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Caspase-3 probes for PET imaging of apoptotic tumor response to anticancer therapy

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Abstract

Apoptosis is a highly regulated process involved in the normal organism development and homeostasis. In the context of anticancer therapy, apoptosis is also studied intensively in an attempt to induce cell death in cancer cells. Caspase activation is a known key event in the apoptotic process. In particular, active caspase-3 and -7 are the common effectors in several apoptotic pathways, therefore effector caspase activation may be a promising biomarker for response evaluation to anticancer therapy. Quantitative imaging of apoptosis *in vivo* could provide early assessment of therapeutic effectiveness and could also be used in drug development to evaluate the efficacy as well as potential toxicity of novel treatments. Positron Emission Tomography (PET) is a highly sensitive molecular imaging modality that allows non-invasive *in vivo* imaging of biological processes such as apoptosis by using radiolabeled probes. Here we describe the development and evaluation of fluorine-18-labeled caspase-3 activity-based probes (ABPs) for PET imaging of apoptosis. ABPs were selected by screening of a small library of fluorine-19-labeled DEVD peptides containing different electrophilic warhead groups. An acyloxymethyl ketone was identified with low nanomolar affinity for caspase-3 and was radiolabeled with fluorine-18. The resulting radiotracer, [^{18}F]MICA-302, showed good labeling of active caspase-3 *in vitro* and favorable pharmacokinetic properties. A μPET imaging experiment in colorectal tumor xenografts demonstrated an increased tumor accumulation of [^{18}F]MICA-302 in drug-treated versus control animals. Therefore, our data suggest this radiotracer may be useful for clinical PET imaging of response to anticancer therapy.

Keywords: Apoptosis, caspase-3 activity, warhead, activity-based probe, small-animal PET imaging.

Introduction

Apoptosis is a highly regulated form of cell death that facilitates the orchestrated removal of damaged and dead cells from healthy tissues. As such, this type of programmed cell death is an essential biological process that plays a key role in normal development and in maintaining normal tissue homeostasis. Disruption of homeostasis, either by excessive or deficient apoptosis is a hallmark of many pathological processes including cardiovascular diseases, neurodegeneration, sepsis, and cancer.¹⁻⁵ Because many anticancer therapeutic strategies aim at promoting apoptosis in tumor cells, early detection of apoptosis is of particular interest for treatment response evaluation in patients and in drug development.^{6, 7} The activation of caspases is a critical component in the signaling cascades that lead to the execution of apoptosis. Both intrinsic (via mitochondria) and extrinsic (activation of death receptors) pathways of apoptosis converge in the activation of effector caspase-3, the most studied and characterized member of this family. Several studies have implicated caspase-3 as an important effector molecule in promoting therapy-induced cell death.^{8, 9} It is also known that dysregulated caspase-3 activity can lead to resistance to drug-induced apoptosis in tumor cells.¹⁰⁻¹² Therefore, methods that allow dynamic imaging of caspase-3 activity may have the potential to predict response to anticancer therapy.

Recent advances in non-invasive optical and nuclear imaging techniques have made it possible to image protease activity, for monitoring disease progression and evaluating therapeutic efficacy.^{13, 14} Positron Emission Tomography (PET) is a highly sensitive molecular imaging technique that does not suffer from the common limitations of optical imaging, such as low sensitivity and low spatial resolution with increased imaging depth. Several PET probes, or radiotracers, targeting active caspase-3 have been developed and extensively explored in recent years. Examples of these radiotracers include radiolabeled peptide derivatives ($[^{18}\text{F}]\text{FB-VAD-FMK}$, $[^{18}\text{F}]\text{C-SNAT}$, $[^{18}\text{F}]\text{CP18}$, $[^{18}\text{F}]\text{-TBD}$) and molecules with a canonical small-molecule architecture ($[^{18}\text{F}]\text{ML-10}$, $[^{18}\text{F}]\text{ICMT-11}$).¹⁵⁻²⁰ Among these $[^{18}\text{F}]\text{ML-10}$ and $[^{18}\text{F}]\text{ICMT-11}$

have been used in clinical trials.²¹⁻²³ The first in human study using [¹⁸F]ML-10 - a low molecular weight (206 Da) molecule based on the alkyl-malonate group of γ -carboxyglutamic acid - indicated high *in vivo* stability and favorable biodistribution and dosimetry profiles.²¹ In patients with brain metastases, [¹⁸F]ML-10 was able to detect responses to whole-brain radiotherapy several weeks earlier than standard anatomical imaging techniques.²² Increased [¹⁸F]ML-10 uptake after successful therapy was found to be associated with radiotherapy-induced tumor apoptosis.²³ Notwithstanding these promising results, [¹⁸F]ML-10 uptake failed to correlate with drug-induced cell death, and the specific target of [¹⁸F]ML-10 remains undefined.²⁴ Therefore, the utility of this probe for early evaluation of treatment response warrants further investigation.

[¹⁸F]ICMT-11 is another example of a small-molecule caspase inhibitor which was evaluated in clinical trials. The probe is a nonpeptidic covalent inhibitor based on the isatin sulfonamide moiety with nanomolar affinity for caspase-3 and -7 (0.5 and 2.5 nM, respectively).¹⁸ Isatins are covalent inhibitors with a dicarbonyl moiety in their structure essential for binding to the nucleophilic active site cysteine. Yet, the high reactivity of the dicarbonyl functionality, may also result in lower probe selectivity.^{25, 26} In addition, complex voxel-wise analysis of [¹⁸F]ICMT-11 PET imaging data is necessary for analysis of heterogeneous tumours.²⁷ Despite these limitations [¹⁸F]ICMT-11 has proceeded into clinical studies. Initial results demonstrated the safety of [¹⁸F]ICMT-11 with a dosimetry profile that lies within the range of other clinically used ¹⁸F PET radiotracers.²⁸ Recently, [¹⁸F]ICMT-11 entered a clinical study for assessing therapeutic response in breast and lung cancer patients, where no significant changes in [¹⁸F]ICMT-11 tumor uptake were observed after therapy.²⁹ This was attributed to the relatively small increase in active caspase-3/-7 levels induced by chemotherapy.²⁹

In spite of some impressive preclinical results and significant investment in clinical evaluation, so far, no caspase-3-targeting PET imaging probe has made it into routine clinical practice for treatment response

assessment in cancer patients. Two important reasons are cited: (1) lack of targeting group specificity for caspase-3, due to the sequence homology among the caspases; and (2) low absolute target uptake, due to rapid tissue diffusion.^{15, 30, 31} In order to yield useful probes, they should target individual caspases in complex samples, and remain bound to the active form of the target caspase. This can potentially be achieved by using activity-based probes (ABPs). Detection of specific active caspases using fluorescent ABPs has been described in detail previously.³²⁻³⁵

Caspases must be catalytically active to kill a cell. Therefore, we reasoned that selective caspase-3 targeting ABPs might be useful to better define the individual role of caspase-3 in apoptosis-related therapy response. ABPs are generally inhibitor-derived compounds capable of forming a covalent bond with the active site of the target enzyme in an activity-dependent manner. Caspases preferentially recognize and process short tetrapeptides with appropriate amino acid sequences within protein substrates, which confers binding specificity to the active caspase.³⁶ It has been demonstrated that caspase-3 highly efficiently processes the Asp-Glu-Val-Asp (DEVD) sequence, when compared to initiator and other effector caspases such as caspase-6 and -7.³⁷ Therefore, recently a PET substrate containing the DEVD epitope was developed and successfully used for the detection of active caspase-3 by PET imaging.³⁸ Different warheads such as halomethyl ketones, acyloxymethyl ketones (AOMKs) and aldehydes have also been incorporated in a series of peptide inhibitors for caspases, including caspase-3.^{15, 39-43} These warheads are relatively strong electrophiles that react with the nucleophilic cysteine thiol at the active site of the enzyme, and they can therefore have a positive impact on inhibitor potency and on selectivity against other cysteine proteases, such as legumain and cathepsins.^{34, 44} Recent studies have additionally identified Michael acceptors as a new class of potent and specific inhibitors for clan CD cysteine proteases, which includes caspase-3.^{45, 46} When coupled with the preferred tetrapeptide recognition sequence for a specific caspase, Michael acceptors did not show cross-reactivity with enzymes that belong to clan CA,

such as calpain and cathepsin B.^{46, 47} Up to this moment, Hight et al. were the only ones to describe a radiolabeled ABP, [¹⁸F]FB-VAD-FMK, for PET imaging of apoptotic effector caspase activity.¹⁵ The authors showed that this radiotracer can be used to predict the response to a multidrug treatment regimen consisting of an inhibitor of mutant BRAF and a phosphoinositide 3-kinase (PI3K) inhibitor. However, this ABP has demonstrated lack of selectivity, consequently acting as a broad spectrum probe targeting not only initiator and effector apoptotic caspases, but also inflammatory caspases at the same time.¹⁵ It is important to note that this might be the result of a combination of two factors, the intrinsic high reactivity of the fluoromethyl ketone (FMK) electrophilic warhead, and the presence of the non-specific VAD peptide recognition sequence, which is efficiently cleaved by inflammatory caspase-1. Therefore, this probe is limited in its utility when applied to anticancer therapies that induce inflammation such as radiation therapy.

Since one of the pivotal hallmarks of cancer is the ability of tumor cells to evade apoptosis, cancer drug development has predominantly focused on triggering apoptosis.⁴⁸ Therefore, in this study we aimed to develop non-invasive imaging tools that allow the evaluation of early efficacy of anticancer therapy. We hypothesized that an ABP based on selective caspase-3 inhibitors would provide a starting point for the design of an apoptosis targeting radiotracer. Here we describe the development and evaluation of caspase-3 targeting PET radiotracers for apoptosis with an ABP architecture. The general structure of the compounds that were designed in this study is depicted in Figure 1. All compounds are fluorinated, containing a galacturonic acid moiety, a typical caspase-3-targeting DEVD sequence and an electrophilic warhead functionality. To systematically probe for the influence of the warhead on inhibitor potency and selectivity, several warhead types were included in the series. A careful selection of these groups was performed, as the degree of reactivity of the electrophilic warhead can have an impact on the selectivity of an ABP.⁴⁹ Therefore, the inhibitors included in this study can be classified as reversible (aldehydes) or

irreversible (Michael acceptors and activated ketones like AOMKs and fluoromethyl ketones (FMKs)), which have distinct modes of binding, thus modulating inhibitor potency.⁴⁹ Among the latter group, the AOMKs are the weakest electrophiles, thus they have shown little cross-reactivity with other bionucleophiles.⁴⁰ A non-reactive amide functionality was used as control. To separate the fluorine tag from the caspase active site, we incorporated the galacturonic acid spacer at the *N*-terminus of the molecule. This moiety was also used to improve the pharmacokinetics of the ABP, as previously described for [¹⁸F]CP18, a radiolabeled DEVD-containing substrate-based probe developed for imaging of caspase-3 activity.⁵⁰ The PET radiotracers were first synthesized and biochemically evaluated in a non-radiolabeled (*i.e.*, ¹⁹F-containing) form. The most promising inhibitor was labeled with ¹⁸F via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). The developed ¹⁸F-labeled ABP was further characterized by evaluating its binding efficiency to human recombinant caspases, cell lysates and tumor homogenates. The preliminary evaluation of the ABP potential for *in vivo* PET imaging, included the determination of the metabolic profile and *in vivo* biodistribution of the ¹⁸F-labeled ABP in normal CD1-/- nude mice. Additionally, a μ PET imaging study was performed in colorectal cancer xenografts treated with targeted therapy to assess the probe's ability to *in vivo* image caspase-3 activation and subsequent tumor response to treatment.

Figure 1. General structure of DEVD peptide inhibitors containing different warhead functionalities (R) at the P1' position.

Results and discussion

Chemistry

The synthetic preparation of target compounds started with the preparation of a general DEV-based, tripeptide precursor common to all target compounds (Scheme 1, compound **5**). The latter was prepared in gram-scale amounts using solution- or liquid-phase peptide synthesis (LPPS) similar to previously described methods.^{51, 52} According to a previously reported procedure, tripeptide **2** was deprotected to obtain **3**, which was equipped with Fmoc-protected β -D-galacturonic acid (Fmoc-SAA) to give **4**.⁵³ Hydrogenative *O*-debenzylation finally led to intermediate **5**.

Scheme 1. Preparation of the Fmoc-SAA-DEV peptide^a

^aReagents and conditions: (a) TAEA, DCM, rt, 30 min; (b) 1-(Fmoc-aminomethyl)-β-D-galacturonic acid, HOBT, EDC.HCl, DIPEA, DCM/DMF, rt, 16 h; (c) Pd/C, H₂, MeOH, 6 h.

The preparation of the Asp-derived, warhead-containing moieties is represented in Scheme 2. The α,β -unsaturated ethyl ester **6** was obtained using the Wittig reaction, as previously reported.⁵⁴ Vinyl sulfone **8** derivatized with *N*-Boc protected Asp was prepared by reaction with the corresponding phosphonate by the Horner-Wadsworth-Emmons condensation reaction.⁵⁵ Acetal protected *N*-Cbz aldehyde moiety **10** was prepared as previously described.⁵⁶ Formation of the acyloxymethyl ketone (AOMK) **12** was performed from a *N*-Boc Asp-derived chloromethyl ketone (CMK), using a procedure described by Krantz et al.^{57, 58} FMK **14** was prepared according to a procedure described for the development of a fluorescent ABP, Bodipy-VAD-FMK.⁵⁹ The control amide **16** was synthesized according to a known procedure.⁶⁰ The deprotection of the *N*-Boc group in **6**, **8**, **12** and **14** was performed under mild acidic conditions for maintaining the *tert*-Bu side chain protection, resulting in the tosylate salts **7**, **9**, **13** and **15**, respectively.⁶¹ Deprotection of the *N*-Cbz protected warheads **10** and **16** was conducted via catalytic hydrogenation to yield the free amines **11** and **17**.⁵⁶

Scheme 2. Preparation of the Asp-derived, warhead-containing moieties^a

^aReagents and conditions: (a) *p*-Toluene sulfonic acid, DCM/Et₂O, rt, 48 h; (b) 1,4-Dihydrobenzene, palladium hydroxide, EtOAc, rt, 24 h; (c) Pd/C, H₂, MeOH, rt, 1 h.

The common peptide **5** was subsequently transformed into finalized ¹⁹F-compounds by introducing the Asp-derived, warhead containing moieties **7**, **9**, **11**, **13**, **15**, **17** (Scheme 3). The obtained **18a-f** were deprotected and *N*-terminal extended with azidoacetic acid to give the protected azides **19a-f**. Finally, CuAAC of the azides **19a-f** with 5-fluoropent-1-yne, was followed by trifluoroacetic acid (TFA) deprotection of the obtained triazoles to give the final fully deprotected inhibitors **1a-f**.

Scheme 3. Preparation of the ^{19}F -DEVD inhibitors^a

^aReagents and conditions: (a) **7**, **9**, **11**, **13**, **15**, or **17**, HOBT, EDC.HCl, DIPEA, DCM/DMF, rt, 16 h; (b) DEA, THF, rt, 8 h; (c) Azidoacetic acid, HOBT, EDC.HCl, DIPEA, DCM/DMF, rt, 16 h; (d) sodium ascorbate, CuSO₄, TBTA, 5-fluoropent-1-yne, *tert*-BuOH/H₂O, rt, 16 h; (e) TFA, TIS, H₂O, rt, 30 min.

Although we used synthetic procedures available from the literature, and reaction conditions which are known to minimize the occurrence of racemization (e.g. hydroxybenzotriazole, sterically hindered bases and short reaction times), in some of the final products a mixture of two epimers was observed. This was most likely due to P1 Asp racemization during the preparation of the Asp-derived, warhead-containing moieties. These products were separated by HPLC and only the inhibitory activity of the most abundant and most active diastereoisomer is reported.

Biochemical evaluation of the inhibitor library

Prototypical caspase inhibitors contain simple warheads attached to a peptide backbone, inhibiting caspases via covalent modification of the cysteine active site. The most commonly used warheads

encompass aldehydes and ketones with their derivatives, such as FMKs or AOMKs, which tend to be highly reactive with cysteine proteases.³⁷ These small groups bind tightly to the S1' pocket in the enzyme, which is in agreement with the observation that caspases recognize small residues in the P1' position of their substrates.³⁷ Therefore, optimization of the P1' probe position can be of special interest for increasing inhibitor affinity and selectivity for individual caspases.³⁷

Inhibitors **1a-f** were examined for their ability to inhibit human and mouse recombinant caspases. The inhibitory potency of these compounds was assessed using a fluorogenic caspase inhibition assay, as previously described.⁶² Two commercially available inhibitors were included as reference compounds: the caspase-3 and -7 specific reversible peptide inhibitor, Ac-DEVD-CHO; and a broad spectrum irreversible pan-caspase inhibitor, zVAD-FMK. All the synthesized inhibitors displayed inhibition potency in the nM range against caspase-3 and -7 recombinant human and mouse caspases, as shown by the IC₅₀ values listed in Tables 1 and 2, respectively.

Table 1. Inhibitory potency of ¹⁹F-DEVD peptide inhibitors **1a-f** against human caspases expressed as IC₅₀ values.

Compound	R	Inhibitory potency IC ₅₀ (nM) ^a					
		caspase-1	caspase-3	caspase-6	caspase-7	caspase-8	caspase-9
1a		> 1,000	20.7 ± 1.4	> 1,000	48.0 ± 1.2	455.0 ±	> 1,000
						29.1	
1b		> 1,000	24.4 ± 1.9	> 1,000	776.1 ±	> 1,000	> 1,000
					22.7		
1c		> 1,000	19.9 ± 2.1	> 1,000	159.7 ±	69.5 ± 2.3	> 1,000
					8.0		
1d		> 1,000	1.0 ± 0.1	20.0 ± 1.9	13.2 ± 0.5	23.6 ± 2.0	> 1,000
1e		> 1,000	7.6 ± 0.8	208.5 ±	68.7 ± 3.9	35.0 ± 2.5	432.8 ±
				17.5			37.7
1f		> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000
zVAD-FMK	n.a.	112.7 ±	154.8 ±	190.5 ±	63.9 ± 2.6	11.5 ± 0.3	19.0 ± 1.3
		3.4	10.2	10.2			
Ac-DEVD-CHO	n.a.	> 1,000	18.8 ± 1.6	> 1,000	35.0 ± 2.6	45.5 ± 2.8	> 1,000

^aValues are the mean ± SD of two independent duplicate determinations. n.a.: not applicable.

Table 2. Mouse caspase inhibition potency of selected compounds **1a-f**.

Compound	Inhibitory potency IC ₅₀ (nM) ^a			
	caspase-1	caspase-3	caspase-7	caspase-8
1a	> 1,000	1.4 ± 0.1	21.7 ± 2.9	475.9 ± 45.7
1b	> 1,000	15.1 ± 0.9	129.2 ± 29.6	> 1,000
1c	> 1,000	1.4 ± 0.1	21.7 ± 2.9	68.4 ± 8.3
1d	10.5 ± 4.4	0.9 ± 0.1	18.1 ± 1.7	4.4 ± 0.1
1e	31.4 ± 4.2	2.9 ± 0.2	57.7 ± 5.0	4.1 ± 0.2
1f	> 1,000	> 1,000	> 1,000	> 1,000
zVAD-FMK	n.d.	84.1 ± 10.7	42.7 ± 3.1	4.4 ± 0.5
Ac-DEVD-CHO	77.2 ± 6.2	8.6 ± 0.5	43.7 ± 6.9	47.2 ± 7.4

^aValues are the mean ± SD of duplicate determinations. n.d.: not determined.

AOMK derivative **1d** was the most potent inhibitor for caspase-3 (IC₅₀ = 1.0 ± 0.1 nM, for human, and IC₅₀ = 0.9 ± 0.1 nM, for mouse caspase-3), which is 9 to 19 times more potent than the reference inhibitor Ac-DEVD-CHO. This is in good agreement with the preference of caspase-3 for bulky and hydrophobic warheads at P1'.³⁷ Furthermore, compound **1d** showed some promiscuity against the other caspases, with 20- and 13- to 20-fold selectivity over executioner caspase-6 and -7, respectively, and 5- to 24-fold selectivity over the initiator caspase-8. In contrast, the binding potency toward human caspase-9 was weak, with IC₅₀ > 1,000 nM. FMK compound **1e** also displayed a good binding potency towards caspase-3 (IC₅₀ = 7.6 ± 0.8 nM, for human caspase). Nevertheless, this inhibitor showed less selectivity for caspase-3 when compared to **1d**. FMK-based inhibitors were among the first to be developed as caspase inhibitors, yet the high reactivity of the FMK group often leads to off-target binding, thus cross-reactivity with other

cysteine proteases. By comparison, the AOMK-based inhibitors are weaker electrophiles, thus they are more suitable for investigating caspases because of lower cross-reactivity with other bionucleophiles.³⁷ Although binding potencies toward human caspase-1 were weak ($IC_{50} > 1,000$ nM), compounds **1d** and **1e** inhibited mouse caspase-1. This difference in affinity between human and murine caspase-1 might be explained by the fact that murine caspase-1 contains 3 additional aspartates between D296 and D314, which could increase the affinity of these two compounds for murine caspase-1.⁶³

Both Michael acceptors **1a** and **1b**, showed weaker inhibitory potency against human caspase-3, compared to the activated ketones (AOMK and FMK) with affinities of 20.7 ± 1.4 nM and 24.4 ± 1.9 nM, respectively. The increased inhibitory potency of compound **1a** (1.4 ± 0.1 nM) against murine caspase-3 compared with the one of inhibitor **1b** (15.1 ± 0.9 nM) was surprising as vinyl sulfones are 2 to 10 times more reactive towards nucleophiles than vinyl esters.⁵⁵ Nevertheless, the vinyl sulfone containing inhibitor **1b** exhibited a higher degree of selectivity for human caspase-3 over caspase-7 (32-fold) comparing to ketone counterparts (13- and 9-fold for AOMK and FMK, respectively), in agreement with previously reported data.⁵⁵ Differences in the size and shape of the S1' subsite between caspase-3 and caspase-7, have been explored for the modulation of inhibition selectivity between caspase-3 and caspase-7 by changing the functionality that occupies the P1' position.⁴³ Another potential advantage of this α,β -unsaturated scaffold tailored to explore the P1' region is the lower cross-reactivity towards other cysteine proteases, as caspases prefer trans stereochemistry in their inhibitors.⁴⁷ The low activity of compounds **1a** and **1b**, taken together with previous studies, yields these Michael acceptor warheads not suitable for caspase inhibition.⁵⁵

Compound **1c** exhibited an inhibitory potency for human caspase-3 ($IC_{50} = 19.9 \pm 2.1$ nM), similar to reference inhibitor Ac-DEVD-CHO, with very good selectivity for human caspase-3 over human caspase-1, -6 and -9. However, inhibitor **1c** displayed low selectivity for human caspase-3 over caspase-8 (3.5-fold),

in good agreement with the preference of caspase-8 for small and aliphatic groups at P1'.⁴² As expected, control compound **1f** showed no caspase inhibition.

Promising work on activity-based probe research has shown that many efforts are directed toward selectivity for a single caspase. Recently, different research groups have independently developed peptide libraries containing both natural and unnatural amino acids in order to provide compounds to distinguish between initiator and effector caspases.^{41, 64, 65} The inhibitors developed by Wolan's lab were successfully converted into fluorescent ABPs and displayed high selectivity for caspase-3 over caspase-7 and -8.³⁴ Despite favorable affinity and selectivity profile obtained with fluorinated DEVD-containing inhibitors, the development of next-generation derivatives, using the same general ABP structure, but displaying improved selectivity profiles, are warranted for maximum selective caspase targeting.

Radiosynthesis

Since compound **1d** demonstrated the highest inhibitory potency against both human and mouse caspase-3 and -7 in the biochemical assays, it was selected as candidate for radiolabeling with ¹⁸F. Compound **1f** which contains an amide in place of the reactive AOMK functional group, was selected as a control radiotracer. The azide precursors for radiolabeling **20a** and **20b** were obtained by TFA deprotection of **19f** and **19d**, respectively. The radiolabeling of compounds **1f** and **1d** was performed by CuAAC of 5-[¹⁸F]fluoro-1-pentyne ([¹⁸F]**22**), prepared by nucleophilic substitution of the tosylate precursor **21** with [¹⁸F]fluoride, with the azide precursors **20a** and **20b**, yielding the ¹⁸F-labeled triazoles [¹⁸F]**MICA-301** and [¹⁸F]**MICA-302**, respectively (Scheme 4).

Scheme 4. Radiosynthesis of [^{18}F]MICA-301 and [^{18}F]MICA-302^a

^aReagents and conditions: (a) $\text{K}[^{18}\text{F}]\text{F}/\text{K2.2.2}$, K_2CO_3 , CH_3CN , 110°C , 3 min; (b) CuSO_4 , sodium ascorbate, TBTA, EtOH, CH_3CN , rt, 20 min.

The optimal conditions for click reaction were 20 min reaction time under stirring at room temperature in the presence of aqueous copper sulfate, sodium ascorbate, and TBTA as copper-stabilizing ligand.¹⁷ Following HPLC purification and sterile formulation, control probe [^{18}F]MICA-301 and ABP [^{18}F]MICA-302 were obtained in 13.4 ± 4.0 ($n=4$, decay-corrected) and $12.6 \pm 2.1\%$ ($n=4$, decay-corrected) isolated radiochemical yield (RCY), respectively. By using a biocompatible mobile phase for purification, an additional purification step was avoided yielding the final products in a total synthesis time of 90 min from end of bombardment (EOB), including formulation. Both tracers were obtained in a radiochemical purity $> 99\%$. The molar activities (A_m) of both radiotracers were in the range of 50-300 GBq/ μmol at EOB. The identity of the radiolabeled compounds was confirmed by co-injection and co-elution on HPLC of

[¹⁸F]**MICA-301** and [¹⁸F]**MICA-302** with the corresponding non radiolabeled 'cold' reference compounds **1f** (t_R = 6.7 min, gradient method A) and **1d** (t_R = 7.2 min, gradient method B), respectively (Supplemental Figure 1).

The partition coefficients ($\log D$ values) of [¹⁸F]**MICA-301** and [¹⁸F]**MICA-302** were determined experimentally. Both [¹⁸F]**MICA-301** ($\log D$ = - 3.29 $\pm$ 0.12) and [¹⁸F]**MICA-302** ($\log D$ = - 3.09 $\pm$ 0.05) were found to be highly hydrophilic, which generally confers poor cell membrane permeability.

Activity-based labeling of recombinant enzymes

To evaluate the affinity and selectivity of the radiolabeled ABPs for caspase-3 we performed a binding assay using equal concentrations (20 nM) of pre-mixed caspase-3 and caspase-6, -7 or -8, and legumain and cathepsin B, followed by addition of probe and subsequent incubation with the enzyme mixture for 10 and 60 minutes. As expected, only the active ABP [¹⁸F]**MICA-302** efficiently labeled caspase-3 within 10 min after incubation (Figures 2a, b and c), whereas [¹⁸F]**MICA-301** demonstrated no binding to any of the enzymes (results not shown). Although [¹⁸F]**MICA-302** predominantly labeled caspase-3, it also demonstrated binding to cathepsin B, and to a smaller extent legumain (Figure 2d). This promiscuous binding profile has been reported before for other caspase-3 ABPs.^{32,40,44} Moreover, zVAD-FMK also inhibited legumain (Figure 2d), which although a lysosomal protease, belongs to clan CD proteases similar to caspases and is not related to cathepsins.⁶⁶

Interestingly, [¹⁸F]**MICA-302** demonstrated a lower degree of cross-reactivity against other caspases compared to fluorescence-based DEVD-AOMK probes.^{41, 66} This might be explained by the fact that [¹⁸F]**MICA-302** was used in low nanomolar concentration, below enzyme concentration. As previously shown, at stoichiometric concentrations a 5(6)-carboxyfluorescein-labeled ABP (FAM-DEVD-AOMK) binds almost exclusively to caspase-3. However, in the presence of excess probe (micromolar concentrations),

promiscuous labeling of caspase-6, -7 and -8 occurs.⁴¹ In general, a high amount of probe is required for fluorescence imaging thereby increasing the risk of off-target binding, while the use of only nmol to fmol amounts are required for imaging when using radiotracers.

Figure 2. Biochemical evaluation of activity-based probes. Direct labeling of recombinant caspase-3 and caspase-6 (a), caspase-3 and caspase-7 (b), caspase-3 and caspase-8 (c), and legumain and cathepsin B (d) with [¹⁸F]MICA-302. The probe (74 kBq) was incubated with recombinant human enzymes for 10 and 60 minutes, the reaction was stopped, enzymes were separated by SDS-PAGE and the gel was scanned for radioactivity. Samples marked with + represent pre-treatment with the pan-caspase inhibitor zVAD-FMK (1 μM) which was added 30 min prior to probe. The location of the caspase-3 p12 small subunit is also indicated. Cropped regions of the gel images containing the expected protein bands are shown.

Activity-based labeling of apoptotic cell lysates

The ability of [¹⁸F]MICA-302 to detect activated caspase-3 *in vitro* was evaluated using lysed HeLa cells treated with staurosporine (STS). Caspase-3-deficient MCF-7 cells treated with STS were used as a negative control.⁶⁷ First, the optimal time window for detection of caspase-3 activity in apoptotic HeLa cells was determined in a biochemical caspase activity assay using a fluorogenic substrate (Figure 3a).

Incubation of HeLa cells with a fluorogenic substrate indicated significant caspase-3 activity within 4 h after treatment with STS ($p < 0.001$), gradually decreasing until 24 h. Pre-incubation with the pan-caspase inhibitor zVAD-FMK successfully abolished caspase-3 activity ($p < 0.001$; Figure 3a). This was confirmed by blotting with a caspase-3 antibody (Figure 3b). Following a 60 min incubation with the cell lysates, autoradiography of the SDS-PAGE gel demonstrated that [^{18}F]MICA-301 did not label any enzymes (results not shown). By contrast, active caspase-3 was detected by [^{18}F]MICA-302 4 to 10 h after STS treatment in HeLa cells (Figure 3c). Processing of procaspase-3 (32 kDa) involves two different cleavage events. First initiator caspases cleave within its interdomain linker, found between the p20 and p10 domains, and then its *N*-terminal prodomain is removed resulting in the formation of the mature active caspase-3 (17 kDa). However, if the prodomain of caspase-3 is not efficiently removed a fragment will run at 19 kDa.⁶⁸ Detection of active caspase-3 by [^{18}F]MICA-302 was highest 4 h post treatment, and two protein bands could be visualized at 19 and 17 kDa. In contrast, in MCF-7 cells no labeling of active caspase-3 was detected (Figure 3c). Although MCF-7 cells do not express caspase-3, it has been demonstrated that apoptosis proceeds via activation of caspase-9, -7 and -6.⁶⁷ In a recent study, using a rhodamine-containing ABP Rho-DEVD-AOMK, additional bands for caspase-6 and -7 were observed at 8 and 14 h post STS treatment in MCF-7 cells.³⁴ This suggests again that the use of lower amounts of radiolabeled ABPs might reduce the risk of off-target binding, when comparing to fluorescent ABPs. Radiotracer specificity was further assessed by addition of the inhibitor zVAD-FMK, before STS treatment or after radiotracer incubation in HeLa cells, which successfully inhibited caspase-3 labeling (Figure 3c). Blotting with a caspase-3 antibody showed that zVAD-FMK indeed inhibited cleaved caspase-3 (Figure 3b). These results show that [^{18}F]MICA-302 labels active caspase-3 in apoptotic cell lysates.

Activity-based labeling of tumor homogenates

Finally, the usefulness of $[^{18}\text{F}]\text{MICA-302}$ to monitor activation of caspase-3 *in vivo* was evaluated using a model of targeted therapy-induced apoptosis. For this, tumor homogenates were prepared from mice bearing therapy-resistant HT29 and therapy-sensitive COLO205 tumor xenografts and treated with a single dose of conatumumab or the corresponding IgG1 isotype control. Incubation of the tumor homogenates with a fluorogenic substrate indicated significant caspase-3 activity in the COLO205 tumors 24 h after treatment with conatumumab ($p = 0.002$), but not after treatment with the control IgG1 antibody. By contrast, very low caspase-3 activity could be detected in the treatment-resistant HT29 tumor homogenates (Figure 4a). Accordingly, blotting with a caspase-3 antibody showed that cleaved

caspase-3 could be detected in COLO205 tumors treated with conatumumab (Figure 4b). After addition of [^{18}F]MICA-301 or [^{18}F]MICA-302 to the homogenates, the samples were run by SDS-PAGE followed by autoradiography of the gels for detection of enzyme labeling. Active caspase-3 could be detected with [^{18}F]MICA-302 in therapy-sensitive COLO205 tumors treated with conatumumab, but not in the tumors treated with IgG1 nor in the resistant HT29 tumors (Figure 4c). A doublet of labeled proteins could be seen as 19 and 17 kDa bands, corresponding to the active caspase-3 as well as to the form of this protease without its *N*-terminal prodomain.⁴⁰ Addition of [^{18}F]MICA-301 to the tumor homogenates did not result in any labeling (results not shown).

Figure 4. Caspase-3/7 activity measurement (a), detection of cleaved caspase-3 (b), and labeling of endogenous active caspase-3 with [^{18}F]MICA-302 (c) in COLO205 and HT29 tumor homogenates 24 h after treatment with targeted therapy. Location of active caspase-3 is shown based on molecular weight. **p=0.002, significantly different from IgG1-treated COLO205.

In vitro stability and *in vivo* metabolic stability studies

Because [^{18}F]MICA-302 demonstrated a relative selective binding to caspase-3 when using a mixture of recombinant enzymes, as well as in the cell lysate and tumor homogenate study, its potential for *in vivo* imaging was investigated. First, the *in vitro* stability of [^{18}F]MICA-301 and [^{18}F]MICA-302 was determined in PBS and in mouse plasma. In the validation study extraction yields of $91.4 \pm 3.0\%$ and $88.4 \pm 2.8\%$ for [^{18}F]MICA-301 and [^{18}F]MICA-302, respectively, were determined and no degradation of the radiotracers

occurred during work-up. As shown in Figure 5, both radiotracers showed good stability in PBS at 37°C up to 120 min, with only the parent radiotracers being detected by radio-HPLC, without signs of degradation ($[^{18}\text{F}]\text{MICA-301}$: $93.5 \pm 1.2\%$ intact; $[^{18}\text{F}]\text{MICA-302}$: $95.8 \pm 2.3\%$ intact). After incubation in mouse plasma for 120 min at 37°C, $[^{18}\text{F}]\text{MICA-301}$ revealed the formation of one minor polar metabolite ($t_R = 4.5$ min, Supplemental Figure 2; $81.8 \pm 2.2\%$ intact), whereas $[^{18}\text{F}]\text{MICA-302}$ remained largely intact ($95.1 \pm 0.3\%$). Additionally, stability of both radiotracers was evaluated *in vivo* following intravenous (i.v.) injection of the radiotracers in normal CD1 nude mice (Figure 5). Radio-HPLC analysis of the plasma revealed good *in vivo* stability of both $[^{18}\text{F}]\text{MICA-301}$ ($92.6 \pm 0.1\%$ intact) and $[^{18}\text{F}]\text{MICA-302}$ ($87.1 \pm 2.5\%$ intact) up to 60- and 120-min post-radiotracer injection (p.i.), respectively, in normal CD1 nude mice. Similar to the *in vitro* stability study, for $[^{18}\text{F}]\text{MICA-301}$ one main polar metabolite could be detected in the plasma ($t_R = 4.5$ min) at 120 min p.i., accounting for 26% of the total plasma activity (Supplemental Figure 2). A previous study has reported on the hydrolysis of a structurally related substrate-based probe, $[^{18}\text{F}]\text{CP18}$, by plasma hydrolases with the formation of a $[^{18}\text{F}]\text{DE}$ radiolabeled fragment.⁵⁰ We therefore hypothesize that similar enzymatic hydrolysis of $[^{18}\text{F}]\text{MICA-301}$ has occurred in our study, with the formation of a polar radiometabolite. However, this metabolite was not identified experimentally. Nevertheless, since the formation of this metabolite was observed only after 120 min p.i. we do not expect this metabolite to represent a confounding factor for radiotracer uptake at the time of imaging (60 min p.i.) in tumor animal models.

Figure 5. Stability of $[^{18}\text{F}]\text{MICA-301}$ (a) and $[^{18}\text{F}]\text{MICA-302}$ (b) in PBS and mouse plasma at 37°C, and after i.v. injection in CD1 nude mice.

In vivo and *ex vivo* biodistribution study

When used for *in vivo* imaging, a radiotracer should demonstrate good pharmacokinetic properties with efficient clearance from the blood and non-target organs and tissues. The biodistribution profile of ABP $[^{18}\text{F}]\text{MICA-302}$ was therefore evaluated by *in vivo* μPET imaging and *ex vivo* biodistribution and compared to $[^{18}\text{F}]\text{MICA-301}$. Figure 6 shows representative $\mu\text{PET}/\text{CT}$ sagittal whole-body images recorded between 30 and 60 minutes and the time-activity curves (TACs) for heart (representative for the blood clearance), muscle, and major excretory organs liver and kidneys up to 60 min p.i. in normal CD1 nude mice.

Figure 6. *In vivo* biodistribution of radioactivity in normal CD1^{-/-} nude mice after intravenous injection of $[^{18}\text{F}]\text{MICA-301}$ (a) and $[^{18}\text{F}]\text{MICA-302}$ (b). Representative sagittal μ PET/CT images (30-60 minutes summed activity) of normal mice. Time-activity curves for heart, muscle, liver and kidneys are shown. Data are presented as mean $\pm$ SD.

For both radiotracers an initial peak uptake of radioactivity was immediately followed by fast blood clearance with almost complete elimination from the blood pool at 60 min p.i., with $[^{18}\text{F}]\text{MICA-302}$ (4.1 ± 1.1 %ID/mL) showing a slower blood clearance profile than $[^{18}\text{F}]\text{MICA-301}$ (1.0 ± 0.4 %ID/mL). The uptake in the liver and kidneys was determined to evaluate the elimination pathway of the radiotracers. The radiotracers were mainly eliminated via the kidneys. $[^{18}\text{F}]\text{MICA-301}$ displayed a higher initial kidney uptake (34.2 ± 8.1 %ID/mL at 6 min p.i.) compared to $[^{18}\text{F}]\text{MICA-302}$ (26.1 ± 10.4 %ID/mL at 2 min p.i.), followed by a washout of activity from the kidneys (Figure 6). As was also shown for the heart/blood pool activity, slower liver elimination was visible for $[^{18}\text{F}]\text{MICA-302}$ compared to $[^{18}\text{F}]\text{MICA-301}$. The lack of significant nonspecific accumulation in other organs, resulted in general low image background at 60 min p.i. Therefore, apoptosis imaging can be performed within 60 min p.i.

An *ex vivo* biodistribution was performed at 15, 30, 60 and 120 min p.i. to validate the μ PET imaging results. The fast blood clearance and renal excretion of $[^{18}\text{F}]\text{MICA-301}$ observed by μ PET were reflected in the biodistribution studies (Supplemental Figure 3). This renal excretion profile has also been reported for $[^{18}\text{F}]\text{CP18}$, a structurally related substrate-based probe for caspase-3 imaging.⁵⁰ $[^{18}\text{F}]\text{MICA-302}$ on the

other hand demonstrated a mixed renal-hepatobiliary clearance with increasing uptake of radioactivity in the small (3.2 ± 0.4 %ID/g at 60 min p.i.) and large intestine (2.7 ± 0.7 %ID/g at 120 min p.i.) (Supplemental Figure 3). Multiple cysteine proteases, including caspases, cathepsins and legumain, are known to digest proteins in the intestine as part of a coordinated physiological homeostatic process.⁶⁹⁻⁷¹ Therefore, the uptake of radioactivity in the intestines might also reflect binding of [^{18}F]MICA-302 to cysteine proteases present in the intestinal mucosa. In addition, the high uptake in the small intestine could also be due in part to the high physiological apoptosis of enterocytes, as described before for other caspase-3/7 PET imaging radiotracers.³¹ Both radiotracers demonstrated low accumulation of radioactivity in the bone, which indicates a lack of defluorination and provides additional evidence for high *in vivo* stability.

***In vivo* small-animal PET imaging in tumor-bearing mice**

The favorable pharmacokinetic properties and high metabolic stability, together with the ability of [^{18}F]MICA-302 to selectively target active caspase-3 *in vitro* led us to evaluate its potential for *in vivo* PET imaging of apoptosis in tumor-bearing mice. The pro-apoptotic effect of anti-DR5 monoclonal antibody conatumumab has previously been established in colorectal cancer xenografts, where it demonstrated an increased activation of caspase-3/7 in tumors.^{72, 73} The tumor targeting properties of ABP [^{18}F]MICA-302 were therefore evaluated in COLO205 human colorectal cancer xenografts before and 24 h after a single dose of conatumumab or IgG1 control, and compared to [^{18}F]MICA-301, which was used as control tracer. Tumor uptake of both radiotracers, represented as TACs, is shown in Figure 7. [^{18}F]MICA-301 and [^{18}F]MICA-302 demonstrated a peak tumor uptake of 2.8 ± 0.2 %ID/mL at 2.5 min p.i. and 3.2 ± 0.2 %ID/mL at 5.0 min p.i., respectively ($p=0.132$), in conatumumab-treated animals followed by a washout of radioactivity. However, the washout of [^{18}F]MICA-301 from the tumor was faster than for [^{18}F]MICA-302, which is consistent with diffusion of the substrate away from tumor apoptotic cells.³⁵ Accordingly, there

was no difference in image-derived tumor uptake values (%ID/ml) of [^{18}F]**MICA-301** between IgG1-control group and conatumumab-treated COLO205 xenografts ($p=0.065$) (Figure 7). Additionally, [^{18}F]**MICA-301** tumor uptake did not significantly change at 24 h after conatumumab treatment when compared to baseline ($p=0.764$).

In contrast, at 60 min p.i. there was a significant increased tumor uptake of [^{18}F]**MICA-302** in conatumumab-treated mice ($1.52\pm0.03\%$ ID/ml) compared to IgG1-control group ($1.08\pm0.30\%$ ID/ml, $p=0.004$) and to baseline ($1.22\pm0.31\%$ ID/ml, $p=0.028$), as seen by higher tumor TACs following treatment with conatumumab suggesting increased radiotracer retention in treated tumors (Figure 7). The tumor uptake in conatumumab-treated mice for [^{18}F]**MICA-302** was in the range of previous caspase-3 PET tracers.^{24, 74} For example, in a similar study in COLO205 tumor-bearing mice treated with a DR5-targeted antibody, in which a radiolabeled isatin sulfonamide analog ([^{18}F]WC-4-116) was used, PET imaging demonstrated a tumor uptake of about 1.2%ID/ml at 30 min post-tracer injection.⁷⁵ In another study, [^{18}F]ICMT-11 PET imaging performed in cyclophosphamide treated 38C13 tumor xenografts showed a tumor activity of approximately 0.5%ID/ml at 60 min p.i.⁷⁴ When different caspase-3 imaging agents were evaluated side-by-side, radiotracer uptake in lymphoma tumors of mice treated with etoposide was approximately 1.5 and 2.3%ID/ml for [^{18}F]ML-10 and [^{18}F]C-SNAT, respectively at 90 min p.i.²⁴ *Ex vivo* gamma counting of tumor radioactivity after imaging revealed a significant 1.86-fold increase in [^{18}F]**MICA-302** tumor uptake in conatumumab versus IgG1-treated tumor-bearing mice ($p=0.025$). For [^{18}F]**MICA-301** there was no difference in tumor uptake 60 minutes after radiotracer injection between treated and control mice (Figure 7 and Supplementary Table 1). [^{18}F]**MICA-302** tumor uptake compares favorably to those for other apoptosis imaging radiotracers such as [$^{99\text{m}}\text{Tc}$]HYNIC-annexin V (1.4 to 1.6-fold increase), [^{111}In]C2Am (1.6-fold increase), [^{18}F]ML-10 (1.3-fold decrease), [^{18}F]C-SNAT (1.9 to 2.1-fold increase), [^{18}F]ICMT-11 (2.0-fold increase), [^{18}F]WC-4-116 (1.3-fold increase) or [^{18}F]CP18 (1.6-fold

increase).^{16, 24, 30, 74-77} Although these results are promising, absolute tumor uptake of [¹⁸F]**MICA-302** is still low. This might be related to a less efficient tracer permeation into the cell. We are currently developing second-generation caspase-3 radiotracers with improved cell permeability, and higher intracellular accumulation for enhanced tumor signal after therapy.

The *ex vivo* biodistribution profiles of both radiotracers measured at 60 min p.i. in IgG1 and conatumumab-treated tumor-bearing mice are summarized in Supplementary Table 1. The increase in [¹⁸F]**MICA-302** uptake in conatumumab-treated mice was restricted to the tumors. Radiotracer clearance was fast yielding low background from non-target organs. Of note was the lower background uptake of [¹⁸F]**MICA-302** in the abdominal region (Supplementary Table 1). This is in clear contrast with the suboptimal pharmacokinetic profile of [¹⁸F]ICMT-11 and [¹⁸F]C-SNAT, displaying high abdominal background in the PET images, which may limit assessment of treatment response to tumors of the upper thoracic region.^{24, 28, 74}

Figure 7. $[^{18}\text{F}]\text{MICA-301}$ (a) and $[^{18}\text{F}]\text{MICA-302}$ (b) *in vivo* μ PET imaging analysis, and *ex vivo* tumor uptake at 60 min p.i. in tumor-bearing mice. COLO205 xenografts were treated with 1.5 mg/kg conatumumab (n=6) or IgG1 (n=6) for 24 h and subsequently subjected to 60 min dynamic $[^{18}\text{F}]\text{MICA-301}$ or $[^{18}\text{F}]\text{MICA-302}$ μ PET imaging. Corresponding coronal μ PET/CT images (30-60 minutes summed activity) of IgG1 and conatumumab-treated COLO205 tumor-bearing mice are shown in the panels. Tumor time-activity curves and *ex vivo* tumor uptake at 60 min p.i. are presented as mean $\pm$ SD. **p=0.004, significantly different from IgG1-treated; *p<0.05, significantly different from baseline and from IgG1-treated.

We further assessed the relationship between $[^{18}\text{F}]\text{MICA-302}$ uptake and apoptosis levels in the tumors. Histological examination of tumor sections showed that conatumumab treatment increased apoptosis

defined by the presence cell shrinkage and chromatin condensation, as seen by hematoxylin and eosin staining, increased caspase-3 activation and increased DNA fragmentation (Supplemental Figure 4). These results establish [^{18}F]MICA-302 as a good marker for the sensitive detection of caspase-3 activity and apoptosis *in vivo*.

A potential limitation of caspase-3 targeting for treatment response assesement is the small increase in the levels of tumor apoptosis to be expected after anticancer therapy in patients. The first clinical study with [^{18}F]ICMT-11, showed unchanged tumor radiotracer uptake in breast and lung cancer patients following chemotherapy.²⁹ The authors highlighted the lack of significant apoptosis induction after therapy. Overall, tumor response to therapy is a complex process. Apoptosis may not be the only mechanism of cell death involved in response to therapy. The type of cell death resulting from anticancer therapy can be determined by the mechanism of action and dosing regimen of the therapy, and the genetic background in the tumors being treated.⁷⁸ It has been increasingly noted that conventional chemotherapeutic agents not only elicit apoptosis but other forms of nonapoptotic death such as necrosis, autophagy, mitotic catastrophe, and senescence.⁷⁹ Therefore, targeting other biomarkers that are present in both apoptosis and necrosis, such as exposure of phospholipids in the cell membrane, may have an advantage over caspase-3 targeting for the detection of response to anticancer therapy. The strengths and weaknesses of both approaches are currently under investigation at ours and a number of other institutions.

Conclusion

We have designed, synthesized, and radiolabeled activity-based probe to allow selective PET imaging of caspase-3 *in vivo*. Based on a small library of DEVD peptides modified with different warhead functions a lead compound containing an AOMK warhead was identified with low nanomolar affinity for caspase-3, and good selectivity over initiator and executioner caspases. Click radiolabeling of the lead compound

with 5- ^{18}F fluoro-1-pentyne proceeded in good radiochemical yields, and the resulting triazole demonstrated selective *in vitro* labeling of caspase-3 when using recombinant caspases, as well as in more complex cellular systems. *In vivo* evaluation of ^{18}F **MICA-302** in normal mice showed high metabolic stability, with no indication of defluorination *in vivo*, and fast blood clearance with predominant renal excretion of activity. A preliminary μPET imaging study in colorectal cancer xenografts demonstrated increased retention of ^{18}F **MICA-302** in tumors early after treatment with targeted therapy. Together, the results identify ^{18}F **MICA-302** as a promising radiotracer for detection of drug-induced apoptosis. However, this study also suggests that these radiolabeled ABPs warrant further development as potential scaffolds for design of next-generation translational molecular imaging probes.

Experimental

General procedures

All reagents were purchased from commercial suppliers and were used without further purification. All solvents used were of laboratory reagent grade, unless otherwise noted. For reactions under anhydrous conditions, anhydrous solvents and nitrogen atmosphere were used. Thin-layer chromatography (TLC) analyses were carried out on Merck Silica Gel 60 F₂₅₄ plates. A Waters Acquity UPLC system coupled to a Waters TUV detector and TQD ESI mass spectrometer, and a Waters Acquity UPLC BEH C₁₈ 2.1 mm x 50 mm (1.7 µm) column was used for mass analysis of the intermediate compounds. The mobile phase was composed of water and acetonitrile (both containing 0.1% formic acid) and the method consisted in 5% organic for 0.20 min followed by a linear gradient of 5 to 95% organic in 2 min, at a flow rate of 0.7 mL/min. Flash chromatography was performed using a Biotage IsoleraOne system, equipped with an internal variable dual-wavelength diode array detector (200-400 nm), using Biotage SNAP KP-Sil or KP-C18-HS cartridges for normal- or reverse-phase purifications, respectively. Reverse-phase high performance liquid chromatography (HPLC) was conducted on a Waters 2545 HPLC equipped with a Waters 2998 diode array detector, a Micromass Quattro microTM model and a Waters 2767 fraction collector. For the purification of the final compounds a Waters Xbridge BEH C₁₈ OBD 19 mm x 100 mm (5 µm) column, at a flow rate of 19 mL/min was used. The chemical purities of each inhibitor and precursor were assessed by analytical reverse-phase HPLC using a Waters Xbridge BEH C₁₈ 4.6 mm x 100 mm (5 µm) column and a 20 min gradient elution consisting of 5 to 95% acetonitrile in water with 0.1% formic acid, at a flow rate of 1 mL/min. The purity of the compounds tested *in vitro* or *in vivo* was 95% or higher, unless otherwise stated.

All the compounds were obtained as oils or amorphous powders, therefore the melting points were not determined. To characterize the synthesized compounds, proton (^1H), carbon (^{13}C) NMR and high-resolution mass spectrometry (HRMS) were used. ^1H and ^{13}C NMR spectra were recorded using a Bruker Avance DRX 400 spectrometer (400 MHz for ^1H ; 100 MHz for ^{13}C) and data were analyzed with MestReNova analytical chemistry software. To assign the signals of some complex structures, DEPT and two-dimensional NMR techniques (COSY, HMQC and HMBC) were applied. All chemical shifts are reported in parts per million (ppm), and multiplicities are indicated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), br (broad). Exact mass analyses of the final compounds were performed by the Biomolecular and Analytical Mass Spectrometry Facility (University of Antwerp) using a Waters Q-TOF II mass spectrometer.

Synthesis of the peptide backbone

General procedure for peptide coupling in solution

A 0.15 M solution of the corresponding *N*- α -Fmoc amino acid (1.1 equiv) was stirred in anhydrous DCM/DMF (10:1 (v/v)). To this solution HOBt (1.1 equiv) and EDC (1.1 equiv) were subsequently added. The mixture was cooled to 0°C and the respective amine (1.0 equiv) was added to the mixture. Then DIPEA (2.1 equiv) was added dropwise and the mixture was stirred for 16 h at room temperature. The DCM was removed *in vacuo*, and the residue was stirred with a 1:1 mixture of distilled water and a saturated NaCl solution (brine). The mixture was extracted with EtOAc and the combined organic extracts were washed with a saturated NaHCO_3 solution, followed by washing with a 0.5 M citric acid solution (3x each) and finally with brine. The organic layer was dried over anhydrous Na_2SO_4 , filtered and evaporated. Finally, the residue was purified by IsoleraOne to obtain the desired peptide.

(5*S*,8*S*,11*S*)-Benzyl 5-(2-(*tert*-butoxy)-2-oxoethyl)-8-(3-(*tert*-butoxy)-3-oxopropyl)-1-(9H-fluoren-9-yl)-11-isopropyl-3,6,9-trioxo-2-oxa-4,7,10-triazadodecan-12-oate (**2**).

Prepared using the procedure previously described by Lee.⁵³ The product was purified by IsoleraOne (60% EtOAc in hexanes) to obtain a pale yellow solid (7.25 g, 85 %), as previously described.⁵³

(*S*)-*tert*-Butyl 4-(((*S*)-2-amino-4-(*tert*-butoxy)-4-oxobutanamido)-5-(((*S*)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl)amino)-5-oxopentanoate (**3**).

N-Fmoc group removal was performed as previously described.⁵¹ Briefly, to a 0.17 M solution of *N*- α -Fmoc peptide **2** (3.36 g, 4.28 mmol) in DCM was added tris(2-aminoethyl)amine (20.0 equiv). The mixture was stirred for 30 min at room temperature, after which a white precipitate was formed. The reaction was monitored by TLC (ninhydrin test for primary amine) to verify the conversion. The reaction mixture was diluted in EtOAc and washed with brine, followed by NaH₂PO₄ phosphate buffer (pH 5.5) wash and brine again. The clear organic layer containing the Fmoc-deprotected peptide was dried over anhydrous Na₂SO₄, the volatiles were evaporated *in vacuo* to obtain the product as a yellow oil (1.69 g, 70%), as previously described.⁵³ The product was used in the following step without further purification.

(*S*)-*tert*-Butyl 4-(((*S*)-2-(((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamido)-4-(*tert*-butoxy)-4-oxobutanamido)-5-(((*S*)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl)amino)-5-oxopentanoate (**4**).

Prepared using the general peptide coupling procedure with 1-(Fmoc-aminomethyl)- β -D-galacturonic acid (1.20 g, 2.80 mmol) and **3** (1.43 g, 2.54 mmol) as starting materials. The product was purified by IsoleraOne

(100% EtOAc in hexanes) to obtain an off-white solid (1.65 g, 67%). ^1H NMR (400 MHz, $\text{MeOD-}d_4$) δ 7.81 (d, J = 7.5 Hz, 2H), 7.68 (d, J = 7.5 Hz, 2H), 7.45 – 7.28 (m, 9H), 5.15 (q, J = 12.2 Hz, 2H), 4.67 (t, J = 6.5 Hz, 1H), 4.50 – 4.37 (m, 3H), 4.34 (d, J = 6.0 Hz, 1H), 4.27 – 4.18 (m, 2H), 4.06 (d, J = 1.3 Hz, 1H), 3.58 – 3.52 (m, 2H), 3.33 (br, 3H), 2.95 – 2.83 (m, 1H), 2.74 (dd, J = 16.6, 6.1 Hz, 1H), 2.41 – 2.25 (m, 2H), 2.24 – 2.12 (m, 1H), 2.12 – 2.01 (m, 1H), 1.97 – 1.78 (m, 1H), 1.43 (s, 18H), 0.92 (d, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $\text{MeOD-}d_4$) δ 174.2, 173.6, 172.6, 172.4, 171.8, 171.5, 159.2, 145.3, 142.6, 137.2, 129.6, 129.5, 129.4, 128.8, 128.2, 126.2, 121.0, 82.6, 81.8, 80.5, 79.6, 71.4, 69.2, 67.8, 59.5, 53.8, 51.2, 43.1, 37.9, 32.5, 31.6, 28.6, 28.4, 28.4, 19.5, 18.6. UPLC MS (ESI+): m/z = 976.1 $[\text{M}+\text{H}]^+$.

((S)-2-((S)-2-((2S,3R,4R,5R,6S)-6-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamido)-4-(*tert*-butoxy)-4-oxobutanamido)-5-(*tert*-butoxy)-5-oxopentanoyl)-L-valine (**5**).

Compound **4** (1.00 g, 1.03 mmol) was dissolved in MeOH (16 mL) and stirred under N_2 atmosphere. A catalytic amount of palladium(II)carbon was added to the mixture and the reaction mixture was flushed with N_2 again. Hydrogen (gas) was bubbled on the reaction mixture for 6 h at room temperature with stirring. The reaction mixture was filtered over a celite pad and the solvent was evaporated. The desired compound was obtained as a white solid (0.90 g, quant). UPLC MS (ESI+): m/z = 885.6 $[\text{M}+\text{H}]^+$.

Synthesis of the warheads

(*E*)-6-*tert*-Butyl 1-ethyl (*S,E*)-4-((*tert*-butoxycarbonyl)amino)hex-2-enedioate (**6**).

This compound was prepared from the corresponding *N*-Boc Asp-derived aldehyde (1.30 g, 4.76 mmol) according to a known procedure.⁵⁴ The obtained residue was purified by IsoleraOne (20% EtOAc in

hexanes), and the desired compound was obtained as a white solid (1.40 g, 86%), as previously described.⁵⁴

(*S,E*)-1-(*tert*-Butoxy)-6-ethoxy-1,6-dioxohex-4-en-3-aminium 4-methylbenzenesulfonate (**7**).

Compound **6** (1.32 g, 3.84 mmol) was solvated in a mixture of DCM/Et₂O (1:1 (v/v), 10 mL). *p*-Toluene sulfonic acid monohydrate (0.69 g, 4.00 mmol) was added to the reaction mixture over a period of 6 min. After 48 h of stirring at room temperature, the solvent was evaporated, the residue was washed twice with cold ether and the supernatant was removed to yield a white solid (1.24 g, 77%), as previously described.⁶¹

tert-Butyl (*S,E*)-3-((*tert*-butoxycarbonyl)amino)-5-(methylsulfonyl)pent-4-enoate (**8**).

This compound was prepared from the corresponding *N*-Boc Asp-derived aldehyde (0.70 g, 2.56 mmol) according to a known procedure.⁵⁵ The obtained residue was purified by IsoleraOne (50% EtOAc in hexanes), and the desired compound was obtained as a white solid (0.54 g, 60%), as previously described.⁶¹

(*S,E*)-5-(*tert*-Butoxy)-1-(methylsulfonyl)-5-oxopent-1-en-3-aminium 4-methylbenzenesulfonate (**9**).

Compound **8** (0.52 g, 1.49 mmol) was solvated in a mixture of DCM/Et₂O (1:1 (v/v), 10 mL). *p*-Toluene sulfonic acid monohydrate (0.29 g, 1.52 mmol) was added to the reaction mixture over a period of 6 min. After 48 h of stirring at room temperature, the solvent was evaporated, the residue was washed twice

with cold ether and the supernatant was removed to yield a white solid (0.56 g, 89%), as previously described.⁶¹

tert-Butyl (S)-3-(((benzyloxy)carbonyl)amino)-4,4-dimethoxybutanoate (**10**).

This compound was prepared from the corresponding *N*-Cbz Asp-derived aldehyde (1.55 g, 5.04 mmol) by the procedure previously described by Kappel and Barany.⁵⁶ After purification by IsoleraOne (40% EtOAc in hexanes), the desired compound was obtained as a yellow oil (1.25 g, 70%), as previously described.⁵⁶

tert-Butyl (S)-3-amino-4,4-dimethoxybutanoate (**11**).

This compound was prepared from **10** (0.30 g, 0.85 mmol) by the procedure previously described by Kappel and Barany.⁵⁶ The title compound was obtained as a yellow oil (0.19 g, quant), as previously described.⁵⁶

(S)-5-(*tert*-Butoxy)-3-((*tert*-butoxycarbonyl)amino)-2,5-dioxopentyl 2,6-dimethylbenzoate (**12**).

This compound was prepared from the previously described *N*-Boc Asp-derived CMK (0.92 g, 2.87 mmol) by a previously described procedure.^{57, 58} Purification of the crude material by IsoleraOne (50% CH₃CN in water) yielded the desired compound (1.01 g, 81%). ¹H NMR (400 MHz, CDCl₃) δ 7.22 – 7.15 (m, 1H), 7.07 – 6.99 (m, 2H), 5.64 (d, *J* = 9.0 Hz, 1H), 5.24 (d, *J* = 17.0 Hz, 1H), 5.11 (d, *J* = 17.0 Hz, 1H), 4.64 – 4.56 (m, 1H), 2.92 (dd, *J* = 17.0, 5.0 Hz, 1H), 2.72 (dd, *J* = 17.1, 5.1 Hz, 1H), 2.38 (s, 6H), 1.45 (d, *J* = 16.4 Hz, 18H). ¹³C NMR (100 MHz, CDCl₃) δ 201.7, 170.6, 169.1, 155.5, 135.8, 132.8, 129.8, 127.8, 82.2, 80.8, 67.0, 54.3, 36.8, 28.4, 28.1, 20.0. UPLC MS (ESI+): *m/z* = 435.6 [M+H]⁺.

(S)-1-(*tert*-Butoxy)-5-((2,6-dimethylbenzoyl)oxy)-1,4-dioxopentan-3-aminium 4-methylbenzenesulfonate (**13**).

Compound **12** (0.61 g, 1.40 mmol) was solvated in a mixture of DCM/Et₂O (1:1 (v/v), 12 ml). *p*-Toluene sulfonic acid monohydrate (0.28 g, 1.46 mmol) was added to the reaction mixture over a period of 6 min. After 48 h of stirring at room temperature, the solvent was evaporated, the residue was washed twice with cold ether and the supernatant was removed to yield a white solid (0.58 g, 81%). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.73 (d, *J* = 8.2 Hz, 2H), 7.31 – 7.20 (m, 3H), 7.10 (d, *J* = 7.9 Hz, 2H), 5.28 (d, *J* = 17.1 Hz, 1H), 5.13 (d, *J* = 17.1 Hz, 1H), 4.60 (dd, *J* = 7.1, 4.4 Hz, 1H), 3.19 (dd, *J* = 18.3, 4.4 Hz, 1H), 3.04 (dd, *J* = 18.3, 7.1 Hz, 1H), 2.38 (s, 9H), 1.52 (s, 9H). ¹³C NMR (100 MHz, MeOD-*d*₄) δ 199.5, 170.4, 170.1, 143.5, 141.7, 136.7, 133.6, 131.0, 129.8, 128.8, 127.0, 84.3, 67.0, 54.6, 35.6, 28.2, 21.3, 20.0. UPLC MS (ESI⁺): *m/z* = 336.5 [M+H]⁺.

tert-Butyl (S)-3-((*tert*-butoxycarbonyl)amino)-5-fluoro-4-oxopentanoate (**14**).

This compound was prepared from the commercially available Boc-L-aspartic acid 4-*tert*-butylester (1.10 g, 3.80 mmol) by the procedure previously described by Witte et al.⁵⁹ The obtained residue was purified by IsoleraOne (5 % EtOAc in toluene) and gave title compound (0.85 g, 62%). ¹H NMR (400 MHz, CDCl₃) δ 5.59 (d, *J* = 8.8 Hz, 1H), 5.16 (dd, *J* = 47.1, 16.5 Hz, 1H), 5.02 (dd, *J* = 47.1, 16.5 Hz, 1H), 4.55 – 4.42 (m, 1H), 2.83 (dd, *J* = 16.9, 5.0 Hz, 1H), 2.68 (dd, *J* = 16.9, 5.2 Hz, 1H), 1.36 (d, *J* = 10.3 Hz, 18H). ¹³C NMR (100 MHz, CDCl₃) δ 203.2 (d, *J* = 16.2 Hz), 170.3, 155.3, 84.0 (d, *J* = 183.0 Hz), 81.9, 80.4, 53.5, 36.6, 28.2, 27.9. UPLC MS (ESI⁺): *m/z* = 328.1 [M+Na]⁺.

(*S*)-1-(*tert*-Butoxy)-5-fluoro-1,4-dioxopentan-3-aminium 4-methylbenzenesulfonate (**15**).

Compound **14** (0.25 g, 0.82 mmol) was solvated in a mixture of CH₂Cl₂/Et₂O (1:1 (v/v), 12 ml). *p*-Toluene sulfonic acid monohydrate (0.16 g, 0.85 mmol) was added to the reaction mixture over a period of 6 min. After 48 h of stirring at room temperature, the solvent was evaporated, the residue was washed twice with cold ether and the supernatant was removed to yield a white solid (0.24 g, 78 %). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.72 (d, *J* = 8.1 Hz, 2H), 7.30 – 7.18 (m, 3H), 7.08 (d, *J* = 8.0 Hz, 2H), 5.07 (dd, *J* = 46.9, 16.0 Hz, 1H), 4.98 (dd, *J* = 46.9, 16.0 Hz, 1H), 4.50 (dd, *J* = 7.0, 4.4 Hz 1H), 2.80 (dd, *J* = 17.9, 5.1 Hz, 1H), 2.60 (dd, *J* = 17.9, 6.2 Hz, 1H), 2.39 (s, 3H), 1.45 (s, 9H).

tert-Butyl (*S*)-4-amino-3-(((benzyloxy)carbonyl)amino)-4-oxobutanoate (**16**).

This compound was prepared from the commercially available Cbz-L-aspartic acid 4-*tert*-butylester (2.00 g, 6.19 mmol) by a previously described procedure.⁶⁰ The desired compound was obtained as a white solid (1.45 g, 73%), as previously described.⁶⁰

tert-Butyl (*S*)-3,4-diamino-4-oxobutanoate (**17**).

Compound **16** (0.10 g, 0.31 mmol) was dissolved in MeOH (4 mL) and stirred under N₂ atmosphere. A catalytic amount of palladium(II)carbon was added to the previous solution and flushed with N₂ again. Hydrogen (gas) was bubbled on the reaction mixture for one hour with stirring at room temperature. The reaction mixture was filtered over a pad of celite and the solvent was evaporated to yield a colorless oil (0.05 g, 89%), as previously described.⁶⁰

Synthesis of the ¹⁹F-inhibitors

General procedure for copper(I)-catalyzed alkyne–azide 1,3-dipolar cycloaddition (CuAAC)

Aqueous sodium ascorbate (0.4 equiv) was added to a 0.1 M aqueous solution of copper(II) sulfate (0.2 equiv). Then 2 volumes of a 0.1 M solution of Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (0.4 equiv) in DMSO/*t*-BuOH (3:1 (v/v)) were added to the previous solution. Azides **19a-f** (1.0 equiv) and 5-fluoropent-1-yne (10.0 equiv, 0.5 M in THF) were dissolved in *t*-BuOH/water (2:1 (v/v)) and added to the mixture. The reaction mixture was stirred at room temperature overnight. After that the volatiles were evaporated and the reaction mixture was diluted with water, the pH adjusted to pH 5, and Chelex 100 (sodium form, 50-100 mesh, 5 g/100 mL) was added, and gently stirred for 1 h and filtered. The filtrate was lyophilized yielding the desired triazoles as amorphous powders.

General procedure for *tert*-butyl ester deprotection of the final peptides

To the *Ot*Bu-protected ¹⁹F-peptides or azide precursors for radiolabeling **19d, f** on ice (0°C) was added TFA/TIS/water (95:2.5:2.5 (v/v), 0.015 M). The reaction was stirred at room temperature for 30 min. The volatiles were evaporated. The residue was co-evaporated with toluene (2x 0.5 mL) yielding the final inhibitors **1a-f** or precursors **20a** and **20b**, respectively.

General procedure for the synthesis of the compounds **19a-c**

Prepared using the general peptide coupling procedure with *N*-terminal Fmoc protected peptide **5** (1.1 equiv) and the corresponding warheads **7, 9** or **11** (1.0 equiv) as starting materials. The obtained products **18a-c** were used without further purification in the next reaction. *N*-terminal Fmoc group was removed as previously described by treatment of compounds **18a-c** (1.0 equiv, 0.05 M) in THF with diethylamine (DEA, 5.0 equiv) for 4 h, followed by another 4 h treatment with fresh solution.⁴⁰ The deprotection of the primary amine was monitored by TLC (ninhydrin test) to verify the conversion. The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc. The resulting solution was

concentrated at reduced pressure to remove the remaining DEA and the resulting residue was used without further purification.

6-(*tert*-Butyl) 1-ethyl (S,E)-4-((S)-2-((S)-2-((S)-2-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-azidoacetamido)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamido)-4-(*tert*-butoxy)-4-oxobutanamido)-5-(*tert*-butoxy)-5-oxopentanamido)-3-methylbutanamido)hex-2-enedioate (**19a**).

Prepared using the general peptide coupling procedure using 2-azidoacetic acid (0.05 g, 0.49 mmol) as starting material. After work-up the obtained residue was purified by IsoleraOne (10% MeOH in EtOAc) yielding the desired compound (0.19 g, 44%). ¹H NMR (400 MHz, CDCl₃) δ 7.81 (d, *J* = 7.7 Hz, 1H), 7.72 (d, *J* = 7.4 Hz, 1H), 7.67 – 7.53 (m, 1H), 7.49 – 7.32 (m, 2H), 6.85 (dd, *J* = 15.7, 5.4 Hz, 1H), 5.92 (dd, *J* = 15.8, 1.6 Hz, 1H), 5.02 – 4.85 (m, 1H), 4.68 – 4.51 (m, 1H), 4.44 – 4.32 (m, 1H), 4.29 (s, 1H), 4.25 – 4.19 (m, 1H), 4.14 (q, *J* = 7.2 Hz, 2H), 4.06 – 3.91 (m, 3H), 3.79 – 3.50 (m, 4H), 3.41 – 3.25 (m, 1H), 2.99 – 2.71 (m, 2H), 2.66 – 2.47 (m, 2H), 2.40 – 2.25 (m, 2H), 2.24 – 2.12 (m, 1H), 2.11 – 2.01 (m, 1H), 2.00 – 1.87 (m, 1H), 1.39 (d, *J* = 3.6 Hz, 27H), 1.23 (t, *J* = 7.0 Hz, 3H), 0.89 (t, *J* = 7.6 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃) δ 173.4, 171.6, 171.1, 170.7, 170.6, 169.9, 169.4, 169.1, 166.2, 145.9, 122.1, 81.8, 81.2, 78.5, 78.2, 77.4, 76.8, 69.7, 68.1, 60.7, 59.0, 53.7, 52.2, 50.4, 47.2, 40.7, 39.5, 36.6, 31.9, 30.4, 28.1, 28.1, 28.1, 27.1, 19.5, 18.1, 14.3. UPLC MS (ESI⁻): *m/z* = 1015.7 [M+FA-H]⁻.

tert-Butyl (3*S*,6*S*,9*S*,12*S*)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-azidoacetamido)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-3-(2-(*tert*-butoxy)-2-oxoethyl)-6-(3-(*tert*-butoxy)-3-oxopropyl)-9-isopropyl-12-((*E*)-2-(methylsulfonyl)vinyl)-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oate (**19b**).

Prepared using the general peptide coupling procedure using 2-azidoacetic acid (0.02 g, 0.19 mmol) as starting material. After work-up the obtained residue was purified by IsoleraOne (10% MeOH in EtOAc) yielding the desired compound (0.09 g, 52%). ^1H NMR (400 MHz, $\text{MeOD-}d_4$) δ 6.89 – 6.79 (m, 1H), 6.70 (dd, J = 15.2, 1.5 Hz, 1H), 4.64 (q, J = 7.6, 7.0 Hz, 1H), 4.44 (d, J = 6.8 Hz, 2H), 4.38 (dd, J = 9.2, 5.0 Hz, 1H), 4.27 – 4.21 (m, 1H), 4.15 – 4.09 (m, 1H), 4.08 (d, J = 1.4 Hz, 1H), 3.67 (dd, J = 14.3, 2.3 Hz, 1H), 3.59 – 3.52 (m, 2H), 3.40 (dd, J = 14.2, 6.7 Hz, 1H), 2.98 (s, 3H), 2.97 – 2.85 (m, 1H), 2.74 (dd, J = 16.5, 6.1 Hz, 1H), 2.66 (dd, J = 7.0, 1.8 Hz, 2H), 2.43 – 2.23 (m, 2H), 2.21 – 2.04 (m, 2H), 1.99 – 1.82 (m, 1H), 1.44 (d, J = 3.7 Hz, 27H), 0.98 (d, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $\text{MeOD-}d_4$) δ 172.8, 172.3, 171.6, 171.5, 171.1, 170.4, 169.3, 144.8, 130.7, 81.3, 80.5, 79.1, 78.2, 74.1, 70.00, 67.7, 66.4, 59.4, 53.0, 50.1, 41.7, 41.4, 38.5, 36.4, 31.2, 30.2, 27.0, 27.0, 26.6, 18.5, 17.5. UPLC MS (ESI+): m/z = 999.6 $[\text{M}+\text{Na}]^+$.

tert-Butyl(4*S*,7*S*,10*S*,13*S*)-13-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-azidoacetamido)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamido)-4-(2-(*tert*-butoxy)-2-oxoethyl)-10-(3-(*tert*-butoxy)-3-oxopropyl)-7-isopropyl-3-methoxy-6,9,12-trioxo-2-oxa-5,8,11-triazapentadecan-15-oate (**19c**).

Prepared using the general peptide coupling procedure using 2-azidoacetic acid (0.04 g, 0.40 mmol) as starting materials. After work-up the obtained residue was purified by IsoleraOne (10% MeOH in EtOAc) yielding the desired compound (0.25 g, 72%). ^1H NMR (400 MHz, CDCl_3) δ 7.81 (d, J = 7.6 Hz, 1H), 7.59 (d, J = 7.2 Hz, 1H), 7.42 (d, J = 6.2 Hz, 1H), 7.19 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.9 Hz, 1H), 4.56 (q, J = 6.4 Hz, 1H), 4.49 – 4.29 (m, 4H), 4.18 (dd, J = 8.5, 6.5 Hz, 1H), 4.06 – 3.98 (m, 3H), 3.84 – 3.71 (m, 1H), 3.69 – 3.53 (m, 3H), 3.37 (d, J = 1.1 Hz, 6H), 3.33 (d, J = 9.2 Hz, 1H), 2.99 – 2.81 (m, 2H), 2.57 – 2.39 (m, 2H), 2.34 (t, J = 7.2 Hz, 2H), 2.22 – 2.03 (m, 2H), 2.02 – 1.87 (m, 1H), 1.42 (d, J = 6.3 Hz, 27H), 0.92 (dd, J = 6.8, 4.8 Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 173.5, 171.3, 171.1, 170.7, 170.6, 170.4, 170.4, 169.3, 104.7, 81.3, 81.2,

78.6, 78.5, 78.4, 73.9, 69.7, 68.00, 59.1, 56.00, 55.2, 53.6, 52.3, 50.5, 47.9, 40.6, 35.4, 31.9, 30.7, 28.2, 28.1, 28.1, 28.1, 27.3, 19.2, 18.2. UPLC MS (ESI+): m/z = 969.7 [M+Na]⁺.

(7*S*,10*S*,13*S*,16*S*,*E*)-13-(2-Carboxyethyl)-7-(carboxymethyl)-16-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-(4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl)acetamido)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-carboxamido)-10-isopropyl-4,9,12,15-tetraoxo-3-oxa-8,11,14-triazaoctadec-5-en-18-oic acid (**1a**).

First, the triazole was prepared using the general procedure for CuAAC with the azide **19a** (0.19 g, 0.20 mmol) and 5-fluoropent-1-yne (4.0 mL, 0.5 M) as starting materials. After lyophilization the obtained product was used without further purification. After side-chain deprotection using the general procedure for *tert*-butyl ester deprotection the residue was purified by preparative reverse-phase HPLC (5 – 40% acetonitrile in water with 0.1% formic acid in 15 min) yielding the desired inhibitor (0.06 g, 43%). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.85 (s, 1H), 6.90 (dd, J = 15.7, 5.3 Hz, 1H), 5.95 (dd, J = 15.7, 1.7 Hz, 1H), 5.20 (s, 2H), 4.75 (t, J = 6.3 Hz, 1H), 4.54 (t, J = 5.9 Hz, 1H), 4.45 – 4.38 (m, 2H), 4.22 (dd, J = 2.9, 1.4 Hz, 1H), 4.19 – 4.12 (m, 3H), 4.10 (d, J = 1.4 Hz, 1H), 3.76 (dd, J = 14.1, 2.8 Hz, 1H), 3.58 – 3.46 (m, 3H), 3.41 – 3.32 (m, 1H), 3.02 – 2.89 (m, 2H), 2.88 – 2.80 (m, 2H), 2.67 (d, J = 7.0 Hz, 2H), 2.53 – 2.32 (m, 2H), 2.25 – 1.90 (m, 5H), 1.26 (t, J = 7.1 Hz, 3H), 0.95 (d, J = 6.8 Hz, 6H). ¹³C NMR (100 MHz, MeOD-*d*₄) δ 176.9, 174.6, 173.7, 173.6, 173.1, 172.9, 171.4, 168.5, 167.7, 148.1, 147.6, 125.2, 122.6, 83.90 (d, J = 164.1 Hz), 79.7, 79.6, 75.4, 71.3, 69.4, 61.7, 60.7, 54.6, 53.09, 51.1, 48.5, 41.8, 39.0, 36.5, 31.4 (d, J = 17.9 Hz), 31.2, 31.1, 27.9, 22.1 (d, J = 5.8 Hz), 19.8, 18.7, 14.5. HRMS (ESI+) = 889.3599 [M+H]⁺. Calcd for C₃₆H₅₃FN₈O₁₇ 889.3591. HPLC t_R = 12.3 min.

(3*S*,6*S*,9*S*,12*S*)-6-(2-Carboxyethyl)-3-(carboxymethyl)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-(4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl)acetamido)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-9-isopropyl-12-((*E*)-2-(methylsulfonyl)vinyl)-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oic acid (**1b**).

First, the triazole was prepared using the general procedure for CuAAC with the azide **19b** (0.09 g, 0.10 mmol) and 5-fluoropent-1-yne (1.9 mL, 0.5 M) as starting materials. After lyophilization the obtained product was used without further purification. After side-chain deprotection using the general procedure for *tert*-butyl ester deprotection the residue was purified by preparative reverse-phase HPLC (5 – 20% acetonitrile in water with 0.1% formic acid in 10 min) yielding the desired inhibitor (0.06 g, 43%). ¹H NMR (400 MHz, D₂O) δ 7.83 (s, 1H), 6.91 (dd, *J* = 15.2, 4.8 Hz, 1H), 6.66 (dd, *J* = 15.3, 1.7 Hz, 1H), 5.24 (s, 2H), 5.05 – 4.92 (m, 1H), 4.78 – 4.72 (m, 1H), 4.57 (t, *J* = 5.9 Hz, 1H), 4.45 (t, *J* = 5.8 Hz, 1H), 4.39 (dd, *J* = 9.1, 5.3 Hz, 1H), 4.27 – 4.19 (m, 2H), 4.09 – 3.93 (m, 1H), 3.81 – 3.63 (m, 2H), 3.59 – 3.40 (m, 3H), 3.09 (d, *J* = 4.3 Hz, 3H), 2.95 – 2.85 (m, 4H), 2.83 (t, *J* = 7.6 Hz, 2H), 2.50 – 2.38 (m, 2H), 2.18 – 1.89 (m, 5H), 0.94 – 0.87 (m, 6H). ¹³C NMR (100 MHz, D₂O) δ 176.7, 173.2, 173.1, 172.9, 170.7, 170.7, 168.3, 166.9, 146.0, 144.0, 129.2, 124.7, 84.1 (d, *J* = 158.6 Hz), 77.8, 77.7, 73.3, 69.4, 67.8, 60.2, 53.2, 52.0, 46.9, 41.7, 40.3, 37.4, 36.0, 30.0, 29.7, 29.2 (d, *J* = 18.9 Hz), 25.9, 20.4 (d, *J* = 5.9 Hz), 18.3, 17.7. HRMS (ESI+) = 895.3163 [M+H]⁺. Calcd for C₃₄H₅₁FN₈O₁₇S 895.3155. HPLC *t*_R = 10.8 min.

(3*S*,6*S*,9*S*,12*S*)-6-(2-Carboxyethyl)-3-(carboxymethyl)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-(4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl)acetamido)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-12-formyl-9-isopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oic acid (**1c**).

First, the triazole was prepared using the general procedure for CuAAC with the azide **19c** (0.25 g, 0.27 mmol) and 5-fluoropent-1-yne (5.3 mL, 0.5 M) as starting materials. After lyophilization the obtained

product was used without further purification. After side-chain and acetal deprotection using the general procedure for *tert*-butyl ester deprotection the residue was purified by preparative reverse-phase HPLC (5 – 20% acetonitrile in water with 0.1% formic acid in 15 min) yielding the desired inhibitor (0.07 g, 33%). ^1H NMR (400 MHz, $\text{MeOD-}d_4$) δ 7.86 (s, 1H), 5.22 (s, 2H), 4.79 – 4.71 (m, 1H), 4.61 – 4.50 (m, 2H), 4.42 (t, J = 5.8 Hz, 2H), 4.28 (dd, J = 9.0, 4.7 Hz, 1H), 4.25 – 4.20 (m, 1H), 4.14 (d, J = 7.2 Hz, 1H), 4.11 (s, 1H), 3.77 (d, J = 13.1 Hz, 1H), 3.65 – 3.46 (m, 3H), 3.42 – 3.33 (m, 1H), 2.91 (s, 2H), 2.84 (t, J = 7.5 Hz, 2H), 2.73 – 2.60 (m, 1H), 2.57 – 2.35 (m, 3H), 2.24 – 1.91 (m, 5H), 0.99 – 0.84 (m, 6H). ^{13}C NMR (100 MHz, $\text{MeOD-}d_4$) δ 173.5, 173.4, 173.0, 173.0, 171.4, 168.5, 168.5, 157.4, 125.3, 98.5, 83.9 (d, J = 164.0 Hz), 79.7, 79.6, 75.4, 71.3, 69.4, 60.6, 60.5, 54.5, 53.1, 52.0, 51.2, 41.8, 31.8, 31.2 (d, J = 19.9 Hz), 28.0, 22.1 (d, J = 5.3 Hz), 19.7, 18.8. HRMS (ESI+) = 819.3178 $[\text{M}+\text{H}]^+$. Calcd for $\text{C}_{32}\text{H}_{47}\text{FN}_8\text{O}_{16}$ 819.3172. HPLC t_{R} = 9.4 min.

(6*S*,9*S*,12*S*,15*S*)-6-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamido)-15-(2-(*tert*-butoxy)-2-oxoethyl)-9-(3-(*tert*-butoxy)-3-oxopropyl)-12-isopropyl-2,2-dimethyl-4,7,10,13,16-pentaoxo-3-oxa-8,11,14-triazaheptadecan-17-yl 2,6-dimethylbenzoate (**18d**).

Prepared using the general peptide coupling procedure with *N*-terminal Fmoc protected peptide **5** (0.52 g, 0.58 mmol) and the corresponding warhead **13** (0.27 g, 0.53 mmol) as starting materials. The obtained residue was purified by IsoleraOne (10% MeOH in EtOAc) yielding the desired compound (0.47 g, 73%). ^1H NMR (400 MHz, CDCl_3) δ 7.83 (d, J = 7.3 Hz, 2H), 7.77 – 7.68 (m, 2H), 7.62 – 7.51 (m, 2H), 7.49 (d, J = 8.2 Hz, 1H), 7.35 (q, J = 8.4, 7.8 Hz, 2H), 7.31 – 7.22 (m, 2H), 7.20 – 7.10 (m, 1H), 7.02 – 6.94 (m, 2H), 5.94 (s, 1H), 5.23 – 5.02 (m, 2H), 5.00 – 4.86 (m, 1H), 4.54 (t, J = 6.6 Hz, 1H), 4.44 – 4.23 (m, 5H), 4.21 – 4.15 (m, 1H), 4.02 (s, 1H), 3.70 – 3.56 (m, 4H), 3.51 – 3.38 (m, 1H), 3.25 (s, 1H), 2.96 – 2.71 (m, 4H), 2.41 – 2.28 (m,

3H), 2.16 – 2.04 (m, 1H), 2.03 (s, 7H), 1.43 – 1.34 (m, 27H), 0.93 (d, J = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 200.8, 173.8, 171.0, 170.8, 170.2, 170.1, 169.5, 169.2, 169.1, 158.1, 143.8, 141.3, 135.6, 132.7, 129.64, 127.7, 127.6, 127.1, 125.2, 120.0, 82.0, 82.0, 81.4, 73.7, 69.5, 67.5, 67.2, 67.0, 59.3, 54.3, 53.0, 50.6, 47.1, 41.7, 36.5, 36.0, 31.9, 29.7, 28.0, 28.0, 26.4, 21.1, 19.9. UPLC MS (ESI+): m/z = 1224.9 $[\text{M}+\text{Na}]^+$.

tert-Butyl(3*S*,6*S*,9*S*,12*S*)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-3-(2-(*tert*-butoxy)-2-oxoethyl)-6-(3-(*tert*-butoxy)-3-oxopropyl)-12-(2-fluoroacetyl)-9-isopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oate (**18e**).

Prepared using the general peptide coupling procedure with *N*-terminal Fmoc protected peptide **5** (0.62 g, 0.70 mmol) and the corresponding warhead **15** (0.24 g, 0.64 mmol) as starting materials. The obtained residue was purified by IsoleraOne (10% MeOH in EtOAc) yielding the desired compound (0.63 g, 92%). ^1H NMR (400 MHz, CDCl_3) δ 7.86 (d, J = 6.9 Hz, 1H), 7.76 – 7.65 (m, 2H), 7.61 – 7.53 (m, 2H), 7.40 – 7.31 (m, 2H), 7.29 – 7.21 (m, 2H), 6.02 (d, J = 16.1 Hz, 1H), 5.31 – 4.94 (m, 2H), 4.93 – 4.74 (m, 1H), 4.55 (dd, J = 12.3, 5.9 Hz, 1H), 4.45 – 4.27 (m, 4H), 4.25 – 4.14 (m, 2H), 4.04 (s, 1H), 3.74 – 3.50 (m, 4H), 3.37 – 3.24 (m, 1H), 2.93 – 2.72 (m, 4H), 2.43 – 2.30 (m, 2H), 2.29 – 2.08 (m, 1H), 2.07 – 1.91 (m, 2H), 1.39 (d, J = 10.7 Hz, 27H), 0.91 (d, J = 5.5 Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3) δ 202.8 (d, J = 17.7 Hz), 173.9, 173.7, 171.1, 171.0, 170.2, 170.0, 169.7, 158.0, 143.9, 141.3, 127.8, 127.2, 125.3, 120.0, 84.4 (d, J = 179.2 Hz), 81.6, 81.5, 78.9, 78.2, 73.9, 69.6, 67.7, 67.3, 59.3, 54.3, 51.4, 50.6, 47.2, 41.9, 36.5, 36.1, 32.0, 30.5, 28.1, 28.1, 26.7, 19.4, 18.3. UPLC MS (ESI+): m/z = 1095.1 $[\text{M}+\text{H}]^+$.

tert-Butyl (3*S*,6*S*,9*S*,12*S*)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-3-(2-(*tert*-butoxy)-2-oxoethyl)-6-(3-(*tert*-butoxy)-3-oxopropyl)-12-carbamoyl-9-isopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oate (**18f**).

Prepared using the general peptide coupling procedure with *N*-terminal Fmoc protected peptide **5** (0.31 g, 0.35 mmol) and the amide **17** (0.06 g, 0.32 mmol) as starting materials. The obtained residue was purified by IsoleraOne (10% MeOH in EtOAc) yielding the desired compound (0.20 g, 59%). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.82 (d, *J* = 7.5 Hz, 2H), 7.70 – 7.66 (m, 2H), 7.44 – 7.38 (m, 2H), 7.37 – 7.29 (m, 2H), 4.71 – 4.59 (m, 2H), 4.47 – 4.40 (m, 2H), 4.39 (dd, *J* = 9.1, 5.1 Hz, 1H), 4.28 – 4.18 (m, 2H), 4.17 – 4.04 (m, 2H), 3.67 (dd, *J* = 14.2, 2.4 Hz, 1H), 3.58 – 3.52 (m, 2H), 3.45 – 3.34 (m, 1H), 3.33 – 3.25 (m, 1H), 2.97 – 2.81 (m, 2H), 2.80 – 2.57 (m, 2H), 2.41 – 2.22 (m, 2H), 2.19 – 2.03 (m, 2H), 1.97 – 1.82 (m, 1H), 1.44 (d, *J* = 3.1 Hz, 27H), 0.97 (dd, *J* = 6.8, 2.9 Hz, 6H) ppm. ¹³C NMR (100 MHz, MeOD-*d*₄) δ 173.7, 172.8, 172.5, 171.9, 171.4, 170.4, 170.3, 170.0, 157.8, 143.9, 141.2, 127.4, 126.8, 124.8, 119.6, 81.2, 81.0, 80.5, 79.1, 78.2, 74.1, 70.0, 67.8, 66.4, 59.5, 52.9, 50.0, 49.7, 41.7, 36.5, 36.4, 31.2, 30.2, 27.0, 26.7, 18.3, 17.6. UPLC MS (ESI+): *m/z* = 1055.6 [M+H]⁺.

General procedure for the synthesis of the compounds **1d-f**

N-terminal Fmoc group was removed as previously described by treatment of compound **18d-f** (1.0 equiv, 0.05 M) in THF with diethylamine (DEA, 5.0 equiv) for 4 h followed by another 4 h treatment with fresh solution.⁴⁰ The deprotection of the primary amine was monitored by TLC (ninhydrin test) to verify the conversion. The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc. The resulting solution was concentrated at reduced pressure to remove the remaining DEA and the resulting residue was used without further purification. Then, using the general peptide

coupling procedure and using 2-azidoacetic acid (1.1 equiv) as starting material, the azides **19d-f** were obtained and were used without further purification in the CuAAC.

(5*S*,8*S*,11*S*,14*S*)-11-(2-Carboxyethyl)-5-(carboxymethyl)-1-(2,6-dimethylphenyl)-14-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-(4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl)acetamido)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-carboxamido)-8-isopropyl-1,4,7,10,13-pentaoxo-2-oxa-6,9,12-triazahexadecan-16-oic acid (**1d**).

First, the triazole was prepared using the general procedure for CuAAC with the azide **19d** (0.30 g, 0.28 mmol) and 5-fluoropent-1-yne (5.7 mL, 0.5 M) as starting materials. After lyophilization, the obtained product was used without further purification. After side-chain deprotection using the general procedure for *tert*-butyl ester deprotection the residue was purified by preparative reverse-phase HPLC (5 – 30% acetonitrile in water with 0.1% formic acid in 15 min) yielding the desired inhibitor (0.13 g, 57%). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.79 (s, 1H), 7.23 – 7.15 (m, 1H), 7.07 – 7.00 (m, 2H), 5.16 (s, 2H), 4.82 (t, *J* = 6.7 Hz, 1H), 4.77 (t, *J* = 6.7 Hz, 1H), 4.52 (t, *J* = 5.9 Hz, 1H), 4.43 (dd, *J* = 9.2, 4.8 Hz, 1H), 4.40 (t, *J* = 5.9 Hz, 1H), 4.22 (dd, *J* = 2.9, 1.4 Hz, 1H), 4.17 (d, *J* = 7.3 Hz, 1H), 4.08 (d, *J* = 1.4 Hz, 1H), 3.77 (dd, *J* = 14.0, 2.7 Hz, 1H), 3.59 – 3.41 (m, 3H), 3.39 – 3.31 (m, 1H), 3.05 – 2.82 (m, 4H), 2.86 – 2.77 (m, 2H), 2.51 – 2.36 (m, 2H), 2.33 (s, 6H), 2.24 – 1.91 (m, 5H), 0.97 (d, *J* = 6.6 Hz, 6H). ¹³C NMR (100 MHz, MeOD-*d*₄) δ 176.8, 174.8, 174.3, 173.8, 173.8, 173.0, 171.3, 170.5, 168.4, 148.0, 136.6, 134.2, 130.7, 128.6, 125.2, 83.9 (d, *J* = 164.0 Hz), 79.7, 79.6, 75.4, 71.3, 69.4, 67.9, 60.7, 54.6, 53.7, 53.0, 51.0, 41.7, 36.6, 35.4, 31.4, 31.2 (d, *J* = 19.6 Hz), 27.9, 22.1 (d, *J* = 5.8 Hz), 20.0, 19.8, 19.0. HRMS (ESI+) = 981.3854 [M+H]⁺. Calcd for C₄₂H₅₇FN₈O₁₈ 981.3853. HPLC *t*_R = 15.0 min.

(3*S*,6*S*,9*S*,12*S*)-6-(2-Carboxyethyl)-3-(carboxymethyl)-12-(2-fluoroacetyl)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-(4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl)acetamido)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-9-isopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oic acid (**1e**).

First, the triazole was prepared using the general procedure for CuAAC with the azide **19e** (0.19 g, 0.20 mmol) and 5-fluoropent-1-yne (4.1 mL, 0.5 M) as starting materials. After lyophilization, the obtained product was used without further purification. After side-chain deprotection using the general procedure for *tert*-butyl ester deprotection the residue was purified by preparative reverse-phase HPLC (5 – 30% acetonitrile in water with 0.1% formic acid in 15 min) yielding the desired inhibitor (0.07 g, 66%). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.87 (s, 1H), 5.23 (s, 2H), 4.84 – 4.73 (m, 2H), 4.56 (t, *J* = 5.9 Hz, 1H), 4.44 (t, *J* = 5.8 Hz, 2H), 4.27 – 4.22 (m, 1H), 4.20 – 4.07 (m, 2H), 3.84 – 3.74 (m, 1H), 3.62 – 3.47 (m, 3H), 3.43 – 3.35 (m, 1H), 3.04 – 2.87 (m, 3H), 2.86 (t, *J* = 7.6 Hz, 2H), 2.44 (q, *J* = 7.1 Hz, 2H), 2.26 – 1.92 (m, 5H), 1.02 – 0.93 (m, 6H). ¹³C NMR (100 MHz, MeOD-*d*₄) δ 176.8, 174.8, 173.8, 173.7, 173.7, 173.0, 171.4, 168.5, 148.1, 125.2, 83.9 (d, *J* = 164.0 Hz), 79.7, 79.6, 75.4, 71.3, 69.4, 60.8, 54.5, 53.1, 51.0, 51.0, 41.8, 36.5, 35.1, 31.5, 31.2 (d, *J* = 19.7 Hz), 28.0, 22.1 (d, *J* = 5.8 Hz), 19.7, 19.1. HRMS (ESI+) = 981.3854 [M+H]⁺. Calcd for C₃₃H₄₈F₂N₈O₁₆ 981.3853. HPLC *t*_R = 10.1 min (85%).

(3*S*,6*S*,9*S*,12*S*)-12-Carbamoyl-6-(2-carboxyethyl)-3-(carboxymethyl)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-(4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl)acetamido)methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-9-isopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oic acid (**1f**).

First, the triazole was prepared using the general procedure for CuAAC with the azide **19f** (0.13 g, 0.14 mmol) and 5-fluoropent-1-yne (2.9 mL, 0.5 M) as starting materials. After lyophilization, the obtained product was used without further purification. After side-chain deprotection using the general procedure

for *tert*-butyl ester deprotection the residue was purified by preparative reverse-phase HPLC (5 – 10% acetonitrile in water with 0.1% formic acid in 15 min) yielding the desired inhibitor (0.08 g, 70%). ^1H NMR (400 MHz, D_2O) δ 7.84 (s, 1H), 5.25 (s, 2H), 4.69 (dd, J = 8.0, 5.6 Hz, 1H), 4.58 (t, J = 5.8 Hz, 1H), 4.46 (t, J = 5.9 Hz, 1H), 4.42 (dd, J = 9.1, 5.5 Hz, 1H), 4.26 (dd, J = 3.3, 1.3 Hz, 1H), 4.22 (d, J = 1.4 Hz, 1H), 4.07 (d, J = 7.5 Hz, 1H), 3.79 – 3.66 (m, 2H), 3.60 – 3.43 (m, 3H), 2.94 (dd, J = 17.0, 5.9 Hz, 2H), 2.89 – 2.76 (m, 4H), 2.55 – 2.36 (m, 2H), 2.19 – 1.90 (m, 5H), 0.90 (dd, J = 6.8, 1.4 Hz, 6H). ^{13}C NMR (100 MHz, D_2O) δ 177.0, 174.5, 174.0, 173.9, 173.1, 173.1, 172.0, 170.7, 168.3, 147.4, 124.7, 84.1 (d, J = 158.9 Hz), 77.8, 77.7, 73.3, 67.8, 59.7, 53.1, 52.0, 49.8, 49.6, 40.4, 35.5, 35.4, 29.9, 29.8, 29.2 (d, J = 19.4 Hz), 25.8, 20.5 (d, J = 5.8 Hz), 18.2, 17.7. HRMS (ESI+) = 834.3276 $[\text{M}+\text{H}]^+$. Calcd for $\text{C}_{32}\text{H}_{48}\text{FN}_9\text{O}_{16}$ 834.3281. HPLC t_{R} = 8.8 min.

(3*S*,6*S*,9*S*,12*S*)-1-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-Azidoacetamido)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-12-carbamoyl-6-(2-carboxyethyl)-3-(carboxymethyl)-9-isopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatetradecan-14-oic acid (**20a**).

The side-chains of azide **19f** (0.25 g, 0.27 mmol) were deprotected using the general procedure for *tert*-butyl ester deprotection. The residue was purified by preparative reverse-phase HPLC (5 – 10% acetonitrile in water with 0.1% formic acid in 15 min) yielding the desired precursor (0.11 g, 52%). ^1H NMR (400 MHz, D_2O) δ 4.87 – 4.80 (m, 1H), 4.74 – 4.66 (m, 1H), 4.41 (dd, J = 9.0, 5.4 Hz, 1H), 4.26 (dd, J = 3.2, 1.2 Hz, 1H), 4.22 (t, J = 1.1 Hz, 1H), 4.08 (d, J = 7.4 Hz, 1H), 4.05 (s, 2H), 3.80 – 3.65 (m, 2H), 3.59 – 3.41 (m, 3H), 3.04 – 2.90 (m, 3H), 2.89 – 2.78 (m, 1H), 2.56 – 2.36 (m, 2H), 2.21 – 2.07 (m, 2H), 2.06 – 1.87 (m, 1H), 0.92 (dd, J = 6.8, 2.7 Hz, 6H). ^{13}C NMR (100 MHz, D_2O) δ 177.0, 174.6, 174.0, 174.0, 173.2, 173.1, 172.0, 170.8, 170.7, 78.0, 77.7, 73.3, 69.5, 68.0, 59.7, 53.1, 51.9, 49.9, 49.6, 40.3, 35.4, 29.9, 29.6, 25.8, 18.2, 17.7. HRMS (ESI+) = 748.2751 $[\text{M}+\text{H}]^+$. Calcd for $\text{C}_{27}\text{H}_{41}\text{N}_9\text{O}_{16}$ 748.2750. HPLC t_{R} = 7.0 min.

(5*S*,8*S*,11*S*,14*S*)-14-((2*S*,3*R*,4*R*,5*R*,6*S*)-6-((2-Azidoacetamido)methyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamido)-11-(2-carboxyethyl)-5-(carboxymethyl)-1-(2,6-dimethylphenyl)-8-isopropyl-1,4,7,10,13-pentaoxo-2-oxa-6,9,12-triazahexadecan-16-oic acid (**20b**).

The side chains of azide **19d** (0.18 g, 0.18 mmol) were deprotected using the general procedure for *tert*-butyl ester deprotection. The residue was purified by preparative reverse-phase HPLC (5 – 30% acetonitrile in water with 0.1% formic acid in 20 min) yielding the desired precursor (0.12 g, 75%). ¹H NMR (400 MHz, MeOD-*d*₄) δ 7.21 (t, *J* = 7.6 Hz, 1H), 7.05 (d, *J* = 7.6 Hz, 2H), 5.09 (s, 2H), 4.83 (t, *J* = 6.7 Hz, 1H), 4.75 (t, *J* = 6.3 Hz, 1H), 4.41 (d, *J* = 4.2 Hz, 1H), 4.23 – 4.19 (m, 1H), 4.17 (d, *J* = 7.3 Hz, 1H), 4.06 (s, 1H), 3.91 (s, 2H), 3.76 (dd, *J* = 14.1, 2.6 Hz, 1H), 3.58 – 3.40 (m, 3H), 3.37 – 3.32 (m, 1H), 3.03 – 2.77 (m, 4H), 2.42 (q, *J* = 6.8 Hz, 2H), 2.34 (s, 6H), 2.23 – 2.10 (m, 2H), 1.98 (dd, *J* = 14.8, 6.7 Hz, 1H), 0.98 (d, *J* = 6.7 Hz, 6H). ¹³C NMR (100 MHz, MeOD-*d*₄) δ 176.9, 174.8, 174.4, 173.8, 173.8, 173.0, 171.4, 170.7, 170.6, 136.6, 134.3, 130.7, 128.6, 79.9, 79.6, 75.4, 71.3, 69.5, 69.5, 60.7, 54.7, 53.8, 52.9, 51.1, 41.6, 36.7, 35.5, 31.4, 31.3, 27.9, 20.0, 19.8, 19.0. HRMS (ESI+) = 895.3221 [M+H]⁺. Calcd for C₃₇H₅₀N₈O₁₈ 895.3229. HPLC *t*_R = 14.2 min.

Pent-4-yn-1-yl 4-methylbenzenesulfonate (**21**).

This compound was prepared from the commercially available pentyn-1-ol (2.42 g, 28.80 mmol) and *p*-toluenesulfonyl chloride (5.00 g, 26.20 mmol) in DCM (25 mL) according to a previously described procedure.⁸⁰ A yellow oil was obtained containing the desired product (5.94 g, 95%), as described before.⁸⁰

Biochemical inhibition assay

The inhibitory activity (IC_{50} value) of the target DEVD peptides **1a-f** towards recombinant human and mouse caspases, was determined in an enzyme inhibition assay. Recombinant human caspase-1, -3, -6, -7, -8 and -9 and their respective peptide-specific fluorogenic substrates, Ac-YVAD-AMC for caspase-1, Ac-DEVD-AMC for caspase-3 and -7, Ac-VEID-AMC for caspase-6, Ac-IETD-AMC for caspase-8, and Ac-LEHD-AMC for caspase-9, were purchased from Enzo Lifesciences. Recombinant mouse caspase-1, -3, -7 and -8 were expressed and purified as previously described.⁸¹ Commercially available reversible, Ac-DEVD-CHO (Enzo Lifesciences), and irreversible, zVAD-FMK (Bachem) inhibitors were used as reference compounds. All assays were performed in black 96-well low-binding microplates at a volume of 150 μ L per well. The assays were performed at 37°C in appropriate caspase assay buffer (50 mM HEPES, 0.1% (w/v) CHAPS, 10 mM KCl, 50 mM sucrose, 1 mM $MgCl_2$, and 10 mM DTT, pH 7.4). For experiments containing caspase-9, 0.70 M sodium citrate was added to the assay buffer. The inhibitors were dissolved in DMSO and diluted with assay buffer so that the final concentration of DMSO in the wells was 0.5%. Equal volumes (10 μ L) of caspase solution and inhibitor in assay buffer were mixed and incubated for 5 min at room temperature followed by the addition of 130 μ L of the peptide substrate to a final concentration of 50 μ M. The final concentrations of human caspases used were the following, 10 nM caspase-1, 2 nM caspase-3, 10 nM caspase-6, 5 nM caspase-7, 3 nM caspase-8 and 30 nM caspase-9. For the mouse caspases, the concentrations used were the following, 50 nM caspase-1, 2 nM caspase-3, 10 nM caspase-7 and 50 nM caspase-8. Control wells contained all reaction components except for the enzyme. Inhibition of the recombinant caspases by the peptide inhibitors was assessed by measuring the

accumulation of the cleaved fluorescent product, 7-amino-4-methylcoumarin (AMC), for 20 min with a Synergy Mx microplate reader (BioTek) with excitation at 360 nm and emission detection at 460 nm. All the experiments were performed in duplicate. The concentration of inhibitor that inhibits caspase activity by 50% (IC₅₀) was calculated by four-parameter logistic nonlinear regression using GraFit (Erithacus). Values were calculated from eleven data points.

HPLC methods for radiochemistry

Semi-preparative isocratic radio-HPLC purification of the radiotracers was carried out using a Knauer HPLC pump and a Smartline UV detector (λ = 254 nm) in line with a Hi-Rad 1000-CD-X CdWO₄ scintillation detector (Scionix). A Phenomenex Luna C₁₈ 250 x 10 mm (5 μ m) HPLC column, a mobile phase of 25 mM NaH₂PO₄ pH 2.5 /ethanol ([¹⁸F]**MICA-301**, 85:15 (v/v), and [¹⁸F]**MICA-302**, 50:50 (v/v)) and a flow rate of 2 mL/min were used.

Quality control of the radiotracers was performed using analytical radio-HPLC and a Shimadzu LC-20AT HPLC pump equipped with a SPD-20A UV/VIS detector (λ = 210 nm, Shimadzu) in series with a NaI-scintillation detector for radiation detection (Raytest) and a Phenomenex Gemini C₁₈ 150 x 4.6 mm (5 μ m) HPLC column. Gradient method A: 5 to 30% CH₃CN in 20 mM NaH₂PO₄ pH 4 over 14 min, with a flow rate of 1 mL/min. Gradient method B: 5 to 80% CH₃CN in 20 mM NaH₂PO₄ pH 4 over 14 min, with a flow rate of 1 mL/min. The recorded data were processed by the GINA-Star 5 software (Raytest).

Radiochemistry

The radiosynthesis of [¹⁸F]**MICA-301** and [¹⁸F]**MICA-302** was carried out in an automated synthesis module (Fluorsynthon III, Comacer Netherlands) specifically adapted to the radiosyntheses, by modification of an existing procedure.⁵⁰ No-carrier added aqueous [¹⁸F]fluoride was produced in an Eclipse HP cyclotron (Siemens) using the ¹⁸O(p,n)¹⁸F reaction by proton bombardment of [¹⁸O]H₂O (Rotem Industries), and

passed through an ion exchange resin (Sep-Pak Accell Plus QMA Light cartridge (Waters)). $[^{18}\text{F}]\text{F}^-$ was eluted from the resin in the reactor vial with 1 mL of a mixture of 0.03 M K_2CO_3 /0.07 M Kryptofix2.2.2 in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (95:5 (v/v)) and evaporated to dryness. The remaining traces of water were removed by azeotropic distillation using CH_3CN . Tosylate precursor **21** (20 mg) in dry CH_3CN was added and reacted with the $[^{18}\text{F}]\text{KF-K2.2.2}$ solution for 3 min at 110°C , to generate 5- $[^{18}\text{F}]\text{fluoro-1-pentyne}$ ($[^{18}\text{F}]\text{22}$). After cooling down to 90°C , the pentyne was distilled under a gentle stream of N_2 from the reaction vessel into a second reactor vial and reacted with a mixture of 4 mg of azide precursor **20a** or **20b**, in the presence of 20 mg of sodium ascorbate, 250 μL of 0.1 M CuSO_4 and 7.5 mg of TBTA in 150 μL ethanol and 100 μL CH_3CN , at room temperature. After 20 min, 2 mL of water was added and the mixture was passed through a Sep-Pak Alumina N Light cartridge (Waters) (preconditioned with 10 mL of water), before injection on the HPLC. The crude reaction mixture was purified by semi-preparative HPLC. The fraction containing $[^{18}\text{F}]\text{MICA-301}$ or $[^{18}\text{F}]\text{MICA-302}$ ($t_R = 18.2$ min and $t_R = 16.0$ min, respectively) was collected, sterile-filtered and diluted with 0.9% NaCl to reduce ethanol concentration to < 10% in the final formulation. The pH was adjusted to pH 6-8 using a sterile 1 M NaOH solution. Radiochemical purity was determined by analytical reverse-phase HPLC using gradient methods A and B. Radiochemical yields (RCY) were calculated from the theoretical initial amount of $[^{18}\text{F}]\text{F}^-$ and decay corrected to end of bombardment (EOB).

Partition coefficient determination

The partition coefficient ($\log D$) of $[^{18}\text{F}]\text{MICA-301}$ and $[^{18}\text{F}]\text{MICA-302}$ was measured using the 'shake-flask' method.⁸² Briefly, approximately 74 kBq of $[^{18}\text{F}]\text{MICA-301}$ or $[^{18}\text{F}]\text{MICA-302}$ were added to a test tube containing a mixture of 2 mL *n*-octanol and 2 mL PBS (0.01 M, pH 7.4). The mixture was shaken well, vortexed for 2 min and centrifuged at 3,000 rpm for 10 min. After separation of the layers, a 0.5 mL aliquot of both layers was taken into separate tubes and counted for radioactivity in an automatic gamma- (γ)

counter (Wizard² 2480, Perkin Elmer). Corrections were made for differences in mass and density between the two phases. The octanol-water partition coefficients were obtained by dividing the octanol containing radioactivity by the PBS containing radioactivity and the $\log_{10}$ of this ratio was calculated. Reported $\log D$ values represent the mean of three determinations and were expressed as mean $\pm$ SD.

Activity-based labeling of recombinant enzymes

The recombinant human caspase-3, -6, -7 and -8 were purchased from Enzo Lifesciences and recombinant human cathepsin B and legumain were purchased from R&D Systems. The direct labeling of recombinant enzymes with [¹⁸F]**MICA-301** and [¹⁸F]**MICA-302** was performed as previously described.^{40, 41, 44, 83} First, enzymes were diluted into 50 μ L of the indicated buffer: 100 mM Tris, 0.1 % (w/v) CHAPS, 10 % (w/v) sucrose and 4 mM DTT (pH 7.4), for caspases; 50 mM acetate, 5 mM MgCl₂ and 2 mM DTT (pH 5.5), for cathepsin B and legumain. Then, the pre-mixed enzymes were labeled with approximately 74 kBq of [¹⁸F]**MICA-301** or [¹⁸F]**MICA-302** for 10 and 60 min at 37°C (with or without 30 min pre-treatment with a pan-caspase inhibitor, 1 μ M zVAD-FMK). Total enzyme concentration used was 20 nM. The reaction was stopped with reducing 4X Bolt LDS sample buffer (Thermo Scientific) and boiled for 5 min. The mixture was loaded in Bolt 12% Bis-Tris Plus gel (Thermo Scientific) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After washing, the gels were immediately exposed overnight to phosphor screen plates (Fujifilm). The exposed plates were imaged in a phosphor imager system (FLA7000, GE Healthcare). The digital images were analyzed using ImageJ software (version 1.51; NIH), and transformed uniformly to produce images to facilitate visualization of the protein bands. Cropped regions of the gel images containing the expected protein bands are shown. The regions containing free probe in the bottom of the gels were cropped from the gel images to improve signal to background ratio. No additional bands other than the ones depicted on the figures were observed.

Preparation of cell lysates

HeLa (ATCC CCL-2) human cervical cancer cells and MCF-7 (ATCC HTB-22) human breast cancer cells were seeded in 6-well plates at a density of 1×10^6 cells per well, and cultured in complete DMEM medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum and 100 U/mL penicillin plus 100 mg/mL streptomycin (Life Technologies), at 37°C under a 5% CO₂ humidified atmosphere. Twenty-four hours later, the media was refreshed and the cells were treated with staurosporine (STS; 1 µM for HeLa, and 5 µM for MCF-7 cells) for up to 24 h, with or without pre-incubation with zVAD(OMe)-FMK (100 µM) or 0.2% DMSO (n=3 for each condition).³⁴ After the end of the treatment period, the cells were collected from the wells, centrifuged at 1,000 *g* for 2 min, and the pellet was washed twice with ice cold PBS. After the last centrifugation step, the pellet was resuspended in 50 µL of hypotonic lysis buffer (50 mM PIPES, 10 mM KCl, 5 mM MgCl₂, 2 mM disodium EDTA, 1% (v/v) Nonidet P-40 (NP-40), 2 mM DTT, pH 7.4) and left to incubate for 10 min on ice, as described before.⁸⁴ The cells were snap-frozen and thawed, passed twice through a 29G needle, centrifuged at 7,000 *g* for 15 min at 4°C, and the supernatant was then transferred to a fresh tube. The total protein concentration of each sample was quantified using the bicinchoninic acid (BCA) protein assay (Thermo Scientific). The samples were stored at - 80°C until further use.

Animals

Immunodeficient CD1-/- nude female mice (Charles River Laboratories), weighing 20 - 25 g were kept under environmentally controlled conditions (12-h light/dark cycle, 20 - 24°C and 40 - 70% relative humidity) in IVC cages with food and water ad libitum. Experimental procedures and protocols were performed in accordance with European Directive 86/609/EEC Welfare and Treatment of Animals and were approved by the local ethical commission (2013-16, University of Antwerp, Belgium).

***In vivo* tumor model**

The xenograft models were generated by subcutaneous injection of COLO205 (2×10^6 in 100 μ L DPBS) or HT29 (10×10^6 in 100 μ L DPBS) tumor cells into the right hind flank of 7- to 8-week old female CD1-/- nude mice. When xenografts reached approximately 200 mm³, mice were treated with a single dose of conatumumab (1.5 mg/kg intraperitoneal, i.p.; Amgen, Inc.; n=16) or the corresponding IgG1 isotype control (1.5 mg/kg i.p.; Amgen, Inc.; n=13). COLO205 tumor-bearing mice were subjected to μ PET-CT imaging with [¹⁸F]**MICA-301** (n=6) or [¹⁸F]**MICA-302** (n=6), before (baseline) and 24 h after therapy, for treatment-response assessment.

Preparation of tumor homogenates

Twenty-four hours after treatment the mice were sacrificed, the tumors were snap-frozen in liquid nitrogen and subsequently homogenized on ice using a Dounce homogenizer in muscle lysis buffer (0.01 M PBS, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, pH 7.4), as described before.⁸⁴ The total protein concentration of each tumor sample was determined using the BCA protein assay. The tumor homogenates were stored at - 80°C.

Caspase activity assay using a fluorogenic substrate in cell lysates and tumor homogenates

Equal amounts of cell lysate or tumor homogenate protein (20 μ g) were transferred to each well of a black 96-well low-binding microplate containing 50 μ M of the fluorogenic substrate, Ac-DEVD-AMC, in 100 μ L of caspase assay buffer. The reaction mixture was incubated in the microplate for 1 h at 37°C. The amount of liberated fluorescent group (AMC) was measured on a Synergy Mx microplate reader (BioTek) with excitation wavelength of 360 nm and emission detection at 460 nm. The time of incubation and the amount of substrate concentration used were previously determined to be in the linear range of substrate utilization. Based on the standard curve prepared from fluorescence reading using free AMC, the data for

caspase activity represent picomoles of liberated AMC and were expressed as mean $\pm$ SD. The reported values represent the mean of two independent measurements.

Caspase labeling with [^{18}F]MICA-301 or [^{18}F]MICA-302 in cell lysates and tumor homogenates

The cell lysates or tumor homogenates (60 μg) were labeled with approximately 74 kBq of [^{18}F]MICA-301 or [^{18}F]MICA-302 for 60 min at 37°C, in 40 μL of caspase assay buffer. No-probe and probe alone controls were run in parallel to account for any background bands that may appear in the gel due to the lysate samples and the probes, respectively. In addition, to validate selectivity of the probes a caspase inhibitor (50 μM zVAD-FMK) was added 30 min prior the incubation with the probes. The reaction was quenched by adding reducing 4X Bolt LDS sample buffer and directly analyzed by SDS-PAGE, as described above. The digital images were analyzed using ImageJ software (version 1.51; NIH), and transformed uniformly to produce images to facilitate visualization of the protein bands. The regions containing free probe in the bottom of the gels were cropped from the gel images to improve signal to background ratio.

Detection of cleaved caspase-3 in cell lysates and tumor homogenates

The samples were denatured by adding 4X Bolt LDS sample buffer and boiling for 5 min. Equal amounts of protein were loaded into a Bolt 12% Bis-Tris Plus gel and proteins were separated by SDS-PAGE. After electrophoresis was complete, proteins were transferred from the gel onto PVDF membranes (Millipore) for antibody staining and detection. The membrane was blocked with 5% (w/v) nonfat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween 20 (TBS-T) for 1 h at room temperature under gentle agitation. The membranes were then incubated with a rabbit anti-caspase-3 primary antibody (1:1,000, Cell Signaling #9662) overnight at 4°C. After washing for 1 h in TBS-T, the membrane was incubated with an anti-rabbit HRP-linked IgG secondary antibody (1:2,000, Cell Signaling) in TBS-T with 5% (w/v) nonfat milk for 1 h at room temperature. For protein detection the

membranes were incubated with the enhanced chemiluminescence substrate (ECL, Cell Signaling). Visualization of the chemiluminescent signal was performed using the Chemidoc XRS+ detection system (BioRad).

***In vivo* small-animal PET imaging studies**

For *in vivo* PET biodistribution studies, normal CD1-/- nude mice were anesthetized using isoflurane (5% for induction, 2% for maintenance), placed on the animal bed in the scanner and injected via lateral tail vein catheterization with 18.5 MBq of [^{18}F]MICA-301 (n=3) or [^{18}F]MICA-302 (n=3). Dynamic whole-body PET images were acquired during 60 min (12x 10 s, 3x 20 s, 3x 30 s, 3x 60 s, 3x 150 s and 9x 300 s frames) using an Inveon small-animal PET/CT scanner (Siemens). Following each PET acquisition, a whole-body CT scan was acquired to obtain anatomic information for segmentation. Throughout the entire PET/CT scanning procedure, the mice were maintained at constant body temperature by using a heating pad. In COLO205 tumor xenografts, treatment-induced changes in the tumors were assessed with [^{18}F]MICA-301 (n=6) or [^{18}F]MICA-302 (n=6) PET imaging just before start of conatumumab or IgG1 treatment (baseline) and 24 h after start of treatment, as described above.

For quantitative analysis, PET data were reconstructed using 3-dimensional ordered subset expectation maximization (OSEM3D, 16 subsets and 2 iterations) and 18 maximum a posteriori (MAP) iterations including scatter and attenuation correction. The PET images were additionally reconstructed on a 128 x 128 x 159 matrix with a voxel size of 0.776 x 0.776 x 0.776 mm. CT-based attenuation and single scatter stimulation (SSS) scatter corrections were applied to the PET data. Volumes of interest (VOIs) were manually drawn on the PET/CT images using PMOD (version 3.3; PMOD Technologies) to delineate the regions with distinct PET time activity pattern: heart, liver, kidney, and muscle. In tumor-bearing mice VOIs were also drawn to delineate the tumors. The average organ or tissue activity per volume was

obtained from the co-registered PET images and the decay-corrected time-activity curves (TACs) were extracted for each target organ. For an absolute measure of tracer uptake, normalized images were scaled according to the percent injected dose ($\%ID/mL = \text{tissue uptake [kBq/mL]} / \text{injected dose [kBq]} \times 100$).

***Ex vivo* biodistribution studies**

Mice were injected with 18.5 MBq of [^{18}F]MICA-301 (n=12) or [^{18}F]MICA-302 (n=12) via the lateral tail vein. At 15, 30, 60 or 120 min (n=3 for each time point) post-radiotracer injection (p.i.) the blood was collected through cardiac puncture and the mice were euthanized by cervical dislocation. The organs and tissues were harvested, weighed and the radioactivity in the samples was measured using an automatic γ -counter (Wizard² 2480, Perkin Elmer). The uptake levels of the tracers were expressed as percentage of the injected dose per gram ($\%ID/g$).

***In vitro* stability evaluation of the radiotracers**

The recovery of [^{18}F]MICA-301 and [^{18}F]MICA-302, and their stability during the work-up were first determined in control experiments using blood spiked *in vitro* with 185 kBq of [^{18}F]MICA-301 or [^{18}F]MICA-302. Sample work-up was identical as described hereafter for the main stability experiment. The stability of [^{18}F]MICA-301 and [^{18}F]MICA-302 was evaluated in PBS (0.01 M, pH 7.4) and in mouse plasma at 37°C for up to 120 min. Approximately, 185-370 kBq of [^{18}F]MICA-301 or [^{18}F]MICA-302 were incubated in 200 μL PBS or mouse plasma. The incubation was quenched by adding cold $\text{MeOH}/\text{CHCl}_3$ (1:1 (v/v), 200 μL), followed by vortexing and centrifugation (5 min, 4,000 g) to remove the proteins. The radioactive contents of the supernatant (100 μL) were analyzed by analytical radio-HPLC. The HPLC eluate was collected in fractions of 30 s, and the radioactivity was counted in an automated γ -counter. All experiments were performed in duplicate.

***In vivo* metabolite analysis**

The blood from the above *ex vivo* biodistribution studies was collected in EDTA-coated tubes and used to evaluate the *in vivo* plasma stability of [^{18}F]MICA-301 (n=3 per time point) and [^{18}F]MICA-302 (n=3 per time point). The plasma fraction was obtained by centrifugation at 4,000 *g* for 7 min, and mixed (200 μL) with an equal volume of cold $\text{MeOH}/\text{CHCl}_3$ (1:1 (v/v)) to enable sample deproteinization. After vigorously vortexing and γ -counting, the samples were centrifuged at 4,000 *g* for 4 min, and the supernatant and the pellet were γ -counted separately to determine the amount of recovered radioactivity in the organic phase. The radioactive contents of the supernatant (100 μL) were analyzed by analytical radio-HPLC using the method described before. The eluate fractions were collected every 30 s and counted for radioactivity in an automated γ -counter.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using Prism (version 6.01; GraphPad Software). Statistical significance between different groups were analyzed by the one-way analysis of variance (ANOVA) followed by Bonferroni correction. Statistical significance between two data sets was evaluated by the unpaired two-tailed Student *t* test. Differences between groups were considered statistically significant if the *p* value was less than 0.05.

Conflicts of interest

There are no conflicts to declare.

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