong>Broadband</strong></td>
<td><strong>In vivo: athymic mice U-87 MG xenograft</strong></td>
<td><strong>Perfusion/metabolism (18F-FDG) Inh. (GM6001)</strong> (60)</td>
<td></td>
</tr>
<tr>
<td><strong>Cy5.5-AF489</strong></td>
<td><strong>Cy5 (FOI)</strong></td>
<td><strong>Inh.</strong></td>
<td><strong>Hydroxamate-PEG-Cy5.5</strong></td>
<td><strong>IC50: MMP-2 (1.5 nm), -9 (2.1 nm), -8 (1.8 nm), 13 (1.7 nm)</strong></td>
<td><strong>30% specific uptake</strong></td>
<td><strong>Broadband</strong></td>
<td><strong>In vivo: athymic mice A-673 (+++), HT-1080 (++), MDA-MB-231 (++), BT-20 (+) xenograft</strong></td>
<td><strong>Inh. (GM6001) Perfusion (Cy5.5) Comparison (MMPSense)</strong> (71)</td>
<td></td>
</tr>
<tr>
<td><strong>64Cu-DOTA-CTT</strong></td>
<td><strong>64Cu (PET)</strong></td>
<td><strong>Inh.</strong></td>
<td><strong>64Cu-DOTA-CTTHWGFTLC</strong></td>
<td><strong>Effective IC50: mouse MMP-2 (18 μM) and human MMP-2 (9 μM) and -9 (9 μM)</strong></td>
<td><strong>Poor</strong></td>
<td><strong>MMP-2/9</strong></td>
<td><strong>In vivo: B16F10 (CS7/B6 mice) or MDA-MB-435 (nu/nu mice) xenograft</strong></td>
<td><strong>STT (linear version of CTT)</strong> (104)</td>
<td></td>
</tr>
<tr>
<td><strong>NPC-Cy5.5</strong></td>
<td><strong>Fe3O4 (MRI)</strong></td>
<td><strong>Ligand</strong></td>
<td><strong>Fe3O4 nanoparticles funct. w/ chlorotoxin and Cy5.5</strong></td>
<td><strong>In cellulo: enters glioma 9 L cells, and not cardiomyocytes Not quantified</strong></td>
<td><strong>MMP-2 (unconfirmed)</strong></td>
<td><strong>In cellulo: 9 L glioma cells (+) vs cardiomyocytes (–)</strong></td>
<td><strong>Nanoparticles w/o Chlorotoxin</strong> (70)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PKM**, pharmacokinetic modifier; **FOI**, fluorescence optical imaging; **athy**, athymic; **Inh.**, inhibitor; **Inc.**, increase; **Sig.**, signal; **w/**, with; **w/o**, without; **NA**, not available; %g-1, % of injected dose per gram; **Patho.**, pathology model; **ctrl.**, control; **xenograft.**, xenograft; **mam.**, mammary; **TMR/TBR**, tumor–muscle ratio and tumor–blood ratio; **TMRh**, tetramethylrhodamine; **AAA**, abdominal aortic aneurism; **bt.**, biotin; **Strep.**, Streptavidin; **PAMAM**, polyamino amine dendrimer; **NZR**, New Zealand rabbits; **r**, relaxivity; **R1**, rate of longitudinal spin relaxation (**R1 = 1/T1**); **n.c.**, not confirmed – usually, authors select a molecule from the literature but do not confirm the specificity of their probe; **QE**, quenching efficiency ([fluorescence increase after activation = 1/(100% – QE)]; (+), target positive; (–), target negative; (±), refers to the comparison of a wild-type cell line against this same cell line modified to change the expression of the target; @, at.
laboratory also indicated an atypical optic behavior by BHQ3/Cy5 probes (data not shown).

The greatest advantage of activatable fluorescence probes is that they have the potential to be fully and directly quantitative, since the activation rate, a measurable quantity, can be related to the enzymatic activity of interest. It will not allow for the determination of unactivated or inhibited MMP concentrations, which may be an asset if it turns out that MMP activity is a better biomarker than MMP concentrations.

3.3. Pharmacokinetics modulation

3.3.1. Functionalized Inhibitors

The simplest method to image a molecular target is by designing a probe that will bind to it, but in order to adopt this straightforward strategy, the concentration of the target must be sufficient. As a rule of thumb, the signal generated by the probe at a concentration equal to that of the target must surpass the sensibility threshold of the imaging modality. For enzymes in vivo, this usually means submicromolar concentrations. Agents for MRI will require a very high relaxivity in order to be detectable in this concentration range. As a comparison point, to modulate $R_1$ identically to 10 µM of Gd–DTPA [relaxivity $r = 3.5$ mm$^{-1}$ s$^{-1}$ at 7 T (41)] with 100 nm of a molecule, then this molecule will require a $r = 350$ mm$^{-1}$ s$^{-1}$. Even fluorescence-based 1:1 binding molecules might prove difficult to visualize at concentration levels similar to that of receptors. Broad spectrum MMP agents are more likely to be successful than single MMP agents because the individual concentrations of each MMP can then be summed up.

Inhibitor-based agents (1:1 binding agents) were the first molecules to be synthesized for PET and SPECT MMP imaging, and some of the initial work concerning these agents was reviewed in details elsewhere (42). Between 2001 and 2004, Hutchin’s group published several papers concerning $^{11}$C-labeled CGS27023, CGS25966, FMAME and BPSAH molecules (43–49). In parallel, Ido’s group published the $^{18}$F-labeled SAV03 agent (50,51). Kopka, Breymolz and Schäfers published two papers on an $^{125}$I-labeled version of CGS27023A, which showed low accumulation but some degree of specificity, and then an $^{18}$F version of CGS 25966 and CGS 27023A (52,53). Oltenfreiter et al. published the $^{11}$Clabeledl-dopa and valine based broad-spectrum MMP imaging agents (54–56), obtaining better results than with the $^{11}$C version introduced by Hutchin’s group. Medina et al. (57) and Ujula et al. (58) screened peptide libraries to develop small cyclic inhibitors of MMP-9, and labeled them with $^{99m}$Tc or $^{68}$Gd, respectively. Following Medina et al.’s work, Sprague et al. prepared a $^{68}$Cu-labeled version of the CTT peptide. While the authors found their molecule to be unstable in vivo, their contribution is noteworthy as it also includes an attempt at quantifying MMP-2/9 activity in tissues (using gel zymography). The authors then used this information to evaluate the chances of successfully imaging MMPs by PET (59). More recently, an $^{18}$F-labeled Marimastat derivative was published by auf dem Keller and colleagues (19). Casalini et al. presented three $^{18}$F-labeled arylsulfone carboxylate inhibitors with different affinity for subsets of the MMP family (60). The authors found that their molecules bound albumin, probably leading to the low specific uptake of 18%. None of these compounds was up to the challenge and in vivo assays, when performed, were disappointing: specificity controls failed, accumulation was low or high background was observed. A general conclusion was that, despite the intense work in the design and synthesis of MMP inhibitors, these were not adequate imaging agents. To our knowledge, the most successful radio-labeled inhibitors for MMP imaging are $[^{111}I]$ RP782 and $[^{99m}$Tc] RP805, which were published together (61), and then further used in several other papers (respectively (62) and (63–68)). For RP782, ex vivo competition control experiments with cold ligand or broadband inhibitor 1,10-phenanthroline showed that >90% of the observed signal was specific in the TOI (Fig. 7 from Zhang et al. (62)).

A few OI and MRI compounds were also based on inhibitors. A barbituric (MMP inhibitor) Cy5.5-labeled agent was published in 2008 (69): results in cells seemed to indicate that the agent was internalized by MMP-2/-9 producing cells. While binding of a molecule to soluble MMPs should not result in cell internalization, it was shown that MMP-2 dependent internalization of nanoparticles may be possible (70). The synthesis of Cy5.5 functionalized inhibitors CGS27023 and CGS25966 was also reported by the same group (37), and results with one of these molecules (Cy5.5-AF489) in xenograft tumor models were recently published (71). Pre-blocking using the inhibitor CGS 27023A indicated that specific uptake was around 30%. A cyclic peptide (72) was also published, but no specificity controls were performed. A Gd–DOTA modified inhibitory peptide named P947 was tested in several studies and was found to be effective for broad-spectrum MMP imaging (73–76). As discussed previously, one would require a very large target concentration or high agent relaxivity to use a 1:1 binding strategy – which is not the case here. Thus, these results were very intriguing, and we will discuss them later in this review.

3.3.2. Labeled antibodies

Labeled monoclonal antibodies (mAB) are an alternative to inhibitor-based molecules. While labeling mABs is often straightforward, clearance is usually problematic unless it is processed in the form of a low molecular weight (MW) fragment (i.e. 15 kDa nanobodies). Even then, the size of the agent remains in the five digits MW, restraining its uptake in healthy tissues. Saji’s group published several papers on monoclonal radionuclide labeling (12,13), one including a biotin/streptavidin agent chase to improve clearance and contrast. While their results are promising, they were less specific than RP805 with only ~38% of specificity as determined by competition controls [Fig. 3 from Temma et al. (12)]. The same group also published a MRI dendritic (PAMAM) Gd-based contrast agent with high relaxivity targeting MMP-14. This agent bears ~44 Gd atoms per molecule, and each Gd atom results in a higher relaxivity than standard Gd–DTPA (15.5 vs. 3.61 mmol$^{-1}$ s$^{-1}$ at 1.5 T), for a total $r = 660$ s$^{-1}$ per mm of agent. These properties greatly increase the contrast generating capabilities of this method, and a 50% specific signal was obtained [Fig. 4c in Sano et al. (13)]. Su et al. (77) published interesting ex vivo and in vivo results obtained with MMP-2 targeted microbubbles in a heart infarct rat model. On the other hand, the complete absence of bubbles in the control areas 10 min after injection is surprising, both because clearance of massive agents is not expected to be fast, and because MMP-2 is naturally present in the healthy heart (20).

Similarly as described above, labeled antibodies are used as 1:1 agents. They can be functionalized to carry several signal/contrast generating groups. Basically, they will supply information which is qualitative, but will not allow for a real quantification.
Figure 2. Specific and non-specific accumulation of imaging probes. A probe injected in the blood circulation may follow a number of different pathways and encounter different events before reaching a tissue of interest (TOI). The probe may be eliminated from the circulation without ever reaching the TOI proximal circulation. This elimination rate and route may vary between an activated (E) and unactivated (E') probe molecule. The probe is most likely to reach tissues with increased blood flow or dense vasculature. Perfusion (P), as well as the exchange rate of the tissue (KIN/OUT) depend upon the state of the tissue (i.e., healthy vs inflamed (EPR)). The exchange rate is also influenced by the nature/activation state of the probe (KIN'/OUT'). Once inside the TOI, most of the kinetics of the molecule depends on the targeting strategy. A) The signal modulation strategy is based on the activation of the probe by MMP-2. The enzymatic cleavage rate is dependent on the affinity of the enzyme for the probe, but also on the concentrations of the probe and the enzyme. In the schematics, three activated probe pools are shown: "Specific", "EPR" and activated distally, "AD". The signal observed in the TOI is the sum of the signal of individual pools. It must be noted that all molecules in these pools are activated by MMP-2, hence could be considered specific in the broader sense. Yet, in the precise context of MMP-2 quantification, this terminology would discard the underlying complexity of the measured signal. For this reason, "Specific" here refers to probe activation which is only related to MMP-2 levels (and based on a standard probe uptake). The "EPR" pool refers to the increase of the probe activation due to both a delayed clearance of the activated probe and to an increased activation rate caused by an increased probe concentration in the TOI (as per classic enzymatic kinetics). Hence, a well perfused tissue containing low MMP-2 concentrations (EPR and MMP+) may accumulate activated probe levels similar to a poorly perfused tissue with high MMP-2 concentrations (Healthy and MMP+++). In the "AD" pool, the probe is activated outside the TOI and then non-specifically accumulates in the TOI. Concentration in this last pool is also modulated by EPR effects. B) The activated cell penetrating peptide probe and solubility switch probes will behave similarly. Upon activation, these molecules will gain a greater affinity for a new compartment. In this case, KON is much larger than KOFF. Non-specific entry of the unactivated probe inside cells or its interaction with cell membranes is still possible – but is expected to be lower. Yet, some ACPP probes were published which only showed a two fold increase in cell penetration (79). Because these strategies also rely on probe activation, the EPR-induced substrate concentration increase will impact the activation rate of the probe. C) The binding strategy will suffer from increased substrate concentration since the equilibrium between free and bound MMP-2/probe depends on the concentrations of both molecules. Note that non-specific binding will also be affected. Finally, all these effects may be present during an experiment. A signal-modulated or binding probe may enter cells (and activation may occur there). An ACPP probe may non-specifically bind molecules in the TOI, more or less efficiently than its activated counterpart. The non-specific binding of a probe molecule may interfere with its signal (e.g., an aggregating fluorescent probe may desagregate and bind a protein – this may influence its fluorescence efficiency). The real quantification of MMP-2 requires the assessment of as many of these effects as possible, and represents a difficult challenge.
3.3.3. **Activatable cell penetrating peptides (ACPP)**

Cell penetrating molecules consist of known peptide sequences which signal for the internalization of a molecule, or a positively charged sequence which will allow the agent to stick to the negatively charged cell membrane to be nonspecifically internalized. In the case of ACPP, the targeted MMP can bind and process the agent to remove an inhibiting moiety (antiICPP), allowing the agent to enter neighbouring cells. After entering the cells, the agent is stuck in the TOI, resulting in a long-lasting contrast. This strategy was used to allow cell access to quantum dots for a MMP-2/7 agent in cells. Using streptavidin-coated quantum dots and biotin-labeled ACPP, the authors demonstrated an efficient delivery of their agent into cells, but no in vivo results were published (78). Watkins *et al.* developed a radio-labeled MMP-14 specific tracer, but in vitro results showed that further work was required since only 27% of the activated probe would enter cells (79). Tien’s group developed a MMP-2/-9 specific ACPP composed of a substrate peptide sequence flanked by poly d-Glu (anti-CPP) and Cy5-poly d-Arg (CPP) (80–83). This agent was tested in PyMT mice spontaneously developing mammary tumors, and in MMP-2/-9/−/− mice. Based on control experiments (uncleavable d-amino acid (d-aa) substrate sequence), 90% of the signal observed in vivo was specific to MMP-2/9 activation. The authors found that their probe generated a better tumor/skin ratio than MMPSense 680 and could be used in vivo to guide tumor resection. While these last reports were encouraging, a recent paper by van Duijnoven *et al.* suggested that ACPP tumor-independent activation may play an important role (59). This paper will be further discussed later in this review.

Since they are based on an enzymatic activation process, each activated target MMP will activate several ACPP molecules, which amplifies the signal. The observed signal/contrast will depend on the activation rate, but this rate is not directly measurable as is the case for activatable fluorescence agents. Hence, while this design supplies information concerning the activity of the targeted MMP, it will not yield MMP concentrations or fully quantitative information. Furthermore, since the agent is trapped within cells, the signal is expected to last for a long time inside high MMP activity tissues. This is double-edged: the final contrast will increase much more over time as the molecules is cleared from other tissues, but at the same time it will become more difficult to perform successive injections and perform longitudinal studies.

3.3.4. **Solubility switch (SS)**

This strategy was introduced along the first MMP targeting MRI contrast agent (84). The authors designed a solubility switch agent by flanking a peptide substrate with a hydrophilic moiety (PEG) and a hydrophobic contrast-generating moiety. Cleavage of the peptide resulted in a drastic reduction of the solubility of the CA, slowing its clearance, and trapping it close to the enzyme. This strategy was first used for MMP-7 (84) and then for MMP-2 (85,86). Recently, the reverse strategy was used to build a MMP-2 and -12 targeted contrast agent (87). The authors observed a pharmacokinetics difference which is in line with previous reports. Finally, a PET/Fluorescent probe (18F, TMR) was prepared and tested in vivo (88). This probe used the TMR intrinsic hydrophobicity and a long PEG chain to create a very efficient solubility switch.

Similarly to ACPP agents, SS agents will supply mostly qualitative information and have a higher signal potential than 1:1 binding agents. Without a reference tissue or a control, it is impossible to determine if a tumor generates more or less active MMP. It might be possible to model the activation of the molecule if its properties are properly characterized a priori, but given the large number of variables in this problem (Fig. 2), the results are likely to be imprecise.

4. **CONTROLS IN MOLECULAR IMAGING**

Refinements in molecular imaging probes should be accompanied by improvement in control experiments; this has unfortunately not been the case. It is our humble opinion that many new probes were presented without sufficient validation. In fact, thorough in vivo controls should be integrated to study designs to fully account for the limitations of a probe. In this section, we will demonstrate the pertinence of closely examining controls in the light of multiple examples from the literature, and discuss the conclusions which were drawn (or not) from them. We will also include a section concerning dynamic imaging, which is of critical importance in the molecular probe characterization process.

4.1. **Probe behavior in vivo: what should you expect and what should you look for?**

Ideally, the distribution of an activatable imaging probe would be restricted to the tissue of interest, and it would then be activated in situ. Unfortunately, this is very improbable. Many other pathways and physiological properties can cause (or prevent) activated probe accumulation. In Fig. 2, we present schematic summarizing different pathways that may lead to ‘activated’ probe in a tissue of interest. We use ‘activated’ in a generic sense here meaning that the injected probe is modified by an event, leading to the formation of a different molecule. Suppose a probe is injected in the blood stream. Blood perfusion (P) or to or in the tissue of interest and excretion (E) of the probe (e.g. via kidneys or liver) will determine the amount of probe available to the tissue. This is a limiting factor in the amount of probe available, and poorly perfused tissues will be characterized by a lower probe concentration. If the target of the probe is extravascular, then the escape from vasculature (KIN) and its retention (KOUT) must be considered. The activation of a molecule (e.g. a probe cleaved by MMP-2) will change its exchange parameters (KIN/KOUT). Blood vessel permeability is high in tumors and inflamed tissues relative to normal, healthy tissues. This is represented by a higher probe concentration in diseased tissues. An additional feature to consider is the presence of the EPR effect in tumors and other inflamed tissues. Blood vessel permeability may result from the presence of activated macrophages and immune cells, causing the accumulation of fluid (oedema) such that any probe may accumulate in inflamed tissues. This is true for nearly almost every high-molecular-weight compound, whether it is targeted or not. EPR is characterized by high permeability (high KIN) and slow excretion from tissue (low KOUT) favoring probe accumulation. At this stage, the probe can be activated by MMP-2, resulting in a specific probe accumulation. Note that, for simplicity, we do not differentiate between MMP-2 produced by tumor cells and MMP-2 produced by
Increased accumulation of a probe, that is, in the presence of MMPS, we show different cases for high or low MMP-2 activity in tissues. If MMP-2 is present in the blood or if the probe is not stable in blood, then activation in the circulation may occur.

(1) We show different cases for high or low MMP-2 activity in tissues. If MMP-2 is present in the blood or if the probe is not stable in blood, then activation in the circulation may occur.

(2) Increased accumulation of a probe, that is, in the presence of EPR effects at the TOI, will allow for the generation of extra signal owing to the influence of probe concentration on any subsequent enzymatic reaction. Probe concentrations are expected to be well below enzyme saturation.

(3) Likewise, the probe may be activated in the vasculature, or in a distal tissue and returned to the blood circulation. Perfusion (P) will then determine the concentration of activated probe reaching the tissue of interest. Activated probe can accumulate in the tissue of interest (K\text{IN}) because of the high blood vessel permeability and the EPR effect. This nonspecific accumulation must be assessed by control experiments in order to determine the portion of specific accumulation. Clearly, molecular imaging of MMP-2 is a dynamic process where concentrations of probe available, activated probe and nonspecific accumulation may follow different time courses.

We have used the term ‘accumulation’ rather than ‘uptake’ to distinguish between the mere presence of a probe and an active mechanism for trapping a probe, as is the case for instance for the GLUT receptors towards \(^{18}\text{F-FDG}\). As a word of caution, the portion of specific ‘uptake’ of \(^{18}\text{F-FDG}\) or other radiotracers could only be assessed with control experiments or, to a lesser extent, by pharmacokinetic modeling. With the advent of MRI–PET systems, perfusion could be assessed with a nonspecific MRI contrast agent and shed some light on the distribution, accumulation and specificity of radiotracers. In the case of MMP targeting, such controls are almost never performed, and when they are, they often refute the specificity of the probe.

In xenografted tumor models, the rim of tumor is known to be more vascularized than the core (89), which means that any probe will accumulate there more quickly. High expression of MMP-2 was shown to be present in healthy tissues surrounding tumors (90), and MMP-2 was suggested to participate in the tissue response to tumor growth (91). Hence, MMPS are more likely to be present at the rim. The concordance of both phenomenon could potentially explain positive results obtained at the rim of tumors (tissues/tumor interface), such as those.
obtained by others and ourselves (80,84,92). Additionally, since the core is fed through diffusion, the probe is also expected to remain longer, whether it is activated in the rim, in the core or outside the tumor. Once more, such results (84) must be considered with great care, and dynamic imaging, as discussed in the next section, can provide significant insight on the kinetics.

Finally, unspecific binding will inherently modify the pharmacokinetics of probes, both inside the tissue (as illustrated in Fig. 2C), and outside the tissue. Casalini and colleagues reported that their hydrophobic inhibitor-based probe 1a bound albumin quite tightly (micromolar affinity) (60). Given the high concentration and size of albumin, the authors suggested that (1) the probe would be largely bound to albumin and (2) this interaction would therefore define the kinetics of their probe by causing unspecific accumulation through the EPR effect. We also reported on how albumin binding by some fluorescent probes may affect their optical properties (40). Hence, special care should be taken to assess unspecific protein binding of novel probes and its effect on accumulation and signal.

4.2. Dynamic imaging

One of the greatest weaknesses of many recent studies is the absence of complete dynamic imaging results. When evaluating a new probe in vivo or testing a probe in a new animal model, it is crucial to include a dynamic signal acquisition (before, during and after the injection of the probe). The pre-injection signal allows for background correction. The first time points provide initial clues on the perfusion of a tissue and exchange rate of a molecule, and reduced substrate/enzyme diffusion in tissues. These results with the activatable probe III showed that maximal activation occurred at roughly 50 min (Fig. 3). Depending on the agent size and affinity for proteins and tissues, biological half-life of compounds can vary significantly – hence, one should expect it to change upon activation. Of course, clearance depends on tissue–blood exchange rate ($K_{in}/K_{out}$) as well as perfusion, such that this information is important in evaluating the specific signal of molecular imaging agents. Our experiments with the preactivated probe V showed that it accumulates in subcutaneous tumors over 10 min, and is eliminated afterwards (Fig. 3). V is similar in size and composition to other imaging molecules presented in this review, including small fluorescent peptide fragments released by many scaffolding agents (31,34). As an additional control, the o-aa control IIIId was injected (Fig. 3). Quenched optical probes such as IIIId have residual signal, and their signal can be measured with proper image analysis. Unlike V, IIIId accumulates slowly over the first 50 min, and is then eliminated – which is perfectly in line with its more hydrophobic nature. This is caused by the quencher, QSY 21, which actually causes probes to aggregate (40) in aqueous media. The behavior of this control is qualitatively similar to that of I, II, and III. Yet, signal intensity informs us that not a small fraction of the probes is activated, for example, at 90 min post injection, there is a ~10-fold increase in signal between III and IIIId ($n = 2$). Hence, neither IIIId nor V would be a perfect control for the overall kinetics of our probes. Although outside the scope of the actual review, a solution to this predicament would be to model the probe activation by considering both the unactivated and activated populations of the probes and their respective pharmacokinetics. Note that o-aa controls
will provide information on $K_{IN}$ and $K_{OUT}$ while $V$ will give insight as to $K_{IN}$ and $K_{OUT}$ (Fig. 2).

In conclusion, dynamic imaging analysis can be used to evaluate the kinetics and biological lifetime, and in the case of EPR-affected tissues, it may allow for pinpointing activation specific contrast. Interpretation of EPR accumulation should be made with caution as probe activated distally may accumulate in the tissue of interest at a later time (Fig. 2). The design of an imaging probe, as is often presented in recent papers, aims to assess only one parameter of interest, such as enzymatic activity. However, imaging probes are sometimes designed, willingly or not, as large macromolecules which can readily accumulate (nonspecifically) by the EPR effect. A robust analysis of dynamic imaging can help detect this unspecific accumulation.

4.3. Controls: what they can tell you, and what they cannot

4.3.1. In vitro specificity controls

Specificity controls assess whether a probe binds or is activated only by its intended target(s). In the first studies of MMP-2 targeted probes, this often consisted of incubating the probe with a panel of MMPs, and assessing their inhibition efficacy (using parameters such as the $IC_{50}$ (52,56,69) or $K_{i}$ (61)) or activation rates of the probe (i.e. $k_{cat}/k_{m}$ (32,33,84,86)) to confirm its preference and/or affinity for the intended target(s). Some groups then expanded the assay by adding enzymes other than MMPs to their test panel, such as cathepsins (34). It is of course not possible to test in vivo against every macromolecule or enzyme of the host. Ex vivo plasma stability assays will provide a first-hand evaluation of the in vivo stability of a probe (40). The two major limitations to this assay are the possibility that (a) the unspecific activation/degradation can occur in situ by membrane enzymes (i.e. MT-MMP1) or other enzymes that are absent from the plasma, or (b) the target is present in the plasma. Most MMPs, including MMP-2, are soluble enzyme such that traces are expected to be present in blood.

A partial solution to this predicament was used by auf dem Keller et al. whereby a Marimastat-based radiotracer was used as a marker in a gel electrophoresis of tissue extract (19). This methodology can reveal whether unspecific binding occurs or not. Similarly for an activatable probe, one could perform a zymography and replace the MMP-2/-9 peptide substrate by the probe to be tested, and assess its specificity. Both assays are limited by the fact that some proteins (including enzymes) are irreversibly denatured in the electrophoresis process (e.g. membrane anchored enzymes). For both assays, the relative intensity between MMP-related bands and other bands would indicate the ratio of specific vs unspecific binding/activation.

4.3.2. Nonspecific accumulation assessed with an inert control probe with different physical properties

An inert control probe (molecule or ion) may be used to assess perfusion and blood vessel permeability in tissues (61,75). It must be kept in mind that the physical properties (e.g. hydrodynamic volume, charge, surface polarity) of a probe will influence the accumulation and excretion from tissues. It was in fact shown that the distribution volume of a molecule in a tissue depends on its nature (95). Thus, such a control experiment provides a first approximation of nonspecific accumulation, but it is not appropriate to perfectly normalize the signal measured with a test probe. Su et al. (61) used $^{201}$Tl to assess myocardial perfusion in a mouse heart ischemia–reperfusion infarct model. It provided the authors with interesting information: despite the low perfusion of an infarcted tissue, their radio-labeled broadband MMP inhibitor RP805 accumulated readily in the infarcted region. Yet a question remains: is the probe accumulating because of an EPR effect? The $^{201}$Tl control does not reach the infarcted region and thus cannot provide information on the clearance of RP805 from that region.

Perhaps the answer to this question lies in dynamic imaging which would elucidate the accumulation and clearance kinetics of RP805. Note that the necrotic core of tumors, which are usually filled only by diffusion of probe molecules from the tumor rim, will retain imaging agents efficiently (89). It is entirely possible that RP805 slowly diffused inside the infarcted regions, and remained there because of an increased retention owing to inflammation. Another convincing example of perfusion controls is supplied by Baeten et al. (96), who used the Angiosense 750 control molecule to correct the distribution of the activatable probes MMPSence 680. Their results show that correcting for the distribution of the activatable probes greatly increases the correlation between the corrected signal and the MMP activity as measured by zymography (96). On the other hand, there are two limitations to this study which were not taken into account: there is a large molecular weight difference between the control (vascular) and activatable probes, and the activation of the MMPSence 680 probe releases a small fluorescent fragment which probably enters into tissues more easily.

Applying a signal correction will allow the user to fully appreciate the evolution of specific signal (Fig. 4). While testing molecule I ($n = 4$), our group performed two analyses: first, the signal of I in low and high MMP-2 activity tumors was compared, and a significant difference was detected (more signal was found in the high MMP-2 activity tumor; paired t-test, $p = 0.0123$). Then, we performed a dynamic normalization of the signal using the control V by dividing the signal of I by that of V at each time point. As discussed previously, V is the activated fragment of I, but it behaves differently from IId, which overall structure is more similar to I. Here, V is used here uniquely as a perfusion control. Applying a dynamic signal correction will allow the user to fully appreciate the evolution of specific signal. Using this normalization, the difference between the tumors was no longer found to be significant ($p = 0.134$), suggesting that some of the initial signal difference was caused by inter-tumoral perfusion variations. In fact, MMP-2 producing tumors were more invasive and their rim was clearly more perfused than that of the MMP-2 KD tumors (Fig. 44 from Lebel R. (94)). This does not necessarily mean that the probe is completely ineffective. Increasing the number of subjects may allow one to observe a significant difference. On the other hand, our tests suggest that some of the signal difference might be explained by a perfusion difference.

In the case of a binding agent, the unbound probe molecules would exit both the TOI and the RR, and contrast between target containing tissues and others is expected to increase – but this outflow will be regulated by $K_{OUT}$ and P (Fig. 2C). Similarly, activatable probes will be activated, but residual signal from unactivated probe will remain an important contaminant (Fig. 2A), especially for low quenching efficiency fluorescent probes (e.g. 95 or 85% quenching efficiency (31,33)). In the case of our probes, the high quenching efficiency (>99%) mitigates this effect.
4.4. Control experiments with an inert and comparable probe

These control probes can be separated in three main categories:

1. probes incorporating scrambled amino acids (for peptide based probes only: the control probe has the same amino acids as the test probe but in a different sequence to nullify the specificity);
2. isomeric control probes – a classic example is peptides with D-aa;
3. pre-activated probes.

Each of these controls has limitations. Scrambled peptides may still be partially cleaved by other enzymes – such that blood and in vivo stability must still be assessed. D Isomers may not be totally stable or resistant to enzymatic cleavage (e.g. the possibility of cleavage at achiral sites, such as Gly residues, has been suggested (82)). A control experiment with a pre-activated probe may help assessing the nonspecific distribution of the test probe. However, as the pharmacokinetics of a pre-activated probe may differ from that of the test probe, care must be taken and alternative controls, such as D-aa versions, should be used to confirm that the distribution of unactivated and pre-activated probes are similar.

Scrambled peptides (SP) are one of the most frequently used control type (38,72,84,86,93). A signal difference between scrambled and normal peptide probes is associated with a difference in the activation state. On the other hand, this control allows for the comparison of unactivated and pre-activated probes are similar.

Scrambled peptides (SP) are one of the most frequently used control type (38,72,84,86,93). A signal difference between scrambled and normal peptide probes is associated with a difference in the activation state. On the other hand, this control allows for the comparison of unactivated and pre-activated probes are similar.

Looking at the full distribution of both probe and controls is paramount to confirming the specific uptake of a new molecule. van Duijnoven et al. tested both a negative (scrambled, non-ACPP) and a positive (pre-activated, CPP) control along their novel ACPP probe (93). The authors compared the accumulation of these molecules in a subcutaneous tumor mouse model. They underline that, while the activation by MMsfs allowed for greater ‘absolute’ accumulation of their probe (ACPP on non-ACPP ratio > 1), this phenomenon was present in all organs, including the muscle in which the activity of MMP-2 and -9 was found to be null by zymography. The authors concluded that activation was present in the vasculature, and that tissue accumulation was unspecific. The similitude in the distribution of the ACPP and the CPP positive control supports this conclusion. The authors also point out that a similar muscle signal increase was observed in a recent study by Olson et al. (83).

Scrambled peptide control can lead to unexpected results. As discussed previously, our group tested several MMP-2 fluorescent probes, including probe I and its scrambled version Is. Our results showed that the scrambled version of the probe was not stable in the conditions used – in fact, its activation was much faster than the MMP-2-specific version (Fig. 5). Yet we had tested this molecule in vitro and found that it was not cleaved by MMP-2, 9, 3 or 7 (40). These new results demonstrate that even control molecules must undergo stability trials. Scrambled peptides may have alternative affinities, such as controls must be properly characterized on their own – and not simply used ‘out of the box’ to validate a test probe. This is also true in the case of binding agents – a switch in the peptide sequence may cause it to bind unexpected targets and impact its pharmacokinetics.

In the case of Ab-based agents, a negative control Ab (directed at a protein that is not present in the animal model) can be used. In order to evaluate their MT1-MMP binding Ab-based radiotracer, Temma et al. (12) compared the kinetics of their tracer to a control radio-labeled Ab. Using dynamic imaging and measuring the tumor/blood distribution ratio (TBR), their experiments allowed for the measurement of specific contrast [in Fig. 3 of Temma et al. (12), subtract dashed line from solid line]. Briefly, no specific contrast was observed in the first 6 h, but it steadily increased between 6 and 48 h (up to ~40% TBR difference). The prerequisite for optimal contrast was further illustrated by the same group in a second publication (13). In this paper, the authors used a pre-targeting strategy by injecting a MT-MMP1 specific Ab labeled with a streptavidin molecule first, then injected a radio-iodinated biotin 72 h later. This strategy allowed for a specific contrast to be visible within the first 6 h. The specific contrast increased to a value similar to the previous study [Fig. 2 of Temma et al. (12), TBR shows a ~50% specific contrast after 6 h (MT1-MMP compared with the untargeted Ab tracer)]. Their method reduces the time at which the maximal contrast is obtained (~24 h instead of 72 h), and allows full capitalization on the unbound Ab clearance. Additionally, their method allows for the use of short half-life radionuclide for MT-MMP1 detection. Instead of requiring the unbound Ab to be cleared, this strategy only requires the smaller iodinated biotin to be removed from blood.

D-Amino acid controls are also widely spread in the MMP imaging literature (40,82). Our group compared D-aa controls with our test probes. The signal intensity observed in vivo led us to believe that these controls were not activated in appreciable amount. It was proposed that peptide sequences containing achiral sites (such as Gly) may be slightly activated in vivo (82).
This possibility is noteworthy as Gly residues are present in most activatable probes peptide sequences (Table 1). While D-aa controls are not activated, note that they still accumulate in tissues, and as described above for the scrambled peptide, can be detected and used as perfusion controls.

In any case, a perfusion control will enable at least a partial assessment of nonspecific accumulation or may explain a negative result where the target is present but the probe cannot be delivered adequately.

4.4.1. In vivo competition and inhibition

Competition and inhibition controls are performed by pre-injecting molecules which will either block the binding site of the target(s) MMP and/or prevent the processing of activatable probes. Broadband MMP inhibitors such as GM6001 (Illomastat) and doxycyclin are sometimes used in studies to confirm that MMPs are responsible for the activation of MMP probes or that these probes bind the catalytic sites of MMPs (17,84). Since cross-specificity is present, it is hazardous to base conclusions concerning an individual MMP based on such controls (17). Inhibitor properties must be taken into account – their distribution and stability may not be appropriate. Performing a titration of the enzymatic activity would allow differentiation between unspecific (non-MMP) activation and inappropriate inhibitor dosage (incomplete inhibition). With this information, MMP-specific probes can be used to assess inhibition efficacy, an important end-point of MMP inhibitor trials (4). Finally, the multifaceted role of MMPs should not be neglected during MMP inhibitors trials: a long-term inhibition treatment may reduce MMP activity, but it may also reduce angiogenesis and inflammation and thus reduce the nonspecific accumulation of a probe in a tissue of interest. In addition, a variation in the distribution of the probe inside a tissue may be interpreted as a variation of the MMP activity.

The pre-injection of a cold ligand (precursor) before the injection of a radiotracer can also yield insight on the binding of a probe (65,97). It is important to note that this type of competitive control confirms that the hot and cold molecule may saturate identical targets (e.g. receptor sites or enzymes), and allows for the assessment of nonsaturatable signal (e.g. increase in perfusion or permeability, affinity for cell membranes, intrinsic cell penetration capabilities). It does not provide any information on the identity of the targeted molecules.

4.4.2. Control with cells not expressing or expressing low levels of the target MMP

Xenograft tumor models are often used to test new probes in vivo. The MMP production of cell lines can be characterized ex vivo, and the most straightforward method consists of comparing two cell lines with a large MMP activity difference (31,98). It is also possible to engineer a cell line and modify its MMP production, and then compare the wild-type and modified line in vivo. (e.g. KD or knockout, KO) (34,86). Obviously, different cell lines can have markedly different properties but even a single MMP may influence tumor properties, including its growth rate, invasiveness and vascularization (17,86). MMP-2 promotes angiogenesis, and the absence of MMP-2 could be accompanied by lower perfusion, leading to a reduction in accumulation. In an experimental setup lacking proper perfusion controls, this lower accumulation can be confounded with decreased activation. Conversely, a probe that accumulated nonspecifically in a well-perfused tumor may accumulate to a lesser extent in a poorly perfused tumor; this would be mistakenly interpreted as a successful probe.

The tumor micro-environment does not consist solely of cancer cells, but includes stromal (i.e. fibroblasts) and immunity cells (macrophage, neutrophiles) as well. These cells can potentially produce their own MMPs and create an important discrepancy between MMP activity of cell culture before injection and extracted tumoral tissues (86). For activatable probes, ex vivo zymography (in situ or on electrophoresis gel) should be performed on tissue slices or extracts to assess real MMP-2 activity levels. A final concern is that cell line phenotype may vary over time and between laboratories. An example of this for MMP-2 is the use of the A549 cell line as either a positive (72) or negative (54) control in the literature.

Chuang et al. tested a TMR/¹⁸F solubility switch MMP-2 probe molecule (88) in vivo by comparing the uptake of their probe in a mouse model of xenografted tumors (HT1080 vs MCF-7, high and low MMP-2 expression respectively). Fig. 6(a) of their paper presents images acquired as early as 15 min post injection, which already reveal an important signal offset between tumors. It is unlikely that clearance from the tumors would also have occurred so quickly from MCF-7 tumors, and early post-injection dynamic imaging is woefully lacking; therefore, there is no warranty that the probe ever entered the tumor to interact with MMP-2. It is thus possible that the difference observed between the tumors is caused, at least in part, by a perfusion difference. The authors use a 3-day broadband MMP inhibitor treatment to evaluate the specificity of their probe. Their results show that an important difference between tumors is still present. Overall, MMP activity clearly has an impact on the distribution of their probe, but the lack of correction for its distribution prevents the reader from fully appreciating its efficacy.

4.4.3. Assessment of probes in models of pathology in comparison with healthy controls

Pathology models are usually selected for their realistic representation of human disease and allow testing MMP probes in a more realistic context. These include spontaneous tumor-generating animals (80–83), ischemia/infarct models (17,32,77), atherosclerosis models (64,74,75,99), and arthritis models (39,100). The underlying biochemical and physiological variations between healthy and pathologic/damaged tissues can be important and may lead to false positive or false negative results. As an example, Klohs et al. (17) have tested the probe MMPsense 680 in a model of cerebral ischemia and compared the signal of the damaged and the unaffected brain hemisphere (17). Molecules with a high molecular weight (such as MMPsense 680) do not cross the intact blood–brain barrier. Thus, a lack of accumulation in the control hemisphere cannot be taken as evidence of local activation of the probe in the infarcted region. While MMP inhibition controls (GM6001) support that MMP activity is required to reach high signal levels, local activation is not demonstrated. Similarly, Lancelot et al. used an atherosclerosis model and compared the signal inside the aorta after an initial imaging session (with the imaging agent), then one week later with the control agent (75). Note that their imaging agent, P947, is a potent broad spectrum inhibitor of MMPs, and is injected in sufficient concentration to generate contrast in MRI. Based on their results [Fig. 6 of Lancelot et al. (75)], the half-life of the CA in the tissue of interest...
is roughly 18 h (comparing 3h and 22h times points), suggesting that it may have an effect for several days (the IC50 is suggested to be roughly 5 μM in Fig. 4 from Lancelot et al. (75) for conditioned media from extracted plaques). Based on the information supplied by the authors, one can estimate the initial concentration of agent to 180 micromolar; hence the CA concentration is above its IC50 for at least 4 days. This level of MMP inhibition is likely to have an impact on subsequent imaging sessions and should be taken into account.

Comparison of ex vivo and in vivo results can also provide valuable information on the usefulness of a probe. Sheth et al. imaged the aorta of mice in a model of abdominal aortic aneurysm and attempted to quantify MMP activity using the MMPSense 680 probe (101). A 60% decrease of MMP-2 activity between treated (doxycycline) and untreated animals was detected by in situ zymography. This value was 75% as determined by in vivo imaging [exposed aorta, Fig. 2c of Sheth et al. (101)] or ~35% ex vivo [extracted aorta, Fig. 5 of Sheth et al. (101)]. The value determined by in vivo imaging with an endoscopic catheter introduced in the aorta was ~20% [Fig. 4 of Sheth et al. (101)]. These results may indicate incomplete MMP inhibition. Otherwise, the signal decrease between treated and untreated animals would be expected to be close to 96% (based on theoretical QE, see Table 1). The discrepancy could also be explained by other factors, such as an EPR difference between animal cohorts. These results suggest that additional controls would be necessary.

Perfusion may vary during progression of pathology. Yoon et al. used the MMPSense 680 probe to evaluate the progression of colon tumors in a mouse model (102). In their study, the authors presented a drastic and very significant increase of signal intensity between normal colon, adenoma and adenocarcinoma. Perfusion control experiments were not performed. It is likely that the size and permeability of a lesion will vary during the evolution of a tumor, and their influence on the measured signal intensities should be evaluated and discussed. It is also possible that pathology eliminates the elimination rate of a probe. Kuge et al., reporting on the Ab based MT1-MMP agent, observed a longer blood life time of their agent in atherosclerosis rabbit model compared with control animals, and attributed this difference to a change to the hepatic and renal functions caused by the pathology (15). As seen in Fig. 2, a decrease in the elimination rate will increase the concentration of a probe in all tissues; hence, the signal in a reference tissue could be used to calibrate the signal observed in the tissue of interest.

4.4.4. MMP-2/9 KO animals

Knockout animal models are very convenient to study the unspecific activation of a probe in vivo. Olson et al. tested a MMP-2/9 ACPP fluorescent probe in PyMT mice, which spontaneously develop breast tumors (82). The authors compared the distribution of their probe between PyMT and MMP-2/-9 KO littermates, and showed that these MMPs account for roughly 70% of the accumulation of their probe inside tumors. In fact, a similar distribution was observed for the activatable probe in KO animals and for the control unactivatable (D-amino acids) probe distributions in non-KO animals. The authors limited the reach of KO animal controls by stating that such controls assess the ‘protease-dependant component of tumor uptake’ – more precisely, the MMP-2 and MMP-9 component. It would have been very interesting if the authors had also extracted the tissues of interest and extracted their agent to assess if it had been activated. This would have allowed them to further quantify the nonspecific activation component. The ratio (activated/unactivated) could have been used as a measurement of the efficacy of their ACPP strategy. However, such assessments are very time- and effort-consuming, and cannot be routinely applied in the study of new probes.

5. SUGGESTIONS ON HOW TO PERFORM A ROBUST MOLECULAR IMAGING STUDY

Much work was devoted to imaging MMPs, but few probes have made it past the proof of concept. After a decade of work, new acceptance requirements for the publication of novel MMP probes (and activatable probes) are needed.

A plasma (not serum or phosphate-buffered saline) stability assay should be performed. If possible, a blood concentration curve from live animals should be acquired, and the candidate probe should be characterized for its stability in vivo. Pathology may affect the levels of circulating enzymes such that blood must be withdrawn from appropriate models at appropriate times.

Complete dynamic imaging should be performed and presented – both the short- (high time resolution), and long-term accumulation/activation of the agent should be shown, along with evidence of its biological half-life. Imaging should be performed at time points up to one half-life of the probe (at least).

Inhibition controls using several inhibitor doses should be performed to confirm that the inhibition is working properly. This can also be used as a perfusion control if the signal of the unactivated agent is sufficient. When discussing these results, the specificity of the inhibitor should be considered.

For activatable probes, scrambled or D-aa control should be used, but the stability of these control probes should be confirmed in vivo (i.e. the scrambled control should not be activated, as was the case for our compound 15).

Whenever possible, results should be corrected for perfusion, even if only a linear first-order approximation is applied. In the case of fluorescent activatable probes, SPECT imaging or MRI using chemical exchange saturation transfer (CEST) agents, it is possible to add a reference moiety instead (33,34,59). MRI using standard T1 or T2 agents or PET would not allow for a control moiety since these modalities cannot differentiate between two different probes or signal moieties. As discussed previously, it is in theory possible to correct the signal acquired from one imaging modality with that of a second modality – but both must be quantitative. Obviously, correcting 2D fluorescence imaging using 3D PET imaging is incorrect, since the fluorophore depth will affect the observed signal (nonquantitative). An example of this inadequate procedure was found (103).

Intra-tumoral injections should be avoided – to be efficient, a probe must be able to reach the tissue of interest without a priori knowledge of its location.

Depending on the quantity of interest (e.g. target concentration, activation state or activity), adequate biochemical/histological characterization of the tissue of interest should be performed to validate imaging studies. This should be achieved by a combination of methods (e.g. zymography, immunoblot, RT-PCR) whose description is beyond the scope of this review. The limits of the method used must be considered and discussed. One good example of this is gelatine zymography, which requires tissue
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6. SUMMARY AND OUTLOOK

MMPs are often selected as a proof of concept model to validate these probes in vitro and in vivo. The closely interwoven roles of MMPs in inflammation and tissue reestructuration (including angiogenesis) are seldom taken into full consideration in imaging studies. EPR was shown to have strong repercussions on probe accumulation, and might lead to artefacts. In this review, we emphasized the possible bias of several recent in vitro studies which lacked critical controls – not to discredit the imaging strategies implemented by the authors, but to encourage higher standards in probe validation in future studies.

During the last decade, innovative imaging strategies and brilliant probe design have been brought forward. The most successful probe designs are being selected and re-used by more than one research group, indicating that the field of molecular imaging is slowly maturing. To accelerate this maturation, new controls before publication. In our humble opinion, absolute quantification of targets of interest will require more than a first-order approximation that will include assumptions on the pharmacokinetics/pharmacodynamics/molecular properties of the system. Nevertheless, each step of this iterative work will bring the field of molecular imaging a little closer to the end point of in vivo accurate absolute quantification.

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