Nintedanib-Containing Dual Conjugates Targeting $\alpha_V\beta_6$ Integrin and Tyrosine Kinase Receptors as Potential Antifibrotic Agents

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CHEMISTRY

General methods

General. All chemicals were of the highest commercially available quality and were used without further purification. Solvents were dried by standard procedures and reactions requiring anhydrous conditions were performed under nitrogen or argon atmosphere H-Gly-2-ClTrt resin (loading 0.58 mmol/g) was purchased from Novabiochem, (2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid from PolyPeptide and all other reagents from Alfa Aesar, TCI, Fluorochem or Merck-Sigma-Aldrich. The automated flash chromatography and HPLC solvents respond to ACS standard and they were used without further purification. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F_{254} pre-coated plates with visualization under short-wavelength UV light and by dipping the plates with molybdate reagent (aqueous H_2SO_4 solution of ceric sulphate/ammonium molybdate) followed by heating. Flash column chromatography was performed using 40-63 μm silica gel and the indicated solvent mixtures. Automated flash column chromatography was carried out with the Biotage Isolera One system using Biotage KP-Sil cartridges (direct phase) and KP-C18-HS cartridges (reverse phase). ESI-mass spectra were recorded on UHPLC/ESI-MS system (ACQUITY Ultra Performance LC; ESI, positive ions, Single Quadrupole analyzer) and are reported in the form of (*m/z*). HPLC purifications were performed on a Prostar 210 apparatus (Varian, UV detection) equipped with C18-10 μm columns (Discovery BIO Wide Pore 10 × 250 mm). Routine NMR spectra were recorded on AV400 (Bruker) or 600MHz (Jeol) NMR spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) with TMS ($CDCl_3$), CD_2HOD, and HOD resonance peaks set at 0, 3.31, and 4.80 ppm, respectively. Multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and b (broad). Coupling constants, *J*, are reported in Hertz. ^{1}H and ^{13}C NMR assignments are corroborated by 1D and 2D experiments (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C DEPT experiments). High resolution mass analysis (ESI) was performed on LTQ ORBITRAP XL Thermo apparatus.
Purity of the final compounds was checked by HPLC and was in the 96 - >99% range.

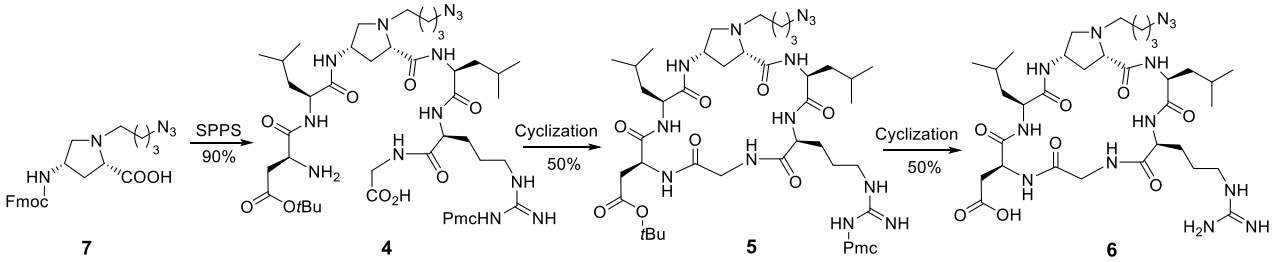
Abbreviations. Amp, 4-amino-L-proline; Boc, *tert*-butoxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Fmoc, 9-fluorenylmethoxycarbonyl; SPPS, solid phase peptide synthesis; DCE, 1,2-dichloroethane; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; TIS, triisopropylsilane; *p*-TsCl, *p*-toluenesulfonyl chloride; DMAP, 4-(dimethylamino)pyridine.

Materials. H-Gly-2-ClTrt resin, Fmoc-Asp(*t*Bu)-OH; Fmoc-Arg(Pmc)-OH, Fmoc-Leu-OH, 2,4,6-collidine, glacial acetic acid, DIPEA, HATU, HOAt, 4-azidobutanol, 3-aminopropanol, *N*-Boc-piperazine were commercially available and were used as such without further purification. Nintedanib was purchased by MedChemExpress. N^{α}-(4-azidobutyl)-4-*N*-(Fmoc)aminoproline (**7**), tosyl derivatives **13** and **15**, and oxindole nucleus **21** were prepared according to reported procedures.[1,2]

General method for HPLC purification. The final conjugates were purified by reverse phase HPLC equipped with a preparative column (C18-10 μm, 21.2 × 250 mm column), with the solvent system H_2O + 0.1% TFA (Solvent A) and ACN (solvent B), using the following method: flow rate 8.0 mL/min; detection at 220 nm, linear gradient from 5% B to 50% B over 23 min, 50% B for 3 min, from 50% B to 5% B over 3 min, room temperature.

Experimental synthetic procedures and characterization data

Synthesis of cyclic peptidomimetic 6



General procedure for cyclopeptide synthesis

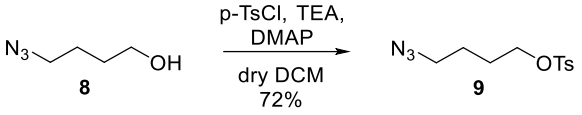
Solid Phase Synthesis. The synthesis of linear hexapeptide H-Asp(*t*Bu)-Leu-1-(4-azidobutyl)Amp-Leu-Arg(Pmc)-Gly-OH (**4**) was performed using the preloaded 2-chlorotrityl-Gly-H resin (loading 0.58 mmol/g). *Resin swelling*: the resin (350.0 mg, 0.203 mmol, 1.0 eq) was swollen in a solid phase reaction vessel with dry DMF (5 mL) under mechanical stirring; after 40 min the solvent was drained, and the resin was washed with DMF (3×). *Peptide coupling:* A preformed solution of Fmoc-Arg(Pmc)-OH (188.7 mg, 0.305 mmol, 1.5 eq) in dry DMF (4 mL) was treated with HATU (154.,3 mg, 0.406 mmol, 2.0 eq), HOAt (55 mg, 0.406 mmol, 2.0 eq) and 2,4,6-collidine (54 µL, 0.406 mmol, 2.0 eq) and stirred for 10 min before adding to the resin. The mixture was shaken at room temperature for 3 h. Completion of the reaction was checked by the Kaiser test. The solution was drained and the resin was washed with DMF (2×), *i*PrOH, (2×), Et_2O (2×), DCM (2×). The resin was washed again with DMF and treated with 20% v/v piperidine in DMF (5 mL) and the mixture was stirred for 40 min. The solution was drained and the resin was washed with DMF (2×), *i*PrOH, (3×), Et_2O (2×), DCM (2×). The couplings of the Fmoc-Leu-OH (107.8 mg, 0.305 mmol, 1.5 eq), the aminoproline nucleus **7** (137.1 mg, 0.305 mmol, 1.5 eq) and Fmoc-Asp(*t*Bu)-OH (125.5 mg, 0.305 mmol, 1.5 eq) were carried out under the same conditions. *Resin cleavage*: the resin was treated with 5 mL of the cleavage mixture DCM:TFE:glacial AcOH (3:1:1) and kept under mechanical stirring for 20 min at room temperature. The solution was recovered and the resin was carefully washed with DCM (2×). This protocol was repeated twice. The combined solution was evaporated under reduced pressure affording the linear hexapeptide **4** (212 mg, AcOH salt, 90% yield) as a colourless glassy solid, which was used in the following step without further purification. MS (ES^+) *m/z* 1104.7 $[M+H]^+$.

In-solution cyclization. To a solution of linear hexapeptide **4** (212 mg, 0.192 mmol, 1 eq) in dry DCM (50 mL), 2,4,6-collidine (78 µL, 0.576 mmol, 3.0 eq) was added. The mixture was stirred under argon at room temperature for 10 min, then was added dropwise to a solution of HATU (221.8 mg, 0.576 mmol, 3.0 eq) and HOAt (79.2 mg, 0.576 mmol, 3.0 eq) in dry DMF (15 mL) and dry DCM (170 mL) to a final peptide concentration of 0.8 mM. The reaction mixture was degassed by argon/vacuum cycles (3×) and left to stir under argon at room temperature for 5 h. After completion, the solution was concentrated under vacuum and extracted with EtOAc (3×). The combined organic layers were dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by reverse phase chromatography (eluent: from 80:20 H_2O+0.1%TFA:ACN to 20:80) affording the protected cyclopeptide **5** as a glassy solid (115.0 mg, TFA salt 50% yield). TLC: EtOAc:MeOH 70:30, R*f* = 0.7. ^{1}H NMR (400 MHz, MeOD): δ 4.71 (m, 1H, H4Amp), 4.48 (m, 3H, αAsp+αLeu), 4.44-4.22 (m, 3H, αArg+H2+αGly), 3.75 (bd, *J* = 16.9 Hz, 1H, αGly), 3.58 (m, 2H, H5Amp), 3.38 (m, 2H, H1'Amp), 3.29-3.12 (m, 4H, H4'+δArg), 2.95 (m, 1H, H3Amp), 2.81 (m, 2H, βAsp), 2.69 (t, *J* = 6.8 Hz, 2H, CH_2 Pmc), 2.59 (s, 3H, CH_3Pmc), 2.58 (s, 3H, CH_3Pmc), 2.13 (m, 3H, CH_3Pmc+H3bAmp), 1.86 (t, *J* = 6.8 Hz, 2H, CH_2Pmc), 1.77-1.59 (m, 12H, βArg+γArg+ γLeu+βLeu+H2'Amp),

1.55 (m, 2H, H3'Amp), 1.47 (s, 9H, *t*Bu), 1.33 (s, 6H, CH_3 Pmc), 0.96 (m, 12H, δLeu). MS (ES^+) *m/z* 1086.7 $[M+H]^+$.

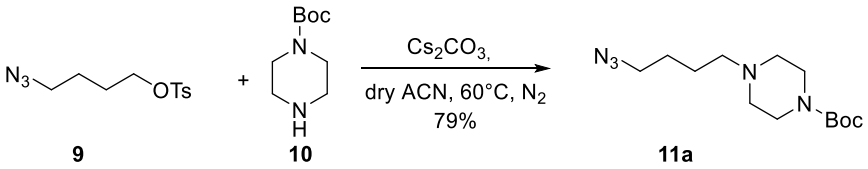
Final deprotection. The protected cyclopeptide **5** (11.0 mg, 0.0083 mmol, 1 eq) was treated with a solution of TFA/TIS/H_2O 95:2.5:2.5 (417 μL). After 1 h, the solvent was removed under vacuum and the residue was washed with Et2O (3 x). The resulted crude was purified by reverse phase chromatography (eluent: from 80:20 H2O+0.1%TFA:ACN to 20:80) affording the protected cyclopeptide **6** as a glassy solid (6.9 mg, TFA salt 63% yield). ^{1}H NMR (400 MHz, MeOD): δ 4.73 (m, 1H, H4Amp), 4.48-4.22 (m, 3H, αAsp+αLeu+αArg+H2+αGly), 3.75 (bd, *J* = 16.8 Hz, 1H, αGly), 3.58 (m, 2H, H5Amp), 3.38 (m, 2H, H1'Amp), 3.29-3.12 (m, 4H, H4'+δArg), 2.95 (m, 1H, H3Amp), 2.81 (m, 2H, βAsp), 2.13 (m, 1H, H3bAmp), 1.77-1.59 (m, 12H, βArg+γArg+γLeu+βLeu+H2'Amp), 1.55 (m, 2H, H3'Amp), 1. 0.96 (m, 12H, δLeu). MS (ES^+) *m/z* 736.4 $[M+H]^+$.

Synthesis of 4-azidobutyl 4-methylbenzenesulfonate 9



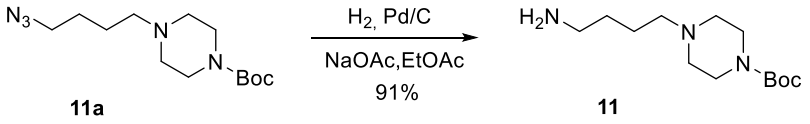
To a solution of 4-azidobutanol **8** (420.0 mg, 3.65 mmol, 1 eq) in dry DCM (10 mL), *p*-TsCl (834.1 mg, 4.38 mmol, 1.2 eq), DMAP (121.0 mg, 0.99 mmol, 0.3 eq) and TEA (509 μL, 4.38 mmol, 1.2 eq) were added. The reaction was kept under stirring for 22 h, then HCl 0.1N (10 mL) was added and the mixture was extracted with DCM (3x). The combined organic layers were dried with $MgSO_4$ and the crude was purified with flash chromatography (Eluent 9:1 DCM:Petroleum Ether), giving compound **9** as a colorless oil (707.0 mg, 72% yield). TLC: Petroleum Ether:EtOAc 70:30, R*f* = 0.8. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 8.5 Hz, 2H, ArH), 7.37 (d, *J* = 8.5 Hz, 2H, ArH), 4.08 (t, *J* = 6.5 Hz, 2H, CH_2), 3.28 (t, *J* = 6.5 Hz, 2H, CH_2), 2.48 (s, 3H, CH_3), 1.59 (m, 2H, CH_2), 1.65 (m, 2H, CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 145.1 (1C, Cq), 133.2 (1C, Cq), 130.1 (2C, CH), 128.1 (2C, CH), 69.9 (1C, CH_2), 50.9 (1C, CH_2), 26.3 (1C, CH_2), 25.2 (1C, CH_2), 21.9 (1C, CH_3). MS (ES^+) *m/z* 270.1 $[M+H]^+$.

Synthesis of 1-Boc-(4-azidobutyl)piperazine 11a



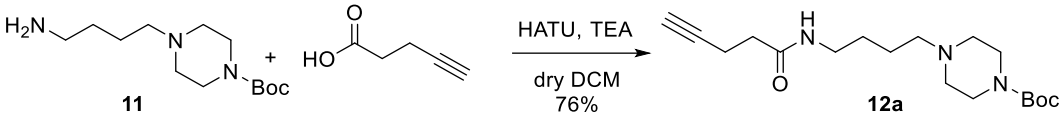
To a solution of compound **9** (113.0 mg, 0.42 mmol, 1 eq) in dry ACN (2 mL) at 60°C, Cs_2CO_3 (163.9 mg, 0.50 mmol, 1.2 eq) and Boc-piperazine **10** (93.8 mg, 0.50 mmol, 1.2 eq) were added. The reaction was kept under stirring for 3 days (adding 2 mL of dry ACN when the solvent was evaporated), then the solvent was removed under reduced pressure. Water (10 mL) was added to the crude and extracted with EtOAc (3x). The combined organic layers were evaporated, and the crude purified with a flash chromatography (eluent: from 100% DCM to 40:60 DCM:EtOAc), giving final compound **11a** as a yellowish liquid (94.2 mg, 79% yield). TLC: Petroleum Ether:EtOAc 50:50, R*f* = 0.4. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.35 (t, *J* = 5.0 Hz, 4H, CH_2NBoc), 3.37 (t, *J* = 6.7 Hz, 2H, CH_2-N_3), 2.29 (m, 6H, CH_2N piperazine), 1.69-1.59 (m, 2H, CH_2CH_2-N_3), 1.52-1.47 (m, 2H, $CH_2CH_2CH_2$-N_3), 1.38 (s, 9H, CH_3 Boc). MS (ES^+) *m/z* 284.2 $[M+H]^+$.

Synthesis of 1-Boc-(4-aminobutyl)piperazine 11



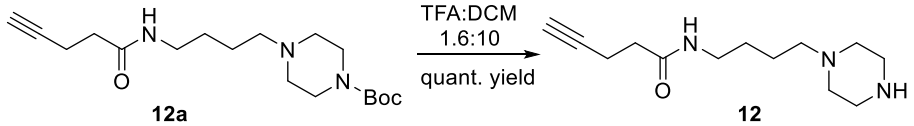
To a solution of azide **11a** (240.0 mg, 0.846 mmol, 1 eq) in EtOAc (50 mL), 10 mmol% of sodium acetate and palladium on carbon were added and the reaction was kept under stirring in hydrogen atmosphere. After 4 h the reaction mixture was filtered to give amine **11** as a colourless oil (217.9 mg, 91% yield). TLC: Petroleum Ether:EtOAc 50:50 R*f* = 0.05. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.42 (bt, *J* = 4.8 Hz, 4H, CH_2NBoc), 2.70 (t, *J* = 7.0 Hz, 2H, CH_2NH_2), 2.38-2.32 (m, 6H, CH_2N piperazine), 1.54-1.43 (m, 13H, $CH_2CH_2CH_2$-NH_2+ CH_2CH_2-NH_2+CH_3Boc). ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.9 (1C, Cq), 79.7 (1C, CH), 58.7 (2C, CH_2), 53.2 (1C, CH_2), 43.4 (1C, CH_2), 42.2 (2C, CH_2), 31.8 (3C, CH_3), 28.6 (1C, CH_2), 24.4 (1C, CH_2). MS (ES^+) *m/z* 258.3 $[M+H]^+$.

Synthesis of substituted Boc-piperazine 12a



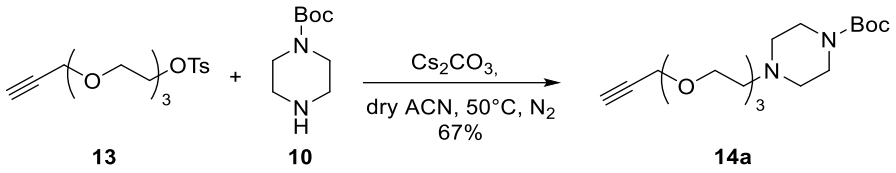
To a solution of amine **11** (199.0 mg, 0.733 mmol, 1.0 eq), 4-pentynoic acid (83.4 mg, 0.85 mmol, 1.1 eq), and HATU (323.2 mg, 0.85 mmol, 1.1 eq) in dry DCM (50 mL), TEA was added (237.3 µL, 1.701 mmol, 2.2 eq). The reaction was kept under stirring in nitrogen atmosphere. After 3 hours the reaction was quenched by adding $NaHCO_3$ saturated solution (pH = 9) and then extracted with DCM (3x). The crude was purified by flash chromatography (eluent: 80:20 EtOAc:MeOH), giving compound **12a** as a glassy solid (199.2 mg, 76% yield). TLC: EtOAc:MeOH 80:20 R*f* = 0.6. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.06 (bs, 1H, NH), 3.42 (bt, *J* = 5.1 Hz, 4H, CH_2-NBoc), 3.27 (m, 2H, OCN-CH_2), 2.51 (td, *J* = 7.2, 2.7 Hz, 2H, Alkyne-CH_2), 2-38-2.34 (m, 8H, CH_2) 1.98 (t, *J* = 2.7 Hz, 1H, CH alkyne), 1.67 (m, 4H, CH_2 piperazine), 1.53 (m, 4H, CH_2), 1.44 (s, 9H, CH_3 Boc). MS (ES^+) *m/z* 338.3 $[M+H]^+$.

Synthesis of substituted piperazine 12



Amine **12** was obtained by deprotection of the corresponding Boc-protected amine **12a** (225.0 mg, 0.874 mmol, 1 eq) using a solution of TFA:DCM 1.6:10 (2.95 mL). The reaction was kept under stirring for 1 h and then quenched by removing the solvent under reduced pressure. The crude was washed with Et_2O (3x), yielding compound **12** (as double TFA salt) as a yellowish oil (409.0 mg, quantitative yield). ^{1}H NMR (400 MHz, MeOD) δ 3.33-3.25 (m, 4H, CH_2), 2.83 (s, 4H, CH_2), 2.51-2.46 (m, 4H, CH_2), 2.43-2.39 (m, 4H, CH_2), 2.28 (m, 1H, CH alkyne), 1.85-1.79 (m, 4H, CH_2). ^{13}C NMR (101 MHz, MeOD) δ 173.2 (1C, Cq), 82.3 (1C, Cq), 69.2 (1C, CH), 56.5 (2C, CH_2), 40.7 (2C, CH_2), 37.8 (1C, CH_2), 37.7 (1C, CH_2), 34.7 (1C, CH_2), 26.2 (1C, CH_2), 19.6 (1C, CH_2), 13.2 (1C, CH_2). MS (ES^+) *m/z* 238.2 $[M+H]^+$.

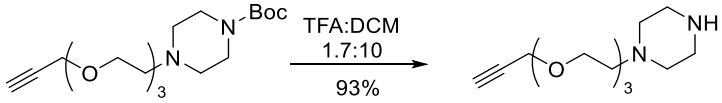
Synthesis of substituted Boc-piperazine 14a



13 10 14a

To a round bottom flask containing compound **13** (125.0 mg, 0.37 mmol, 1 eq) in 1 mL of ACN dry, Boc-piperazine **10** (81.6 mg, 0.44 mmol, 1.2 eq) and Cs_2CO_3 (142.7 mg, 0.44 mmol, 1.2 eq) were added. The mixture was left to stir under nitrogen atmosphere at 50 °C for two days. The reaction was quenched by removing the solvent under reduced pressure. Water (10 mL) was added and then extracted with DCM (3x). The crude was purified by flash chromatography (eluent: 95:5 EtOAc:MeOH•NH_3) giving compound **14a** as a yellowish glassy solid (87.7 mg, 67% yield). TLC: EtOAc: MeOH•NH_3 90:10 R*f* = 0.6. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.16 (d, *J* = 2.4 Hz, 2H, CH_2-alkyne), 3.67-3.57 (m, 10H, CH_2), 3.39 (t, *J* = 4.7 Hz, 4H, CH_2NBoc), 2.56 (t, *J* = 5.8 Hz, 2H, OCH_2CH_2N), 2.41 (m, 5H, NCH_2 piperazine + CH alkyne), 1.41 (s, 9H, CH_3 Boc). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.7 (Cq), 79.8 (Cq), 79.7 (Cq), 74.7 (1C, CH), 70.8 (1C, CH_2), 70.6 (1C, CH_2), 70.5 (1C, CH_2), 69.2 (1C, CH_2), 69.0 (1C, CH_2), 58.5 (1C, CH_2), 57.9 (2C, CH_2), 53.5 (2C, CH_2), 28.6 (1C, CH_3). MS (ES^+) *m/z* 357.3 $[M+H]^+$.

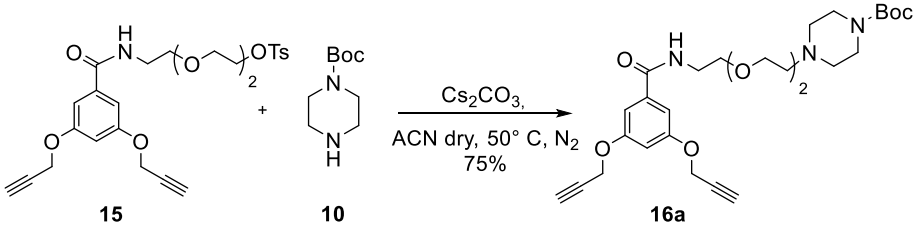
Synthesis of substituted piperazine 14



14a 14

Compound **14a** (169.0 mg, 0.47 mmol, 1 eq) was deprotected as described for compound **12**, using 9 mL of solution of TFA:DCM, to give piperazine **14** as a glassy solid (214.3 mg, 93% yield). ^{1}H NMR (600 MHz, MeOD) δ 4.16 (d, *J* = 2.5 Hz, 2H, alkyne-CH_2), 3.86 (t, *J* = 4.9 Hz, 2H, CH_2), 3.69-3.61 (m, 12H, CH_2), 3.59 (bt, *J* = 5.6 Hz, 2H, CH_2NH), 3.47 (t, *J* = 4.9 Hz, 2H, OCH_2CH_2N), 2.83 (t, *J* = 2.5 Hz, 1H, CH). ^{13}C NMR (151 MHz, $CDCl_3$): δ 79.2 (1C, Cq), 74.8 (1C, CH), 69.9 (1C, CH_2), 69.9 (1C, CH_2), 68.8 (1C, CH_2), 64.0 (1C, CH_2), 57.7 (2C, CH_2), 56.3 (1C, CH_2), 48.7 (2C, CH_2), 40.5 (1C, CH_2). MS (ES^+) *m/z* 257.2 $[M+H]^+$.

Synthesis of substituted Boc-piperazine 16a

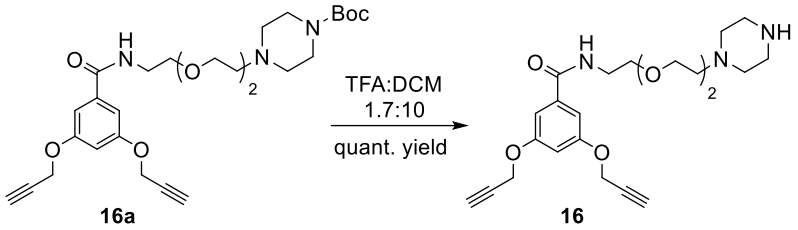


15 10 16a

Compound **16a** was synthesized as described for compound **14a**, starting from tosyl derivative **15** (150.0 mg, 0.29 mmol, 1 eq) and Boc-piperazine **10** (65.2 mg, 0.35 mmol, 1.2 eq). The reaction was kept under stirring at 50 °C under nitrogen for three days. The solvent was evaporated, water was added (10 mL) and extracted with DCM (3x) The organic layers were collected and concentrated under vacuum, affording a crude residue which was purified by flash chromatography (eluent: EtOAc:MeOH•NH_3 99.2:0.8) to give **16a** (105.2 mg, 75% yield) as a yellowish oil. TLC: EtOAc:MeOH•NH_3 93:7. R*f*= 0.5. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (bd, *J* = 2.2 Hz, 2H, ArH), 6.74 (t, *J* = 2.2 Hz, 1H, ArH), 4.71 (d, *J* = 2.4 Hz, 4H, $ArOCH_2$), 3.72–3.58 (bm, 12H, OCH_2), 2.57 (m,

4H, CH_2 piperazine), 2.62-2.55 (m, 4H, $OCNCH_2$+CH alkyne), 2.42 (bs, 4H, CH_2 piperazine), 1.46 (s, 9H, CH_3Boc). MS (ES^+) *m/z* 530.3 $[M+H]^+$.

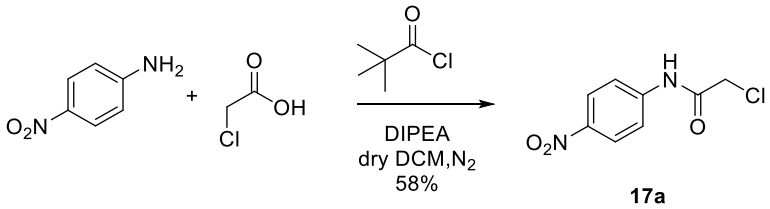
Synthesis of substituted piperazine 16



16a **16**

Compound **16a** (89.3 mg, 0.169 mmol, 1.0 eq) was deprotected as described for compound **14**, using 3.5 mL of solution of TFA:DCM, to give piperazine **16** as a glassy solid (91.7 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 (d, *J* = 2.3 Hz, 2H, ArH), 6.84 (t, *J* = 2.3 Hz, 1H, ArH), 4.80 (d, *J* = 2.5 Hz, 4H, $ArOCH_2$), 3.85 (m, 2H, CH_2), 3.70–3.66 (m, 6H, CH_2 piperazine + CH_2), 2.58 (m, 10H, CH_2 piperazine + CH_2), 3.38 (m, 2H, CH_2), 3.02 (t, *J* = 2.5 Hz, 2H, CH alkyne). MS (ES^+) *m/z* 430.3 $[M+H]^+$.

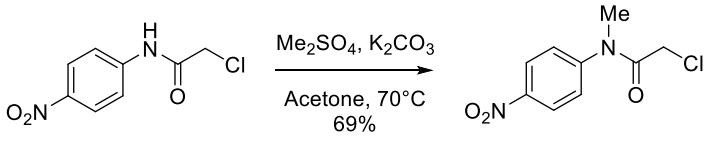
Synthesis of 2-chloro-*N*-(4-nitrophenyl)acetamide 17a



17a

To a solution of 2-chloroacetic acid (1.03 g, 7.38 mmol, 1.7 eq) in dry DCM (10 mL), DIPEA (1.53 mL, 8.69 mmol, 2.0 eq) was added at 0 °C. The mixture was left to stir at 0 °C for 10 min, then pivaloyl chloride (802.00 µL, 6.52 mmol, 1.5 eq) was added dropwise. The reaction was left to stir at room temperature for 1 h, then 4-nitroaniline (600.00 mg, 4.34 mmol, 1.0 eq) was added. After 4 h, the solvent was removed under reduced pressure. Then, water (10 mL) was added and the crude was extracted with EtOAc (3x), dried with $MgSO_4$ and filtered. The solvent was evaporated, and the residue was purified by flash chromatography (eluent: 100% DCM), giving compound **17a** (644.30 mg, yield 58%). TLC: 100% DCM, R*f* = 0.3. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (m, 2H, ArH), 7.79 (m, 2H, ArH), 4.28 (s, 2H, CH_2). MS (ES^+) *m/z* 215.0 $[M+H]^+$.

Synthesis of 2-chloro-*N*-methyl-*N*-(4-nitrophenyl)acetamide 17

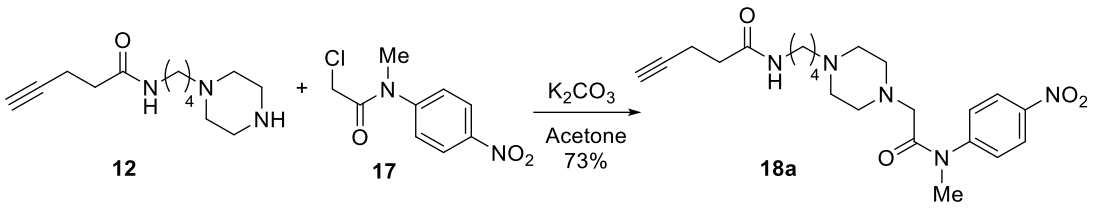


17a **17**

To a solution of compound **17a** (380.2 mg, 1.77 mmol, 1.0 eq.) in acetone (25 mL), K_2CO_3 (406.0 mg, 2.93 mmol, 1.7 eq) was added and the reaction was left to stir at room temperature for 10 min. Then, Me_2SO_4 (278.1 µL, 2.93 mmol, 1.7 eq) was added and the mixture was kept under stirring at 70 °C for 30 h. After completion, the reaction was quenched with saturated solution of NH_4Cl and HCl 10% until pH 3, then extracted with EtOAc (3x). The combined organic layers were dried with $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude was purified by flash chromatography (eluent: 50:45:5 Petroleum Ether:DCM:EtOAc), giving compound **17** as a light-yellow solid (450.0 mg, yield 69%). TLC: 100%

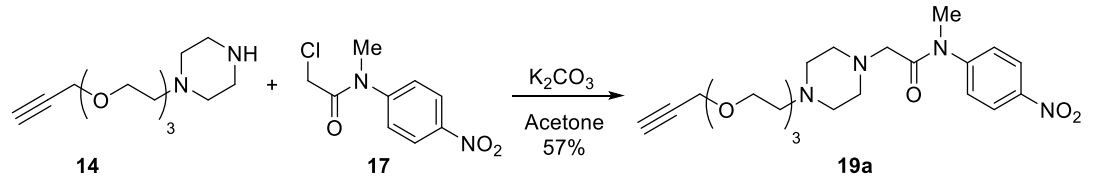
DCM, R*f* = 0.4. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (m, 2H, ArH), 7.50 (m, 2H, ArH), 3.96 (s, 2H, CH_2), 3.41 (s, 3H, CH_3). MS (ES^+) *m/z* 229.1 $[M+H]^+$.

Synthesis of nitro-derivative 18a



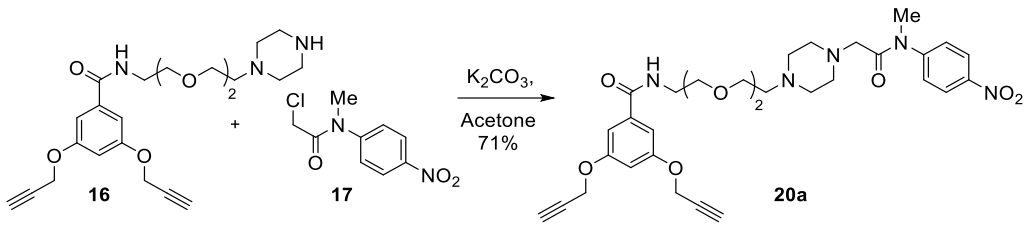
To a solution of amine **12** (409.1 mg, 0.871 mmol, 1.0 eq) and compound **17** (258.7 mg, 1.132 mmol, 1.3 eq) in acetone (20 mL), K_2CO_3 (481.3 mg, 3.482 mmol, 4 eq) was added. The reaction was left under stirring for 22 h, then the solvent was removed under reduced pressure and the residue was dissolved in DCM and filtered. The crude was purified by flash chromatography (gradient: from 100% EtOAc to 8:2 EtOAc:MeOH•NH_3) giving compound **18a** as a yellowish oil (271.4 mg, 73% yield). TLC: 9:1 EtOAc:MeOH•NH_3, R*f* = 0.6. ^{1}H NMR (400 MHz, MeOD) δ 8.29 (m, 2H, ArH), 7.46 (m, 2H, ArH), 6.23 (m, 1H, NH), 3.37 (s, 3H, CH_3), 3.27 (m, 2H, OCN-CH_2), 3.08 (s, 2H, NCH_2CON), 2.57-2.34 (m, 14 H, Alkyne-CH_2 + 4CH_2 piperazine + CH_2-N-Piperazine+CH_2CON), 1.99 (t, *J* = 2.8 Hz, 1H, CH Alkyne), 1.57-1.52 (m, 4H, CH_2) ^{13}C NMR (101 MHz, MeOD) δ 169.3 (1C, Cq), 149.7 (1C, Cq), 127.4 (2C, CH), 125.2 (1C, Cq), 124.9 (2C, CH), 119.0 (1C, Cq), 83.3 (1C, Cq), 69.4 (1C, CH), 60.6 (2C, CH_2), 57.9 (2C, CH_2), 53.0 (1C, CH_2), 39.3 (2C, CH_2), 37.6 (1C, CH2), 35.6 (1C, CH_3), 27.6 (1C, CH_2), 24.3 (1C, CH_2), 15.1 (1C, CH_2). MS (ES^+) *m/z* 430.3 $[M+H]^+$.

Synthesis of nitro-derivative 19a



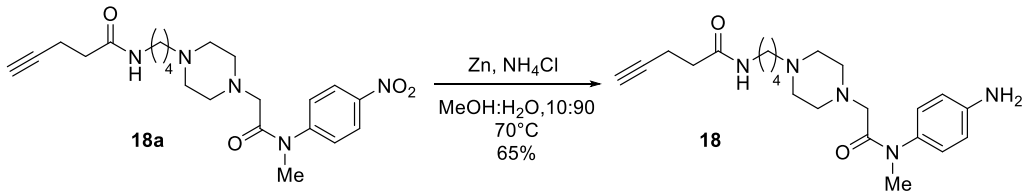
Compound **19a** was obtained as described for compound **18a**, using amine **14** (214.3 mg, 0.44 mmol, 1.0 eq) and compound **17** (131.3 mg, 0.57 mmol, 1.3 eq). The crude was purified by flash chromatography (gradient: from 100% EtOAc to 9:1 EtOAc:MeOH•NH_3) giving compound **19a** as a yellowish oil (113.9 mg, 57% yield). TLC: 9:1 EtOAc:MeOH•NH_3, R*f* = 0.8. ^{1}H NMR (400 MHz, MeOD) δ 8.28 (m, 2H, ArH), 7.46 (m, 2H, ArH), 4.21 (d, *J* = 2.5 Hz, 2H, Alkyne-CH_2), 3.72-3.58 (m, 12H, CH_2), 3.37 (s, 3H, CH_3), 3.06 (s, 2H, NCH_2CON), 2.57 (t, *J* = 5.7 Hz, 2H, CH_2), 2.48 (m, 6H, CH_2), 2.45 (t, *J* = 2.5 Hz, 1H, CH alkyne). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.4 (1C, Cq), 149.7 (1C, Cq), 127.4 (1C, Cq), 125.0 (2C, CH), 79.8 (2C, CH), 74.7 (1C, Cq), 70.8 (1C, CH), 70.6 (1C, CH_2), 70.5 (1C, CH_2), 69.3 (1C, CH_2), 69.0 (1C, CH_2), 60.7 (1C, CH_2), 58.6 (1C, CH_2), 57.8 (1C, CH_2), 53.5 (1C, CH_2), 53.2 (1C, CH_2), 37.6 (1C, CH_3). MS (ES^+) *m/z* 449.4 $[M+H]^+$.

Synthesis of nitro-derivative 20a



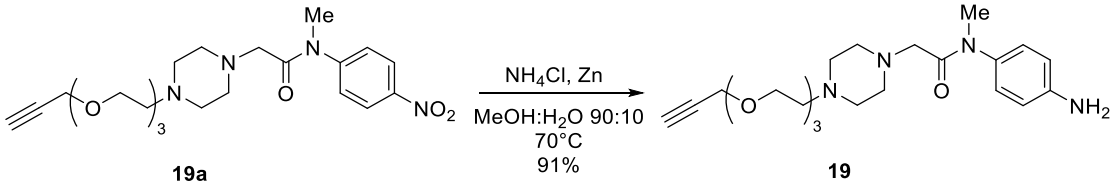
Compound **20a** was obtained as described for compound **18a**, using amine **16** (196.0 mg, 0.298 mmol, 1.0 eq) and compound **17** (88.7 mg, 0.338 mmol, 1.3 eq). The crude was purified by flash chromatography [linear gradient to elution from 100% EtOAc to 90:10 EtOAc:MeOH•NH_3], giving compound **20a** (131.8 mg, yield 71%). TLC: 9:1 EtOAc:MeOH•NH_3, R*f* = 0.6. ^{1}H NMR (400 MHz, MeOD) δ 8.32 (m, 2H, ArH), 7.59 (m, 2H, ArH), 7.10 (d, *J* = 2.3 Hz, 2H, ArH), 6.80 (dd, *J* = 2.3, 2.3 Hz, 1H, ArH) 4.79 (d, *J* = 2.3 Hz 4H, ArOCH_2), 3.70-3.53 (m, 10H, CH_2), 3.39-3.54 (bs, 3H, CH_3), 3.12 (bs, 2H, NCH_2CON), 3.00 (t, *J* = 2.4 Hz, 2H, CH alkyne), 2.52 (t, *J* = 5.7 Hz, 2H, CH_2), 2.44 (m, 8H, CH_2 piperazine). MS (ES$^+$) *m/z* 622.6 $[M+H]^+$.

Synthesis of aniline derivative 18



To a solution of nitro-derivative **18a** (250 mg, 0.582 mmol, 1 eq) in 24 mL of a solution of H_2O:MeOH 10:90, NH_4Cl (68.5 mg, 1.281 mmol, 2.2 eq) and zinc powder (342.5 mg, 5.238 mmol, 9 eq) were added. The reaction was kept under stirring at 70 °C under reflux for 7 h, then the reaction was filtered on paper and cotton and the solvent was removed under reduced pressure. The crude was purified by flash chromatography (gradient: from 100% EtOAc to 60:40 EtOAc:MeOH•NH_3), giving aniline derivative **18** as a glassy solid (151.9 mg, 65% yield). TLC: 85:15 EtOAc:MeOH•NH_3, R*f* = 0.3. ^{1}H NMR (400 MHz, MeOD) δ 6.99 (m, 2H, ArH), 6.76 (m, 2H, ArH), 3.23 (t, *J* = 6.8 Hz, 2H, CH_2), 3.21 (s, 3H, CH_3), 3.09 (s, 2H, CH_2), 2.93 (m, 4H, CH_2), 2.80 (m, 3H, NH_3+), 2.68 (m, 4H, CH_2), 2.51-2.36 (m, 2H, CH_2), 2.42-2.37 (m, 2H, CH_2), 2.31 (bt, *J* = 2.4 Hz, 1H, CH Alkyne), 1.66 (m, 2H, CH_2), 2.33 (m, 2H, CH_2). ^{13}C NMR (101 MHz, MeOD) δ 174.1 (1C, Cq), 171.2 (1C, Cq), 149.6 (1C, Cq), 133.5 (1C, Cq), 128.9 (2C, CH), 116.7 (2C, CH), 83.6 (1C, Cq), 70.4 (1C, CH_2), 59.5 (1C, CH_2), 58.1 (2C, CH_2), 53.2 (2C, CH_2), 52.2 (1C, CH_2), 39.6 (1C, CH_2), 38.0 (1C, CH_2), 36.0 (1C, CH_3), 27.9 (1C, CH_2), 23.3 (1C, CH_2), 15.7 (1C, CH_2), 15.7 (1C, CH_2). MS (ES$^+$) *m/z* 401.5 $[M+H]^+$.

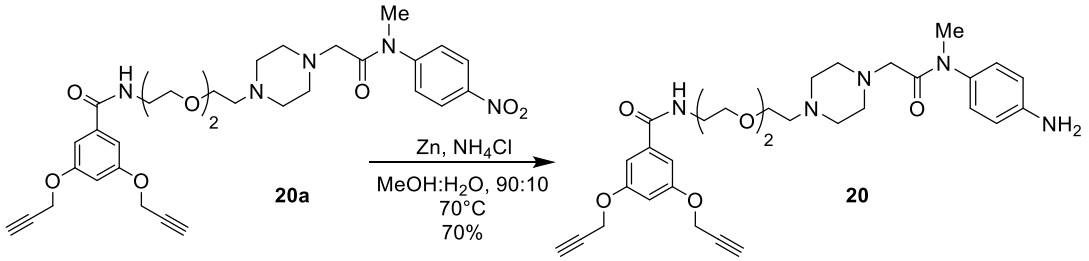
Synthesis of aniline derivative 19



Aniline derivative **19** was synthesized as described for compound **18**, starting from nitro-derivative **19a** (100.0 mg, 0.22 mmol, 1.0 eq). The crude was purified by flash chromatography (gradient: from 100% EtOAc to 60:40 EtOAc:MeOH•NH_3), giving aniline derivative **19** as a glassy solid (82.1 mg, 91% yield). TLC: 85:15 EtOAc:MeOH•NH_3, R*f* = 0.8. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.97 (m, 2H, ArH), 6.75 (m, 2H, ArH), 4.89 (bs, 3H,

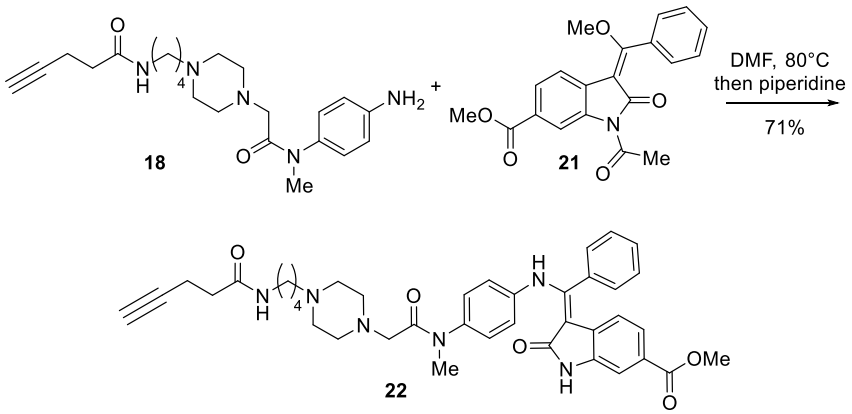
NH_3^+), 4.19 (d, *J* = 2.4 Hz, 2H, alkyne-CH_2), 3.69-3.58 (m, 10H, CH_2), 3.19 (s, 3H, CH_3), 2.97 (s, 2H, NCH_2CON), 2.88 (t, *J* = 2.4 Hz, 1H, CH alkyne), 2.72-2.60 (m, 6H, CH_2), 2.52 (m, 4H, CH_2). 13C NMR (101 MHz, MeOD) δ 170.3 (1C, Cq), 148.3 (1C, Cq), 132.5 (1C, Cq), 127.8 (2C, CH), 115.5 (2C, CH), 79.5 (1C, CH), 74.9 (1C, Cq), 70.3 (1C, CH_2), 70.1 (1C, CH_2), 68.9 (1C, CH_2), 68.0 (1C, CH_2), 58.9 (1C, CH_2), 57.8 (2C, CH_2), 57.3 (2C, CH_2), 52.8 (1C, CH_2), 52.4 (1C, CH_2), 36.8 (1C, CH_3). MS (ES^+) *m/z* 419.3 $[M+H]^+$.

Synthesis of aniline derivative 20



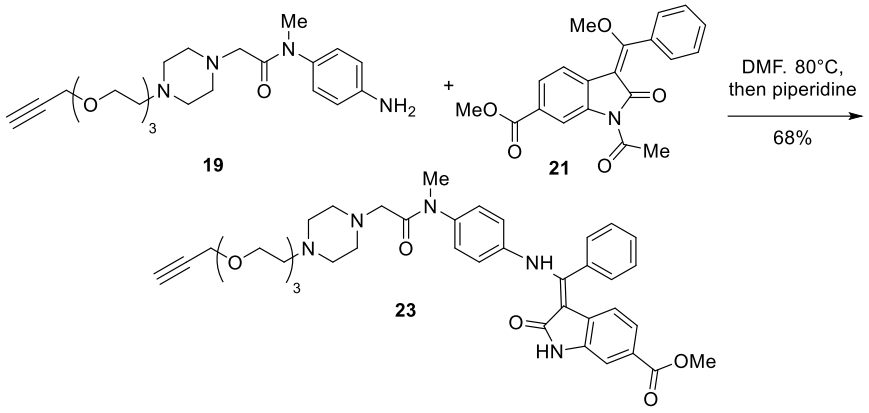
Aniline derivative **20** was synthesized as described for compound **18**, starting from nitro-derivative **20a** (131.0 mg, 0.21 mmol, 1.0 eq). The crude was purified by flash chromatography (gradient: from 100% EtOAc to 80:20 EtOAc:MeOH•NH_3), giving compound **20** (88.5 mg, yield 70%). ^{1}H NMR (400 MHz, MeOD) δ 7.08 (d, *J* = 2.3 Hz, 2H, ArH), 6.96 (m, 2H, ArH), 6.80 (t, *J* = 2.3 Hz, 1H, ArH), 6.75 (m, 2H, ArH), 4.76 (d, *J* = 2.3 Hz, 4H, $ArOCH_2$) 3.67-3.54 (m, 12H, CH_2,), 3.17 (m, 3H, CH_3), 3.02 (t, *J* = 2.3 Hz, 2H, CH alkyne), 2.92 (bs, 2H, CH_2), 2.59-2.39 (m, 10H, CH_2 piperazine+CH_2). MS (ES^+) *m/z* 592.7 $[M+H]^+$.

Synthesis of nintedanib-linker nucleus 22



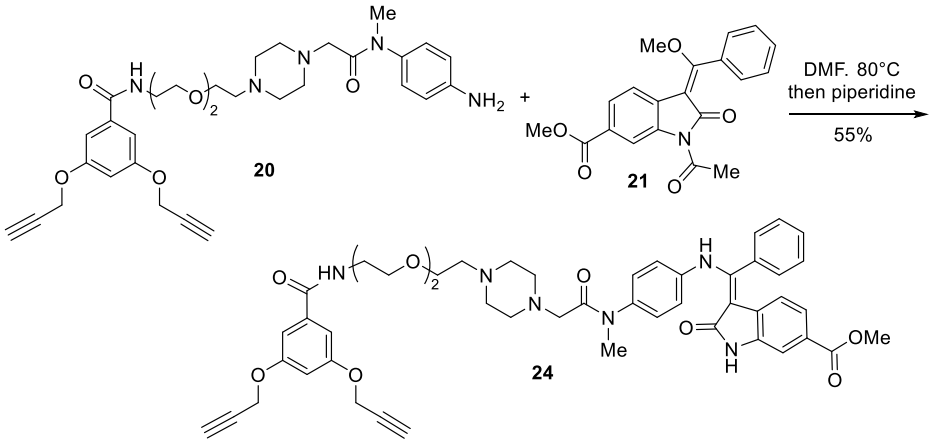
To a solution of aniline **18** (151.9 mg, 0.354 mmol, 1 eq) in DMF (35 mL) at 80 °C under reflux, 2-oxindole **21** (136.7 mg, 0.289 mmol, 1.1 eq) was added. The reaction was stirred for 30 h, then piperidine (78.1 μL, 0.354 mmol, 1 eq) was added and the system was left to lower to room temperature. After 2 h, the solvent was removed under reduced pressure and the crude was purified by flash chromatography (gradient: from 98:2 EtOAc:MeOH•NH_3 to 95:5), giving compound **22** as a yellowish oil (147.7 mg, 71% yield). TLC: EtOAc:MeOH•NH_3 85:15, R*f* = 0.3. ^{1}H NMR (400 MHz, MeOD) δ 7.64-7.55 (m, 4H, ArH), 7.49 (m, 2H, ArH), 7.29 (m, 1H, ArH), 7.12 (m, 2H, ArH), 6.94 (m, 2H), 5.97 (m, 1H, ArH), 3.87 (s, 3H, CH_3 ester), 3.37 (s, 3H, CH_3 amide), 3.24-3.16 (m, 4H, CH_2), 2.84 (s, 2H, CH_2), 2.52-2.36 (m, 12H, CH_2), 2.29 (t, *J* = 2.3 Hz, 1H, CH alkyne), 1.54 (m, 4H, CH_2). MS (ES^+) *m/z* 677.4 $[M+H]^+$.

Synthesis of nintedanib-linker nucleus 23



Compound **23** was synthesized as described for compound **22**, starting from aniline **19** (82.1 mg, 0.20 mmol, 1 eq). The crude was purified by flash chromatography (gradient: from 98:2 EtOAc:MeOH•NH_3 to 90:10), giving compound **23** as a yellow oil (90.0 mg, 68% yield). TLC: EtOAc:MeOH•NH_3 8:2, R*f* = 0.8. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63-7.52 (m, 4H, ArH), 7.46-7.37 (m, 3H, ArH), 6.99 (m, 2H, ArH), 6.81 (m, 2H, ArH), 6.01 (d, *J* = 8.3 Hz, 1H, ArH), 4.20 (d, *J* = 2.4 Hz, 2H, CH_2-alkyne), 3.87 (s, 3H, CH_3 ester), 3.72-3.61 (m, 12H, CH_2), 3.19 (s, 3H, CH_3N), 2.81 (s, 2H, NCH_2CON), 2.64-2.56 (m, 8H, CH_2), 2.44 (t, *J* = 2.4 Hz, 1H, CH alkyne). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1 (1C, Cq), 169.6 (1C, Cq), 167.5 (1C, Cq), 158.5 (1C, Cq), 140.2 (1C, Cq), 138.5 (1C, Cq), 135.6 (1C, Cq), 132.5 (1C, Cq), 130.8 (1C, CH), 129.8 (Cq), 129.3 (2C, CH), 128.7 (2C, CH), 128.1 (1C, CH), 125.3 (2C, CH), 124.1 (2C, CH), 123.0 (1C, CH), 118.4 (1C, CH), 110.5 (1C, Cq), 79.8 (1C, Cq), 77.4 (1C, CH), 74.8 (1C, CH_2), 70.8 (1C, CH_2), 70.6 (1C, CH_2), 70.5 (1C, CH_2), 69.3 (1C, CH_2), 68.9 (1C, CH_2), 59.7 (1C, CH_2), 58.6 (2C, CH_2), 57.8 (2C, CH_2), 53.5 (1C, CH_2), 53.2 (1C, CH_2), 52.1 (1C, CH_3), 37.5 (1C, CH_3). MS (ES^+) *m/z* 696.3 $[M+H]^+$.

Synthesis of nintedanib-linker nucleus 24

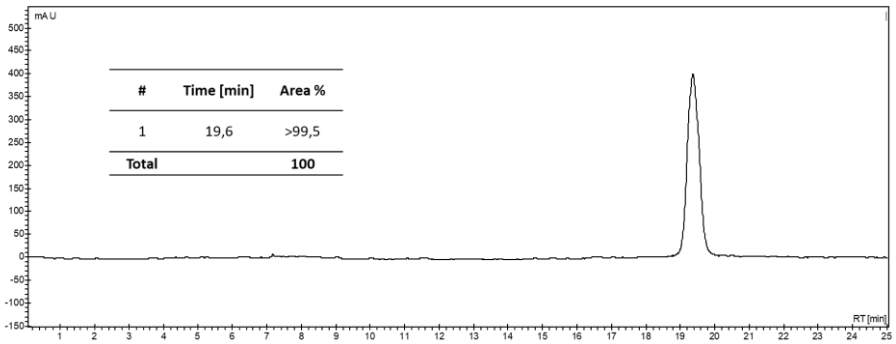


Compound **24** was synthesized as described for compound **22**, starting from aniline **20** (88.5 mg, 0.146 mmol, 1 eq). The crude was purified by flash chromatography (gradient: from 95:5 EtOAc:MeOH•NH_3), giving compound **24** as a yellow oil (67.8 mg, 55% yield). TLC: EtOAc:MeOH•NH_3 95:5, R*f* = 0.2. ^{1}H NMR (400 MHz, MeOD) δ 7.66-7.56 (m, 4H, ArH), 7.51 (m, 2H, ArH), 7.30 (m, 1H, ArH), 7.11 (m, 4H, ArH), 6.95 (m, 2H, ArH), 6.79 (t, *J* = 2.3 Hz, 1H, ArH), 5.97 (d, *J* = 8.5 Hz, 1H, ArH), 4.77 (d, *J* = 2.4 Hz, 4H, $ArOCH_2$), 3.85 (s, 3H, CH_3 ester), 3.67-3.60 (m, 8H, CH_2), 3.56 (t, *J* = 5.6 Hz, 2H, CH_2), 3.17 (bs, 3H, NCH_3), 3.01 (t, *J* = 2.4 Hz, 2H, CH alkyne), 2.81 (bs, 2H, piperazine-CH_2CON), 2.58-2.28 (m, 10H, CH_2 piperazine+CH_2). MS (ES^+) *m/z* 869.4 $[M+H]^+$.

HPLC chromatograms of final compounds

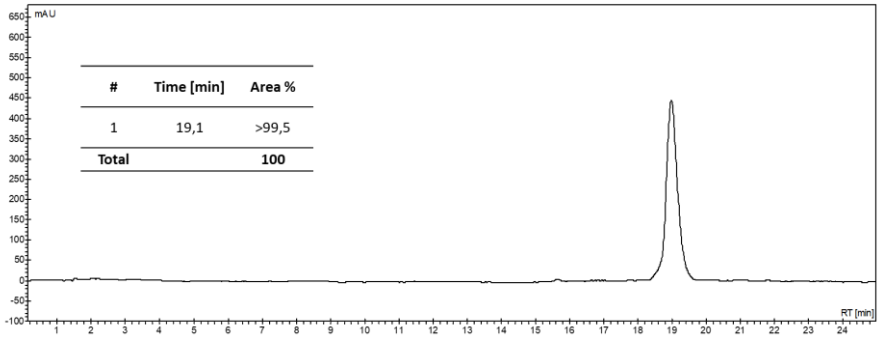
General method for purity determination. The purity of the final compound was determined by reverse phase HPLC (C18-10 µm column Discovery BIO Wide Pore 21.2 × 250 mm), with the solvent system H2O + 0.1% TFA (Solvent A) and ACN (solvent B), using the following method: flow rate 3.0 mL/min; detection at 220 nm, linear gradient from 5% B to 50% B over 23 min, 50% B for 3 min, from 50% B to 5% B over 3 min, room temperature.

Compound 1



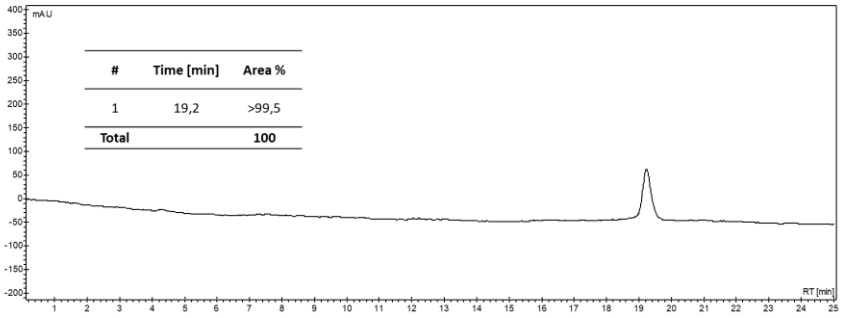
#	Time [min]	Area %
1	19,6	>99,5
Total		**100**

Compound 2



#	Time [min]	Area %
1	19,1	>99,5
Total		**100**

Compound 3



#	Time [min]	Area %
1	19,2	>99,5
Total		**100**

Figure S1. Purity of the final compounds **1**-**3** determined by RP-HPLC

BIOLOGY

Cytofluorimetric assay of $\alpha_v\beta_6$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ expression. Cytofluorimetric analysis was performed to confirm the presence of integrin receptors on the surface of the three selected cell lines: L929 murine fibroblasts, K562 human erythroleukemia cells, and human MDA-MB-231 breast cancer cells. We found that L929 cells express high level of $\alpha_V\beta_6$ (>85% of cell population), $\alpha_V\beta_3$ (>90% of cell population), and $\alpha_V\beta_5$ (>25% of cell population) integrin receptors. K562 cells (Figure S2), as found in previously published papers, do not express $\alpha_V\beta_6$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin receptors and were used as negative expressing cells. We found that MDA-MB-231 cells express only $\alpha_V\beta_3$ integrin receptors (>50% of cell population) and were used as $\alpha_V\beta_6$ negative expressing cells.

Methods. L929, K562 and MDA-MB231 cells were allowed to grow in standard conditions, for 24h and next were collected using Accutase® solution and resuspended in PBS with 1% BSA. After blocking, cells were incubated with 1 µg/50 µL of anti-$\alpha_V\beta_6$ (BS-5791R Bioss antibodies, USA), anti-$\alpha_V\beta_3$ (ABIN674784 Antibodies online) or anti-αvβ5 (SC-13588 Santa Cruz, USA), for 1 h at 4 °C. Primary antibodies recognize human, mouse and rat forms of integrin receptors. Followed by 1 h incubation with FITC-conjugated anti-rabbit (24549933) secondary antibodies were used for anti-$\alpha_V\beta_6$ and anti-$\alpha_V\beta_3$ antibodies, while PE-conjugated anti-mouse (22549814) were used (Immunotools). Cells were analyzed using a BD FACSCanto™ flow cytometry system.

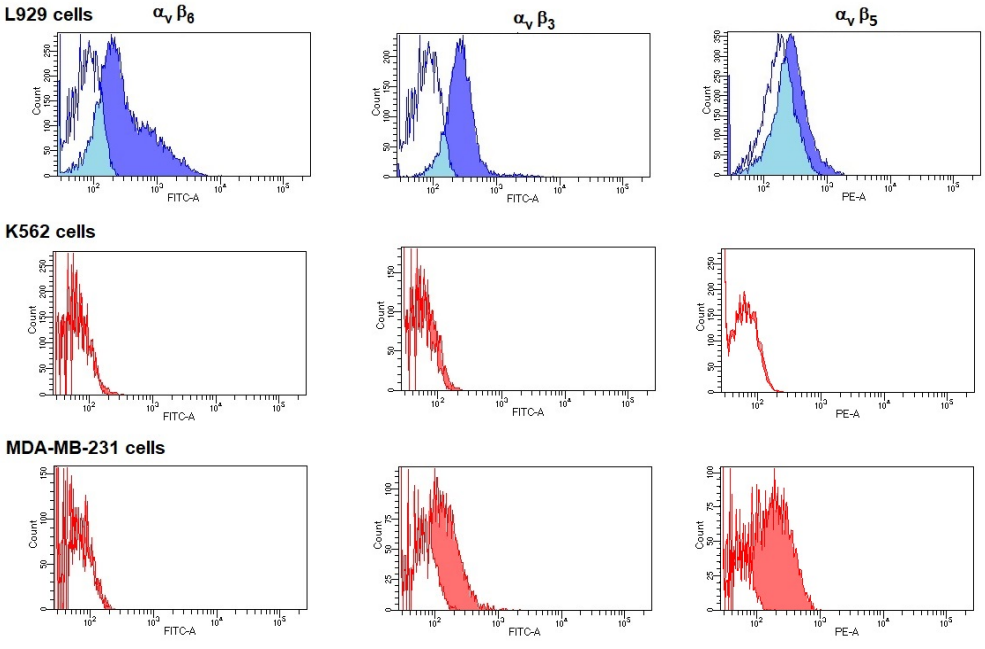


Figure S2. Cytofluorimetric analysis of $\alpha_v\beta_6$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ expression on L929 cells, K562 cells and MDA-MB231 cells. In each graph the *x* axis represents the intensity of fluorescence (FITC-A channel) and the *y* axis represents the number of counted cells. Cell lines were stained with anti-$\alpha_v\beta_6$, anti-$\alpha_v\beta_3$, or anti-$\alpha_v\beta_5$ antibody followed by FITC or PE-conjugated anti-immunoglobulin (full histograms). As a negative control (open histogram), cells were stained with FITC or PE-conjugated secondary antibody only.

Inhibition of cell proliferation assay. The cytotoxic and cytostatic effect of c(AmpLRGDL)-Nintedanib conjugates in comparison with compound **6**, nintedanib or their combination were studied using the MTT assays. Cytostatic effect was evaluated as percentage of growth inhibition according to National Cancer Institute (NCI) as following: [(Tt24x)-(Ct0) / (Ct24)-(Ct0)] x 100 for Tt24x>Ct0. We found that L929 cells exposed to nintedanib at 10 μM exhibited less than 50% inhibition of proliferation compared to untreated cells. The inhibitory effect registered with nintedanib was similar to that observed both with integrin ligand **6** alone and with the nintedanib and compound **6** in combination (**N**/**6**). A weaker cytostatic effect was given by conjugates **1** and **2**, and even lower by compound **3**. Conversely, in any of the treatments we found a Tt24x<Ct0, thus c(AmpLRGDL)-Nintedanib conjugates, compound **6**, nintedanib or their combination did not show direct cytotoxicity for L929 cells. The weaker cytostatic effect obtained by the treatments with the conjugated compounds might be explained by the reduction of drug internalization.

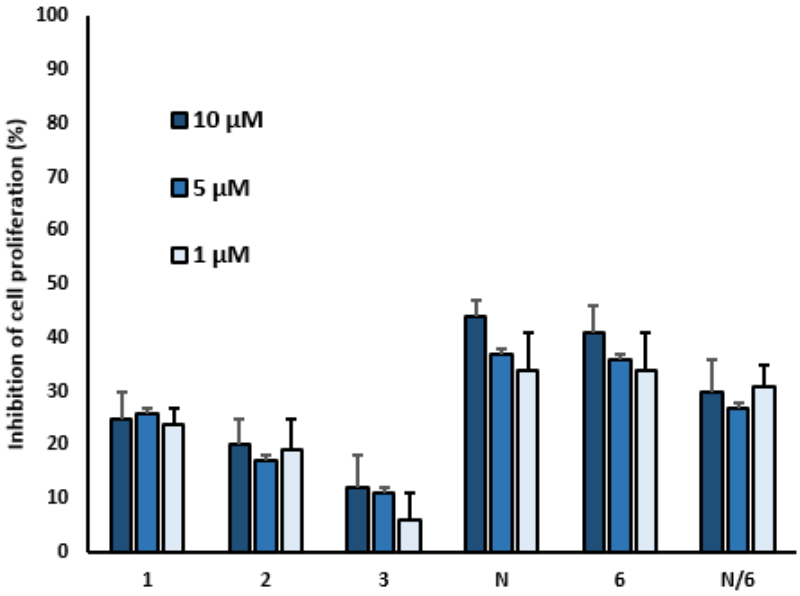


Figure S3. Inhibition of L929 cell proliferation. Cells were exposed for 24 h to 1, 5 and 10 μM concentration of conjugates **1**-**3**, integrin ligand **6**, nintedanib **N** and combination of **N** and **6** (**N**/**6**). Representative of three independent experiments.

Methods. The cells were plated in 96-well plate at a density of $2x10^4$cells/mL per well and maintained for 24 h at 37 °C in a 5% CO_2 incubator and cultured as monolayers. After 24 hours, cells were treated with 1–10 μM of the tested compounds for 24 h. Following 24h of drug exposure, cell viability was determined by the (3-(4,5-imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic assay as described previously. The MTT (Sigma, St Louis, Missouri, USA) was dissolved in PBS in a concentration of 5 mg/ml and added to

the cultured cells. After 3 hours of incubation at 37 °C, formazan crystals were solubilized by DMSO (100 µL). Absorbance of the converted dye was measured at a wavelength of 540nm on ELISA reader (VersaMax Microplate Reader Orleans, USA).

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