

Supporting Information

Molecular Scaffolds as Double-Targeting Agents For the Diagnosis and Treatment of Neuroblastoma

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1. General information

Following compounds were purchased from Sigma-Aldrich Inc.: Amino-protected Fmoc aminoacids, piperidine, *N,N,N',N'*-Tetramethyl-O-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate, O-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), 1-Hydroxybenzotriazole hydrate (HOBT), Diisopropyl Ethyl amine (DIPEA), Trifluoroacetic acid (TFA), Triisopropyl silane (TIPS), O,O'-Bis[2-(*N*-Succinimidyl-succinylamino)ethyl]polyethylene glycol 2KDa, Poly(ethylene glycol) 2-aminoethyl ether acetic acid 3500 Da, Fmoc-NH-Lys-NH-Mtt Wang resin, Sephadex G-25, as well as, the solvents used in the condensation, deprotection and release stages of peptide synthesis, such as *N,N'*-dimethylformamide (DMF) and dichloromethane (DCM). Also other coupling reagents for functionalization, conjugation or benzyl guanidine synthesis, have been purchased from Sigma-Aldrich Inc: *N,N'*-methanediylidenebis (propan-2-amine) (DIC), *N*-hydroxysuccinimide (NHS), *N,N*-bis(tert-butyloxycarbonyl) guanidine, (3-aminophenyl)methanol, Triphenylphosphine (TPP), Diisopropyl azodicarboxylate (DIAD), O,O'-Bis[2-(*N*-Succinimidyl-succinylamino) ethyl]polyethylene glycol 2000 Da, aminopropyl triethoxysilane (APTES), ammonium nitrate, cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS). Cy7-NHS, was purchased from Lumiprobe and all other chemicals (absolute ethanol, acetone, ethyl acetate heptane, dry solvents etc.) were of the best quality commercially available and they have been employed as received.

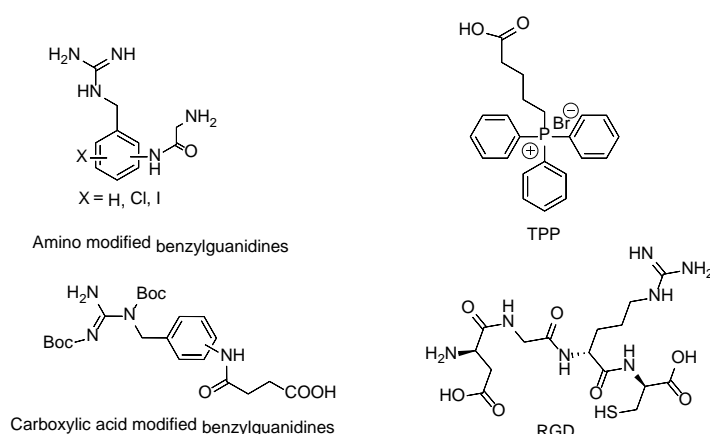
2. Characterization techniques

Fourier transform infrared spectroscopy (FTIR) in a Thermo Nicolet nexus equipped with a Goldengate attenuated total reflectance device. Thermogravimetry analysis (TGA) were performed in a Perkin Elmer Pyris Diamond TG/DTA analyzer, with 5 °C/min heating ramps, from room temperature to 600 °C. The hydrodynamic size of mesoporous nanoparticles was measured by means of a Zetasizer Nano ZS (Malvern Instruments) equipped with a 633 nm “red” laser. Mass spectra were acquired with a Voyager DE-STR Biospectrometry MALDI-TOF mass spectrometer. Scanning electron microscopy (SEM) analyses were made on a JEOL 6400-LINK AN10000 microscope (Electron Microscopy Centre, UCM). The samples underwent Au metallization

previous to observation. Liquid NMR experiments were made in a Bruker AV 250MHz. For in vivo experiments, the distribution of particles and the quantification of the signal in the area of the tumor were observed through fluorescent image acquisition in an IVIS Lumina XRMS instrument (Perkin Elmer).

3. Synthesis of analogues

First step for affording the ligands compounds is the synthesis and modification of the single targeting agents for further inclusion in the double ligands. For this aim, benzyl guanidine analogues have been synthesized and functionalized with final amine or acid groups. Further, other kinds of small molecules such as triphenylphosphine or RGD, have been also synthesized or modified to be included in the ligands compounds as alternative vectorization agent able to interact with typical receptors of tumor cells.



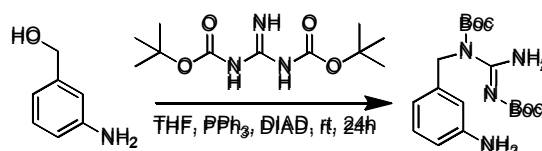
Scheme S1. Targeting moieties for scaffold functionalization.

3.1 Synthesis of single targeting agents

- Synthesis of starting materials :

(3-amino benzyl) - (N, N-bis (tert-butoxycarbonyl) guanidine for synthesis of

S3

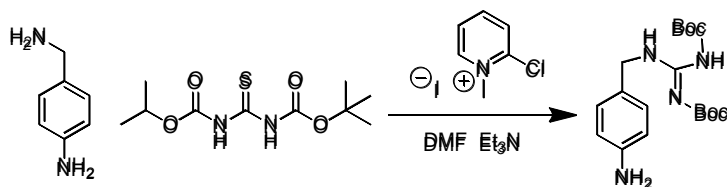


Scheme S2. Synthesis of the protected *meta* amino benzyl guanidine.

3-aminobenzylalcohol (1 g, 8.12 mmol) was solved in 10 mL of THF and stirred under inert atmosphere together with *N, N*-bis (tert-butyloxycarbonyl) guanidine (2.32 g, 8.93 mmol) and triphenylphosphine (TPP) (4.26 g, 16.24 mmol). DIAD (3.20 mL, 16.24 mmol) was added dropwise later. The resulting yellow mixture was stirred at room temperature overnight. The solvent was evaporated and the resulting crude was reconstituted in ethyl acetate. The organic phase was washed with water (3x15mL) and brine (2x20mL). The organic phase was dried and the crude purified by silica column chromatography. Yield 60-70%.

^1H NMR (250 MHz, CDCl_3) δ 9.41 and 9.35 (s, br, 2H, NH_2), 7.18 – 7.04 (m, 1H, $\text{CH}_{(\text{Ar})}$), 6.64 – 6.52 (m, 3H, $\text{CH}_{(\text{Ar})}$), 5.09 (s, 2H, CH_2), 3.62 (s, 2H, NH_2), 1.48 (s, 9H, $3\times\text{CH}_3(\text{BOC})$), 1.32 (s, 9H, $3\times\text{CH}_3(\text{BOC})$). ^{13}C NMR (63 MHz, CDCl_3) δ 173.98 ($\text{C}=\text{O}_{\text{BOC}}$), 173.93($\text{C}=\text{O}_{\text{BOC}}$), 155.42($\text{C}=\text{N}$), 146.69($\text{C}_{(\text{Ar})}$), 140.44($\text{C}_{(\text{Ar})}$), 129.47 ($\text{CH}_{(\text{Ar})}$), 117.56 ($\text{CH}_{(\text{Ar})}$), 114.10 ($\text{CH}_{(\text{Ar})}$), 113.81 ($\text{CH}_{(\text{Ar})}$), 84.58 ($2\times\text{C}_{(\text{BOC})}$), 48.11 (CH_2), 28.70 ($3\times\text{CH}_3(\text{BOC})$), 28.19 ($3\times\text{CH}_3(\text{BOC})$). FTIR (cm^{-1}): 3458; 3369; 3060; 2967; 2920; 1715; 1588; 1495; 1283; 1140; 1107; 977; 589. ESI(-): 363m/z [M-1]

(3-amino benzyl) - (N, N-bis (tert-butoxycarbonyl) guanidine for synthesis of S4

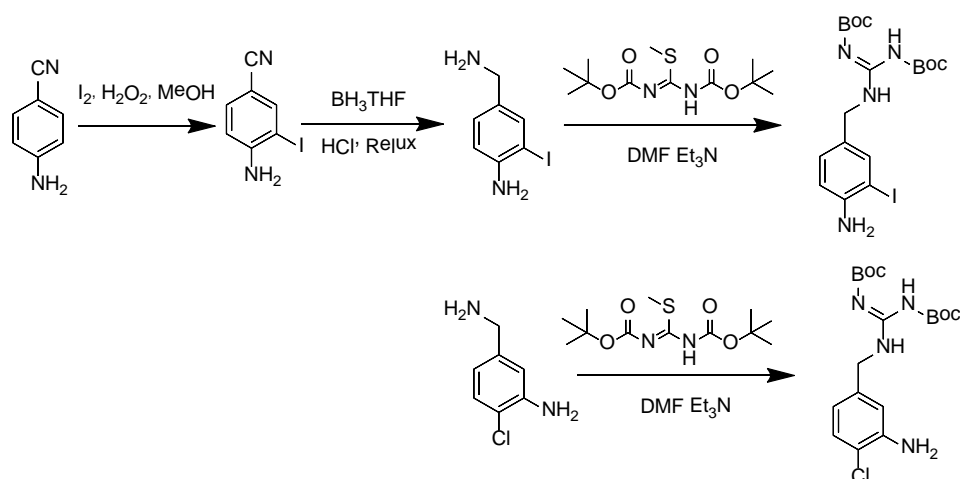


Scheme S3. Synthesis of the protected *para* amino benzyl guanidine.

A solution of 4-(aminomethyl)aniline (100 mg, 0.8 mmol) in anhydrous DMF (200 μL) was treated with *N, N*-bis (tert-butyloxy-carbonyl) thiourea (249 mg, 0.9 mmol) and triethylamine (250 μL 1.8 mmol) in an inert atmosphere. Mukaiyama reagent (209 mg, 0.8 mmol) was dissolved in DMF (400 μL) also in a nitrogen atmosphere and added over the previous mixture dropwise. The reaction was stirred at room temperature overnight. When the reaction came to an end, the mixture was partitioned with water and ethyl acetate. The organic phase was extracted and washed with brine (1x 5 mL), dried and concentrated to dryness. The resulting crude was purified by silica column chromatography. Yield 60-70% .

^1H NMR (250 MHz, CDCl_3) δ 11.52 (s, 1 H, NH, amide), 8.42 (s, broad, 1H, amide), 7.10 (d, $J = 8.5$ Hz, 2H, 2xCH (Ar)), 6.65 (d, $J = 8.5$ Hz, 2H, 2xCH (Ar)), 4.48 (d, $J = 4.9$ Hz, 2H, CH_2), 3.67 (s, 2H, NH_2), 1.52 (s, 9H, 3x CH_3 (BOC)), 1.46 (s, 9H, 3x CH_3 (BOC)). ^{13}C NMR (63 MHz, CDCl_3) δ 164.07 (C = O), 156.24 (C = O), 153.53 (C = N), 146.36 (NH_2 -CAr), 129.72 (2xCHAr), 127.35 (CH_2 -CAr), 115.67 (2xCHAr), 83.44 (C (BOC)), 79.75 (C (BOC)), 45.25 (CH_2), 28.73 (3x CH_3 (BOC)), 28.47 (3x CH_3 (BOC)). ESI (+): 365.1 m/z [M + 1].

(4-amino-3-iodobenzyl) - (N2, N3-bis (tert-butoxycarbonyl) guanidine for synthesis SI



Scheme S4. Synthesis of the protected halobenzyl guanidines.

Step I: To a solution of 4-aminobenzonitrile (500 mg, 4.23 mmol) in methanol (50 mL), I_2 was slowly added (644.53 mg, 2.54 mmol). The mixture was stirred for 30 minutes at room temperature and then half a milliliter of H_2O_2 in THF 5 mL of THF were slowly added over 20 minutes. The reaction was allowed to stir for three days at room temperature, until the total conversion of the starting product was confirmed by TLC. The reaction was terminated by the addition of an aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$, that mixture was left stirring 20 minutes, and then extracted with dichloromethane. The organic phase was washed with brine and then dried in MgSO_4 and concentrated to dryness. The residue was purified by silica column chromatography. The resulting yellow solid was washed with heptane to a fine powder. Yield 250 mg, 24%.

^1H NMR (250 MHz, CDCl_3) δ 7.90 (d, $J = 1.8$ Hz, 1 H, CH_{ar}), 7.40 (dd, $J = 8.4, 1.9$ Hz, 1H, CH_{ar}), 6.70 (d, $J = 8.4$ Hz, 1H, CH_{ar}), 4.64 (s, 2H, NH_2). ^{13}C NMR (63 MHz, CDCl_3) δ 150.63 ($\text{C}_{\text{ar-NH } 2}$), 142.93 ($\text{C}_{\text{ar-H}}$), 133.42 ($\text{C}_{\text{ar-H}}$), 113.64 ($\text{C}_{\text{ar-H}}$), 112.23 (CN), 101.89 ($\text{C}_{\text{ar-CN}}$), 81.99 ($\text{C}_{\text{ar-I}}$). ESI (-): 242.7 [$\text{M}-1$].

Step II: To a solution of 4-amino-3-iodobenzonitrile in THF (4 mL) was added $\text{BH}_3\cdot\text{THF}$ (1 M solution in THF 2.5 mL) drop-wise. The reaction mixture was refluxed for 3 h and 2 N HCl (1.0 mL) was added. The resulting mixture was refluxed for an additional 1 h and then concentrated in vacuo in order to afford the crude, which was re-crystalized in MeOH for characterization to give 150 mg (74 %) of yellow powder.

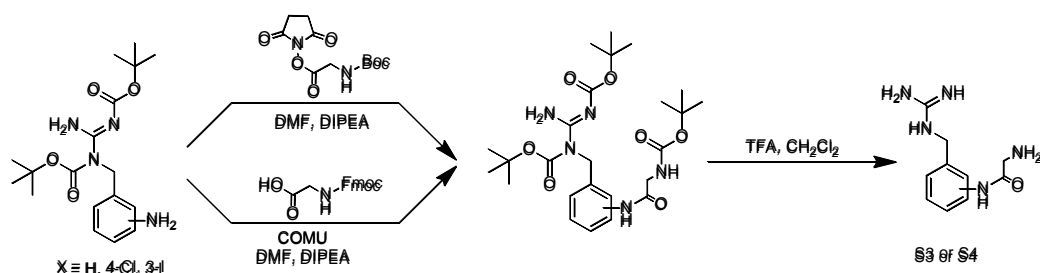
^1H NMR (250 MHz, DMF) δ 8.90 (s, broad, 3H, $^+\text{NH}_3$), 7.88 (d, $J = 2.0$ Hz, 1H, CH_{ar}), 7.42 (dd, $J = 8.3, 2.1$ Hz, 1H, CH_{ar}), 6.93 (d, $J = 8.3$ Hz, 1H, CH_{ar}), 5.81 (s, broad, 6H, NH_3), 4.06 (q, $J = 5.6$ Hz, 2H, CH_2). ^{13}C NMR (63 MHz, DMF) δ 151.64 ($\text{C}_{\text{ar-}^+\text{NH}_3}$), 149.11(C_{ar}), 140.44 (CH_{ar}), 131.17 (CH_{ar}), 124.75 (C_{ar}), 114.86 (CH_{ar}), 82.93($\text{C}_{\text{ar-I}}$), 42.30 (CH_2). ESI (-, m/z): 283.2 [$\text{M}^+\text{+Cl}$]

Step III: For the preparation of the different protected halogenated aminobenzylguanidines, the aminohalobenzylaniline was stabilized as a hydrochloride (2 mmol), which was dissolved in DMF (5 mL), then 2,2 mmol (2.2 mmol) was added to the reaction. 638mg) of commercial 1,3-Bis (tert-butoxycarbonyl) -2-methyl-2-thiopseudourea and 836 μL (6 mmol, 3 equiv) of NEt_3 maintaining the reaction under stirring for 16 h. Next, the following general processing method was applied: dilution with 100mL of AcOEt, washing with 2x50mL of a solution of NH_4Cl saturated in water, washing with 50mL of brine, drying over MgSO_4 , filtration and evaporation in a rotary evaporator. Finally, the crude obtained was purified by SiO_2 column chromatography using Heptane / AcOEt (1: 1) as mobile phase, obtaining the corresponding aminobenzyl guanidines.

(4-amino-3-iodobenzyl) - (N2, N3-bis (tert-butoxycarbonyl) guanidine (SI) ^1H NMR (250 MHz, CDCl_3) δ 11.51 (s, 1H, NH), 8.43 (s, 1H, NH), 7.59 (d, $J = 2.0$ Hz, 1H, CH_{ar}), 7.10 (dd, $J = 8.2, 2.0$ Hz, 1H, CH_{ar}), 6.71 (d, $J = 8.2$ Hz, 1H, CH_{ar}), 4.44 (d, $J = 5.1$ Hz, 2H, CH_2), 4.10 (s, 2H, NH_2), 1.52(s, 9H, $3\times\text{CH}_3(\text{BOC})$), 1.47 (s, 9H, $3\times\text{CH}_3(\text{BOC})$). ^{13}C NMR (63 MHz, CDCl_3) δ 164.00 (C=O), 156.30 (N=C), 153.50

(C=O), 146.75(C_{ar}-NH₂), 139.20 (C_{ar}-H) , 129.72 (C_{ar}), 129.05 (C_{ar}-H), 115.09 (C_{ar}-H), 84.35(2xC_(BOC)), 83.60 (C_{ar}-I), 79.83(C_(BOC)), 44.28 (CH₂), 28.72 (3xCH₃(BOC)), 28.48 (3xCH₃(BOC)). ESI(+): 491.1 m/z [M+1]
(4-amino-3-chlorobenzyl) - (N2, N3-bis (tert-butoxycarbonyl) guanidine (S3)) ¹H NMR (250 MHz, CDCl₃) δ 8.38 (s, 1H), 7.14 (d, *J* = 1.9 Hz, 1H), 6.94 (dd, *J* = 8.2, 1.9 Hz, 1H), 6.65 (d, *J* = 8.2 Hz, 1H), 4.40 (d, *J* = 5.1 Hz, 2H), 3.98 (s, 2H), 1.45 (s, 9H), 1.40 (s, 9H). ¹³C NMR (63 MHz, CDCl₃) δ 163.7, 156.0, 153.3, 142.6, 129.4, 128.0, 127.7, 119.3, 116.1, 83.3, 79.5, 44.3, 28.4 (3C), 28.2 (3C). MS (ESI+) Calc for C₁₈H₂₈³⁵ClN₄O₄ ESI (+) : (M+H)⁺ m/z = 399.2

- Synthesis of analogues with terminal amine group for single agents synthesis.



Scheme S5. Deprotection of protected derivatives S3 and S4 with terminal amine.

To a solution of Boc-protected aminobecilguanidine S3 or S4 (50 mg) in DMF (2 mL), diisopropylethylamine (100 μ L) was added. 2,5-dioxopyrrolidin-1-yl (tert-butoxycarbonyl) glycinate (50 mg) dissolved in 1 mL of DMF was added dropwise, over the reaction, which was allowed to react at 80 ° C overnight. The reaction ended with the addition of 10mL water and 10mL ethyl acetate, the organic phase was washed with NaHCO₃ (5%) 3x10 mL, NaHSO₄ (20%) 3x10 mL, and brine 3x10 mL, dried with anhydrous sodium sulfate and concentrated to dryness. The crude was purified by column chromatography on silica gel.

Then, on a solution of the corresponding analogue in stage I in dichloromethane (2mL), 2 mL of TFA were slowly added at room temperature over inert atmosphere. The resulting mixture was heated at 65 ° C for 48 hours and once the reaction was completed the mixture was concentrated to dryness. The resulting crude was washed with chloroform and dried in a vacuum oven.

2- (tert-Butoxycarbonyl) amino-N- (3 - ((1,2-bis (tert-butoxycarbonyl) guanidino) methyl) -phenyl) acetamide

^1H NMR (250 MHz, CDCl_3) δ 9.49 y 9.37 (s, br, 2H, NH_2), 8.13 (s, 1H, NH), 7.46 (d, $J = 7.7$ Hz, 1H, CH_{ar}), 7.26 – 7.18 (m, 2H, $2\times\text{CH}_{\text{ar}}$), 6.94 (d, $J = 7.8$ Hz, 1H, CH_{ar}), 5.24 (s, 1H, NH), 5.14 (s, 2H, CH_2), 3.91 (d, $J = 5.9$ Hz, 2H, CH_2 , glycine), 1.48 (s, 9H, $3\times\text{CH}_3(\text{BOC})$), 1.47 (s, 9H, $3\times\text{CH}_3(\text{BOC})$), 1.31 (s, 9H, $3\times\text{CH}_3(\text{BOC})$). ^{13}C NMR (63 MHz, CDCl_3) δ 167.97 (C=O), 164.09 (C=O, BOC), 161.33 (C=O, BOC), 155.24 (C=N), 151.70 (C=O, BOC), 140.29 (C_{ar}), 137.90 ($\text{C}_{\text{ar}}\text{-NH}$), 129.31 (CH_{ar}), 122.97(CH_{ar}), 118.74 (CH_{ar}), 118.22 (CH_{ar}), 84.70 ($\text{C}_{(\text{BOC})}$), 79.48 ($\text{C}_{(\text{BOC})}$), 77.64 ($\text{C}_{(\text{BOC})}$), 47.81 (CH_2), 32.30 (CH_2 , glycine), 28.71 ($6\times\text{CH}_3(\text{BOC})$), 28.19 ($3\times\text{CH}_3(\text{BOC})$).

2-Amino-N- (3- (guanidinomethyl) phenyl) acetamide (S3)

^1H NMR (250 MHz, MeOD) δ 7.87 (s, 1H, NH), 7.62 (s, 1H, CH_{ar}), 7.45 (d, $J = 7.8$ Hz, 1H, CH_{ar}), 7.32 (t, $J = 7.9$ Hz, 1H, CH_{ar}), 7.06 (d, $J = 7.6$ Hz, 1H, CH_{ar}), 4.36 (s, 2H, CH_2), 3.82 (s, 2H CH_2 , glycine). ^{13}C NMR (63 MHz, MeOD) δ 178.91 (C=O), 158.51 (C=N), 148.72 (C_{ar}), 147.98 (C_{ar}), 130.33 (CH_{ar}), 123.95 (CH_{ar}), 120.03 (CH_{ar}), 119.31 (CH_{ar}), 45.51 (CH_2), 41.84 (CH_2 , glycine). ESI (+) (M) $^+$ $m/z = 222.1$

2- (tert-Butoxycarbonyl)amino-N-(3 - ((1,2-bis (tert-butoxycarbonyl) guanidino) methyl) -phenyl) acetamide

^1H NMR (250 MHz, CDCl_3) δ 12.50 (s, 1H, NH, amide), 8.37 (s, 1H, NH, amide), 8.09 (s, 1H, NH, amide), 7.50 (d, $J = 8.4$ Hz, $2\times\text{CH}_{\text{ar}}$), 7.31 (d, $J = 5.7$ Hz, 2H, $2\times\text{CH}_{\text{ar}}$), 5.28 – 5.12 (m, 1H, NH, amide), 4.54 (d, $J = 5.7$ Hz, 2H, CH_2), 3.95 (d, $J = 6.2$ Hz, 2H, CH_2 , glycine), 1.51 (s, 9H, $3\times\text{CH}_3(\text{BOC})$), 1.48 (s, 9H, $3\times\text{CH}_3(\text{BOC})$), 1.28 (s, 9H, $3\times\text{CH}_3(\text{BOC})$).

2-Amino-N- (4- (guanidinomethyl) phenyl) acetamide (S4)

^1H NMR (250 MHz, MeOD) δ 7.68 (d, $J = 8.6$ Hz, 2H, $2\times\text{CH}_{\text{ar}}$), 7.40 (d, $J = 8.2$ Hz, 2H, $2\times\text{CH}_{\text{ar}}$), 4.51 (s, 1H, CH_2), 3.83 (s, 1H, CH_2 , glycine). ESI (+) (M) $^+$ $m/z = 222.1$

Fmoc-protected Glycine homologation of guanidines was carried out in a 0.20mmol scale to which were added 65mg (0.22mmol, 1.1 equiv) Fmoc-glycine and 102.8 mg (0.24mmol, 1.2 equiv) of COMU as coupling reagent, 104 μL DIPEA (0.6mmol, 3 equiv) as base and 10mL of anhydrous DMF. The reaction started at 0°C and 30 minutes

later heated to 50°C for overnight. The reaction ended with the addition of 10mL water and 10mL ethyl acetate, the organic phase was washed with NaHCO₃ (5%) 3x10 mL, NaHSO₄ (20%) 3x10 mL, and brine 3x10 mL, dried with anhydrous sodium sulfate and concentrated to dryness. The crude was purified by column chromatography on silica gel with Heptane/AcOEt (1:1) as mobile phase. All compounds were obtained as isomers mixtures (Yield: 43–59%). The deprotection of these analogues followed a two–step procedure. First, the guanidines were treated with CF₃COOH, Et₃SiH y CH₂Cl₂ (0.5:1:3mL) at room temperature under inert atmosphere for 2h. Then the mixture was partially evaporated and the crude precipitated and triturated with Et₂O. The solid was then dissolved in a mixture of 20% dimethylamine in acetone (1mL) to proceed to Fmoc cleavage, maintaining the reaction for 2h at RT. Finally the solvent was evaporated and the desired compound obtained after precipitation and trituration with Et₂O.

2–(Fluorenylmethoxycarbonyl)amino–N–(4–((2,3–bis(*tert*-butoxycarbonyl)guanidino)methyl)–2–iodophenyl) acetamide.

¹H NMR (250 MHz, CDCl₃) δ 8.51 (s, 1H), 8.09 (d, *J* = 8.6 Hz, 2H), 7.69 (d, *J* = 7.2 Hz, 2H), 7.63 (d, *J* = 1.8 Hz, 1H), 7.53 (d, *J* = 7.0 Hz, 2H), 7.38 – 7.16 (m, 7H), 5.48 (s, 1H), 4.45 (d, *J* = 6.3 Hz, 4H), 4.17 (t, *J* = 6.4 Hz, 1H), 3.95 (s, 2H), 1.44 (s, 9H), 1.41 (s, 9H). ¹³C NMR (63 MHz, CDCl₃) δ 167.7, 163.9, 157.0, 156.5, 153.6, 144.0 (2C), 141.8 (2C), 138.6, 137.3, 136.0, 129.3, 128.2 (2C), 127.6 (2C), 125.3 (2C), 122.1, 120.50 (2C), 83.8, 79.9, 47.5, 44.0, 28.7 (3C), 28.5 (3C).

2–Amino–N–(2–iodo–4–(guanidinomethyl)phenyl)acetamide

¹H NMR (250 MHz, MeOD) δ 7.91 (d, *J* = 1.6 Hz, 1H), 7.57 (d, *J* = 8.2 Hz, 1H), 7.40 (dd, *J* = 8.2, 1.8 Hz, 1H), 4.42 (s, 2H), 3.99 (s, 2H).

2–(Fluorenylmethoxycarbonyl)amino–N–(5–((2,3–bis(*tert*-butoxycarbonyl)guanidino)methyl)–2–chlorophenyl)acetamide.

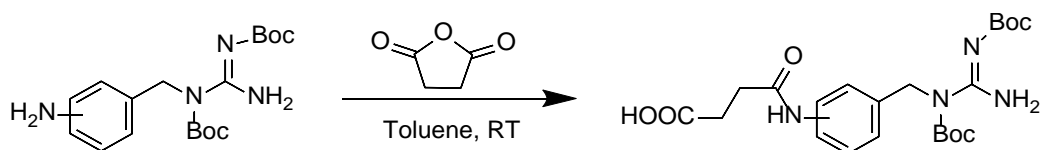
¹H NMR (250 MHz, CDCl₃) δ 8.52 (s, 1H), 8.22 (d, *J* = 8.2 Hz, 2H), 7.69 (d, *J* = 7.2 Hz, 2H), 7.52 (d, *J* = 7.0 Hz, 2H), 7.37 – 7.11 (m, 8H), 4.49 (d, *J* = 5.3 Hz, 2H), 4.43 (d, *J* = 6.7 Hz, 2H), 4.17 (t, *J* = 6.7 Hz, 1H), 3.97 (s, 2H), 1.44 (s, 9H), 1.41 (s, 9H). ¹³C NMR (63 MHz, CDCl₃) δ 163.6, 156.3, 153.3, 143.7 (2C), 141.5 (2C), 128.6, 127.9

(2C), 127.4, 127.2 (2C), 125.1 (2C), 123.4, 121.9, 120.2 (2C), 83.5, 79.7, 67.6, 47.2, 44.1, 28.40 (3C), 28.18 (3C).

2-Amino-N-(2-chloro-5-(guanidinomethyl)phenyl)acetamide

^1H NMR (250 MHz, MeOD) δ 7.91 (d, $J = 8.4$ Hz, 1H), 7.46 (d, $J = 1.8$ Hz, 1H), 7.30 (dd, $J = 8.4, 1.9$ Hz, 1H), 4.41 (s, 2H), 3.98 (s, 2H).

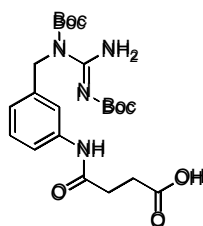
- Synthesis of analogues with terminal acid group for the synthesis of double agents DFS, DF and DFL.



Scheme S6 . Synthesis of succinic acid terminal derivate for the synthesis of DFS, DF and DFL.

A solution of succinic anhydride (1.1 Equivalent) in toluene (10 mL) was treated with a solution of the corresponding analogue (100 mg) also in toluene. The resulting mixture was stirred at room temperature overnight. When the reaction is complete (followed by TLC), the resulting powder was suspended in the mixture. The solid was filtered and washed with water (30 mL). The resulting powder was dried under reduced pressure at 40 ° C. The process was quantitative.

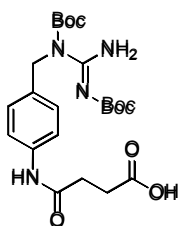
4-((3-((1,2-Bis(*tert*-butoxycarbonyl)guanidino)methyl)phenyl)amino)-4-oxobutanoic acid.



^1H NMR (250 MHz, CDCl_3) δ 9.38 and 9.08 (s, br, 2H, NH_2), 8.09 (s, 1H, NH, amide), 7.42 (d, $J = 7.8$ Hz, 1H, $\text{CH}_{(\text{Ar})}$), 7.16 – 7.08 (m, 2H, $\text{CH}_{(\text{Ar})}$), 6.80 (d, $J = 7.5$ Hz, 1H, $\text{CH}_{(\text{Ar})}$), 5.03 (s, 2H, CH_2), 2.67 – 2.51 (m, 4H, 2x CH_2 , succinic), 1.40 (s, 9H, 3x $\text{CH}_3(\text{BOC})$), 1.25 (s, 9H, 3x $\text{CH}_3(\text{BOC})$). ^{13}C NMR (63 MHz, CDCl_3) δ 175.85 (C=O), 175.56 (C=O), 170.56 (C=O), 154.95 (C=N), 143.16 (C=O), 139.56 (C_{Ar}), 137.99 (C_{Ar}),

128.99 (CH_{Ar}), 122.30 (CH_{Ar}), 118.60 (CH_{Ar}), 117.75 (CH_{Ar}), 84.75 (2xC_(BOC)), 47.62 (CH₂), 31.81 (CH₂, succinic), 29.51 (CH₂, succinic), 28.38 (3xCH₃(BOC)), 27.89 (3xCH₃(BOC)). FTIR (cm⁻¹): 3372; 3329; 2977; 2927; 1721; 1625; 1625; 1608; 1286; 1246; 1137; 1120; 981; 944; 615; 504. ESI(-): 462.9 m/z [M-1]

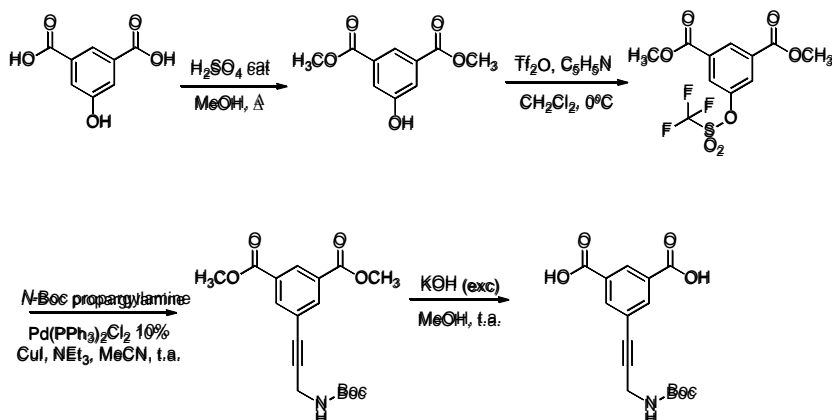
4-((4-((2,3-bis(tert-butoxycarbonyl)guanidino)methyl)phenyl)amino)-4-oxobutanoic acid



¹H NMR (250 MHz, CDCl₃) δ 11.56 (s, 1H, NH, amide), 8.62 (t, *J* = 4.1 Hz, 1H, NH, amide), 8.01 (s, 1H, NH, amide), 7.42 (d, *J* = 8.5 Hz, 2H, 2xCH_(Ar)), 7.17 (d, *J* = 8.0 Hz, 2H, 2xCH_(Ar)), 4.54 (d, *J* = 4.7 Hz, 2H, CH₂), 2.80 – 2.70 (m, 2H, CH₂, succinic), 2.69 – 2.59 (m, 2H, CH₂, succinic), 1.49 (s, 9H, 3xCH₃(BOC)), 1.48 (s, 9H, 3xCH₃(BOC)). ¹³C NMR (63 MHz, CDCl₃) δ 170.56 (C=O), 169.63 (C=O), 163.52 (C=N), 156.38 (C=O), 153.27 (C=O), 137.18 (C_{Ar}), 132.59 (C_{Ar}), 128.20(2xCH_{Ar}), 120.26 (2xCH_{Ar}), 83.55 (C_(BOC)), 79.84 (C_(BOC)), 56.50 (CH₂, guanidine), 31.94(CH₂), 29.74(CH₂), 28.37 (3xCH₃(BOC)), 28.18. (3xCH₃(BOC)). ESI(-): 463.1 m/z [M-1]

3.1 Synthesis of double targeting agents

- Synthesis of Rigid analogues DR.



Scheme S7. Synthesis of starting material for Double-Rigid analogues (DR).

To a solution of 25g of the commercial 5-hydroxyisophthalic acid in 100mL of MeOH, H₂SO₄ was added in catalytic amount. The mixture was then refluxed for 24 to complete the esterification. After the reaction, which was monitored by TLC, it was poured into 250mL of water, producing a precipitate; After filtration of the solid and drying, dimethyl the corresponding 6-hydroxyisophthalate was obtained with a 95% yield that was used without further purification. (*Tetrahedron Lett.*, 2007, 5899)

The transformation of the alcohol to triflate was carried out in an inert atmosphere starting from 840mg (4mmol) of the diester, which was dissolved in 15mL of anhydrous CH₂Cl₂ and at a temperature of 0 °C. Then, and following this order, 0.4 mL (5mmol, 1.2 equiv) of anhydrous pyridine and 4.4 mL of a 1.0M solution of (CF₃SO₂)₂O in CH₂Cl₂ were added to the reaction. The reaction was maintained under stirring for 24 h. After that, the following general processing method was applied: dilution with 100mL of AcOEt, washing with 2x50mL of a solution of NH₄Cl saturated in water, washing with 50mL of brine, drying over MgSO₄, filtration and evaporation in a rotary evaporator. Finally, the crude obtained was purified by SiO₂ column chromatography using Heptane / AcOEt (2: 1) as a mobile phase, obtaining 1.14g (83%)

Dimethyl 5- (trifluoromethanesulfonyloxy) isophthalate.

¹H NMR (250 MHz, CDCl₃) δ 8.71 (t, *J* = 1.4 Hz, 1H), 8.11 (d, *J* = 1.4 Hz, 2H), 3.99 (s, 6H). ¹³C NMR (63 MHz, CDCl₃) δ 164.5 (2C), 149.4, 133.2 (2C), 130.5, 126.5 (2C), 118.8 (q, *J* = 320.8 Hz) 53.1 (2C).

From 667 mg (1.95mmol) of the triflate, the Sonogashira reaction was carried out. For the specific case of the reaction with the commercial N-Boc-propargylamine, 455 mg (2.93mmol, 1.5equiv) of the alkyne, 133mg of the catalyst Pd(PPh₃)₂Cl₂ (0.19mmol, 0.1equiv), 36 mg of CuI (0.19) were used. mmol, 0.1equiv) and 430 μL of NEt₃ (3.12mmol, 1.6equiv) as base in 2mL of CH₃CN, under inert atmosphere and at for 16h. Once the reaction was finished and after applying the general processing method, the crude obtained was purified by SiO₂ column chromatography using Heptane / AcOEt (7: 3) as the mobile phase, obtaining the corresponding product.

Dimethyl 5-(3-(*N*-(*tert*-butoxycarbonyl)amino)propin-1-yl)isofthalate:

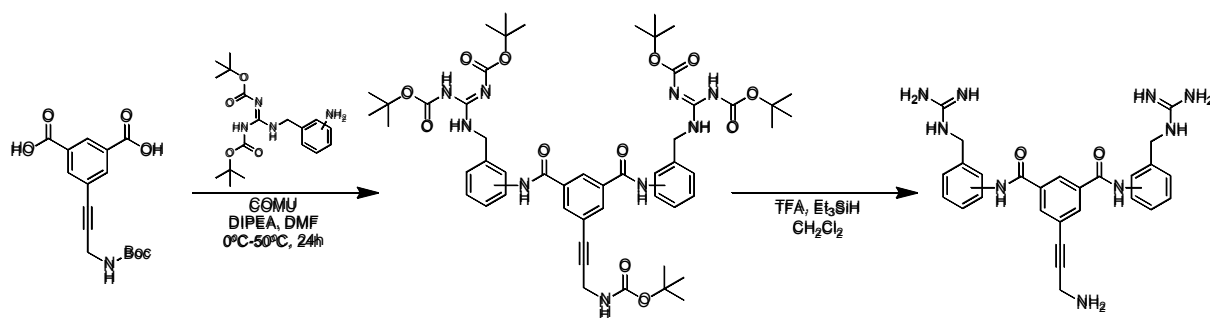
^1H NMR (250 MHz, CDCl_3) δ 8.56 (t, $J = 1.6$ Hz, 1H), 8.21 (d, $J = 1.6$ Hz, 2H), 4.90 (bs NH, 1H), 4.15 (d, $J = 5.5$ Hz, 2H), 3.92 (s, 6H), 1.45 (s, 9H). ^{13}C NMR (63 MHz, CDCl_3) δ 165.6 (2C), 155.4, 136.7 (2C), 131.0 (2C), 130.3, 123.9, 87.7, 81.2, 80.3, 52.6 (2C), 31.2, 28.5 (3C). MS (ESI+) Calc for: $\text{C}_{18}\text{H}_{22}\text{NO}_6$ ($\text{M}+\text{H}$) $^+$: 348.14, found: 348.1

Hydrolysis of the ester groups was carried out from 1.44mmol dissolved in 25mL of MeOH, on which 29mmol (20equiv) of KOH was added, the reaction was maintained for 6h at room temperature. After the reaction was complete, 50mL of H_2O was added and the solution was brought to pH = 5 with HCl. Then, after applying the general processing method, the different isophthalic acids (96-99%) were obtained, which were used for the next step without further purification.

5-(3-(N-(tert-butoxycarbonyl)amino)propin-1-yl)isoftalic acid

^1H NMR (250 MHz, Acetone- d_6) δ 8.63 (t, $J = 1.6$ Hz, 1H), 8.25 (d, $J = 1.6$ Hz, 2H), 6.57 (s, 1H), 4.18 (d, $J = 5.5$ Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (63 MHz, Acetone- d_6) δ 166.1 (2C), 156.28, 137.0 (2C), 132.5, 130.9 (2C), 125.0, 89.9, 80.7, 79.4, 31.2, 28.5(3C). MS (ESI+) Calc for $\text{C}_{16}\text{H}_{16}\text{NO}_6$ ($\text{M}-\text{H}^+$) $^-$ m/z = 318.10, found: 317.8

Coupling with single protected guanidines S3 or S4: Synthesis of DR3 or DR4, respectively.



Scheme S8. Coupling and deprotection with isophthalic acid derivative, synthesis of Double Rigid (DR) targeted ligands.

For the coupling reaction with Bis-(*tert*-butoxycarbonyl)guanidinobenzylanilines, 0.16mmol of the isophthalic diacid, 0.35mmol (2.2 equiv) of the corresponding protected aminobenzylmethylguanidine, 164mg (2.4 equiv) of COMU was used as coupling reagent and 83 μL of DIPEA (0.48 mmol, 3 equiv) as base. The reaction was carried out in anhydrous DMF, starting at 0°C followed by heating at 50°C for 18 h.

Once the reaction was finished and after applying the general processing method, the crude obtained was purified by SiO₂ column chromatography using Heptane/AcOEt (1:1) as mobile phase, obtaining the different compounds provided as mixtures of isomers. (Yield: 64–79%)

1,3-Bis-(3-(3-(1,2-Bis-(tert-butoxycarbonyl)guanidino)methyl)phenyl)-5-(3-(N-tert-butoxycarbonyl-3-amino)propin-1-yl) isophthalamide:

¹H NMR (250 MHz, CDCl₃) δ 9.65-9.38 (m, 4H), 9.06 (bs, 2H), 8.34 (bs, 1H), 8.01 (bs, 2H), 7.60 (d, *J* = 7.9 Hz, 2H), 7.47 (bs, 2H), 7.17 (t, *J* = 7.9 Hz, 2H), 6.83 (t, *J* = 8.7 Hz, 2H), 5.58 (bs, 1H), 5.18 – 4.88 (m, 4H), 4.16 (d, *J* = 4.9 Hz, 2H), 1.46 (s, 9H), 1.40 (s, 18H), 1.32 (s, 18H). ¹³C NMR (63 MHz, CDCl₃) δ 164.8, 163.9, 161.5, 155.3, 139.7, 138.7, 135.6, 133.8, 129.2, 122.6, 119.3, 118.1, 84.8, 79.9, 72.7, 70.7, 28.6, 28.2, 22.1. MS(TOF/TOF) Calc for: C₅₂H₆₉N₉O₁₂ (M+Na⁺): 1084.5114, found: 1084.687

1,3-Bis-(3-(4-(2,3-Bis(tert-butoxycarbonyl)guanidino)methyl)phenyl)-5-(3-(N-tert-butoxycarbonyl-3-amino)propin-1-yl)isophthalamide:

¹H NMR (250 MHz, CDCl₃) δ 11.50 (s, 2H), 9.00 (bs, 1H), 8.58 (bt, *J* = 5.2 Hz, 2H), 8.18 (bs, 1H), 7.87 (s, 2H), 7.52 (d, *J* = 8.4 Hz, 4H), 7.06 (d, *J* = 8.4 Hz, 4H), 5.37 (bs, 1H), 4.45 (d, *J* = 4.8 Hz, 4H), 4.24 – 4.13 (m, 1H), 4.01 (d, *J* = 5.3 Hz, 2H), 1.42 (s, 18H), 1.39 (s, 18H), 1.38 (s, 9H). ¹³C NMR (63 MHz, CDCl₃) δ 164.6, 163.5, 156.4, 153.3, 137.5, 135.1, 133.7, 133.4, 127.9, 123.8, 120.8, 83.5, 79.9, 28.5, 28.3, 28.2. MS(TOF/TOF) Calc for: C₅₂H₆₉N₉O₁₂ (M+Na⁺): 1084.5114, found: 1084.642

For the deprotection of the protective groups (Boc) of this family of compounds, 50mg of the protected compound was dissolved in a mixture composed of CF₃COOH, (iPr)₃SiH and CH₂Cl₂ (0.5: 1: 3mL) maintaining the stirring for 12 h. After that time, the reaction mixture was evaporated in a rotary evaporator, the obtained residue was triturated with Et₂O and the precipitate was filtered under vacuum; the solid obtained was used for the next step without further purification.

1,3-Bis - ((3-guanidinomethyl) phenyl) -5- (3-aminopropin-1-yl) isophthalamide

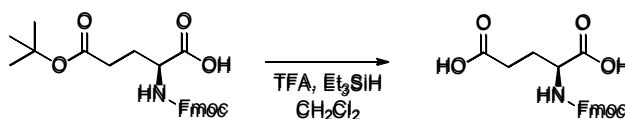
¹H NMR (250 MHz, MeOD) δ 8.56 (t, *J* = 1.7 Hz, 1H), 8.26 (d, *J* = 1.7 Hz, 2H), 7.83 (d, *J* = 1.6 Hz, 2H), 7.66 (dd, *J* = 8.2, 1.0 Hz, 2H), 7.43 (td, *J* = 7.9, 3.1 Hz, 2H), 7.18 (bd, *J* = 7.5 Hz, 2H), 4.47 (s, 4H), 4.12 (d, *J* = 2.3 Hz, 2H). ¹³C NMR (63 MHz, MeOD)

δ 166.8, 158.9, 158.8, 140.2, 138.6, 137.2, 134.9, 130.4, 128.4, 124.5, 123.9, 121.6, 120.9, 85.9, 83.5, 73.3, 71.1, 45.8. MS(TOF/TOF) Calc for: $C_{27}H_{29}N_9O_2$ ($M+H^+$): 512.2517, found: 512.453

1,3-Bis - ((4-guanidinomethyl) phenyl) -5- (3-aminopropin-1-yl) isophthalamide

1H NMR (250 MHz, MeOD) δ 8.56 (t, $J = 1.5$ Hz, 1H), 8.25 (d, $J = 1.6$ Hz, 2H), 7.79 (d, $J = 8.5$ Hz, 4H), 7.39 (d, $J = 8.6$ Hz, 4H), 4.43 (s, 4H), 4.13 (s, 2H). ^{13}C NMR (63 MHz, MeOD) δ 166.8, 158.7, 139.4, 137.3, 134.9, 134.0, 129.0, 128.3, 123.9, 122.4, 85.9, 83.5, 45.5. MS(TOF/TOF) Calc for: $C_{27}H_{29}N_9O_2$ ($M+H^+$): 512.2517, found: 512.425

- Synthesis of the Flexible ligands DFS, DF, DFL.
- ✓ Synthesis of DFS.

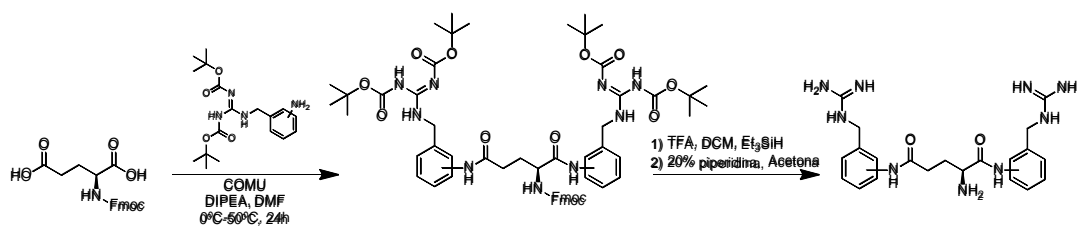


Scheme S9. Precursor preparation for DFS ligands.

From 1.5 g of the commercial amino acid Fmoc-Glu (OtBu) OH the deprotection of the tert-butyl group was carried out, dissolving it in one composed of CF₃COOH, (iPr)₃SiH and CH₂Cl₂ (3.0: 1.5: 7.0mL) maintaining the Stirring for 12 h, according to the procedure described. After that time, the reaction mixture was evaporated in a rotary evaporator, the obtained residue was triturated with Et₂O and the precipitate was filtered under vacuum; the solid obtained was used for the next step without further purification.

(9H-Fluoren-9-yl-methoxycarbonyl)-(L)-glutamic acid:

1H NMR (250 MHz, DMSO) δ 12.37 (bs, 2H), 7.89 (d, $J = 7.3$ Hz, 2H), 7.73 (d, $J = 7.0$ Hz, 2H), 7.42 (t, $J = 7.0$ Hz, 2H), 7.33 (dt, $J = 7.3, 3.7$ Hz, 2H), 4.34 – 4.15 (m, 3H), 3.99 (td, $J = 9.4, 4.7$ Hz, 1H), 3.45 (bs, NH), 2.32 (t, $J = 7.5$ Hz, 2H), 1.99 (td, $J = 12.7, 7.6$ Hz, 1H), 1.88 – 1.68 (m, 1H).



Scheme S10. Coupling with single guanidines S3 or S4: Synthesis of DFS3 or DFS4, respectively

The preparation Double Flexible Short bis-targeted guanidines was achieved by reacting 0.35 mmol (2.2 equiv) of corresponding Bis-(*tert*-butoxycarbonyl)aminobenzylguanidine, with 59.1 mg (0.16 mmol, 1.0 equiv) of the Fmoc protected glutamic acid, 164 mg (2.4 equiv) of the coupling reagent COMU and 83 μ L of DIPEA (0.48 mmol, 3 equiv) as base. The reaction was carried out in anhydrous DMF, starting at 0°C followed by heating at 50°C for 24 h. Once the reaction was finished and after applying the general processing method, the crude obtained was purified by SiO₂ column chromatography using Heptane / AcOEt (1:1) as mobile phase, obtaining the different expected compounds as complex isomers mixtures.

Then, the deprotection of the protective groups (Boc) of this family of compounds was dissolved 50 mg of the protected compound in a mixture composed of CF₃COOH, (iPr)₃SiH and CH₂Cl₂ (0.5: 1: 3 mL) maintaining the stirring for 12 h. After that time, the reaction mixture was evaporated in a rotary evaporator, the obtained residue was triturated with Et₂O and the precipitate was filtered under vacuum; the solid obtained was used for the next step without further purification. For the deprotection of the Fmoc protecting group in this series, the Boc-deprotected compound was split in a mixture composed of 20% piperidine in DMF at room temperature for 2 h. After the partial evaporation of the solvent and applying the general processing method, the crude obtained was purified by Sephadex G-25 size exclusion column chromatography, using H₂O as eluent; then, after lyophilization, the different compounds provided completely unprotected were obtained.

(S)-2-amino-1,5-bis(3-(guanidinomethylphenyl)pentanodiamide. (Main isomer)

¹H NMR (250 MHz, MeOD) δ 7.73–7.62 (m, 1H), 7.57 – 7.29 (m, 4H), 7.23–7.17 (m, 1H), 7.08 (t, *J* = 7.0 Hz, 1H), 4.49–4.36 (m, 4H), 2.72–1.82 (m, 4H), 1.80 – 1.12 (m, 5H). ¹³C NMR (63 MHz, MeOD) δ 181.6, 176.9, 173.8, 163.4, 162.9, 158.8, 140.5,

140.1, 139.5, 138.5, 137.5, 131.0, 130.4, 129.6, 129.0, 128.3, 124.2, 123.8, 120.6, 120.0, 115.9, 59.0, 58.3, 45.9, 45.4, 34.2, 30.5, 28.7, 26.6, 24.5.

(S)-2-amino-1,5-bis(4-(guanidinomethylphenyl)pentanediamide. (Main isomer):

¹H NMR (250 MHz, MeOD) δ 7.55–7.42 (m, 2H), 7.40 – 7.29 (m, 2H), 7.23–7.06 (m, 4H), 4.43–4.19 (m, 4H), 2.71–1.72 (m, 4H), 1.71 – 1.11 (m, 5H). ¹³C NMR (63 MHz, MeOD) δ 176.9, 173.7, 163.4, 162.8, 158.8, 158.6, 139.8, 138.4, 136.6, 133.1, 130.7, 129.3, 128.9 (2C), 128.9 (2C), 121.5 (2C), 120.5 (2C), 58.9, 58.3, 46.0, 45.6, 45.4, 34.2, 28.6, 26.6, 24.3, 23.5.

✓ Synthesis of Double-Flexible (DF), Double-Flexible-Long (DFL).

For affording the ligands compounds DF and DFL main structure, peptide solid phase synthesis have been assumed as best strategy. For this aim, classic approximation of Fmoc protection /coupling methodology have been applied using commercialized Wang Resin and protected aminoacids or small molecules needed for each case. The protocol concerns the multi-step functionalization and coupling on the resin with the molecules needed in order to build the desired ligand structure. Finally the coupling of the targeting agents has to be done before the release and purification steps.

The general methodology of each type of reactions in solid phase is described below;

I Mtt deprotection: The resin was suspended in a solution of TFA (1%) in DCM. The suspension mixture was shaken in the solid phase reactor by wrist-shaker for 2h. The resin was then filtered and washed thoroughly with DMF and took to the next step.

II Amino group Fmoc deprotection: A solution of piperidine (20%) in DMF was added to the peptide functionalized resin. The suspension mixture was shaken in the solid phase reactor by wrist-shaker overnight. The obtained solid was filtered and washed with DMF.

III) Peptide amide bond formation: A solution of HOBt (3 eq), HBTU (3 eq) and the corresponding amino acid (2 eq) in DMF (2 mL) was added to the washed resin, and after slight shaking DIPEA (6 eq) was finally added. The mixture was shaken in the reactor with a wrist-shaker overnight. The obtained solid was filtered and washed with DMF.

IV) Final peptide release from the resin: Once the last amino acid of the chain was added, the solid was filtered, washed with DMF and dried under vacuum. A solution of trifluoroacetic acid (95%), triisopropylsilane (2.5%) and water (2.5%) was added dropwise. The mixture was stirred in wrist-shaker for 4 hours, then filtered and washed with one milliliter of the same previous mixture. Both filtered solutions were then mixed.

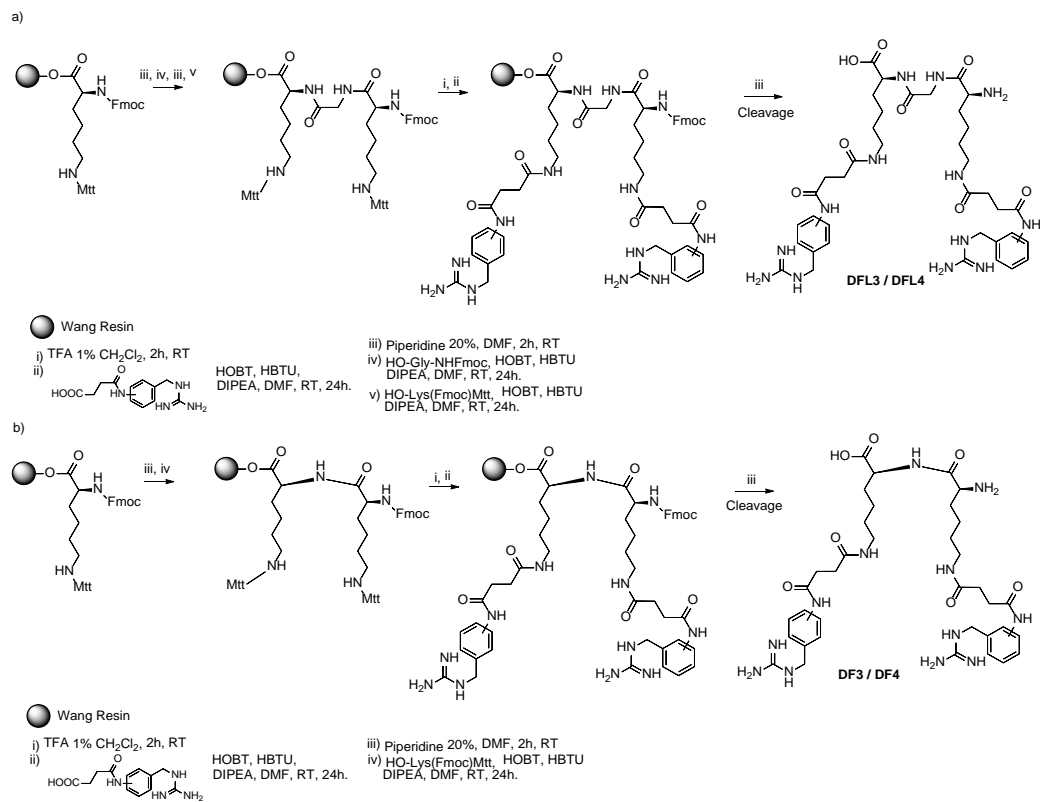
The product was obtained in all cases by precipitation of the filtered solution with cold ether. The product was then added dropwise to cold ether. After one hour, the suspension was centrifuged at $T = 4\text{ }^{\circ}\text{C}$; 14000 r.p.m. The obtained solid was dried overnight in a vacuum, at room temperature.

V) Final peptide-like ligand isolation by chromatography: The obtained solid was dissolved in the minimum amount of water and purified by flash column for molecular exclusion chromatography (stationary phase: Sephadex® G-25; mobile phase: water). All phases were then frozen at $-80\text{ }^{\circ}\text{C}$ and lyophilized.

The preparation of ligands was accomplished from the the guanidine analogues with bearing a terminal carboxylic group modification which were coupled with the orthogonal-protected Lysine-modified Wang's resin: [*Wang*]-Lys(Fmoc)Mtt.

Briefly, the preparation of DF ligands started from the [*Wang*]-Lys(Fmoc)Mtt resin, which was subjected to Mtt deprotection followed by amide coupling with the guanidine analogue. Then, after Fmoc cleavage a second HO-Lys(Fmoc)Mtt unit was incorporated to the growing sequence; followed by deprotection of Mtt group, coupling with a second guanidine fragment and Fmoc deprotection. Finally, following the peptide cleavage and purification protocol, were obtained and isolated both DF ligands.

Similarly to the preparation of double guanidine ligands, there were also prepared a series of hybrids analogues containing either triphenylphosphine (TPP), moieties together with an aminobenzylguanidine analogue.



Scheme S11. Solid phase synthesis of DFL a) and DF b) ligands.

DF-3

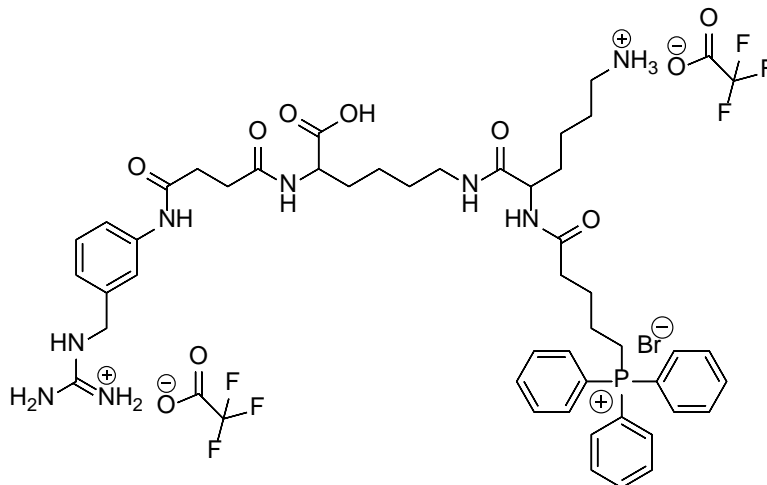
TOF/TOF m/z: 767 [M⁺+1] (100%);

DF-4

TOF/TOF m/z: 767 [M⁺+1] (100%);

Similarly to the preparation of double guanidine ligands, there were also prepared a series of hybrids analogues containing either triphenylphosphine (TPP and RGD)

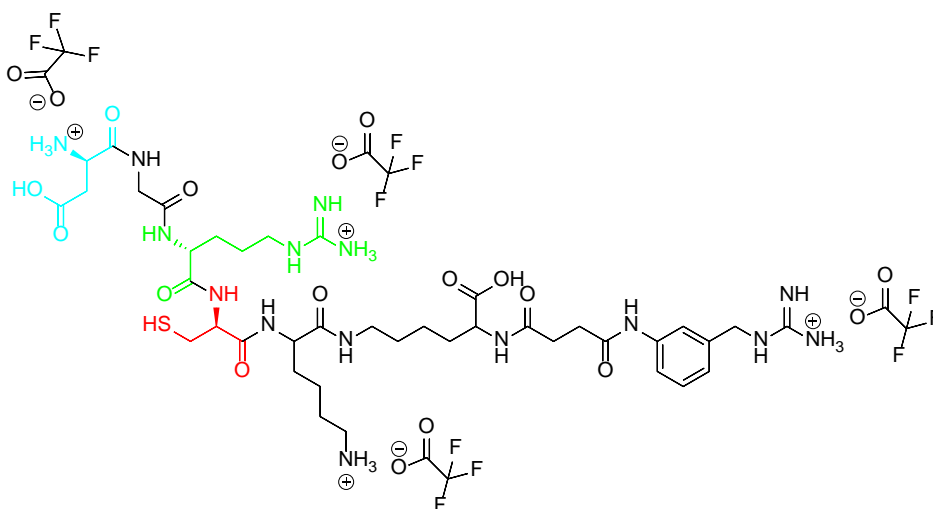
DFS3-TPP



Scheme S12. Structure of S3-TPP DF ligand.

^1H NMR (250 MHz, D_2O) δ 7.88 – 7.54 (m, 15H, CH_a , TPP), 7.47 – 7.20 (m, 3H, $3\times\text{CH}_{\text{ar}}$, benzylguanidine), 7.19 – 7.01 (m, 1H, CH_{ar} , benzylguanidine), 4.36 (d, $J = 18.2$ Hz, 2H, CH_2 , benzylguanidine), 4.19 – 3.99 (m, 2H, $2\times\text{CH}$, Lys), 3.37 – 3.13 (m, 4H, $2\times\text{CH}_2$, Lys), 2.65 (d, $J = 4.9$ Hz, 4H, $2\times\text{CH}_2$, succinic), 2.26 (t, $J = 5.5$ Hz, 2H, $\text{CH}_2\text{-P}$), 1.85 – 1.47 (m, 10H, $5\times\text{CH}_2$), 1.48 – 1.11 (m, 8H, $4\times\text{CH}_2$). TOF/TOF z/m ; 865 [$\text{M}^+ + 1$] (68 %); 1167 [$\text{M} + \text{Br} + 2\times\text{TFA}$] (100%).

DFS3-RGD



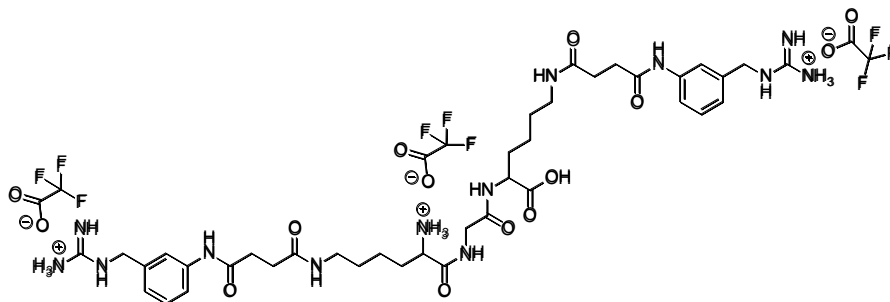
Scheme S13. Structure of S3-RGA DF ligand.

^1H NMR (250 MHz, D_2O) δ 7.37 – 7.19 (m, 3H, CH_{ar}), 7.10 – 7.00 (m, 1H, CH_{ar}), CH, Asp coupled with water signal, 4.32 (s, 2H, CH_2 , benzylguanidine), 4.10 – 3.99 (m, 2H, 2xCH, Lys), 3.75 (s, 1H, CH, Arg), 3.66 – 3.50 (m, 2H, CH_2 , Gly), 3.49 – 3.40 (m, 1H, CH, Cys), 3.17 – 3.00 (m, 2H, CH_2 , Arg), 2.95 – 2.73 (m, 6H, 2x CH_2 , Cys, Asp,), 2.60 (s, 4H, 2x CH_2 , succinic), 1.79 – 1.37 (m, 10H, 3x CH_2 , Lys, 2x CH_2 , Arg), 1.34 – 0.98 (m, 6H, 3x CH_2 , Lys). TOF/TOF z/m ; 865 [M^+ -Fmoc-NH- $\text{C}_2\text{H}_3\text{COOH}$ aspartic] (100%); 976 [M^+ -Asp+TFA] (51%); 1041 [M^+ -SH] (22%).

✓ Synthesis of Double Large Flexible analogues DFL3 and DFL4.

Synthesis of DFL3 and DFL4 analogues were carried out following the general method of solid phase Fmoc/coupling strategy.

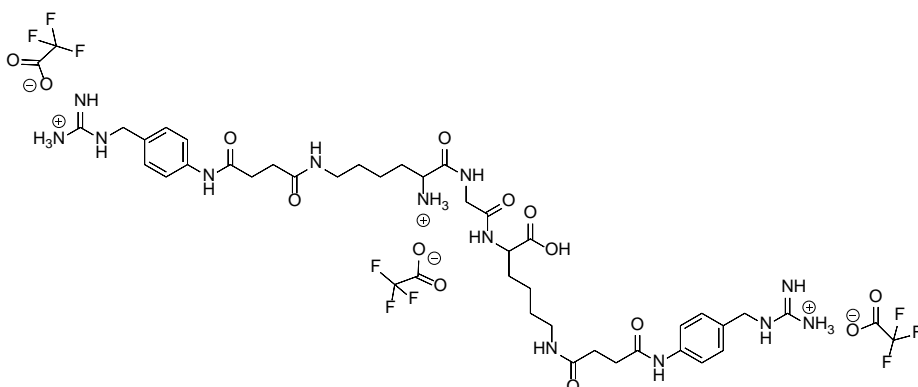
DFL3



Scheme S14. Structure of DFL3 ligand.

^1H NMR (250 MHz, D_2O) δ 7.41 – 7.11 (m, 6H), 7.04 (d, $J = 7.3$ Hz, 2H), 4.35 – 4.13 (m, 6H), 2.78 (t, $J = 7.5$ Hz, 4H), 2.59 (d, $J = 4.4$ Hz, 8H), 1.86 – 1.63 (m, 4H), 1.53 (dt, $J = 15.0, 5.6$ Hz, 6H), 1.40 – 1.21 (m, 2H). TOF/TOF z/m ; 826 [M^+ +3 H^+]

DFL4

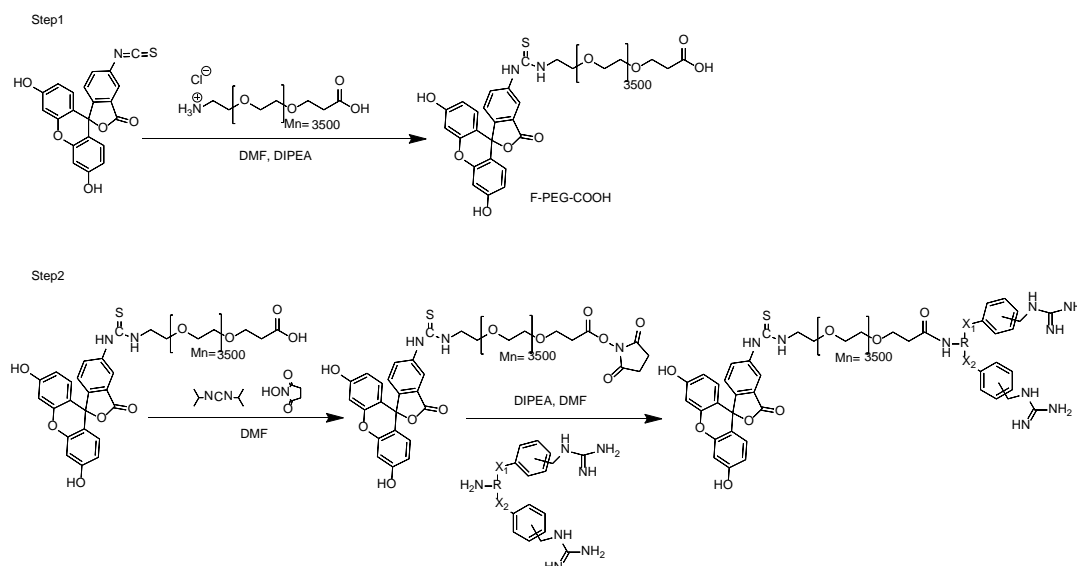


Scheme S15. Structure of DFL4 ligand.

^1H NMR (250 MHz, D_2O) δ 7.40 (d, J = 8.5 Hz, 4H), 7.31 (d, J = 8.4 Hz, 4H), 4.37 (s, 4H), 4.25 (d, J = 4.7 Hz, 2H), 2.90 (d, J = 7.7 Hz, 4H), 2.67 (d, J = 5.4 Hz, 8H), 1.81 (d, J = 4.7 Hz, 6H), 1.61 (s, 4H), 1.39 (d, J = 7.3 Hz, 2H). TOF/TOF z/m ; 826 [$\text{M}^+ + 3\text{H}^+$]

Preparation of conjugate compounds with linker and nanoparticle anchoring

3.1 General methodology for fluorescent labeling for conjugates with linker.



Scheme S16. General methodology of fluorescent labeling of analogues.

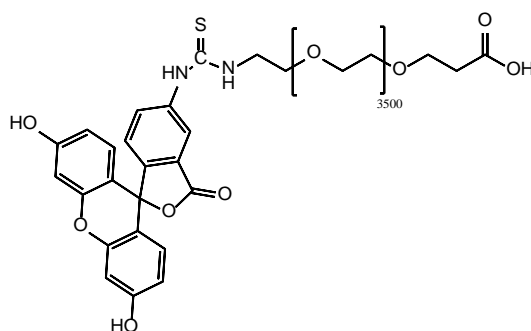
The evaluation of prepared targeting ligands was performed after their incorporation onto a fluorescent PEG chain. This modification would allow to sort ligands by affinity, as the great molecular weight of the PEG hybrids will force the same uptake mechanism in all examples. For such modification a commercial amino-acid bifunctional

polyethylene glycol (PEG) of about 3500 Da was first reacted with fluorescein isothiocyanate (FITC) throughout its amino group. Then, after purification, the carboxylic group present was transformed into an activated *N*-hydroxysuccinimide ester, suitable for anchoring all prepared ligands.

For the fluorescent labeling of the ligand compounds, a polyethylene glycol (PEG) of approximately 3500 Da previously functionalized at the end of the chain with an amino and an acid group at the other, was reacted with fluorescein isothiocyanate (FITC), forming a thiourea bond.

In a second step, the already labeled PEG derivative binds with the analogue under study, through the formation of an amide bond between the amine of the derivative to the study and the *N*-hydroxysuccinimide ester of the fluorescein-labeled PEG. All this following synthesis scheme below.

Step 1 , F-PEG-COOH



Scheme S17. Structure of F-PEG-COOH.

A solution of *O*-(2-Aminoethyl)-*O'*-(2-carboxyethyl) polyethylene glycol hydrochloride (3500 Da) (50 mg in 2 mL of DMF) was treated with 18 μ L of diisopropylethylamine. The mixture was stirred at room temperature for one hour in a nitrogen atmosphere. After that time, a solution of fluorescein isothiocyanate (65 mg in 1 mL of DMF) was added to the mixture slowly. The reaction mixture was allowed to stir overnight at room temperature. The final product was obtained by precipitation in cold ether and subsequent purification by molecular exclusion column (G-25 Sedaphex in water)

^1H NMR (250 MHz, D_2O) δ 7.71-7.50 (m, 2H, CH_{Ar} , Fluorescein), 7.31 – 7.05 (m, 3H, CH_{Ar} , Fluorescein), 6.57 (m, 4H, CH_{Ar} , Fluorescein), 3.82 (s, broad, 2H, CH_2 , PEG),

3.79 –3.36 (s, broad, 248 H, CH₂, PEG), 3.29 (s, broad, 2H, CH₂, PEG-CH₂-COOH).TOF/TOF m/z: 3859 (100%).

Step 2; Acid activation and ligand compound coupling ; An equivalent of F-PEG-COOH was dissolved in 2mL of DMF together with 1.5 equivalents of N-hydroxysuccinimide (NHS). The mixture in an inert atmosphere was stirred together with 6 equivalents of diisopropylcarbodiimide (DIC) and 6 equivalents of diisopropylethylamine (DIPEA) for 4 hours.

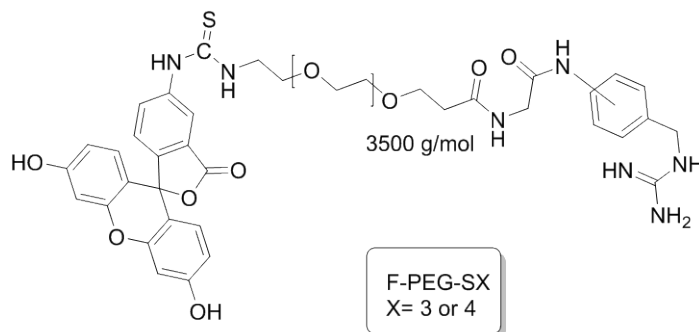
The corresponding analogue with the available amino group (1.5 equivalents) is dissolved in 1 mL of DMF and activated by adding 6 equivalents of DIPEA in an inert atmosphere.

The solution containing the analogue is added to that containing the activated acid slowly, the final mixture is allowed to stir at room temperature overnight.

The final product was obtained by precipitation in cold ether and subsequent purification by molecular exclusion column (G-25 Sedaphex in water).

Following the method described above derivate F-PEG-S3, F-PEG-S4, F-PEG-SI and F-PEG-SI with their deprotected compounds with amine terminal group.

F-PEG-S3



Scheme S17. Structure of F-PEG-COOH.

F-PEG-S3

¹H NMR (250 MHz, Acetone) δ 11.95 (s, NH, broad), 10.23 (s, OH, broad), 10.07 (s, OH, broad), 9.77 (s, NH, broad), 8.79 (s, 1H, NH), 8.25 (s, 1H, NH), 7.77 (s, 1H, CH_{ar}, benzylguanidine), 7.64 (dd, *J* = 2.4, 1.6 Hz, 1H, CH_{ar}, benzylguanidine), 7.31-7.22 (m, 1H, CH_{ar}, benzylguanidine), 7.15 – 7.04 (m, 3H, 3CH_{ar}, 2 x fluorescein, 1 x benzylguanidine), 6.82-6.73 (m, 3H, CH_{ar}, fluorescein), 6.72 – 6.61 (m, 4H, 4xCH_{ar},

fluoresceín), 5.36 (d, broad, $J = 4.6$ Hz, 2H, CH₂, benzylguanidine), 4.44 (s, broad, 2H, CH₂, glycine), 3.64 (s, broad, 360H, PEG). TOF/TOF z/m : 4122 [M].

F-PEG-S4

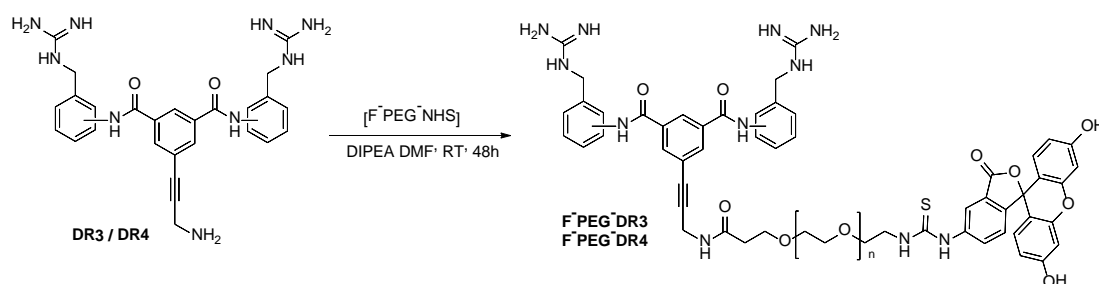
¹H NMR (250 MHz, , Acetone) δ 11.95 (s, NH, broad), 10.23 (s, OH, broad), 10.07 (s, OH, broad), 9.77 (s, NH, broad), 8.79 (s, 1H, NH), 8.25 (s, 1H, NH), 7.67 (s, broad, 2H, CH_{ar}, benzylguanidine), 7.42 (s, broad, 1H, CH_{ar}, benzylguanidine), 7.20 – 7.11 (m, 3H, 3CH_{ar}, 2 x fluorescein), 6.81-6.75 (m, 3H, CH_{ar}, fluorescein), 6.69 – 6.59 (m, 4H, 4xCH_{ar}, fluoresceín), 5.51 (s, broad, CH₂, benzylguanidine), 4.44 (s, broad, 2H, CH₂, glycine), 3.66 (s, broad, 360H, PEG). TOF/TOF z/m : 4111 [M]

F-PEG-SI

¹H NMR (250 MHz, D₂O) δ 7.82 (br. s, 1H), 7.60 (br. s, 1H) 7.59-7.49 (m, 3H), 7.32-7.19 (m, 6H), 6.81 (s, 1H), 6.67 – 6.62 (m, 6H), 4.26 (s, 2H), 3.76 (s, 2H), 3.63 (br. s, >200 H). MS (TOF/TOF) m/z : 4312.872

F-PEG-SI

¹H NMR (250 MHz, D₂O) δ 7.62 (br. s, 1H), 7.59-7.49 (m, 4H), 7.22-6.98 (m, 6H), 7.13 6.79 (s, 1H), 6.61 – 6.52 (m, 6H), 4.12 (s, 2H), 3.69 (s, 2H), 3.64 (br. s, >200 H).



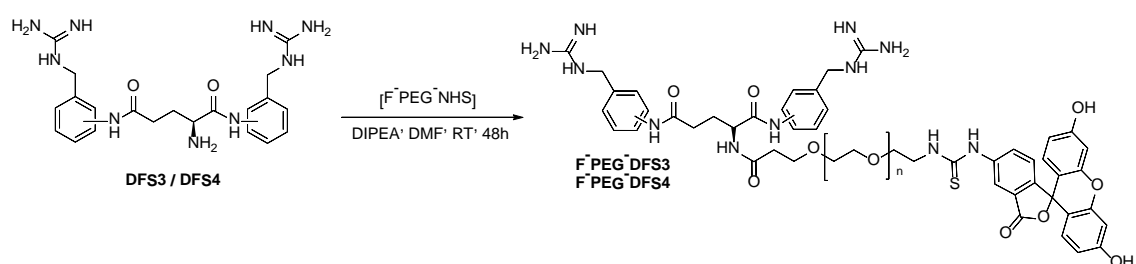
Scheme S18: Coupling of Double Rigid Ligands with Fluorescent PEG.

F-PEG-DR3

¹H NMR (250 MHz, MeOD) δ 8.54 (t, $J = 1.6$ Hz, 1H), 8.16 (d, $J = 1.6$ Hz, 2H), 8.08 – 7.98 (m, 1H), 7.76 (br. s, 2H), 7.64 (br. d, $J = 8.9$ Hz, 2H), 7.62 – 7.56 (m, 4H), 7.36 (t, $J = 7.8$ Hz, 1H), 7.19 (dd, $J = 5.7, 3.0$ Hz, 1H), 7.10 (d, $J = 7.7$ Hz, 1H), 6.97 (d, $J = 8.9$ Hz, 4H), 6.52-6.49 (br. s, 6H), 6.47-6.46 (m, 2H), 4.40 (s, 4H), 3.94 – 3.88 (m, 4H), 3.64 (s, CH₂). MS (TOF/TOF) m/z : 4661.439

F-PEG-DR4

^1H NMR (250 MHz, MeOD) δ 8.53 (t, $J = 1.6$ Hz, 1H), 8.19 (d, $J = 1.6$ Hz, 2H), 8.08 – 8.03 (m, 1H), 7.78 (d, $J = 8.5$ Hz, 8H), 7.64 (dd, $J = 5.8, 3.1$ Hz, 8H), 7.39-7.30 (m, 6H), 7.26 – 7.20 (m, 3H), 7.00 (d, $J = 9.4$ Hz, 8H), 6.57 (br. s, 6H), 6.55-6.52 (m, 2H), 4.40 (s, 4H), 3.98 – 3.90 (m, 4H), 3.66 (s, CH_2). MS (TOF/TOF) m/z : 4559.085



Scheme S19: Fluorescein coupling to DFS analogues.

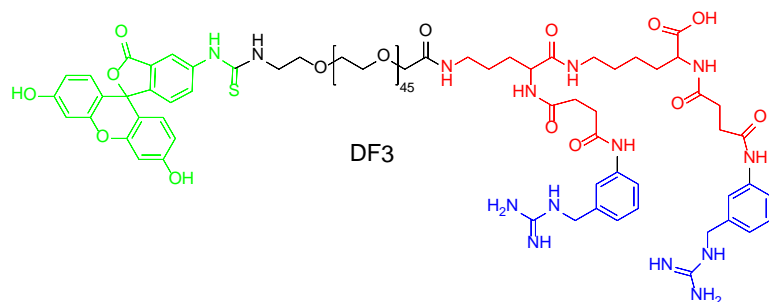
F-PEG-DFS3

^1H NMR (250 MHz, D_2O) δ 7.70 (s, 1H), 7.50 (br. s, 2H), 7.40 – 6.88 (m, 6H), 6.58-6.37 (s, 4H), 4.89 (m, 1H), 4.31 (s, 4H), 4.10 (s, 4H), 3.58 (s, $>200\text{H}$), 2.60-2.30 (m, 4H), 2.19-2.01 (m, 2H). MS (TOF/TOF) m/z : 4658.223

F-PEG-DFS4

^1H NMR (250 MHz, D_2O) δ 7.70 (s, 1H), 7.43 – 6.86 (m, 8H), 6.55-6.36 (m, 4H), 4.88 (m, 1H), 4.30 (s, 4H), 4.12 (s, 4H), 3.56 (s, $>200\text{H}$), 2.58-2.29 (m, 4H), 2.21-2.00 (m, 2H). MS (TOF/TOF) m/z : 4525.381

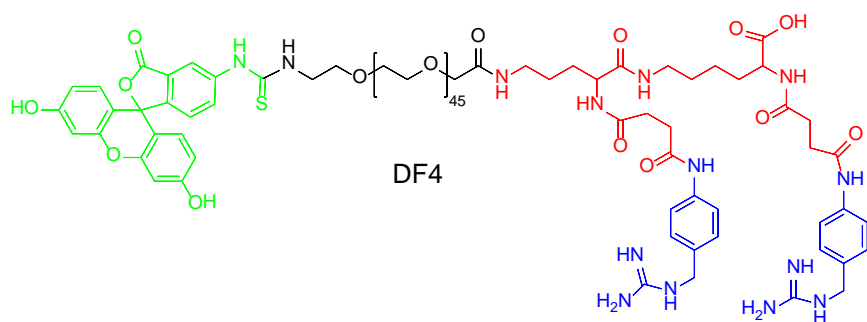
F-PEG-DF3



Scheme S20: F-PEG-DF3 structure.

TOF/TOF m/z; 4325 [M]

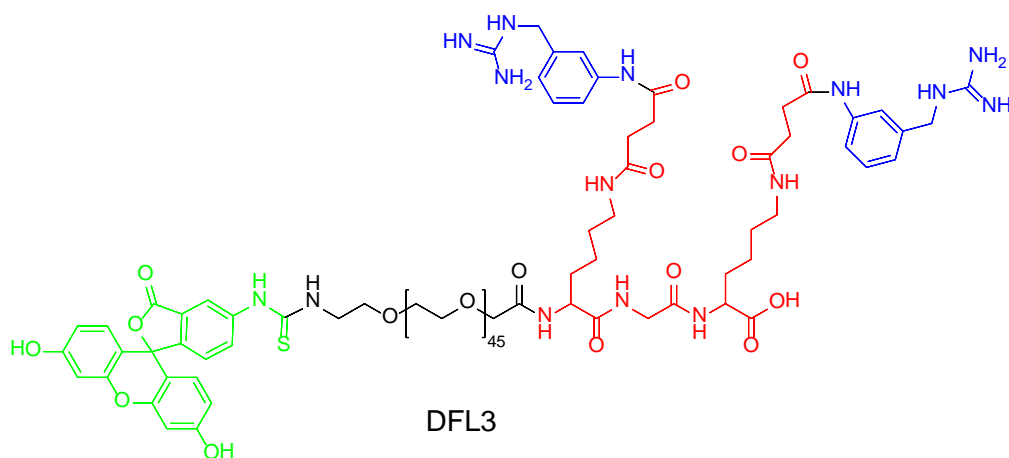
F-PEG-DF4



Scheme S21: F-PEG-DF4 structure.

TOF/TOF m/z; 4551 [M]

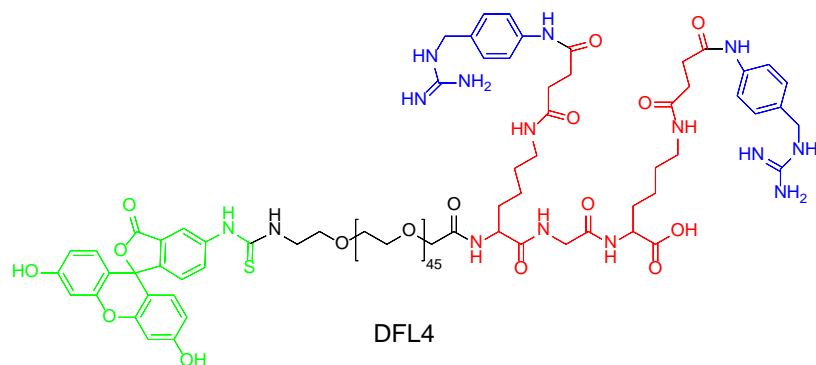
F-PEG-DFL3



Scheme S22: F-PEG-DFL3 structure.

TOF/TOF m/z; 4510 [M]

F-PEG-DFL4

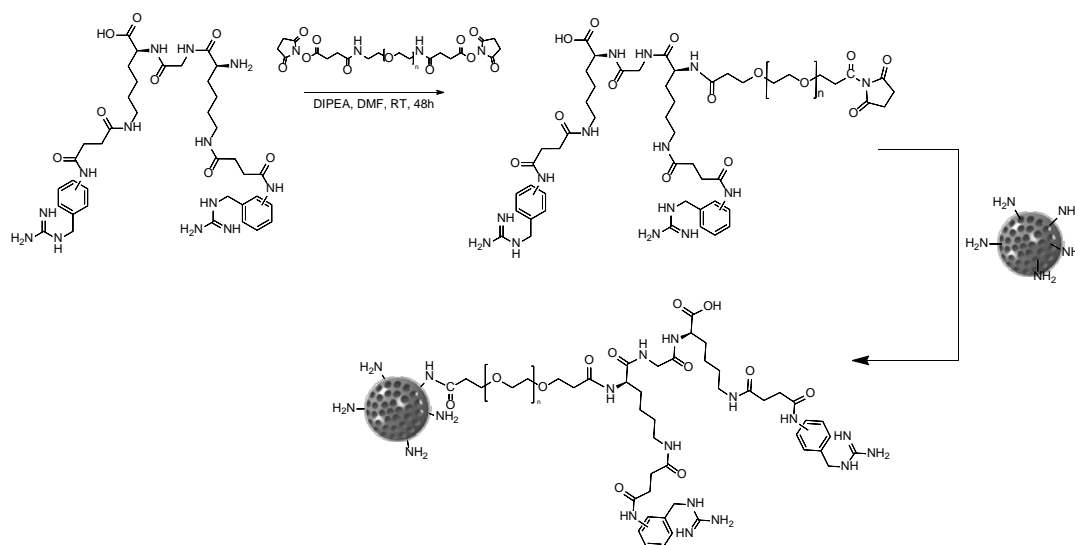


Scheme S22: F-PEG-DFL4 structure.

TOF/TOF m/z; 4551 [M]

4. General methodology for grafting scaffolds ligands with linker on nanoparticle surface.

In order to study the ability of these targeting systems to transport drugs or therapeutic nanoparticles selectively to the neuroblastoma tumor environment, mesoporous silica nanoparticles were synthesized, as a model nanocarrier, labeled with a fluorophore compatible with the *in vivo* study by fluorescence, in this case Cy7.



Scheme S23: Ligand PEGylation and grafting on silica mesoporous nanoparticle's surface.

For this aim nanoparticles were functionalized with primary amine group. On the other hand, ligand compounds were functionalized with *bis*-NHS acid PEG chain in one of the end, keeping the other extreme for anchoring on the nanoparticle surface. The materials were characterized by DLS and Z-Pot, and FTIR.

	Z-Pot (eV)	RSD %	Size (nm)	SD
NP-NH ₂	17.0	4.49	135.0	12.9
NP-PEG-DFL3	18.8	2.03	164.0	5.66
NP-PEG-DFL4	16.5	1.55	255.0	5.88

Table S1. Characterization of Nanoparticles for “*in vivo*” assays.

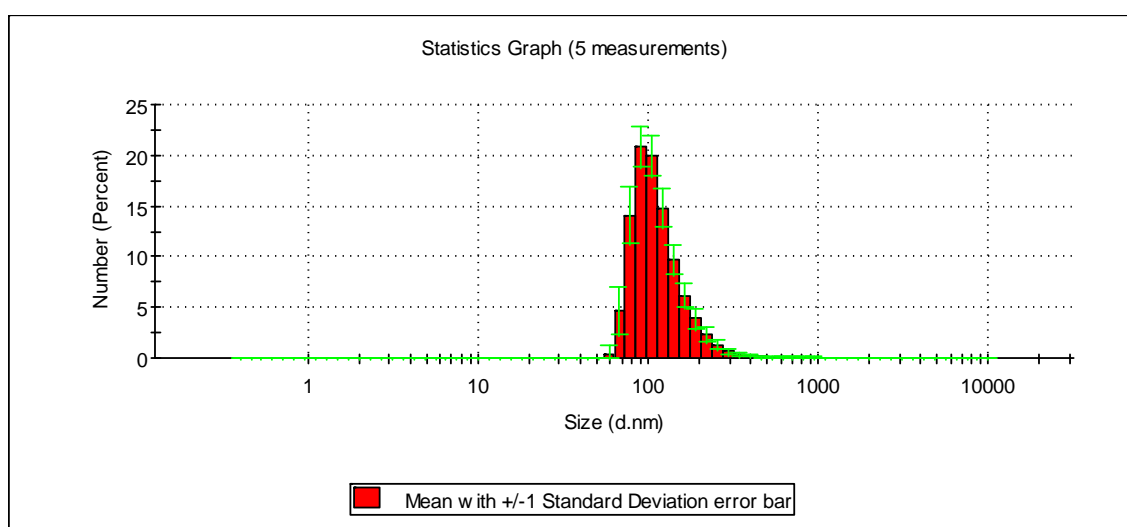


Figure S1. NP-NH₂ Size statistics graph.

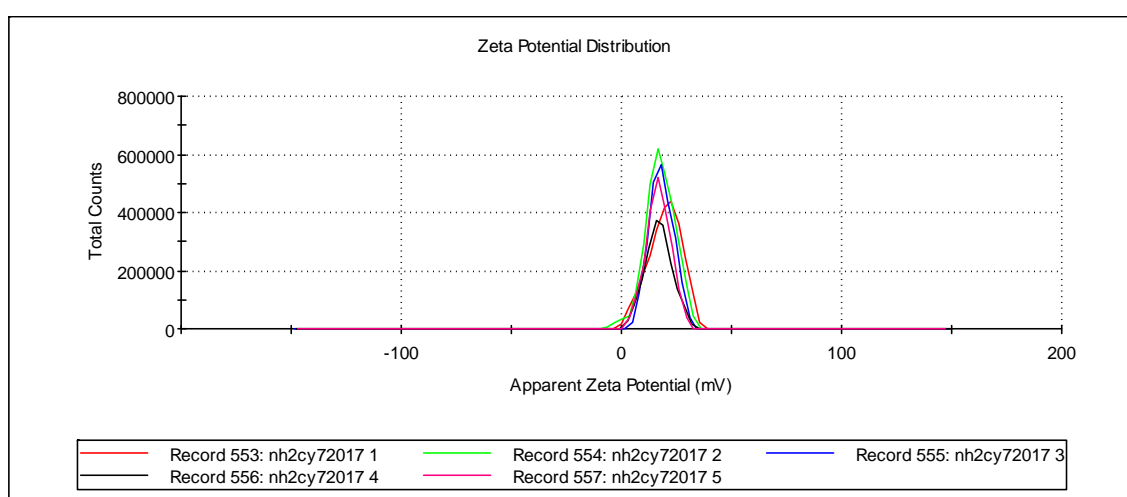


Figure S2. NP-NH₂ Zeta Potential distribution.

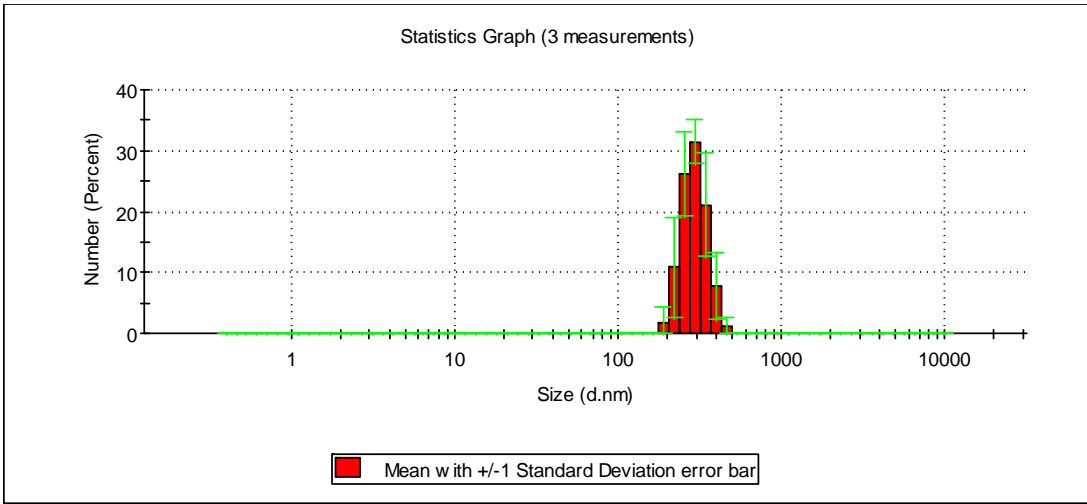


Figure S3. NP-PEG-DFL4, Size statistics graph.

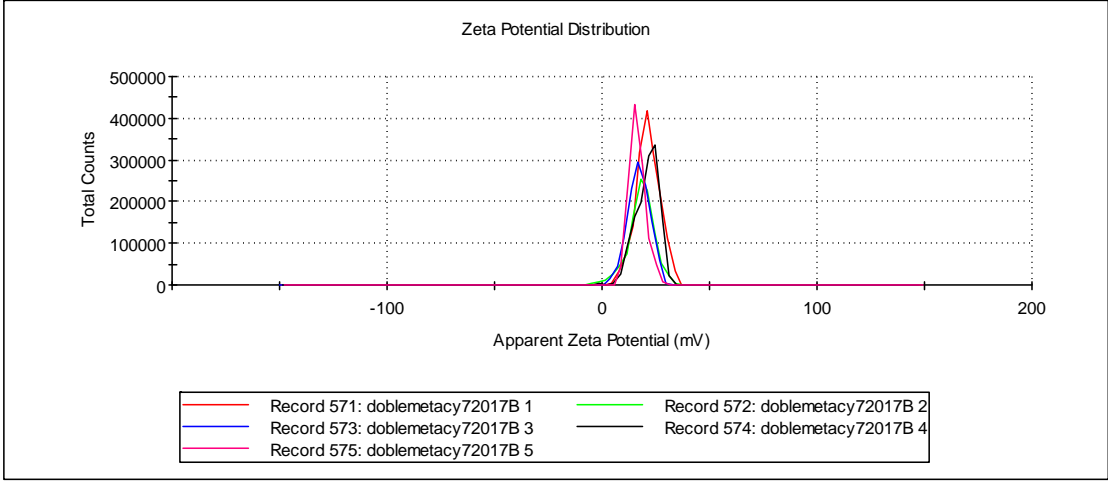


Figure S4. NP-PEG-DFL4, Zeta Potential distribution.

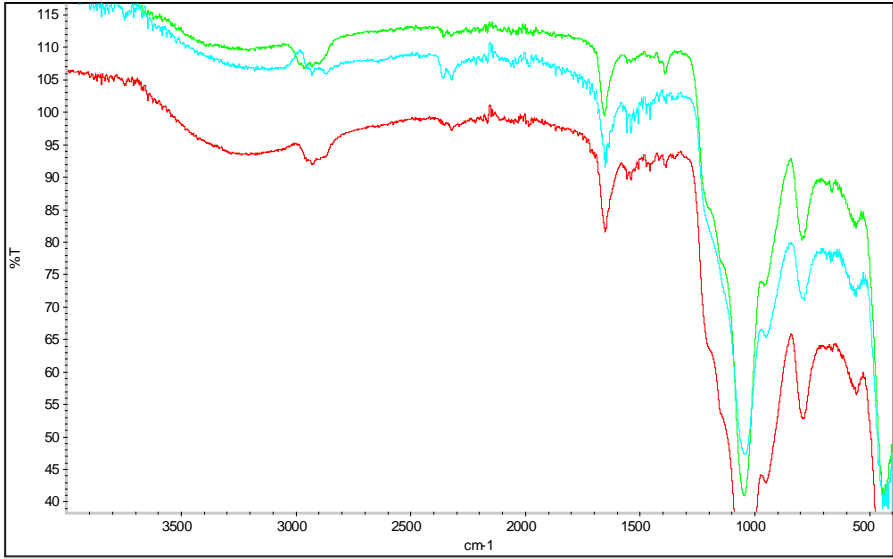


Figure S5. FTIR of NP-NH2 (Green), NP-PEG-DLF3 (blue), NP-PEG-DFL4 (red).

5. in vitro cellular uptake

The *in vitro* cellular uptake was evaluated in neuroblastoma cells by attaching a fluorophore (fluorescein in this case) to the corresponding ligand. Bidimensional cell cultures of neuroblastoma cells (NB 1691-luc) were incubated two hours in the presence of different concentrations of the marked ligand compound (from 50 $\mu\text{g mL}^{-1}$ to 6.25 $\mu\text{g mL}^{-1}$). The cellular uptake was measured by flow cytometry observing the percentage of cells showing fluorescence at the wavelength of fluorescein ($\lambda_{\text{ex}} = 492 \text{ nm}$ and $\lambda_{\text{em}} = 520 \text{ nm}$), as well as the average of intensity per cell.

An increased cellular uptake in neuroblastoma cells was observed for both *meta* (DFS3, DF3, DLF3, DR3) and *para* (DFS4, DF4, DFL4, DR4) conjugates compared to the prior art compound S3 according to previous work.^[1] At 50 $\mu\text{g mL}^{-1}$, the cellular uptake for most of the conjugates was so high that they could not be distinguished. Therefore, some of the measurements were repeated at lower conjugate concentrations (25 $\mu\text{g mL}^{-1}$, 12.5 $\mu\text{g mL}^{-1}$, and 6.25 $\mu\text{g mL}^{-1}$).

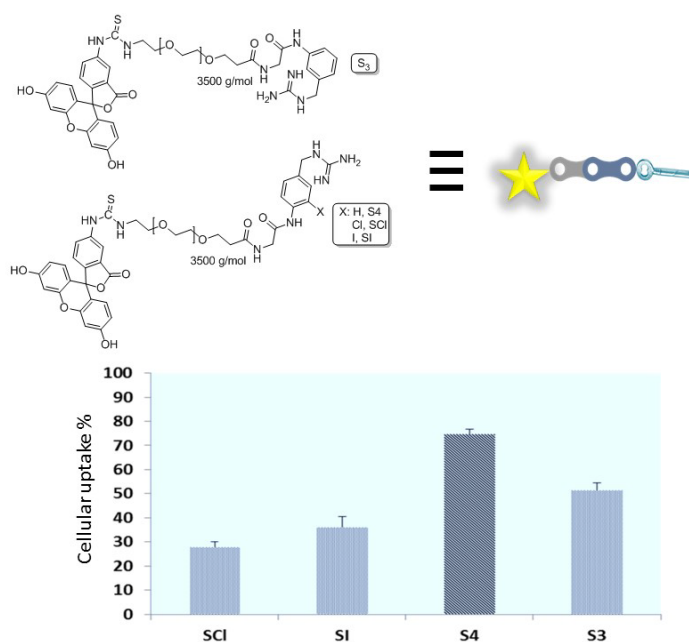
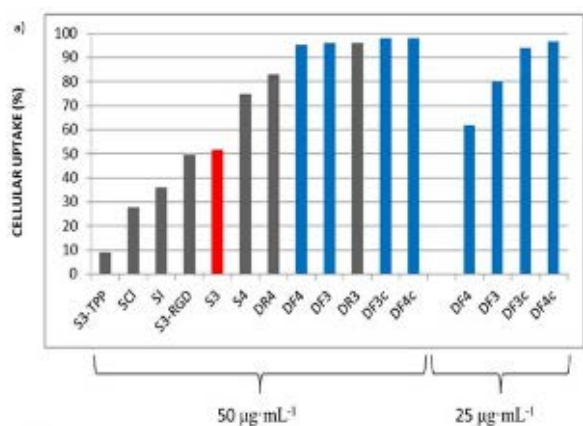


Figure S6. Structure and Cellular uptake of single analogues on NB cells.



Single Agents	Cellular uptake (%)		Double agents	Cellular uptake (%)	
	12.5 µg mL ⁻¹	6.25 µg mL ⁻¹		12.5 µg mL ⁻¹	6.25 µg mL ⁻¹
F-PEG-S3	5	1	F-PEG-DFL3	82	55
F-PEG-S4	3	1	F-PEG-DFL4	100	83

Figure S7. Comparison graphic of single versus double ligands Cellular uptake at 50 µg mL⁻¹, 25 µg mL⁻¹, 12.5 µg mL⁻¹ and 6.25 µg mL⁻¹ on NB cells.

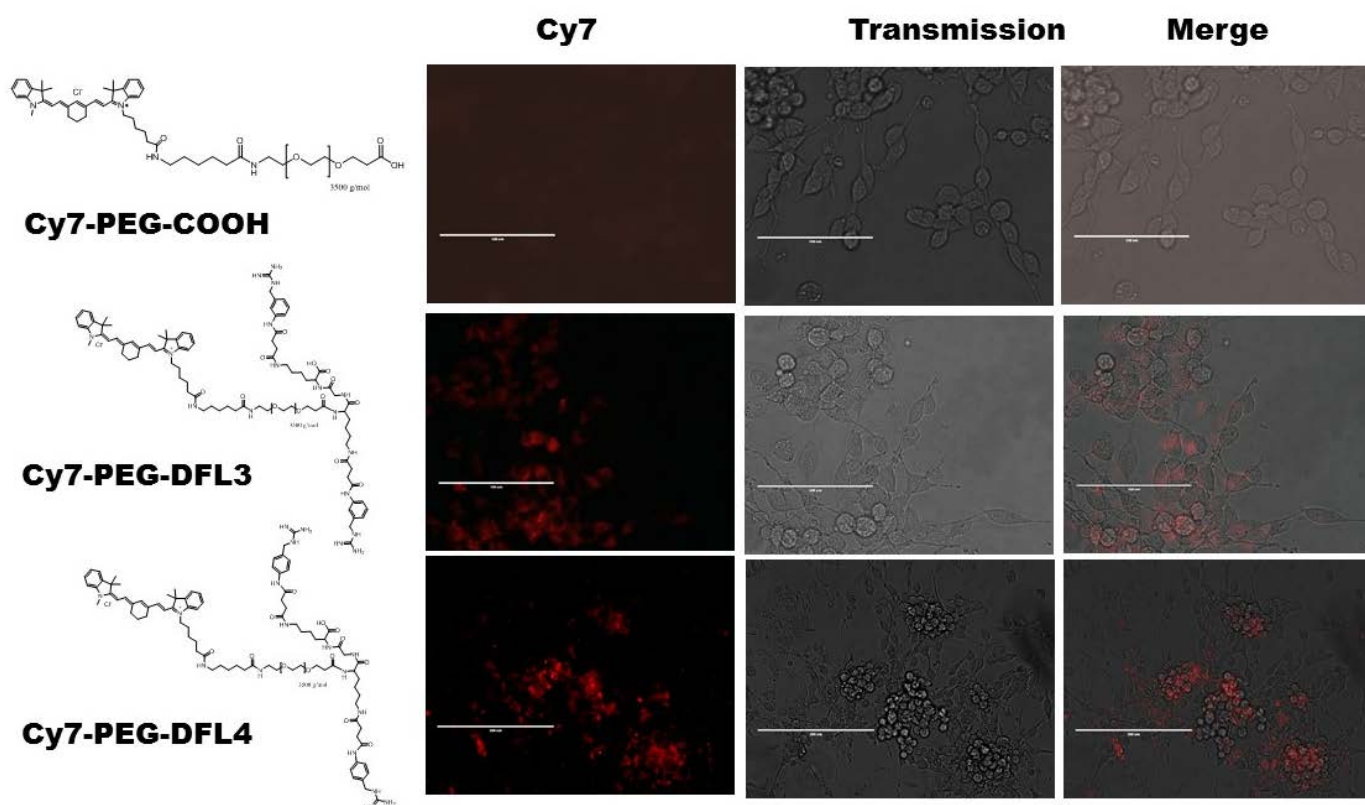


Figure S8. Microscopy pictures of Neuroblastoma cells 2h hours of incubation with 25 µg/mL of analogues Cy7-PEG-COOH, Cy7-PEG-DFL3 and Cy7-PEG-DFL4 respectively, washed with PBS buffer for eliminating non-internalized nanoparticles in the membrane cell surface. Preliminary qualitative assay of targeting effect of the double system with Cy7.

6. *in vivo*

Neuroblastoma cells (cell line NB1691-luc) were implanted subcutaneously into the flanks of immune-deficient NSG mice (NOD/SCID gamma mice), originally obtained from Jackson Laboratories (Bar Harbor, ME). The recommendations of FELASA (Federation of Laboratory Animal Science Associations) and the Spanish and European laws and rules of animal experimentation were followed.

Three weeks after the inoculation, the presence of subcutaneous tumors as well as their degree of development were demonstrated by bioluminescence. The mice were distributed homogeneously in four groups (n=5), each group receiving the different preparations of the study (intravenous injection of 1 mg/mouse in 0.2 ml saline).

The preparations contained mesoporous nanoparticles labeled with Cy7 fluorophore ($\lambda_{ex} = 750 / \lambda_{em} = 773$ nm). The distribution of particles and the quantification of the signal in the area of the tumor were observed through fluorescent image acquisition in an IVIS Lumina XRMS instrument (Perkin Elmer) at 24 and 72 h after injection of the nanoparticles. The presence and localization of NB-Luc derived tumors was confirmed through the detection of a luminescent signal after i.p. injection of 1.25 mg per mice D-luciferin (Perkin Elmer). Image analysis was performed with Living Images 4.4 software (Perkin Elmer). Animal studies were in accordance with the guidelines of the EU on animal care (2010/63/EU) and approved by an institutional ethics committee (FELASA) of Hospital Universitario Niño de Jesús de Madrid. The animals were sacrificed 72 hours after the infusion. The fluorescence results of the nanoparticles are summarized in **Figure S8**. The fluorescence level has been quantified in photons/cm². In the graphs the data were normalized in front of the fluorescence of the whole mice.

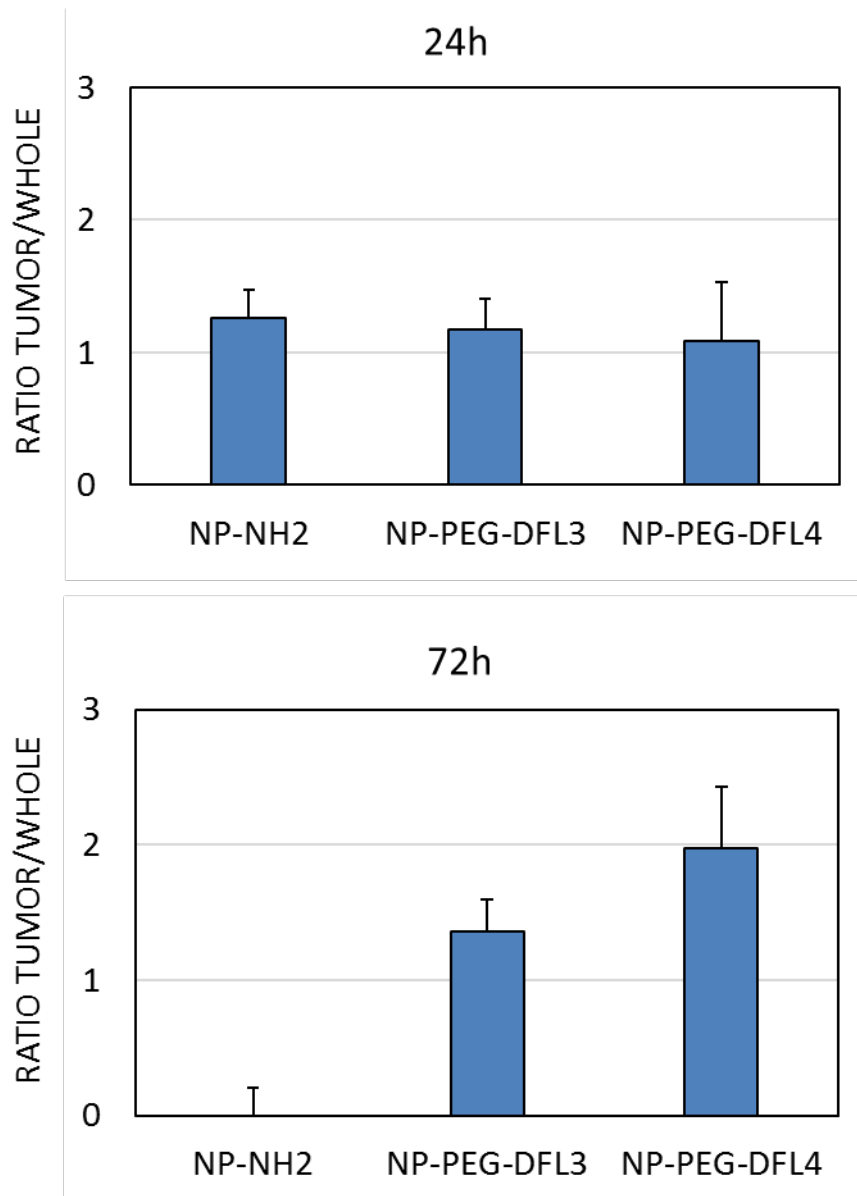


Figure S9. Relative fluorescence of tumor area vs. whole mice injected with the indicated compounds and measured at 24h and 72h.

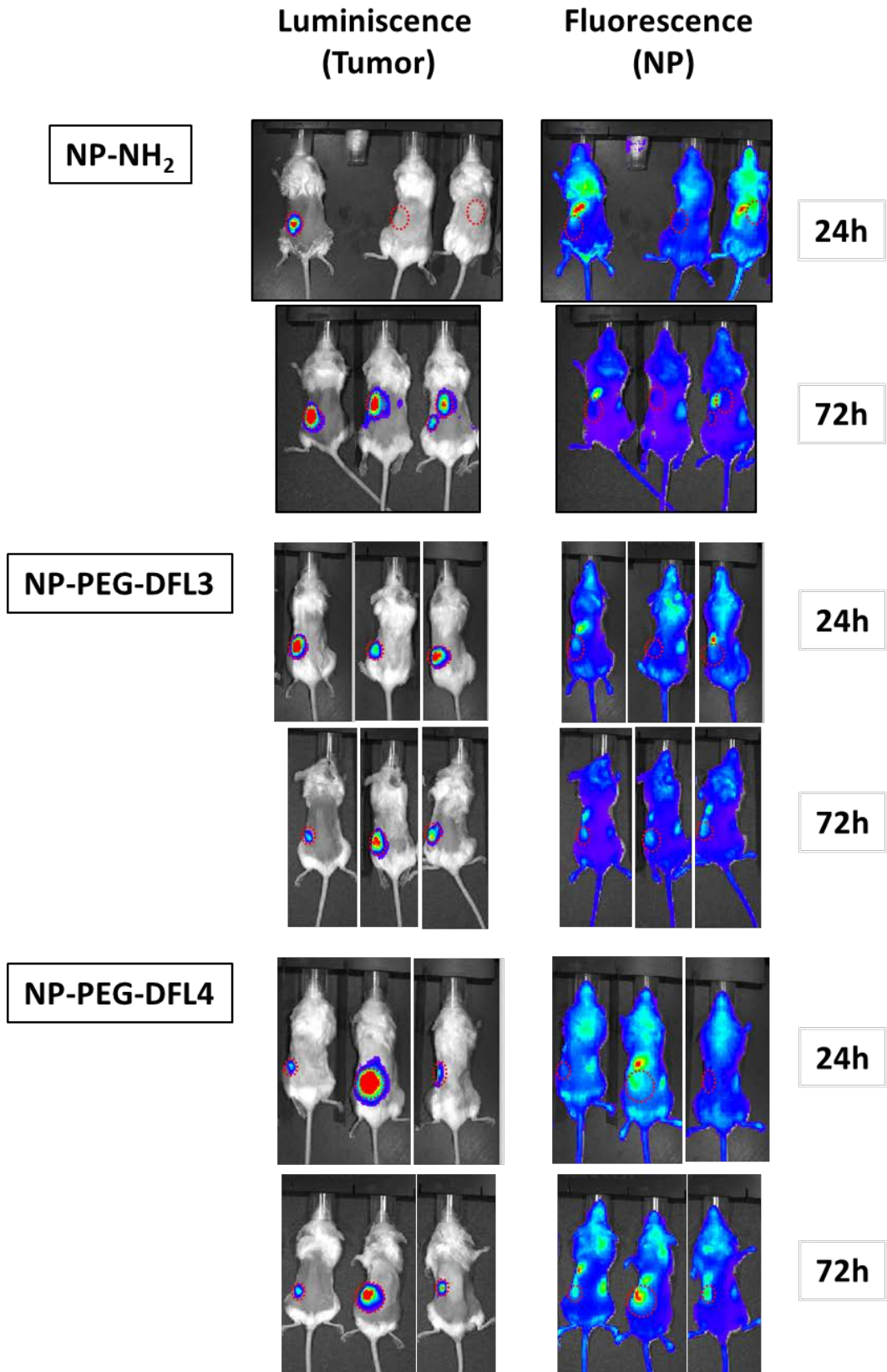


Figure S10. Representative photographs of mice inoculated with human neuroblastoma cells and subsequently treated with functionalized NP as indicated. Images show relative luminiscence (tumor cells) and fluorescence (NP) at 24 and 72h after NP injection.