Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Targeted imaging of integrins in cancer tissues using photocleavable Ru(II) polypyridine complexes as mass-tags

Experimental Section and Figures

General

All reagents and solvents were obtained from commercial suppliers and used without further purification, unless otherwise stated. $RuCl_3 \cdot 3H_2O$, 2,2':6',2'' terpyridine, 22,2'-bipyridine, D-biotin, triethylamine (EtN_3), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), KPF_6, ethanol, diethyl ether and acetonitrile were purchased from Sigma-Aldrich, cyclicRGDfK peptide was purchased from GL Biochem (Shanghai) Ltd.

Synthesis of ruthenium compounds

The Ru(II) compounds **1** $[Ru^{II}(terpy)(bpy)Cl]PF_6$ and **2** $[Ru^{II}(terpy)(bpy)D\text{-biotin}]^{2+}$ were synthesized according to previously established procedures[1–3].

$[Ru^{II}[(terpy)(bpy)(D\text{-biotin})cyc(RGDfL)]]Cl_2$ (**3**)

Compound **3** was synthesized activating compound **2** $[Ru^{II}[(terpy)(bpy)D\text{-biotin}]]Cl_2$ (65 mg, 0.08 mmol) *via* EDC (153 mg, 0.8 mmol) and sulfo-NHS (174 mg, 0.8 mmol) treatment at room temperature overnight in dark. Afterwards, the coupling reaction was accomplished by adding cyc(RGDfK) peptide (50 mg, 0.08 mmol) with 0.5% EtN_3 (pH = 7) for 1 h to form compound **3** with an orange color. The final product was purified on a C18 column (flow at 30 mL/min with a linear gradient from 0 to 90% of solvent B within 30 minutes, solvent A is water with 0.1% trifluoroacetic acid, and solvent B is acetonitrile with 0.1% trifluoroacetic acid) with a yield of 50% (55 mg, 0.04 mmol) and analyzed by HPLC-MS.

LC-MS analysis and preparative HPLC

LC-high-resolution mass spectrometry (MS) was performed on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) coupled to an analytical high-performance liquid chromatography (HPLC) Shimadzu

instrument (Shimadzu 20 series) equipped with a C18-column (Waters ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 50 mm). Suitable linear gradients (0.35 mL/min) of water (0.1% v/v formic acid, solvent A) and acetonitrile (0.1% v/v formic acid, solvent B) were applied for the analysis of all compounds. Preparative reversed phase HPLC was performed on a Buchi instrument (Buchi Reveleris X2) equipped with a C18-column (Grace Reveleris SRC C18 Cartridges, 40 μm, 12 g). Suitable linear gradients (30 mL/min) of water (0.1% v/v trifluoroacetic acid (TFA), solvent A) and acetonitrile (0.1% v/v trifluoroacetic acid (TFA), solvent B) were applied for the purification of compounds **2** and **3**.

Spectrophotometric studies

UV-visible absorption spectra of compound **3** were recorded using a V-650 (Jasco) spectrophotometer. Stock solutions of **3** were prepared in Milli-Q water and diluted to 100 µM for analysis.

Integrin binding studies

The affinity and selectivity of integrin ligands were determined by a solid-phase binding assay applying a previously described protocol[4,5] that involves coated extracellular matrix (ECM) proteins and soluble integrins. The following compounds were used as internal standards: *cilengitide*[6], *c*(f(*N*Me)VRGD) (αvβ3 – 0.54 nM, αvβ5 – 8 nM, α5β1 – 15.4 nM). Flat-bottomed 96-well ELISA plates (*BRAND*, Wertheim, Germany) were coated overnight at 4 °C with ECM protein (1) (100 µL per well) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Afterwards, each well was washed with PBS-T buffer (phosphate-buffered saline/Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.01% Tween 20, pH 7.4; 3 × 200 µL) and blocked for 1 h at room temperature with TS-B buffer (Tris-saline/bovine serum albumin (BSA) buffer, 20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.5, 1% BSA; 150 µL/well). Meanwhile, a dilution series of the compounds and internal standard was prepared in an extra plate, ranging from 20 µM to 256 pM in 1:5 dilution steps. After washing the assay plate three times with PBS-T (200 µL), 50 µL aliquots of the dilution series were transferred to each well from B-G in 6 appropriate concentrations. Well A was filled with 100 µL of TSB solution (blank), and well H was filled with 50 µl of TS-B buffer. Then, 50 µl of a solution of human integrin (2) in TS-B buffer was transferred to wells H–B and incubated for 1 h at r.t. The plate was washed three times with PBS-T buffer, and then primary antibody (3) (100 µL per well) was added to the plate. After incubation for 1 h at r.t., the plate was washed three times with PBS-T. Then, secondary peroxidase-conjugated antibody (4) (100 µL/well) was added to the plate and incubated for 1 h at r.t. Details on the respective solutions (1-4) for each integrin ligand are provided in the supplementary material. The plate was then washed three times with PBS-T, developed by the addition of SeramunBlau (50 µL/well, Seramun Diagnostic GmbH, Heidesee, Germany) and incubated for approx. 1 min at r.t. in the dark. The reaction was stopped with 3 M

H_2SO_4 (50 µL/well), and the absorbance was measured at 450 nm with a plate reader (*infinite M200 Pro, TECAN*). The IC_{50} value of each compound resulted from a sigmoidal fit of two data rows (serial dilution rows) done by *OriginPro 9.0G* statistical software. All IC_{50} values were referenced to the affinity of the internal standard.

αvβ3
(1) 1.0 µg/mL human vitronectin; Merck Millipore.
(2) 2.0 µg/mL, human αvβ3-integrin, R&D.
(3) 2.0 µg/mL, mouse anti-human CD51/61, BD Biosciences.
(4) 2.0 µg/mL, anti-mouse IgG-POD, Sigma-Aldrich.

αvβ5
(1) 5.0 µg/mL; human vitronectin, Merck Millipore.
(2) 3.0 µg/mL, human αvβ5-integrin, R&D.
(3) 1:500 dilution, anti-αv mouse anti-human MAB1978, Merck Millipore.
(4) 2.0 µg/mL, anti-mouse IgG-POD, Sigma-Aldrich.

α5β1
(1) 0.5 µg/mL; human fibronectin, Sigma-Aldrich.
(2) 2.0 µg/mL, human α5β1-integrin, R&D.
(3) 1.0 µg/mL, mouse anti-human CD49e, BD Biosciences.
(4) 2.0 µ g/mL, anti-mouse IgG-POD, Sigma-Aldrich.

Preparation of frozen human tumor tissue samples by cryosection

Human tissue sample. Human tumor tissue samples were obtained from the Department of Otorhinolaryngology, University of Groningen. Tissue samples were collected from hypopharyngeal squamous cell carcinoma tissue immediately after surgical resection, which did not interfere with the diagnostics or treatment of the patients. Patients gave informed consent for scientific analysis of the removed tissue. First, tissue samples were snap frozen in isopentane on dry ice. Subsequent processes were performed on dry ice until preparation of the cryosections was completed. Without thawing, each tissue was cut into sections with a cryotome (Thermo, Cryostar NX70) at -25°C. Human tumor tissue sections of 4 µm thickness for IHC and hematoxylin staining were cut and mounted on IHC microscope glass slides, and 10 µm thick sections for LDI-MSI were cut and mounted on ITO coated glass slides. Then sections were dried for 30 min using a fan. Remaining tissue samples were frozen at -80 °C for future use.

Immunohistochemistry (IHC) and hematoxylin staining of the integrin $\alpha v\beta_3$ in tumor tissue sections

Initially, sections were fixated with 50 mL acetone at room temperature for 10 min. After drying, sections were rehydrated in PBS. In the first incubation step, control sections were incubated with 50 µL PBS buffer (pH 7.4), and the others were incubated with 50 µL of the first monoclonal antibody (Mab) LM609 (anti-integrin $\alpha v\beta_3$ antibody, abcam) (1 mg/mL stock solution) at room temperature in serial dilution: 1:2000, 1:4000, 1:5000, 1:8000, and 1:10000. After 1 h, sections were washed in PBS and then incubated with 0.3% H_2O_2 in methanol for 20 min to inhibit endogenous peroxidase and then washed in demi-water for 2 min. The second incubation step was implemented by incubating sections with 50 µL of the secondary antibody (Rabbit Anti-Mouse IgG(H+L), Human ads-HRP, Southern Biotech) for 30 min at 1:100 diluted PBS solution with 5% normal human serum. In this protocol, a third antibody was utilized to enhance staining/amplification of the signal. The third incubation step was implemented by incubating sections with 50 µL of the third antibody (Goat Anti-Rabbit IgG(H+L), Mouse/Rat/Human ads-HRP, Southern Biotech) for 30 min at 1:100 diluted PBS solution with 5% normal human serum. Sections were washed three times with PBS buffer after each incubation step. Subsequently, sections were incubated with ImmPACT NovaRED (peroxidase (HRP) substrate kit, Vector) with a mixture of 1 mL NovaRED diluent, 16 µL reagent 1, 10 µL reagent 2, 10 µL reagent 3, and 10 µL reagent 4 for 15 min followed by a wash step in water. All of the above incubation steps were processed in the dark. Subsequently, sections were counterstained with hematoxylin according to Mayer for 1 min and washed under tap-water for 5 min. Finally, they were dehydrated and embedded in mounting medium DePeX (Serva). Stained tissue sections were dried in a fuming cupboard and the dried sections were used for further analysis.

Laser Desorption Ionization Mass Spectrometry (LDI-MS)

Stock solutions of compound **3** were prepared in Milli-Q water and diluted to 2 µg/mL for analysis. 1 µL of this dilution was spotted in duplicate onto a polished steel MALDI target plate. (Matrix Assisted) Laser Desorption/Ionization ((MA)LDI) time-of-flight (TOF) MS was performed on a Bruker UltrafleXtreme MALDI TOF-TOF instrument (Bruker Daltonics, Bremen, Germany) using ImageFlex (version 3.4, Bruker

Daltonics). Reflector positive MALDI-TOF spectra were recorded between *m/z* 300 and 3000 under the following conditions: 140 ns delayed extraction; signal deflection up to *m/z* 300; 2 kHz Smartbeam-II UV laser (Nd:YAG; λ = 355 nm) operating with the "4_large" parameter set; 5 GS/s digitizer sampling rate; ion source 1, 2, and lens voltages of 20.00, 17.73, and 7.7 kV, respectively.

Laser Desorption Ionization Mass Spectrometry Imaging (LDI-MSI)

Initially, tissue sections were dried under a vertical electric fan for 30 min and fixed with acetone for 10 min. After drying, sections were rehydrated in PBS. In the incubation step, three sections were incubated with 50 μL PBS buffer, compound **2** (2 mg/mL) and compound **3** (2 mg/mL), in the dark, respectively. After 1 h, sections were washed three times with PBS buffer and twice with 50%, 70% and 90% of ethanol successively to remove smaller molecules, such as small peptides, and lipids. Then, the sections were dried for several minutes under a fan for further LDI-MSI measurement. The MSI results were compared with the results obtained from IHC and hematoxylin staining detection of the integrin $\alpha v\beta_3$ by visual inspection of corresponding images. The parameters of MALDI-MS instrument were set the same as those described above in the LDI-MS measurement of compound **3**. During the LDI-MSI, the shots per raster spot is 100 and the diameter of the laser is 20 μm for every sample. The raster width for X and Y are both at 75 μm. Processing of the collected MSI data was performed using SCiLS Lab version 2014b (version 2.02.5378, SCiLS GmBH) and Cardinal R package in R (version 3.6.1, 2019-07-05). The "Isotope simulation" module of Xcalibur Qual Browser (Thermo Fisher Scientific) 3.0.63 was used to generate theoretical isotope distribution of compounds based on elemental composition using "pattern" output style.

Figures

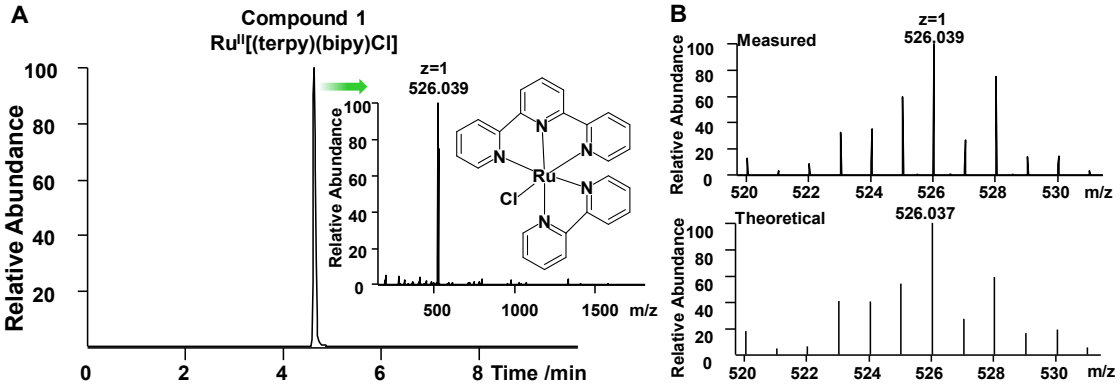


Figure S1. LC-MS analysis of purified compound **1.** (A) shows the extracted LC-MS chromatogram of compound **1** $[Ru^{II}(terpy)(bipy)Cl]PF_6$. The inset shows the mass spectrum and chemical structure of $[Ru^{II}(terpy)(bipy)Cl]^+$. (B) Mass spectra showing the isotopic pattern of measured (top) and theoretical (bottom) singly charged ion of $[Ru^{II}(terpy)(bipy)Cl]^+$.

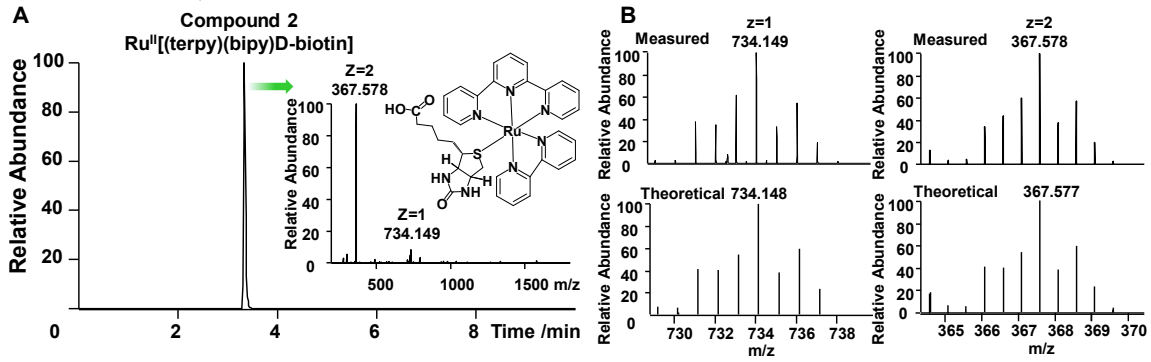


Figure S2. LC-MS analysis of purified compound **2**. The inset shows the mass spectrum and chemical structure of $[Ru^{II}[(terpy)(bipy)(D\text{-}biotin)]^{2+}$. (B) shows the isotopic pattern distribution of measured (top) and theoretical (bottom) singly charged (left) and doubly charged (right) ions of $[Ru^{II}[(terpy)(bipy)(D\text{-}biotin)]]^{2+}$.

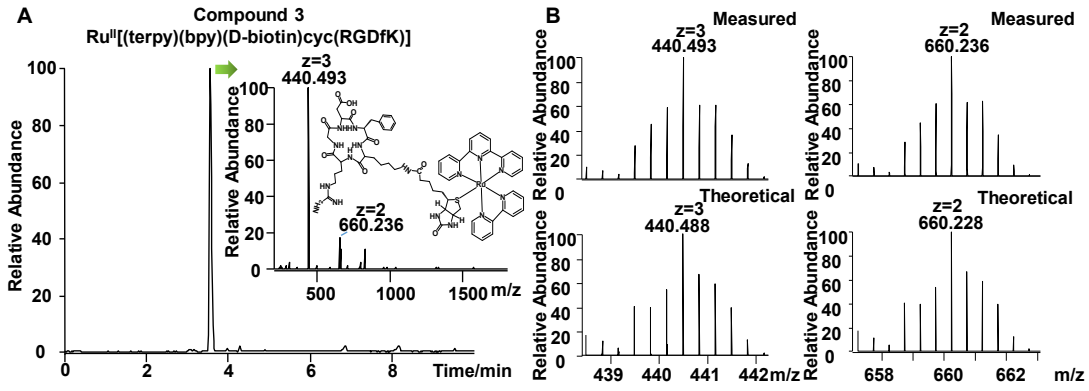


Figure S3. LC-MS analysis of purified compound **3** $[Ru^{II}[(terpy)(bpy)(D\text{-}biotin)cyc(RGDfK)]^{2+}$. (A) shows the total ion chromatogram of purified compound **3**. The inset shows the mass spectrum and chemical structure of $[Ru^{II}[(terpy)(bpy)(D\text{-}biotin)cyc(RGDfK)]^{2+}$. (B) shows the isotopic pattern distribution of measured (top) and theoretical (bottom) triply (left) and doubly (right) charged ions of $[Ru^{II}[(terpy)(bpy)(D\text{-}biotin)cyc(RGDfK)]^{2+}$.

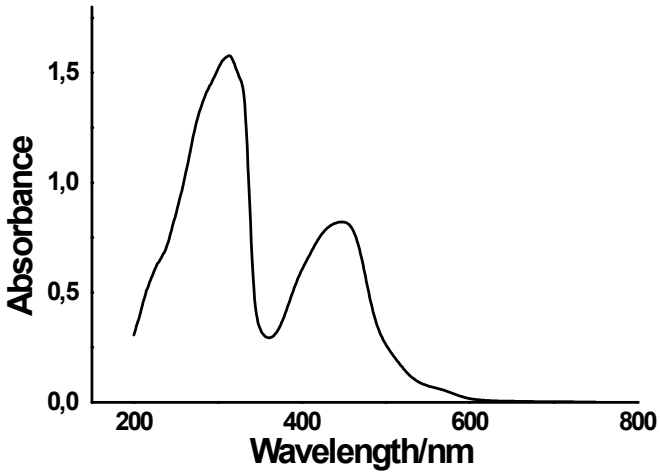
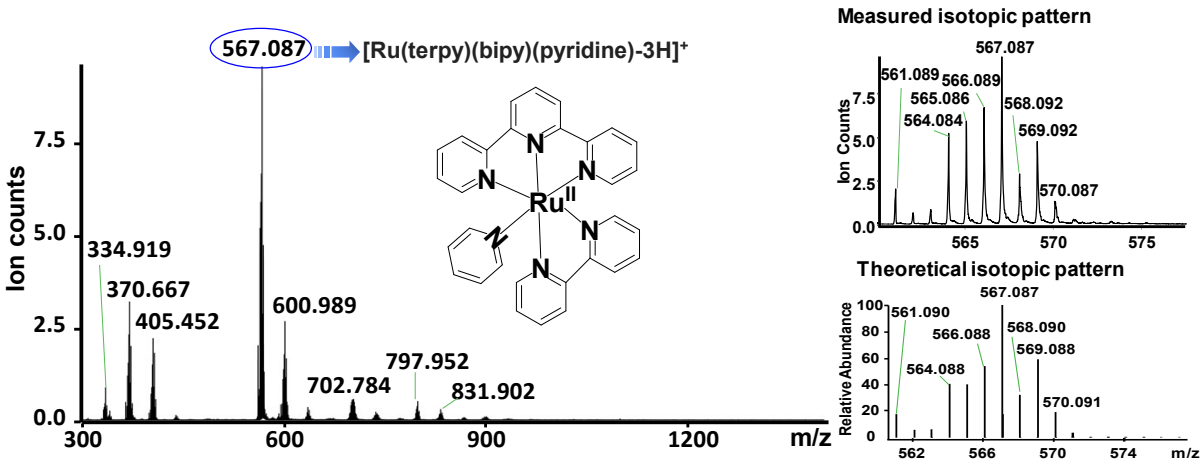


Figure S4. UV-visible absorption spectrum of compound **3** $[Ru^{II}[(terpy)(bpy)(D\text{-biotin})(cyc(RGDfK))]]^{2+}$ (100 μM) in Milli-Q water.

A



B

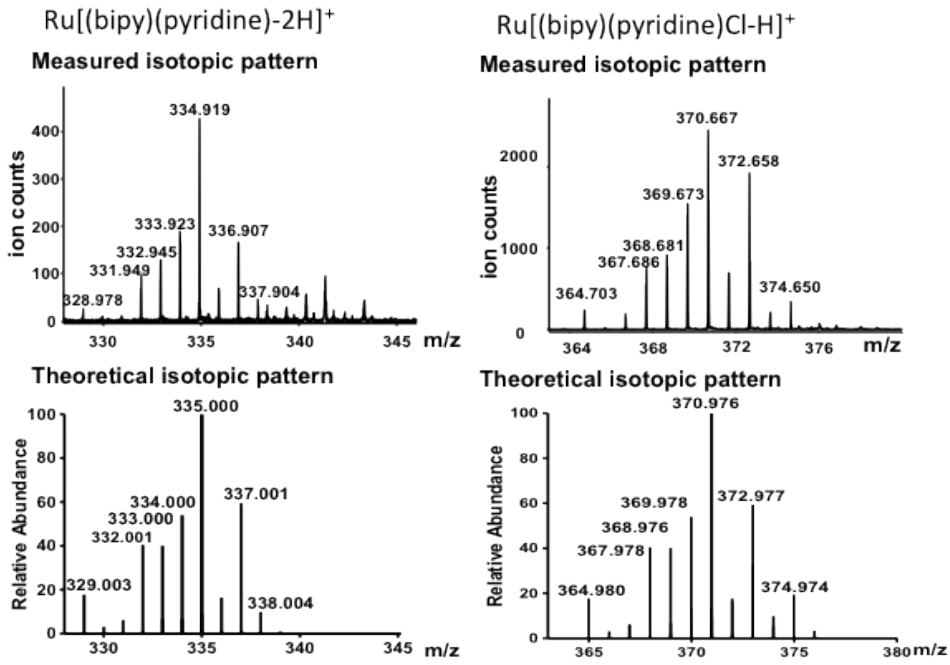


Figure S5. LDI-MSI spectrum of mass-tag compound **3** $[Ru^{II}(terpy)(bpy)(D\text{-biotin})cyc(RGDfK)]^{2+}$. Insets show the isotopic pattern distribution measured (top) and theoretical (bottom) for the resulting $[Ru(terpy)(bipy)(pyridine)\text{-}3H]^{+}$ fragment (A) and for secondary additional Ru(II) fragments (B).

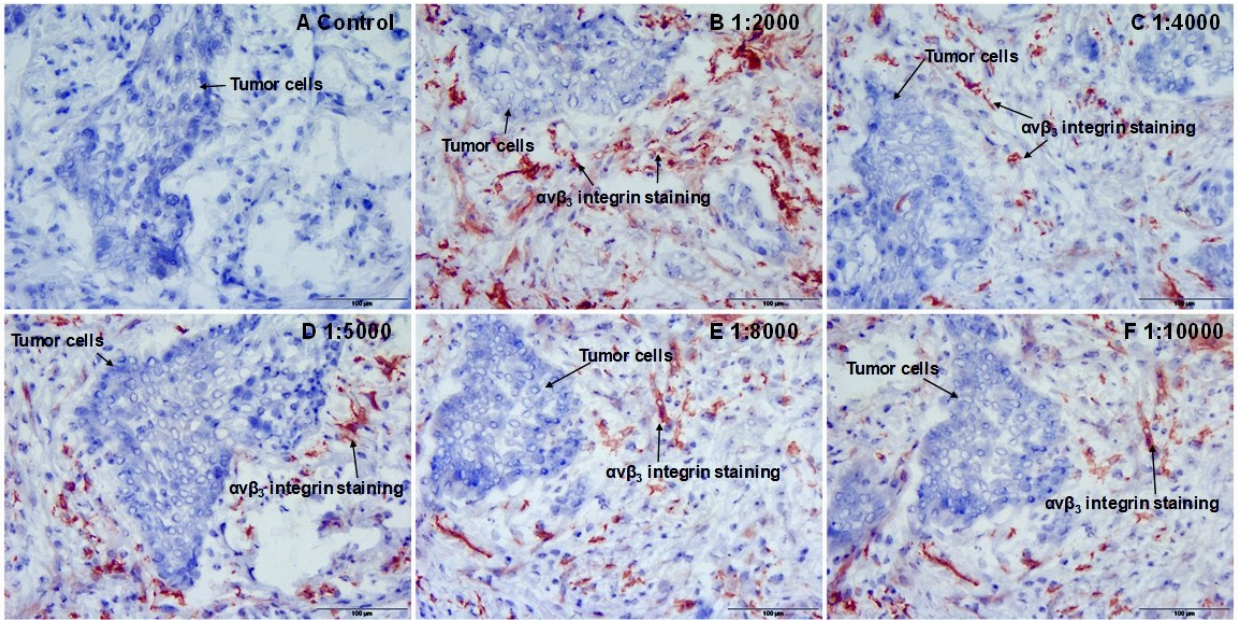


Figure S6. Immunohistochemistry (IHC) and hematoxylin staining used for the assessment of $\alpha v\beta_3$ integrin expression with different dilutions of Mab LM609 in hypopharynx tumor tissue. (A) negative control, (B) dilution 1:2000 (C) 1:4000, (D) 1:5000, (E) 1:8000, (F) 1:10000. The crimson color in B-F represents positive staining signal. Bars represent 100 μm.

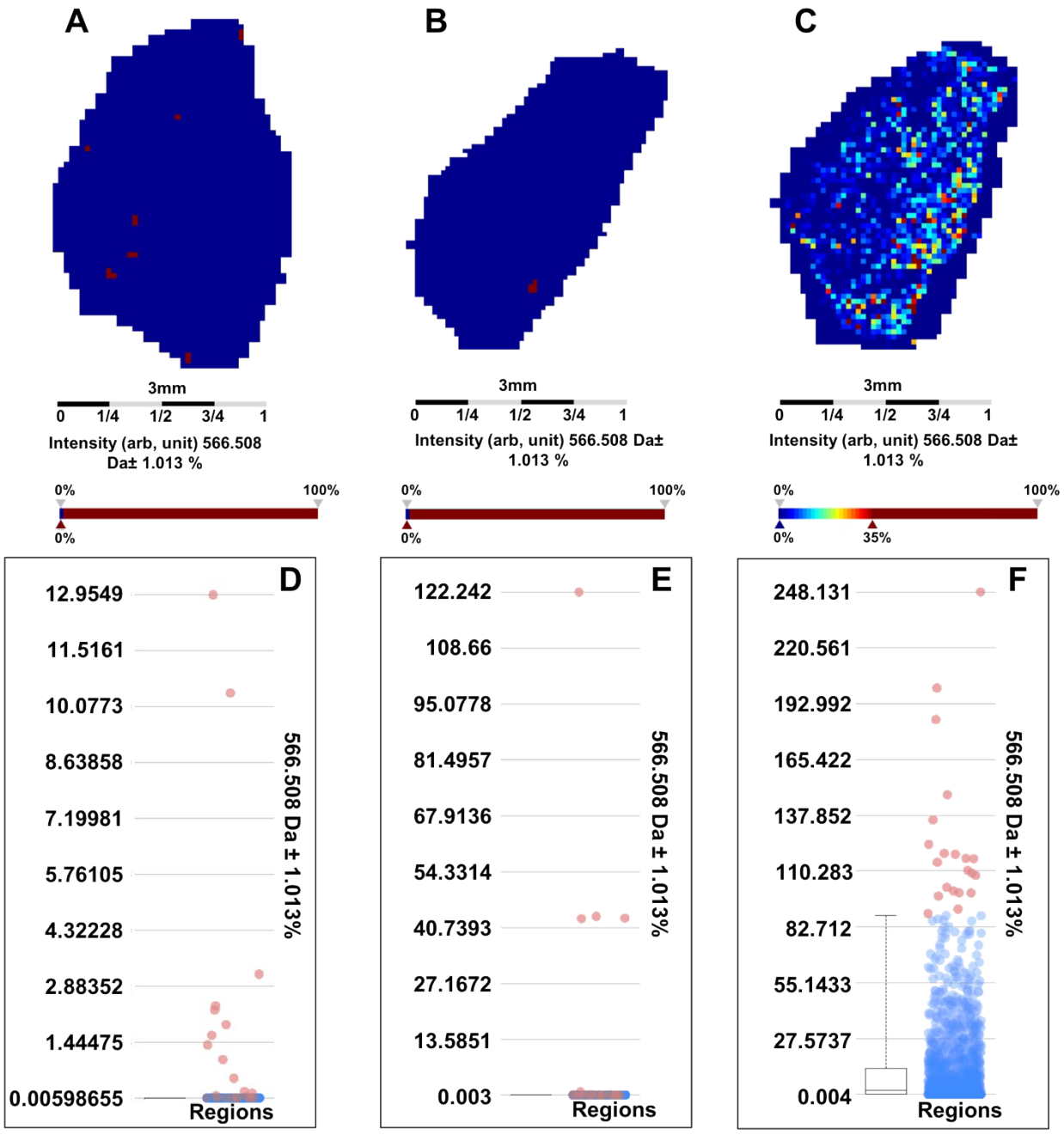
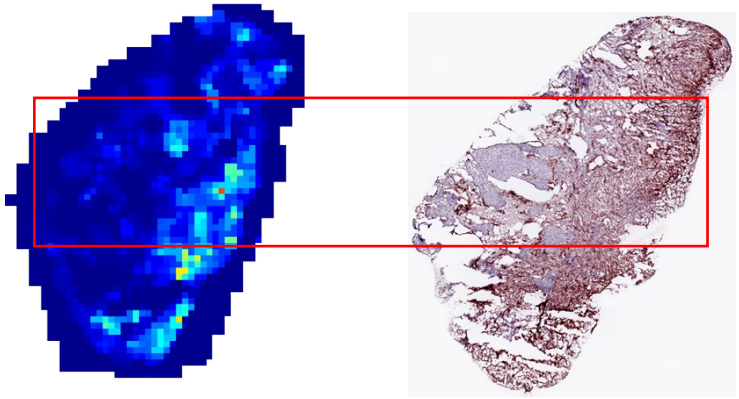


Figure S7. LDI-MSI images of mass-tag from hypopharynx tumor tissue sections incubated with (A) PBS buffer (pH 7.4), (B) compound 2 $[Ru^{II}(terpy)(bpy)D\text{-}biotin]Cl_2$ and (C) compound 3 $[Ru^{II}[(terpy)(bpy)((D\text{-}biotin)cyc(RGDfK))]]^{2+}$ without "weak denoising" option of the SCiLS software. The corresponding pixel intensity distribution of mass-tag signal (m/z = 566.508±1.013%) related to the image of the tissue sections incubated with: (D) PBS (pH 7.4), (E) compound **2** $[Ru^{II}[(terpy)(bpy)(D\text{-}biotin)]]^{2+}$ and (F) compound **3** $[Ru^{II}[(terpy)(bpy)(D\text{-}biotin)cyc(RGDfK)]]^{2+}$. The distribution is shown with box plot (left) and with bee-swarm plot indicating the intensity of each pixel in corresponding images of (A), (B) and (C). Blue dots are pixel intensity within 95% of the distribution, while red dots are outliers exceeding this limit.

A)



B)

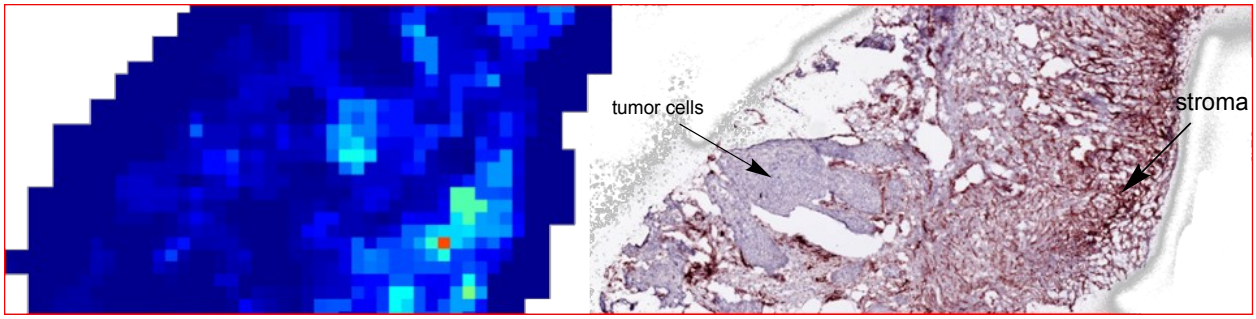


Figure S8. A) LDI and IHC/haematoxylin stained adjacent sections from hypopharynx tumor tissue. The red outlined rectangular shape facilitates the visualization of corresponding areas in the two sections. **B)** Magnification of the outlined area.

References

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