

Article

Synthesis and Anti-Angiogenic Activity of Novel c(RGDyK) peptide-based JH-VII-139-1 conjugates

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amide, carbamate

Abstract: Peptide–drug conjugates are delivery systems for selective delivery of cytotoxic agents to target cancer cells. In this work, the optimized synthesis of JH-VII-139-1 and its c(RGDyK) peptide conjugates is presented. The low nanomolar SRPK1 inhibitor, JH-VII-139-1, which is an analogue of Alectinib, was linked to the $\alpha_v\beta_3$ targeting oligopeptide c(RGDyK) through amide, carbamate and urea linkers. The chemostability, cytotoxic and antiangiogenic properties of the synthesized hybrids were thoroughly studied. All conjugates retained mid nanomolar-level inhibitory activity against SRPK1 kinase and two out of four conjugates, **geo75** and **geo77** exhibited antiproliferative effects with low micromolar IC_{50} values against HeLa, K562, MDA-MB231 and MCF7 cancer cells. The activities were strongly related to the stability of the linkers and the release of JH-VII-139-1. *In vivo* zebrafish screening assays demonstrated the ability of all synthesized conjugates to significantly inhibit width angiogenesis in zebrafish embryos. Flow cytometry experiments were used to test the cellular uptake of a fluorescein tagged hybrid in MCF7 and MDA-MB231 cells that revealed a receptor mediated endocytosis process. In conclusion, most conjugates retained the inhibitory potency against SRPK1 as JH-VII-139-1 and demonstrated antiproliferative and antiangiogenic activities. Further animal model experiments are needed to uncover the full potential of such peptide conjugates in cancer therapy and angiogenesis-related diseases.

Keywords: Cancer; Angiogenesis; Peptide Drug Conjugates; PDCs; Hybrid Molecules; RGD peptide; Receptor Mediated Endocytosis; Integrins; SRPK1.

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Last-name

Received: date

Accepted: date

Published: date

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1. Introduction

Angiogenesis is described as the formation of new blood vessels from pre-existing vessels [1, 2]. It is a vital physiological process for growth, development, and wound repair by which various materials, oxygen and nutrients are delivered to tissues and cellular wastes are removed. In cancer, abnormal angiogenesis is related to cell growth, tumorigenesis, and metastases [3–5]. However, recent findings implicate angiogenesis in many other diseases [6] such as diabetic retinopathy, neovascular glaucoma,[7] autoimmune diseases, multiple sclerosis,[8] rheumatoid arthritis,[9] cardiovascular diseases,[10] atherosclerosis,[11] and cerebral ischemia. Angiogenesis is regulated by many pathways and signaling molecules such as the vascular endothelial growth factor (VEGF), the transforming growth factor (TGF)- β , the angiopoietin-1 and 2, the placental growth factor (PGF), $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins,[12] the Notch, Akt, Jak/STAT, ephrin/Eph signaling pathways and others [13,14]. VEGF binds to receptor VEGFR and this interaction predominantly regu-

lates pathological and physiological angiogenesis [15–17]. The VEGFR signaling is monitored at multiple levels [18]. For example, serine-arginine (SR) protein kinases (SRPKs) regulate the splicing of proangiogenic VEGF165, by phosphorylation of the serine/arginine splicing factor 1 (SRSF1) [19]. Therefore, targeting SRPK1 kinase to regulate angiogenesis in angiogenic disorders has been proposed by several research groups. Bates et al has used the SRPK1 inhibitor SRPIN340 to reduce proangiogenic VEGF165 and inhibit melanoma tumour growth *in vivo* [20]. Oltean et al demonstrated that SPHINX and SRPIN340 treatment changed the expression of VEGF165 towards the anti-angiogenic splice isoform VEGF165b and decreased tumour growth in orthotopic PC3 models in mice [21].

In previous research efforts by our group, the cyclic pentapeptide c(RGDyK) which is a potent integrin $\alpha_v\beta_3$ ligand was employed for the targeted delivery of various anti-cancer drugs and bioactive molecules such as the gemcitabine, [22] the triterpenoids cucurbitacins, [23] and platinum complexes [24]. c(RGDyK) peptide potently binds $\alpha_v\beta_3$ integrins ($IC_{50} = 3.8 \pm 0.42$ nM) and less potently $\alpha_v\beta_5$ ($IC_{50} = 503 \pm 55$ nM), $\alpha_v\beta_6$ ($IC_{50} = 86 \pm 7$ nM), and $\alpha_5\beta_1$ integrins ($IC_{50} = 236 \pm 45$ nM) [25]. In earlier studies, the SRPIN803 inhibitor which binds to SRPK1 and CK2 kinases was coupled to c(RGDyK) peptide aiming to form a new conjugate with improved antiangiogenic activities compared to both the peptide and the SRPIN803 [26]. Although the antiangiogenic activities of the synthesized SRPIN803-c(RGDyK) hybrids and SRPIN803 were evident in zebrafish embryos, an unfavorable stability profile of SRPIN803 was observed as SRPIN803 and its derivatives undergo a retro-Knoevenagel reaction. As a next step, we intended to develop new anti-angiogenic compounds based on the more potent SRPK1 inhibitor, JH-VII-139-1 [27]. JH-VII-139-1 came up from the optimization of FDA approved ALK inhibitor Alectinib, [28] which previously was shown to bind to SRPK1. Gray and coworkers has demonstrated that JH-VII-139-1 potently inhibits SRPK1 with an IC_{50} value of 1.1 nM and blocks angiogenesis in an age-related macular degeneration (AMD) animal model [27]. In this work, the synthesis of JH-VII-139-1 peptide conjugates with c(RGDyK) and the properties of the new hybrid compounds against SRPK1, cancer cell growth and angiogenesis are reported.

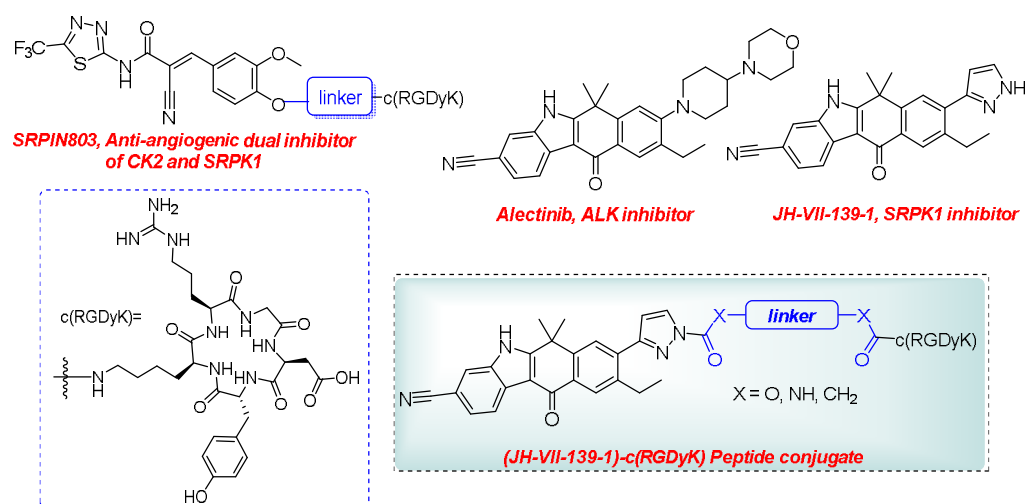


Figure 1. General structures of alectinib, JH-VII-139-1 and c(RGDyK) peptide conjugates that carry an SRPK1 inhibitor.

2. Materials and Methods

2.1. Chemistry

2.1.1 General Experimental Details. All reactions were carried out under an atmosphere of Argon unless otherwise specified. Commercial reagents were used without further purification. Reactions were monitored by TLC and using UV light as a visualizing agent

and aqueous ceric sulfate/phosphomolybdic acid, ethanolic *p*-anisaldehyde solution, potassium permanganate solution, and heat as developing agents. The ^1H and ^{13}C NMR spectra were recorded at 500 and 126 MHz (Agilent) with tetramethylsilane as an internal standard. Chemical shifts are indicated in δ values (ppm) from internal reference peaks (TMS ^1H 0.00; CDCl_3 ^1H 7.26, ^{13}C 77.16, $(\text{CD}_3)_2\text{SO}$ ^1H 2.50, ^{13}C 39.52, $(\text{CD}_3)_2\text{CO}$ ^1H 2.05, ^{13}C 29.84, 206.26). LC-MS analysis was performed on a LC-20AD Shimadzu connected to Shimadzu LCMS-2010EV equipped with C18 analytical column (Supelco discovery C18, $5\mu\text{m}$ 250 x 4.6mm).

2.1.2 Synthetic Procedures

7-Methoxy-1,1-dimethyl-3,4-dihydronaphthalen-2(1H)-one, 2. To a solution of 7-methoxy-2-tetralone (5 g, 28.5 mmol) in THF (20 mL) were added tetrabutylammonium bisulfate (1.53 g, 4.5mmol), methyl iodide (5.25 mL, 85.5mmol), and 50% aqueous KOH solution (32.5 mL) and stirred at room temperature for 2 hours. The mixture was diluted with EA and washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (gradient elution PS – EA, 15:1 to 10:1) to afford the desired product, **2** (5.3 g, 91%) as a white solid. The spectral data were in accordance with those reported in the literature [29]. **2**: ^1H NMR (500 MHz, CDCl_3) δ 7.09 (d, J = 8.3 Hz, 1H), 6.88 (d, J = 2.5 Hz, 1H), 6.74 (dd, J = 8.3, 2.5 Hz, 1H), 3.81 (s, 3H), 3.03 (t, J = 6.9 Hz, 2H), 2.66 (t, J = 6.9 Hz, 2H), 1.42 (s, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ 214.7, 158.8, 145.0, 129.1, 127.5, 112.3, 111.4, 55.4, 48.0, 37.6, 27.8, 26.9. ESI-MS, positive mode: m/z calcd mass for $\text{C}_{13}\text{H}_{16}\text{O}_2\text{Na}$ $[\text{M}+\text{Na}]^+$ = 227.10, was found 227.05.

6-Bromo-7-methoxy-1,1-dimethyl-3,4-dihydronaphthalen-2(1H)-one, 3. To a solution of **2** (2.00 g, 9.8 mmol) in CH_3CN (40 mL) was added *N*-bromosuccinimide (1.92 g, 10.8 mmol). After stirring for 2.5 h at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (gradient elution PS – EA, 15:1 to 13:1) to yield **3** (2.55 g, 92%) as a white solid. ^1H -NMR data was in accordance with those reported in the literature [30]. **3**: ^1H NMR (500 MHz, CDCl_3) δ 7.35 (s, 1H), 6.83 (s, 1H), 3.90 (s, 3H), 3.01 (t, J = 6.9 Hz, 2H), 2.65 (t, J = 6.9 Hz, 2H), 1.43 (s, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ 213.8, 155.1, 144.2, 132.7, 129.0, 110.0, 109.7, 56.5, 48.0, 37.2, 27.5, 26.9.

3-Hydrazinylbenzonitrile, 5. 4-Cyanoaniline (472 mg, 4 mmol) was added to a round bottom flask containing 10 mL 6N HCl. The solution was vigorously stirred at 0 °C for 10 min. Sodium nitrite (280 mg, 4 mmol) dissolved in 2 mL water was added dropwise to the aniline solution, and the resulting mixture was stirred at 0 °C for an additional 1h. To a beaker containing SnCl_2 (1.9 g, 10 mmol) was added 10 mL of concentrated HCl, and the solution was sonicated and cooled to 0 °C. The SnCl_2 solution was then added dropwise to the diazonium salt solution, and the mixture was stirred for additional 90 min at room temperature. Then, sodium hydroxide (7.2 g, 0.18 mol) dissolved in 20 mL water was added to quench the reaction. The solution was extracted with 20 mL of diethyl ether three times, and the organic layers were combined and concentrated under reduced pressure to give the crude product, **5** (300mg, 56%), which was further purified by recrystallization in hot ethanol. The spectral data were in accordance with those reported in the literature [31]. **5**: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 7.27 – 7.20 (m, 2H), 7.08 (d, J = 1.1 Hz, 1H), 7.01 (dd, J = 8.4, 1.1 Hz, 1H), 6.93 (d, J = 7.4 Hz, 1H), 4.12 (s, 2H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 152.9, 129.7, 119.7, 119.4, 116.1, 113.2, 111.4.

9-Bromo-8-methoxy-6,6-dimethyl-6,11-dihydro-5H-benzo[b]carbazole-3-carbonitrile, 6a. A mixture of **3** (2.8 g, 9.89 mmol), **5** (1.58 g, 11.9 mmol), and trifluoroacetic acid (48 mL) was refluxed at 80°C for 2 h. The reaction mixture was cooled and neutralized by saturated aqueous Na_2CO_3 , then by NaHCO_3 and extracted with EA. The combined organic layers were washed with brine, dried over Na_2SO_4 , and concentrated under reduced pressure. The residue was resuspended in EA and precipitated solid (undesired regioisomer) was filtered off. The filtrate was evaporated under reduced pressure and purified by column chromatography (isocratic elution PS - EA 4:1) to yield **6a** as an orange solid (1.9 g, 50%).

6a: ^1H NMR (500 MHz, acetone- d_6) δ 10.79 (s, 1H), 7.75 (s, 1H), 7.67 (d, J = 8.1 Hz, 1H), 7.55 (s, 1H), 7.34 (dd, J = 8.2, 1.2 Hz, 1H), 7.31 (s, 1H), 4.08 (s, 2H), 3.96 (s, 3H), 1.77 (s, 6H). ^{13}C NMR (126 MHz, acetone- d_6) δ 155.5, 145.4, 144.8, 136.6, 134.2, 131.2, 130.6, 127.7, 122.6, 119.8, 116.2, 111.1, 110.1, 106.8, 104.1, 56.7, 37.1, 31.1, 25.9. ESI-MS, negative mode: m/z calcd mass for $\text{C}_{20}\text{H}_{16}\text{BrN}_2\text{O}$ $[\text{M}-\text{H}]^-$ = 379.04, was found 380.85.

9-Bromo-8-methoxy-6,6-dimethyl-6,11-dihydro-5H-benzo[b]carbazole-1-carbonitrile, 6b: ^1H NMR (500 MHz, DMSO) δ 11.68 (s, 1H), 7.68 (d, J = 7.9 Hz, 1H), 7.54 (s, 1H), 7.46 (d, J = 7.1 Hz, 1H), 7.26 (s, 1H), 7.21 (t, J = 7.6 Hz, 1H), 4.23 (s, 2H), 3.91 (s, 3H), 1.70 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 154.1, 143.7, 143.6, 136.4, 133.0, 126.3, 125.7, 124.7, 120.8, 119.4, 116.2, 110.4, 108.9, 103.2, 99.8, 56.4, 35.9, 30.8, 25.3.

6a **9-Bromo-8-methoxy-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[b]carbazole-3-carbonitrile, 7.** To a solution of **6a** (2.1 g, 4.96 mmol) in THF (21.4 mL) and water (2.14 mL) was gradually added DDQ (3.38 g, 14.9 mmol). After stirring for 3 h at 0 °C, the reaction mixture was evaporated under reduced pressure and the residue was dissolved in EA. The solution was washed with aqueous NaOH, water, and brine, dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. The residue was purified with column chromatography (gradient elution PS - EA 3:1 to 1:2) to afford **7** (1.58 g, 81%) as yellow solid. ^1H -NMR data was in accordance with those reported in the literature [31]. **7:** ^1H NMR (500 MHz, acetone- d_6) δ 8.42 (s, 1H), 8.41 (s, 1H), 7.92 (d, J = 0.6 Hz, 1H), 7.57 (dd, J = 8.1, 1.4 Hz, 1H), 7.50 (s, 1H), 4.09 (s, 3H), 1.91 (s, 6H). ^{13}C NMR (126 MHz, acetone- d_6) δ 178.6, 160.2, 159.9, 150.7, 136.9, 131.8, 128.9, 127.3, 125.7, 123.0, 120.4, 117.0, 111.4, 110.8, 110.6, 106.8, 57.2, 38.1, 30.4. ESI-MS, negative mode: m/z calcd mass for $\text{C}_{20}\text{H}_{14}\text{BrN}_2\text{O}_2$ $[\text{M}-\text{H}]^-$ = 393.02, was found 394.85.

Cs2CO3 **9-Ethyl-8-methoxy-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[b]carbazole-3-carbonitrile, 8.** To a degassed mixture of **7** (1.52 g, 3.85 mmol), Cs_2CO_3 (3.76 g, 11.5 mmol), $\text{Pd}(\text{dppf})\text{Cl}_2$ (57.7 mg, 0.08 mmol) and DMF (55.7 mL) in a Schlenk flask under argon atmosphere was added a solution of triethylborane in THF (11.5 mL, 1M solution, 11.5 mmol) and the reaction was stirred at 86 °C overnight. The reaction mixture was then cooled, diluted with EA, washed several times with water, dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (gradient elution PS - EA 2:1 to 1:2) to yield **8** (870 mg, 66%) as yellow crystalline solid. **8:** ^1H NMR (500 MHz, acetone- d_6) δ 11.69 (s, 1H), 8.47 (d, J = 8.1 Hz, 1H), 8.12 (s, 1H), 7.90 (s, 1H), 7.57 (dd, J = 8.1, 0.5 Hz, 1H), 7.34 (s, 1H), 4.02 (s, 3H), 2.71 (q, J = 7.5 Hz, 2H), 1.88 (s, 6H), 1.24 (t, J = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, acetone- d_6) δ 180.2, 161.9, 160.2, 149.1, 136.8, 132.2, 129.1, 127.4, 125.7, 125.5, 123.0, 120.6, 116.8, 111.1, 108.3, 106.4, 56.2, 37.8, 30.7, 23.6, 14.3. ESI-MS, positive mode: m/z calcd mass for $\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}_2$ $[\text{M}]^+$ = 344.15, was found 344.95.

9-Ethyl-8-hydroxy-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[b]carbazole-3-carbonitrile, 9. Pyridine (141 μL , 1.74 mmol) was slowly added to a hydrochloric acid solution in 1,4-dioxane (436 μL , 4M, 1.74 mmol) at 0 °C under argon. The mixture was stirred for 5 minutes and then concentrated under reduced pressure to form white highly hygroscopic pyridinium chloride salt. Both **8** (12 mg, 34.8 μmol) and pyridinium chloride (1.74 mmol) were quickly transferred to a sealed tube and stirred at 170 °C overnight. The reaction mixture was dissolved in water, neutralized by saturated aqueous NaHCO_3 , and extracted with EA three times. The combined organic layers were washed with aqueous NaHCO_3 and brine, dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (gradient elution of PS - EA 2:1 to 1:2) to yield **9** as a beige solid (9.5 mg, 83%). **9:** ^1H NMR (500 MHz, DMSO- d_6) δ 12.67 (s, 1H), 10.20 (s, 1H), 8.32 (dd, J = 8.1, 0.4 Hz, 1H), 7.98 (d, J = 0.6 Hz, 1H), 7.94 (s, 1H), 7.59 (dd, J = 8.1, 1.4 Hz, 1H), 7.10 (s, 1H), 2.62 (q, J = 7.5 Hz, 2H), 1.70 (s, 6H), 1.19 (t, J = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 179.2, 159.4, 159.3, 147.9, 135.6, 129.4, 127.8, 126.8, 124.7, 123.3, 121.7, 120.2, 116.4, 111.8, 109.4, 104.4, 36.2, 30.3, 22.5, 13.9. ESI-MS, positive mode: m/z calcd mass for $\text{C}_{21}\text{H}_{18}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}]^+$ = 330.14, was found 330.90.

3-Cyano-9-ethyl-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[b]carbazol-8-yl trifluoromethanesulfonate, 10. A suspension of **9** (480 mg, 1.45 mmol) and K_2CO_3 (402 mg, 2.91

mmol) in DMF (13.7 mL) was slowly (portionwise) treated with *N*-phenyl-bis(trifluoromethanesulfonimide) (1.04 g, 2.91 mmol) at 0 °C. After 24 h, the reaction mixture was diluted with EA and poured into water. The layers were partitioned, and the organic phase washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by silica gel chromatography (gradient elution PS - EA 3:1 to 1:1) to afford **10** (540 mg, 80% yield) as a **sub** yellow solid. **10**: ¹H NMR (500 MHz, acetone-*d*₆) δ 11.87 (s, 1H), 8.44 (d, *J* = 8.1 Hz, 1H), 8.37 (s, 1H), 7.94 (d, *J* = 1.3 Hz, 1H), 7.84 (s, 1H), 7.60 (dd, *J* = 8.1, 1.3 Hz, 1H), 2.89 (q, *J* = 7.6 Hz, 2H), 1.91 (s, 6H), 1.36 (t, *J* = 7.6 Hz, 3H). ¹³C NMR (126 MHz, acetone-*d*₆) δ 178.8, 160.3, 151.5, 149.1, 137.1, 136.3, 132.9, 129.5, 128.9, 126.1, 123.2, 120.7, 120.4, 119.7, 117.2, 111.2, 107.2, 37.9, 30.4, 23.4, 14.2. ESI-MS, positive mode: *m/z* calcd mass for C₂₂H₁₇F₃N₂O₄S [M]⁺ = 462.08, was found 462.95

1-(Tetrahydro-2H-pyran-2-yl)-1H-pyrazole, **12**. A mixture of pyrazole (14.3 g, 0.21 mol), 3,4-dihydro-2H-pyran (29 mL, 0.32 mol), and trifluoroacetic acid (0.1 mL, 3 mmol) was refluxed for 2 h. After quenching with sodium hydride (0.2 g, 8 mmol), the mixture was subjected to column chromatography (gradient elution PS - EA 20:1 to 5:1) to **yield 12 (3.2 g, 95%) as a colorless oil**. The spectral data were in accordance with those reported in the literature [32]. **12**: ¹H NMR (500 MHz, CDCl₃) δ 7.61 (d, *J* = 2.4 Hz, 1H), 7.56 (d, *J* = 1.4 Hz, 1H), 6.32 – 6.29 (m, 1H), 5.40 (dd, *J* = 9.7, 2.5 Hz, 1H), 4.06 (dd, *J* = 12.2, 2.6 Hz, 1H), 3.73 – 3.67 (m, 1H), 2.23 – 2.05 (m, 3H), 1.77 – 1.55 (m, 3H).

(1-(Tetrahydro-2H-pyran-2-yl)-1H-pyrazol-3-yl)boronic acid, **S1**. To a solution of 1-(tetrahydropyran-2-yl)-1H-pyrazole (7.6 g, 0.052 mol) in THF (50 mL), cooled to -78 °C, was added *n*-butyllithium (1.6 M in hexane, 33 mL, 0.052 mol) dropwise. Bulky precipitate formed and the mixture turned yellow. After 30 min, trimethyl borate (12.7 mL, 0.055 mol) was added over 10 min, and the reaction mixture was stirred at -78 °C for 1 h. The reaction was quenched with hydrochloric acid (2 M in water, 0.104 mol, 52 mL) and was left to return to ambient temperature in 1 h. The mixture was extracted with EA and the organic layers were dried over Na₂SO₄ and concentrated *in vacuo*. The residue was crystallized with PS - EA (12:1) to **yield S1 (130 mg, 40%) as a white solid**. **S1** was used to the next step without further purification.

3-Borono-1H-pyrazol-1-ium trifluoroacetate, **13**. To a solution of **S1** (110 mg, 0.56 mmol) in dichloromethane (1 mL), trifluoroacetic acid (0.5 mL) was added at room temperature, and the reaction solution was stirred for 24 hours. The reaction solution was concentrated, and the residue was crystallized with PS - DCM - dioxane (5:10:1) to afford **13** (70 mg 55%) as white powder. The spectral data were in accordance with those reported in the literature [33]. **13**: ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.28 (s, 2H), 7.52 (d, *J* = 1.6 Hz, 1H), 6.70 (d, *J* = 1.7 Hz, 1H).

9-Ethyl-6,6-dimethyl-11-oxo-8-(1H-pyrazol-3-yl)-6,11-dihydro-5H-benzo[*b*]carbazole-3-carbonitrile, **JH-VII-139-1**. A degassed mixture of **10** (76 mg, 0.164 mmol), K₂CO₃ (182 mg, 1.31 mmol), Pd(dppf)Cl₂ (6 mg, 0.008 mmol), **13** (85 mg, 0.378 mmol), tBuXPhos (5.6 mg, 0.013 mmol), dioxane (5.06 mL) and H₂O (1.26 mL) was stirred in a microwave tube at 70 °C in microwave for two hours. The reaction mixture was then cooled, diluted with EA, washed three times with water, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (gradient elution PS - EA 1:1, 1:2, 1:4) to **yield JH-VII-139-1 (50 mg, 80 %) as brown crystalline solid**. **JH-VII-139-1**: ¹H NMR (500 MHz, DMSO-*d*₆) δ 13.08 (s, 1H), 8.35 (d, *J* = 8.1 Hz, 1H), 8.13 (s, 1H), 8.03 (s, 1H), 7.90 (s, 1H), 7.83 (s, 1H), 7.63 (d, *J* = 8.1 Hz, 1H), 6.65 (s, 1H), **2.94 (s, 2H)**, 1.80 (s, 6H), 1.19 (t, *J* = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 180.3, 160.6, 149.2, 146.2, 141.7, 137.5, 136.9, 132.4, 131.8, 129.1, 128.4, 127.6, 125.7, 123.1, 120.5, 117.0, 111.4, 106.7, 106.1, 37.4, 30.3, 27.2, 15.8. ESI-MS, negative mode: *m/z* calcd mass for C₂₄H₁₉N₄O [M-H]⁻ = 379.15, was found 379.00.

(Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis(4-nitrophenyl) **decarbonate**, **16**. To a stirred solution of triethyleneglycol (62 mg, 0.412 μmol) in DCM (1.3 mL) and triethylamine (344 μL, 2.47 mmol), at 0 °C under argon, was added 4-nitrophenyl chloroformate

sub?

?? the highlighted quantities do not seem correct

??

??

??

2,94 (m, 2H)

dicarbonate

(250 mg, 1.23 mmol). As the solution turned yellow, the mixture was stirred at room temperature for 2 hours. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel with PS - EA (4:1, 3:1, 2:1, 1:1, EA) to afford **15** (198 mg, 76%) as a colorless oil. The spectral data were in accordance with those reported in the literature [34]. **16**: ¹H NMR (500 MHz, CDCl₃) δ 8.27 (d, *J* = 9.0 Hz, 4H), 7.38 (d, *J* = 9.0 Hz, 4H), 4.45 (dd, *J* = 5.4, 3.8 Hz, 4H), 3.83 (dd, *J* = 5.3, 4.0 Hz, 4H), 3.74 (s, 4H).

2-(2-(2-(((4-Nitrophenoxy)carbonyl)oxy)ethoxy)ethoxy)ethyl 3-(3-cyano-9-ethyl-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[*b*]carbazol-8-yl)-1H-pyrazole-1-carboxylate, **17**. To a stirred solution of (ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis(4-nitrophenyl) dicarbonate **16** (53 mg, 0.11 mmol) and triethylamine (31 μL, 0.22 mmol) in dry DMF (1.2 mL) was added dropwise a solution of **JH-VII-139-1** (12 mg, 31.5 μmol) in DMF (1.2 mL) at 0 °C under argon. As the solution turned yellow, the mixture was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel with PS - EA (gradient elution 1:1, 1:2, 1:3, 1:4) to give **17** (22mg, 55% yield) as a sub yellow solid. **17**: ¹H NMR (500 MHz, CDCl₃) δ 9.52 (s, 1H), 8.54 (d, *J* = 8.1 Hz, 1H), 8.35 (s, 1H), 8.29 – 8.23 (m, 3H), 7.77 (s, 1H), 7.72 (s, 1H), 7.58 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.40 – 7.35 (m, 2H), 6.65 (d, *J* = 2.8 Hz, 1H), 4.68 – 4.65 (m, 2H), 4.44 – 4.41 (m, 2H), 3.93 – 3.90 (m, 2H), 3.83 – 3.80 (m, 2H), 3.76 – 3.70 (m, 4H), 2.87 (q, *J* = 7.6 Hz, 2H), 1.75 (s, 6H), 1.24 (t, *J* = 7.6 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 180.4, 159.0, 156.2, 155.6, 152.6, 149.5, 145.6, 144.7, 142.0, 135.8, 135.5, 132.1, 131.9, 128.5, 127.7, 127.4, 125.9, 125.5, 123.1, 121.9, 120.2, 115.8, 111.5, 110.5, 106.5, 70.9, 68.9, 68.9, 68.4, 67.5, 36.5, 30.7, 26.5, 15.5. ESI-MS, negative mode: *m/z* calcd mass for C₃₈H₃₈N₇O₈ [M][−] = 721.24, was found 719.95.

Conjugate geo75. To a stirred solution of **17** (3.48 mg, 4.83 μmol) and c(RGDyK) (2.3 mg, 3.71 μmol) in dry DMF (1.15 mL), under argon, was added DIPEA (2.59 μL, 14.8 μmol) and the mixture was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the residue was purified by HPLC (method 1, supplementary material) to yield **geo75** as white solid (2.5 mg, 56 %). **geo75**: ¹H NMR (500 MHz, DMSO-*d*₆) δ 13.03 (s, 1H), 9.09 (s, 1H), 8.47 (d, *J* = 2.7 Hz, 1H), 8.33 (d, *J* = 8.1 Hz, 2H), 8.27 (s, 1H), 8.20 (d, *J* = 9.3 Hz, 2H), 8.16 (s, 2H), 8.12 (d, *J* = 9.2 Hz, 1H), 8.01 (s, 1H), 7.91 (s, 1H), 7.66 – 7.59 (m, 1H), 7.13 (s, 3H), 7.01 (d, *J* = 2.7 Hz, 1H), 6.92 (d, *J* = 8.4 Hz, 2H), 6.58 (d, *J* = 8.4 Hz, 2H), 4.54 (m, 2H), 4.47 (s, 1H), 4.23 (m, 1H), 4.13 (m, 1H), 3.98 (s, 2H), 3.80 – 3.76 (m, 2H), 3.62 – 3.57 (m, 2H), 3.53 (s, 4H), 3.16 (m, 3H), 3.04 (dd, *J* = 12.3, 6.2 Hz, 2H), 2.95 – 2.85 (m, 4H), 2.70 (m, 1H), 2.50 (m, 2H) 2.11 (m, 1H), 1.78 (s, 6H), 1.62 (m, 3H), 1.43 (s, 4H), 1.30 (d, *J* = 7.1 Hz, 2H), 1.16 (t, *J* = 7.5 Hz, 3H), 1.10 (m, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 178.9, 173.5, 172.0, 170.9, 170.8, 170.6, 167.8, 160.4, 156.8, 156.1, 155.5, 155.0, 148.8, 145.6, 140.9, 135.9, 135.0, 132.4, 131.3, 129.8, 128.0, 127.9, 127.7, 127.4, 126.3, 125.0, 121.6, 120.0, 116.6, 114.8, 110.5, 109.5, 104.8, 69.9, 69.7, 69.6, 68.9, 68.0, 67.4, 63.0, 54.9, 53.3, 50.8, 48.6, 40.6, 38.9, 36.4, 31.8, 29.9, 29.0, 28.8, 27.3, 26.0, 24.5, 22.9, 15.3. ESI-MS, positive mode: *m/z* calcd mass for C₅₉H₇₁N₁₃O₁₅ [M+H]⁺ = 1202.52, was found 1202.30.

Bis(2,5-dioxopyrrolidin-1-yl) glutarate, **19** was synthesized and characterized by our group in a previous work.²⁶

2,5-Dioxopyrrolidin-1-yl 5-(3-(3-cyano-9-ethyl-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[*b*]carbazol-8-yl)-1H-pyrazol-1-yl)-5-oxopentanoate, **20**. To a stirred solution of bis(2,5-dioxopyrrolidin-1-yl) glutarate **19** (18.9 mg, 57.8 μmol) and **JH-VII-139-1** (11 mg, 28.9 μmol) in DMF (0.5 mL) was added triethylamine (16.6 μL, 116 μL) under argon. The reaction was then stirred at room temperature for 48 hours. The solvent was removed under vacuum and the residue was chromatographed on silica gel with DCM - Acetone (gradient elution 4:0.1 to 4:0.3) to give **20** (8 mg, 47 %) as white solid. **20**: ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.84 (s, 1H), 8.57 (d, *J* = 2.8 Hz, 1H), 8.34 (d, *J* = 8.1 Hz, 1H), 8.17 (s, 1H), 8.02 (s, 2H), 7.62 (d, *J* = 8.2 Hz, 1H), 7.14 (d, *J* = 2.8 Hz, 1H), 3.32 – 3.36 (m, 2H), 3.00 (q, *J* = 7.4 Hz, 2H), 2.87 (t, *J* = 7.4 Hz, 2H), 2.82 (s, 4H), 2.11 – 2.04 (m, 2H), 1.81 (s, 6H), 1.23 (t, *J* = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 178.7, 171.1, 170.2, 168.7, 154.6, 145.7, 140.9, 134.5, 131.4, 129.4, 128.0, 126.6, 124.8, 121.6, 120.1, 119.5, 118.1, 117.9, 116.7, 110.9, 109.5,

104.5, 36.5, 32.2, 29.9, 29.4, 26.4, 25.4, 19.1, 15.4. ESI-MS, positive mode: m/z calcd mass for $C_{33}H_{30}N_5O_6$ $[M+H]^+=592.21$, was found 592.00.

Conjugate geo77. To a stirred solution of **20** (3.82 mg, 6.46 μ mol) and c(RGDyK) (4 mg, 6.46 μ mol) in DMF (2 mL), under argon, was added DIPEA (4.5 μ L, 25.8 μ mol) and the mixture was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the residue was purified by HPLC (method 1, supplementary material) to yield **geo77** as a white solid (3.2 mg, 45%). **geo77**: ^1H NMR (500 MHz, DMSO- d_6) δ 12.97 (s, 1H), 9.11 (s, 1H), 8.54 (d, J = 2.8 Hz, 1H), 8.40 (d, J = 8.7 Hz, 1H), 8.35 (d, J = 8.1 Hz, 1H), 8.20–8.30 (m, 3H), 8.21–8.12 (m, 3H), 8.03 (s, 1H), 8.01 (s, 1H), 7.91 (t, J = 5.6 Hz, 1H), 7.68–7.59 (m, 2H), 7.00–7.25 (m, 3H), 6.93 (d, J = 8.5 Hz, 2H), 6.59 (d, J = 8.4 Hz, 2H), 4.56–4.46 (m, 2H), 4.33–4.26 (m, 1H), 4.23–4.11 (m, 2H), 3.20–3.16 (m, 4H), 3.09–3.03 (m, 2H), 2.95–3.42 (m, 4H), 2.75–2.69 (m, 1H), 2.21 (t, J = 7.4 Hz, 2H), 2.06–2.13 (m, 1H), 1.98–1.90 (m, 2H), 1.81 (d, J = 1.7 Hz, 6H), 1.54–1.70 (m, 3H), 1.53–1.42 (m, 2H), 1.41–1.33 (m, 2H), 1.22 (t, J = 7.5 Hz, 3H), 1.20–1.11 (m, 2H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 178.9, 173.5, 172.0, 171.5, 171.4, 170.9, 170.8, 170.6, 167.7, 160.4, 156.7, 155.5, 154.4, 145.6, 140.9, 135.8, 134.7, 131.2, 129.8, 129.4, 128.3, 127.9, 127.6, 126.7, 125.0, 123.9, 121.7, 120.0, 116.5, 114.8, 110.8, 109.5, 104.8, 54.9, 53.3, 50.8, 48.6, 42.9, 40.6, 38.3, 36.4, 35.9, 34.3, 32.9, 31.8, 29.8, 29.0, 28.5, 27.2, 26.5, 24.5, 23.0, 20.2, 15.5. ESI-MS, positive mode: m/z calcd mass for $C_{56}H_{66}N_{13}O_{11}$ $[M+H]^+=1096.49$, was found 1096.00.

Bis(4-nitrophenyl) ((ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))dicarbamate, 22. 2,2'-(Ethylenedioxy)bis(ethylamine) (1.00 g, 6.75 mmol) and pyridine (2.18 mL, 27.0 mmol) were dissolved in CH_2Cl_2 (250 mL). The reaction flask was cooled to 0 °C and then *p*-nitrophenyl chloroformate (5.44 g, 27.0 mmol) was added. The solution warmed to room temperature while stirring for 24 h. The reaction was filtered, and the solvent was evaporated leaving a white solid. The crude product was dissolved in CH_2Cl_2 (150 mL) and washed with aqueous 1 M $NaHSO_4$ (3 $\times$ 30 mL). The organic layer was dried with Na_2SO_4 , and the solvent was evaporated. The resulting solid was crystallized twice from CH_2Cl_2 – hexanes (1:1) providing **22** (2.19 g, 68%) as a white solid. **22**: ^1H NMR (500 MHz, acetone- d_6 , DMSO- d_6) δ 8.27 (d, J = 9.1 Hz, 4H), 7.91 (t, J = 5.3 Hz, 2H), 7.43 (d, J = 9.2 Hz, 4H), 3.63 (s, 4H), 3.60 (t, J = 5.8 Hz, 4H), 3.34 (q, J = 5.8 Hz, 4H). ^{13}C NMR (126 MHz, acetone- d_6 , DMSO- d_6) δ 157.12, 153.93, 144.86, 125.54, 122.86, 70.48, 69.66, 41.28.

4-Nitrophenyl (2-(2-(2-(3-(3-cyano-9-ethyl-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[b]carbazol-8-yl)-1H-pyrazole-1-carboxamido)ethoxy)ethoxy)ethyl)carbamate, 23. To a stirred solution of bis(4-nitrophenyl) ((ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))dicarbamate **22** (30.2 mg, 63.1 μ mol) and triethylamine (17.6 μ L, 126 μ mol) in dry DMF (0.8 mL) was added dropwise a solution of JH-VII-139-1 (1eq) in dry DMF (0.8 μ L) at 0 °C under argon. As the solution turned yellow, the mixture was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel with DCM - Acetone (gradient elution 4:0.1, 4:0.2, 4:0.3, 4:0.4, 4:0.6) to give **23** (15 mg, 66 %) as a beige solid. **23**: ^1H NMR (500 MHz, acetone- d_6) δ 11.79 (s, 1H), 8.49 (d, J = 8.1 Hz, 1H), 8.39 (d, J = 2.6 Hz, 1H), 8.29 (s, 1H), 8.22 (d, J = 9.1 Hz, 2H), 8.01 (s, 1H), 7.93 (s, 1H), 7.90 (s, 1H), 7.61 (d, J = 8.1 Hz, 1H), 7.37 (d, J = 9.1 Hz, 2H), 6.99 (s, 1H), 6.88 (d, J = 2.6 Hz, 1H), 3.74 (t, J = 5.5 Hz, 2H), 3.69–3.60 (m, 8H), 3.35 (q, J = 5.6 Hz, 2H), 3.02 (q, J = 7.5 Hz, 2H), 1.91 (s, 6H), 1.26 (d, J = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, acetone- d_6) δ 178.9, 159.4, 156.3, 153.2, 152.9, 149.2, 145.2, 141.0, 135.8, 135.4, 131.5, 128.9, 127.9, 127.7, 127.6, 126.5, 124.6, 124.5, 121.9, 121.7, 119.3, 115.8, 110.2, 108.7, 105.6, 69.9, 69.0, 40.6, 39.7, 36.3, 29.4, 26.1, 14.6. ESI-MS, positive mode: m/z calcd mass for $C_{38}H_{38}N_7O_8$ $[M+H]^+=720.27$, was found 720.00.

Conjugate geo85. To a stirred solution of **23** (3 mg, 4.2 μ mol) and c(RGDyK) (2 mg, 3.23 μ mol) in dry DMF (1 mL), under argon, was added DIPEA (2.25 μ L, 12.9 μ mol) and the mixture was stirred at room temperature for 24 hours. The solvent was removed under reduced pressure, and the residue was purified by HPLC (method 1, supplementary material) to yield **geo85** (3.4 mg, 88%) as a white solid. **geo85**: ^1H NMR (500 MHz, DMSO- d_6) δ 13.05 (s, 1H), 9.12 (s, 1H), 8.44 (d, J = 2.7 Hz, 1H), 8.40–8.30 (m, 3H), 8.27 (s, 1H), 8.23 (d,

$J = 9.2$ Hz, 2H), 8.17 (s, 1H), 8.13 – 8.08 (m, 3H), 8.02 (s, 1H), 7.95 (s, 1H), 7.65 (d, $J = 9.9$ Hz, 1H), 7.63 (dd, $J = 8.2, 1.3$ Hz, 1H), 7.30 – 6.98 (br, 3H) 6.94 (d, $J = 8.5$ Hz, 2H), 6.91 (d, $J = 2.7$ Hz, 1H), 6.60 (d, $J = 8.4$ Hz, 2H), 6.00 (t, $J = 5.5$ Hz, 1H), 5.86 (t, $J = 5.7$ Hz, 1H), 4.60 – 4.53 (m, 1H), 4.52 – 4.47 (m, 1H), 4.32 – 4.25 (m, 1H), 4.23 – 4.19 (m, 1H), 4.19 – 4.13 (m, 1H), 3.62 – 3.54 (m, 4H), 3.52 – 3.46 (m, 4H), 3.20 (dd, $J = 14.2, 4.8$ Hz, 2H), 3.12 – 3.05 (m, 4H), 2.97 – 2.92 (m, 2H), 2.91 – 2.86 (m, 2H), 2.74 (dd, $J = 16.9, 2.8$ Hz, 1H), 2.53 – 2.51 (m, 2H), 2.10 (d, $J = 14.2$ Hz, 1H), 1.80 (d, $J = 2.5$ Hz, 6H), 1.73 – 1.66 (m, 2H), 1.65 – 1.62 (m, 1H), 1.57 – 1.49 (m, 2H), 1.48 – 1.41 (m, 2H), 1.35 – 1.30 (m, 2H), 1.17 (t, $J = 7.5$ Hz, 3H), 1.15 – 1.10 (m, 1H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 179.0, 173.7, 172.0, 171.0, 170.7, 170.5, 167.6, 160.4, 158.1, 156.7, 155.5, 153.0, 149.4, 145.5, 140.8, 135.9, 135.5, 131.1, 129.8, 129.6, 128.3, 128.0, 127.6, 126.0, 124.9, 121.6, 120.0, 116.6, 114.8, 109.5, 109.3, 104.8, 70.1, 69.5, 55.0, 53.2, 50.7, 48.6, 42.9, 40.6, 36.4, 35.8, 31.9, 29.9, 29.8, 29.4, 27.3, 26.0, 24.6, 23.0, 15.19, 4 peaks are missing due to overlapping. ESI-MS, positive mode: m/z calcd mass for $\text{C}_{59}\text{H}_{74}\text{N}_{15}\text{O}_{13}$ $[\text{M}+\text{H}]^+=1200.55$, was found 1199.80.

(*S*)-14-(4-((*tert*-butoxycarbonyl)amino)butyl)-1-(3-(3-cyano-9-ethyl-6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[*b*]carbazol-8-yl)-1H-pyrazol-1-yl)-1,12-dioxo-5,8-dioxo-2,11,13-triazapentadecan-15-oic acid, **24**. To a stirred solution of **23** (11 mg, 15.3 μmol) and *N* ϵ -Boc-L-lysine (3.8 mg, 15.3 μmol) in DMF (0.8 mL) was added triethylamine (8.5 μL , 61 μmol) at ambient temperature, under argon. As the solution turned yellow, the mixture was stirred at room temperature for 24 hours. After condensation of the mixture under reduced pressure, the residue was subjected to flash column chromatography (gradient elution DCM - MeOH 10:1, 10:2, 10:3) to yield **24** (8 mg, 63%) as white solid. **24**: ^1H NMR (500 MHz, DMSO- d_6) δ 8.40 (d, $J = 2.7$ Hz, 1H), 8.33 (d, $J = 8.1$ Hz, 1H), 8.23 (t, $J = 4.9$ Hz, 1H), 8.17 (s, 1H), 8.10 (s, 1H), 7.94 (s, 1H), 7.58 (dd, $J = 8.1, 1.3$ Hz, 1H), 6.87 (d, $J = 2.7$ Hz, 1H), 6.57 (s, 1H), 6.07 – 6.01 (m, 2H), 3.95 (dd, $J = 12.6, 7.3$ Hz, 1H), 3.61 (t, $J = 5.7$ Hz, 2H), 3.58 (dd, $J = 5.7, 4.0$ Hz, 2H), 3.53 (dd, $J = 6.1, 4.1$ Hz, 2H), 3.51 – 3.46 (m, 3H), 3.38 (t, $J = 5.7$ Hz, 2H), 3.13 – 3.07 (m, 4H), 2.96 – 2.91 (m, 2H), 2.87 (dd, $J = 12.9, 6.8$ Hz, 2H), 1.80 (d, $J = 3.7$ Hz, 6H), 1.66 – 1.59 (m, 2H), 1.53 – 1.46 (m, 2H), 1.37 – 1.31 (m, 11H), 1.18 (t, $J = 7.6$ Hz, 3H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 178.7, 174.8, 160.4, 157.6, 155.3, 152.7, 149.2, 145.4, 140.4, 136.1, 135.2, 131.1, 129.4, 127.7, 127.6, 125.9, 124.5, 121.4, 119.8, 116.6, 109.3, 109.0, 104.4, 77.1, 70.0, 69.4, 68.5, 53.1, 36.3, 32.2, 31.0, 29.8, 29.7, 28.7, 28.1, 25.7, 22.5, 21.8, 14.9. ESI-MS, positive mode: m/z calcd mass for $\text{C}_{43}\text{H}_{55}\text{N}_8\text{O}_9$ $[\text{M}+\text{H}]^+=827.40$, was found 826.95.

Compound 25. In a stirred solution of **24** (7 mg, 8.32 μmol) and NHS (1.44 mg, 12.5 μmol) in dry DMF (0.5 mL), was added EDCi (4.8 mg, 25 μmol) under argon at room temperature. After 3 hours and upon starting material consumption (TLC monitoring), c(RGDyK) (3.6 mg, 5.83 μmol) was added and the reaction was stirred for 48 hours at room temperature. The mixture was then concentrated under reduced pressure and the residue purified by HPLC (method 1, supplementary material) to afford conjugate **25** (3.5 mg, 30%) as white solid. **25**: ^1H NMR (500 MHz, DMSO) δ 13.10 (s, 1H), 9.15 (s, 1H), 8.49 – 8.31 (m, 6H), 8.24 – 8.16 (m, 6H), 8.04 (s, 1H), 7.96 (s, 1H), 7.91 (s, 2H), 7.70 – 7.62 (m, 2H), 7.27 (s, 2H), 6.98 – 6.85 (m, 3H), 6.77 – 6.70 (m, 1H), 6.60 (d, $J = 8.2$ Hz, 2H), 6.16 (d, $J = 8.4$ Hz, 2H), 6.10 (s, 1H), 4.59 – 4.46 (m, 2H), 4.32 – 4.26 (m, 1H), 4.22 – 4.10 (m, 2H), 4.07 – 4.00 (m, 1H), 3.62 – 3.54 (m, 4H), 3.53 – 3.45 (m, 4H), 3.20 (d, $J = 9.3$ Hz, 3H), 3.14 – 3.04 (m, 6H), 3.00 – 2.91 (m, 4H), 2.89 – 2.83 (m, 2H), 2.73 (d, $J = 15.5$ Hz, 1H), 2.57 – 2.53 (m, 2H), 2.12 – 2.06 (m, 1H), 1.80 (s, 6H), 1.72 – 1.58 (m, 3H), 1.57 – 1.44 (m, 6H), 1.40 – 1.29 (m, 13H), 1.23 – 1.10 (m, 6H). ^{13}C NMR (126 MHz, DMSO) δ 179.1, 173.7, 172.5, 172.0, 171.1, 170.1, 170.6, 167.7, 160.5, 157.6, 156.8, 155.5, 153.0, 149.5, 145.5, 140.8, 135.9, 135.5, 131.2, 129.9, 129.7, 128.4, 128.1, 127.7, 126.1, 125.0, 121.7, 120.1, 118.2, 116.7, 114.8, 109.5, 109.4, 104.8, 77.3, 70.1, 69.6, 69.6, 68.7, 55.0, 52.9, 50.8, 48.6, 40.4, 38.2, 36.4, 33.2, 29.9, 29.9, 29.3, 28.5, 28.3, 26.0, 24.6, 23.0, 22.6, 15.2, 3 peaks are missing due to overlapping. ESI-MS, negative mode: m/z calcd mass for $\text{C}_{70}\text{H}_{93}\text{N}_{17}\text{O}_{16}$ $[\text{M}-\text{H}]=1426.70$, was found 1426.10

Conjugate geo107. Compound **25** (1.8 mg, 1.26 μmol) was dissolved in a $\text{H}_2\text{O}/\text{ACN}/\text{TESH}/\text{TFA}$ (0.36 mL, 80/180/20/80) mixture and stirred for 2 hours at room tem-

perature. The mixture was then freeze dried and subjected to HPLC (method 1, supplementary material) to yield **geo107** (1.5mg, 90%) as beige solid. **geo107**: ^1H NMR (500 MHz, DMSO- d_6) δ 9.02 (s, 1H), 8.46 – 8.36 (m, 4H), 8.34 (d, J = 8.1 Hz, 1H), 8.28 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 9.7 Hz, 1H), 8.17 (s, 1H), 8.10 (d, J = 9.2 Hz, 1H), 8.07 – 7.89 (m, 5H), 7.66 (d, J = 9.1 Hz, 1H), 7.63 (d, J = 8.7 Hz, 1H), 7.49 – 7.24 (br, 3H), 6.96 – 6.90 (m, 3H), 6.86 (d, J = 8.4 Hz, 1H), 6.60 (d, J = 8.3 Hz, 2H), 6.26 – 6.14 (m, 2H), 4.58 – 4.46 (m, 2H), 4.35 – 4.28 (m, 1H), 4.20 – 4.11 (m, 2H), 4.08 – 0.2 (m, 1H), 3.60 – 3.54 (m, 4H), 3.53 – 3.44 (m, 4H), 3.26 – 3.21 (m, 3H), 3.14 – 3.03 (m, 6H), 2.98 – 2.80 (m, 4H), 2.74 – 2.68 (m, 3H), 2.10 – 2.04 (m, 1H), 1.80 (s, 6H), 1.62 – 1.55 (m, 3H), 1.54 – 1.42 (m, 6H), 1.27 – 1.20 (m, 4H), 1.19 – 1.13 (m, 6H). ^{13}C NMR (126 MHz, DMSO) δ 179.0, 173.9, 172.3, 170.8, 166.6, 166.4, 166.3, 160.6, 157.6, 157.1, 157.0, 155.8, 155.5, 153.0, 149.5, 145.6, 140.8, 136.1, 135.5, 131.2, 129.9, 129.8, 129.7, 128.1, 127.7, 126.1, 125.0, 121.6, 120.1, 116.7, 115.1, 114.9, 109.5, 109.3, 104.8, 70.1, 69.6, 68.7, 55.8, 52.7, 49.8, 46.0, 40.5, 38.9, 36.5, 32.9, 29.9, 28.5, 27.6, 26.0, 25.2, 25.1, 22.2, 22.1, 15.2, 3 peaks are missing due to overlapping. ESI-MS, positive mode: m/z calcd mass for $\text{C}_{65}\text{H}_{86}\text{N}_{17}\text{O}_{14}$ $[\text{M}+\text{H}]^+=1328.65$, was found 1327.9.

3',6'-Dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5(6)-carboxylic acid, **28**. 1,3-Dioxo-1,3-dihydroisobenzofuran-5-carboxylic acid **26** (1 g, 5.1 mmol) and resorcinol **27** (1.15 g, 10.4 mmol) in methanesulfonic acid (5 mL) were placed in a pressure tube equipped with a magnetic stirrer bar. The reaction mixture was stirred at 80 °C overnight and then poured to cold water (500 mL) with stirring and filtered. The solid residue was heated at reflux in EtOH (200 mL), H_2O was added until precipitation, and the mixture cooled to room temperature, filtered, and freeze-dried for 24 h to yield 1.9 g (97 %) of crude 5(6)-carboxyfluorescein as an orange powder. The crude compound was used without further purification. The spectral data were in accordance with those reported in the literature [35]. **28**: ^1H NMR (500 MHz, DMSO- d_6) δ 10.14 (s, 2H), 8.40 (s, 1H), 8.29 (dd, J = 8.0, 1.4 Hz, 1H), 8.22 (d, J = 8.0 Hz, 0.7H), 8.11 (d, J = 8.0 Hz, 0.7H), 7.65 (s, 0.7H), 7.39 (d, J = 8.0 Hz, 1H), 6.70 – 6.65 (m, 2.2H), 6.66 – 6.58 (m, 3.2H), 6.56 – 6.52 (m, 4.8H), regioisomer ratio present: 60 – 40 (5/6).

2,5-Dioxopyrrolidin-1-yl 3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5(6)-carboxylate, **29**. 5(6)-Carboxyfluorescein **28** (200 mg, 0.531 mmol) was dissolved in dry DMF (2.5 mL). EDCi (306 mg, 1.59 mmol) and N -hydroxysuccinimide (122 mg, 1.06 mmol) were added to the mixture. The reaction was stirred under argon for 2 hours and after completion, was washed with aqueous KH_2PO_4 three times (EA was moderately used as additional organic solvent, since EA is not dissolving fluorescein derivatives). Organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The residue was then subjected to column chromatography (gradient elution EA, EA - MeOH 10 - 1, 10 -2) and yielded **29** as dark brown solid (120 mg, 48 %). The spectral data were in accordance with those reported in the literature [35]. **29**: ^1H NMR (500 MHz, DMSO- d_6) δ 10.18 (s, 2H), 8.54 (s, 1H), 8.43 (dd, J = 8.1, 1.6 Hz, 1H), 7.56 (d, J = 8.1 Hz, 1H), 6.72 – 6.65 (m, 4H), 6.57 – 6.53 (m, 2H), 2.93 (s, 4H), regioisomer ratio present: 72 – 28 (5/6). ESI-MS, positive mode: m/z calcd mass for $\text{C}_{25}\text{H}_{15}\text{NO}_9$ $[\text{M}]^+=473.07$ was found 473.85.

5(6)-Carboxyfluorescein tagged conjugates **geo106**. To a stirred solution of **geo107** (2 mg, 1.51 μmol) and **geo98** (0.7 mg, 1.51 μmol) in dry DMF (1.33 mL), under argon, was added DIPEA (2.62 μL , 15.1 μmol) and the mixture was stirred at room temperature for 48 hours. The solvent was removed under reduced pressure, and the residue was purified by HPLC (method 5, supplementary material) to yield **geo106** (1.6 mg, 63%) as a yellow solid. **geo106**: ^1H NMR (500 MHz, DMSO- d_6) δ 9.10 (s, 1H), 8.83 (s, 1H), 8.72 (s, 1H), 8.63 (s, 1H), 8.45 (d, J = 12.9 Hz, 1H), 8.39 (s, 1H), 8.34 (d, J = 8.0 Hz, 1H), 8.29 (s, 1H), 8.23 – 8.15 (m, 2H), 8.13 (d, J = 8.1 Hz, 1H), 8.04 – 8.02 (m, 2H), 7.96 (s, 1H), 7.88 (s, 1H), 7.83 (s, 1H), 7.63 (t, J = 6.9 Hz, 2H), 7.35 – 7.30 (m, 1H), 7.19 (s, 2H), 6.96 – 6.90 (m, 2H), 6.86 (d, J = 8.2 Hz, 1H), 6.69 – 6.56 (m, 4H), 6.55 – 6.51 (m, 1H), 6.17 (d, J = 8.0 Hz, 1H), 6.16 – 6.04 (m, 1H), 4.92 – 4.79 (m, 26H), 4.69 – 4.63 (m, 1H), 4.60 – 4.53 (m, 1H), 4.49 (dd, J = 13.8, 7.3 Hz, 1H), 4.38 – 4.25 (m, 2H), 4.23 – 4.16 (m, 1H), 4.14 (d, J = 9.3 Hz, 1H), 4.12 – 4.04 (m, 2H), 3.63 – 3.54 (m, 4H), 3.52 – 3.46 (m, 4H), 3.17 (s, 1H), 3.14 – 3.10 (m, 2H), 3.09 – 3.03 (m, 2H), 2.94

(q, $J = 15.0$, 2H), 2.80 – 2.73 (s, 3H), 2.09 (d, $J = 9.3$ Hz, 1H), 2.04 – 1.96 (m, 2H), 1.80 (s, 6H), 1.59 – 1.51 (m, 3H), 1.50 – 1.43 (m, 3H), 1.40 – 1.36 (m, 2H), 1.31 – 1.22 (m, 7H), 1.17 (t, $J = 7.4$ Hz, 4H), 1.16 – 1.07 (m, 2H). ESI-MS, negative mode: m/z calcd mass for $C_{86}H_{95}N_{17}O_{20}$ [M/2-H] = 841.84, was found 841.60.

2.2 Chemostability Assays

2.2.1 Stability in buffer solutions

The stability of the conjugates was examined by performing chemostability tests at two different pH values, 7.4 and 5.2. Stock solutions of the conjugates were prepared (0.32–0.47 mM) by dissolving 0.19–0.28 mg of each conjugate with 5 μ L DMSO and 500 μ L of the relevant buffer solution (phosphate buffer pH 7.4 or acetate buffer pH 5.2). The solution was incubated at 37°C. Aliquots were collected at 0, 1, 3, 5, 7, 24, and 48 h and analyzed by LC-MS. Results are presented as the mean $\pm$ SD for three independent experiments.

2.2.2 Stability in Dulbecco's Modified Eagle Medium

c(RGDyK)-based conjugates (200 μ g dissolved in 5 μ L DMSO) were added to 0.5 mL of DMEM (+ 10% fetal bovine serum). Solutions were maintained at 37 °C and at predetermined time points (0, 1, 2, 3, 4, 5, 24, 48 and 72 hours), 50 μ L of aliquot was removed and quenched with 150 μ L acetonitrile/H₂O1:1 v/v solution (+ 0.1 % formic acid) and analyzed by LC-MS. Results are presented as the mean $\pm$ SD for three independent experiments.

2.2.3 Stability in human plasma

c(RGDyK)-based conjugates (250 μ g dissolved in 5 μ L DMSO) were added to 0.5 mL of human plasma. Solutions were maintained at 37 °C and at predetermined time points (0.25 + 0, 1, 2, 3, 4, 5, 24 and 48 hours), 50 μ L of aliquot was removed and quenched with 150 μ L ice cold acetonitrile (+ 0.1 % formic acid) and centrifuged at 10000 rpm for 10 minutes. 50 μ L of supernatant was added to 50 μ L of ultrapure water (+ 0.1 % formic acid) and analyzed by LC-MS. Results are presented as the mean $\pm$ SD for three independent experiments.

2.3 In vitro kinase assays

Human SRPK1 and a fragment of the N-terminal domain of turkey Lamin B Receptor (LBR) comprising amino acids 62–92 (LBRNt(62–92)) were subcloned into pGEX-2T and expressed in bacteria as GST fusion proteins as previously described [36]. Kinase assays were performed in a reaction mixture containing 0.5 μ g GST-SRPK1, 1.5 μ g GST-LBRNt(62–92) as substrate, 25 μ M ATP, 1 μ Ci of [γ -³²P]ATP, 12 mM Hepes pH 7.5, 10 mM MgCl₂ and increasing concentrations of the inhibitors as indicated, in a final volume of 25 μ L. Reactions were carried out for 30 min at 30°C. The final concentration of dimethyl sulfoxide (DMSO) was adjusted to 4% irrespectively of the inhibitor concentration.

Phosphorylated GST-LBRNt(62–92) was detected by autoradiography using Super RX (Fuji medical X-ray film) and incorporation of radioactivity was measured by excising the radioactive bands from the SDS-PAGE gel and scintillation counting.

2.4 Cell toxicity

2.4.1 Cell Culture

HeLa, MCF7, MDA-MB-231 and K562 cancer cell lines were provided by ATCC (American Type Culture Collection). Cells were incubated at 37 °C with 5% CO₂. HeLa, MCF7 and MDA-MB-231 cells were maintained in DMEM, while K562 cells were maintained in RPMI medium, both supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics/antimycotics.

2.4.2 MTT assays

HeLa, MCF7, MDA-MB-231 and K562 cells were seeded in 96-well plates (3×10³ cells per well) and grown in medium supplemented with 10% FBS as monolayers at 37 °C in a 5% CO₂ incubator. One day after seeding, cells were exposed to increasing concentrations of the inhibitors for 48 h. The viability of the cells was estimated by an (3-(4,5-imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic assay as described previously [37] and the percentage growth inhibition (GI₅₀, TGI, IC₅₀) was calculated according

to National Cancer Institute recommendations as referred to Leonidis et al.²⁶ Values shown represent the means $\pm$ SE of three independent experiments.

2.5. Fluorescence Microscopy

MCF7 and MDA-MB-231 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂. Cells were seeded in 24well plate-5x10⁴ cells per well on coverslips and after 24 h they were treated with JH-VII-139-1-c(RGDyK) hybrid compound, **Geo106** (5 μ M), for 2, 24 and 48 h. To test the effect of okadaic acid (endocytosis inhibitor), cells were preincubated, prior to addition of **Geo106**, with okadaic acid (100 nM) for 30 min. After the incubation period, the cells coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Following the quenching of paraformaldehyde (100 mM Tris-HCl pH 7.5), the cells were washed with PBS and DNA was stained with DAPI. The coverslips were mounted with mounting medium (0.01% *p*-phenylenediamine and 50% glycerol in PBS) and visualized with Zeiss LSM 780 confocal microscope, using the Zen 2011 software.

2.6. Zebrafish Angiogenesis Assay

2.6.1 Zebrafish screening assays

Maintenance and breeding of the *Tg(kdrl:gfp)* zebrafish transgenic line was carried out in accordance with the European Directive 2010/63 for the protection of animals used for scientific purposes and the Recommended Guidelines for Zebrafish Husbandry Conditions [38]. Only embryos up to 72 h post fertilization were used in these experiments.

For the compound treatment assay: Zebrafish embryos were raised in E3 medium up to 24hpf; they were then dechlorinated and transferred to a 12-well plate (10 embryos/well). Candidate compounds were initially dissolved in DMSO and added at E3 medium at the following final concentrations: 10 μ M JH-VII-139-1 and c(RGDyK), and 1.3 μ M **geo75**, **geo77**, **geo85**, **geo107**. Treated and DMSO control embryos were kept at 28°C and imaged at 72hpf.

2.6.2 Zebrafish Imaging Analysis

Embryos were anesthetized with 0.4% tricaine methanesulfonate (MS222), and images were acquired by Leica DMIRE2 with a mounted Hamamatsu ORCA-Flash4.0LT digital camera. For quantification of the antiangiogenic effect of compounds, a) the total length of 8 intersegmental vessels (ISVs) per embryo flanking the yolk extension was measured and b) the width from each of the above ISVs was measured at three different points i.e., the top, the middle and the bottom point of each ISV and then the average width was estimated. Length and widths were measured using the open-source software ImageJ 1.53a (<http://imagej.nih.gov/ij>). A total of 20 embryos from independent experiments were measured. Statistically significant differences in total length, total width, total width from the top of ISVs, total width from the middle of ISVs, and total width from the bottom of ISVs, were determined by a two-tailed Student's *t*-test, using Microsoft Excel 16.64, considering $p^{**} < 0.01$ and $p^{***} < 0.001$.

3. Results

3.1. Synthesis of Compounds

3.1.1. Optimized synthesis of SRPK1 inhibitor, JH-VII-139-1

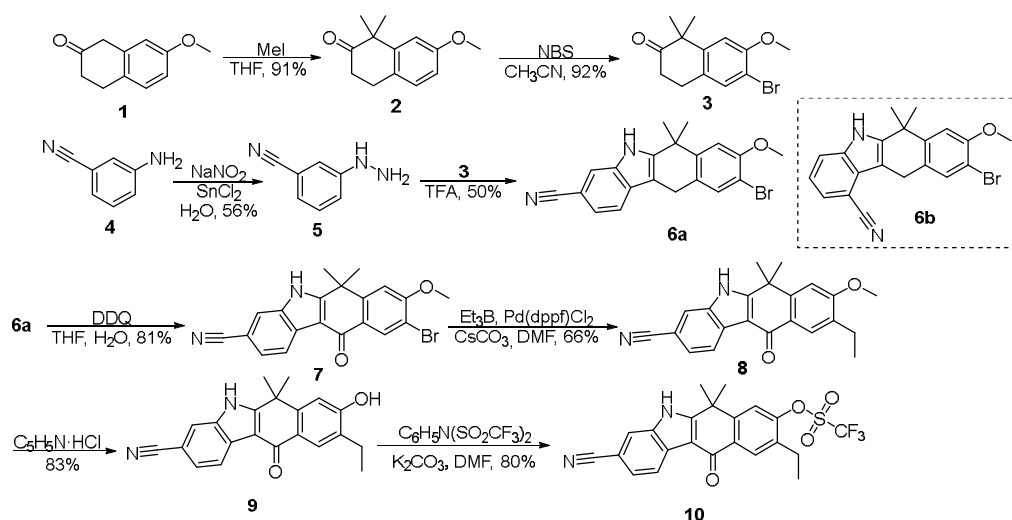
The synthesis of the Alectinib core structure that eventually leads to JH-VII-139-1, is highlighted in Scheme 1. Most Alectinib analogues, including JH-VII-139-1 are fused polycyclic indoles that combine the indole moiety with a polysubstituted tetralone. To synthesize the polycyclic scaffold **6a**, Fischer indole synthesis was implemented based on Kinoshita et al [30]. Initially, 7-methoxy-2-tetralone **1** was dimethylated with MeI to produce **2**, which was brominated with NBS to give the **desirable** tetralone **3**. Then, 3-aminobenzonitrile was converted to 3-hydrazinylbenzonitrile **5**, which reacted with **3** in a Fischer indole synthesis reaction. After removing the unwanted regioisomer **6b** by filtration, **6a** was

desired

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isolated by column chromatography in satisfying yield (**a** = 50%) compared to similar indole synthesis of the literature [30]. Oxidation of **6a** with DDQ afforded **7** which was subsequently alkylated with Et₃B, Pd(dppf)Cl₂ and CsCO₃ in a Suzuki-Miyaura reaction resulting in **8**. To modify the scaffold and insert the required pyrazole moiety that will lead to the synthesis of JH-VII-139-1, Zeisel-Prey ether cleavage with pyridinium chloride in high temperatures was performed to form phenol **9**. Finally, **9** reacted with *N*-phenylbis(trifluoromethanesulfonylimide) to produce the triflate **10** which was subsequently coupled to 1*H*-pyrazole-3-boronic acid **13** (Scheme 2).

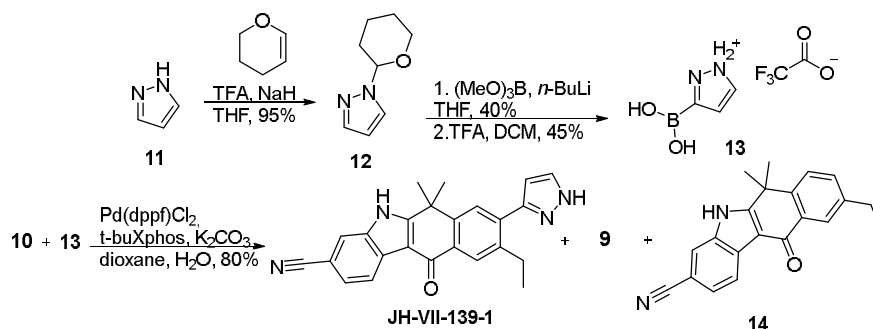


Scheme 1. Synthesis of triflate derivative **10**.

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The synthesis of 1*H*-pyrazole-3-boronic acid **13**, as well as the synthesis of JH-VII-139-1 are outlined in Scheme 2. Pyrazole was protected with a DHP group leading to the formation of **12**, which was then selectively borylated with (MeO)₃B and *n*-BuLi [29,30]. The DHP protected boronic acid was then deprotected in acidic conditions to give **13** that reacted with triflate **10**. The handling of **10** proved to be difficult compared to the iodide analogue used in the JH-VII-139-1 synthesis proposed by Gray et al [27], since it was easily hydrolyzed in H₂O and heat. To address the hydrolysis of triflate **10**, dioxane was used as the sole solvent, which unfortunately led to no reaction. The existence of H₂O in the reaction proved to be crucial, therefore the reaction was examined at different temperatures. The reaction was also tested under different conditions and solvents where in general the starting material **10** was consumed, leading to the hydrolyzed byproduct **9** and the byproduct **14** as depicted in Table 1. After several attempts, microwave irradiation improved the yield of JH-VII-139-1 in 80% and reduced the formation of **9** to 12%. It should be noted that the yield 80% for JH-VII-139-1 is significantly improved compared to the 48% yield reported by Hatcher et al using a similar methodology [27].



Scheme 2. Synthesis of SRPK1 inhibitor, JH-VII-139-1.

Table 1. Suzuki-Miyaura coupling conditions for JH-VII-139-1. Reaction conditions: All reactions were carried out using triflate **10** (1.2 equiv), boronic acid **13** (1 equiv), Pd(dppf)Cl₂ as catalyst (5%) and tBuXPhos (10%).

Temp. (°C)	Solvent	Base	Time (h)	Ratio (%) [*] 10	Ratio (%) [*] 14: 9: JH-VII-139-1	Isolated yield for JH-VII-139-1 (%)
85	DME/H ₂ O	K ₂ CO ₃	24	0	40: 57: 3	ND
120	DME/H ₂ O	Cs ₂ CO ₃	24	0	12: 80: 8	ND
120 (MW)	dioxane	K ₂ CO ₃	2	96	0: 4: 0	0
140 (MW)	dioxane	K ₂ CO ₃	1	90	0: 10: 0	0
85	DME/H ₂ O	K ₂ CO ₃	24	0	22: 64: 14	8
85 (MW)	dioxane/H ₂ O	K ₂ CO ₃	2	0	0: 45: 55	50
100 (MW)	dioxane/H ₂ O	K ₂ CO ₃	2	0	0: 82: 18	17
60 (MW)	dioxane/H ₂ O	K ₂ CO ₃	6	30	0: 4: 66	59
70 (MW)	dioxane/H₂O	K₂CO₃	2	0	0: 12: 88	80

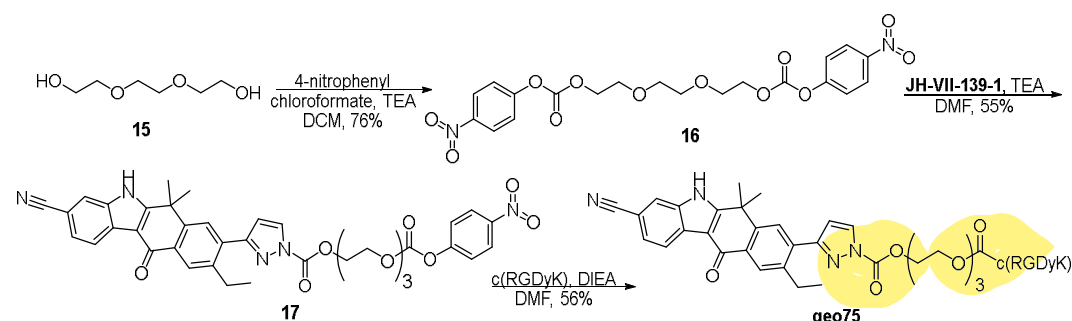
^{*} Ratio % of JH-VII-139-1 to **9**, **14** as determined by integration of the corresponding peaks in the LCMS chromatogram (254 nm).

ND= Not determined, MW = microwave irradiation

3.1.2. Synthesis of (JH-VII-139-1)-c(RGDyK) peptide-drug conjugates

double carbamate

Our first efforts focused on the conjugation of JH-VII-139-1 with c(RGDyK) through a carbonate linker. The pentapeptide c(RGDyK) was prepared using solid-phase peptide synthesis (SPPS) and head to tail cyclization based on Davis et al. methodology following by semipreparative HPLC purification [39]. The synthesis of **geo75** conjugate is summarized in scheme 3.



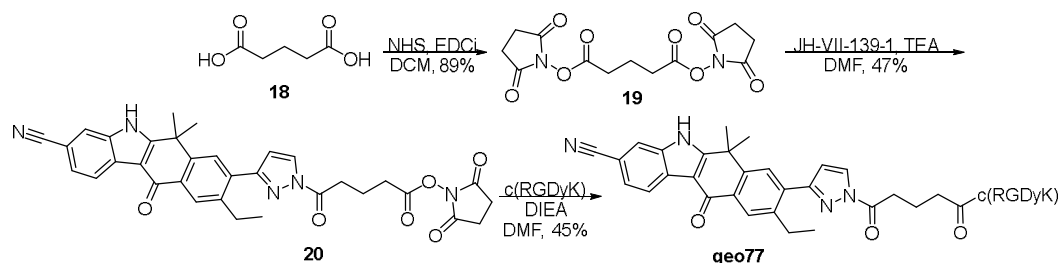
Scheme 3. Synthesis of conjugate **geo75** bearing carbonate linker.

Starting from triethylene glycol **15**, the highly reactive dinitrophenyl carbonate **16** was obtained after reaction with 4-nitrophenyl chloroformate, Et₃N in DCM. Then, dropwise addition of JH-VII-139-1 solution was performed to a solution of **16**, TEA and DMF to give the monosubstituted **17** in 55% yield. Careful handling and low concentration of reactants reduced the unwanted double nucleophilic acyl substitution of **16** by JH-VII-139-1. Finally, **17** was coupled to the ε-amino group of lysine on c(RGDyK) under basic DIPEA conditions to afford conjugate **geo75** in 56% yield. Conjugate **geo75** was purified by semipreparative reverse-phase HPLC and its purity was examined by LC-MS and it was found greater than 98%.

higher

After achieving conjugation of JH-VII-139-1 and c(RGDyK) through a dicarbamate linker, linkers with different length or greater chemical stability were used for the preparation of new derivatives such as the diamide conjugate **geo77** (scheme 4). The synthesis started with the commercially available glutaric acid **18** which reacted with NHS and EDCi to quantitatively form the activated NHS glutarate **19**, which then reacted with JH-VII-139-

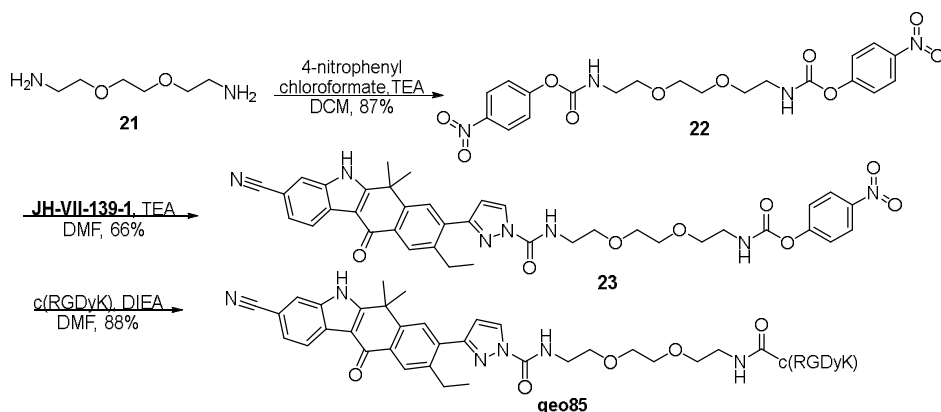
produced 1 in basic conditions and afforded the JH-VII-139-1 derivative in 47% yield. The subsequent reaction of **20** with oligopeptide c(RGDyK) and DIPEA eventually produces conjugate **geo77** in 45% yield, which was purified by HPLC with purity greater than 98%.



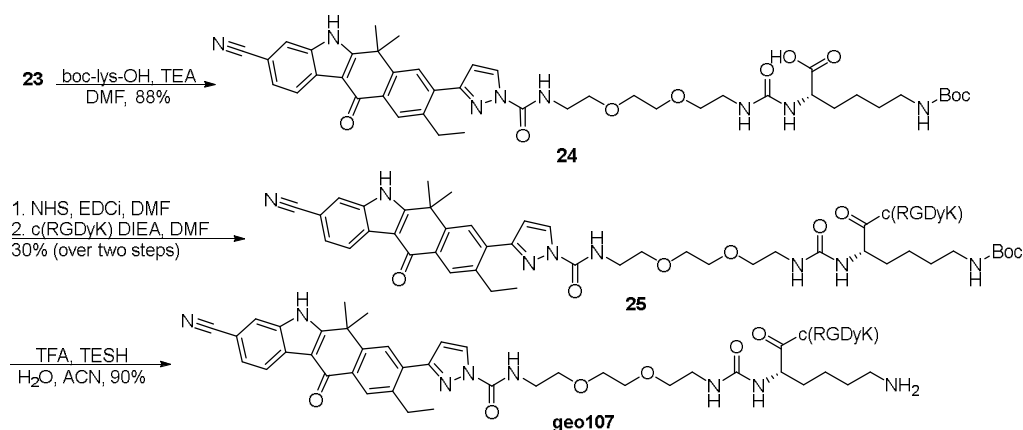
Scheme 4. Synthesis of conjugate **geo77** bearing amide linker.

In an attempt to increase the chemical stability, triethylene glycol in **geo75** synthesis was replaced to 2,2'-(ethylenedioxy)bis(ethylamine) that could lead to a urea linker. The synthetic procedure is outlined in scheme 5. In detail, highly reactive dicarbamate **22** was formed in a similar fashion by reacting 2,2'-(ethylenedioxy)bis(ethylamine) with 4-nitrophenyl chloroformate and EDCi. Then, JH-VII-139-1 reacted with **22** in basic conditions, and after careful handling **23** was obtained in good yield (66%). Compound **23** was then coupled to c(RGDyK) by forming a second urea moiety providing **geo85** in 88% yield. Conjugate **geo85** proved to be easier to handle and was isolated in greater overall yield compared to **geo75**, as urea derivatives were more stable and didn't hydrolyze as readily as their carbamate counterparts. Conjugate **geo85** was isolated after semipreparative HPLC with purity greater than 97%.

Afterwards, a linker that would increase the distance between JH-VII-139-1 and c(RGDyK) and could carry another bioactive moiety was employed. Lysine was a good candidate as it could extend the triethylene glycol chain and bear both a carboxylic and an amino group. This approach that eventually led to the synthesis of conjugate **geo107** is highlighted in scheme 6. The synthesis starts with a nucleophilic attack by *N*-Boc-*L*-lysine to the highly reactive 4-nitrophenyl carbamate of **23** that produces *boc*-protected **25** in 88% yield. The carboxyl group is then subjected to *in-situ* NHS activation and direct amidation with c(RGDyK) oligopeptide and DIPEA to form *boc*-protected **25** in 30% yield over two steps. The final conjugate **geo107** is obtained after a rapid *boc*-deprotection with TFA and triethylsilane (TESH) followed by semi-preparative reverse phase HPLC purification (purity of **geo107** > 97%).

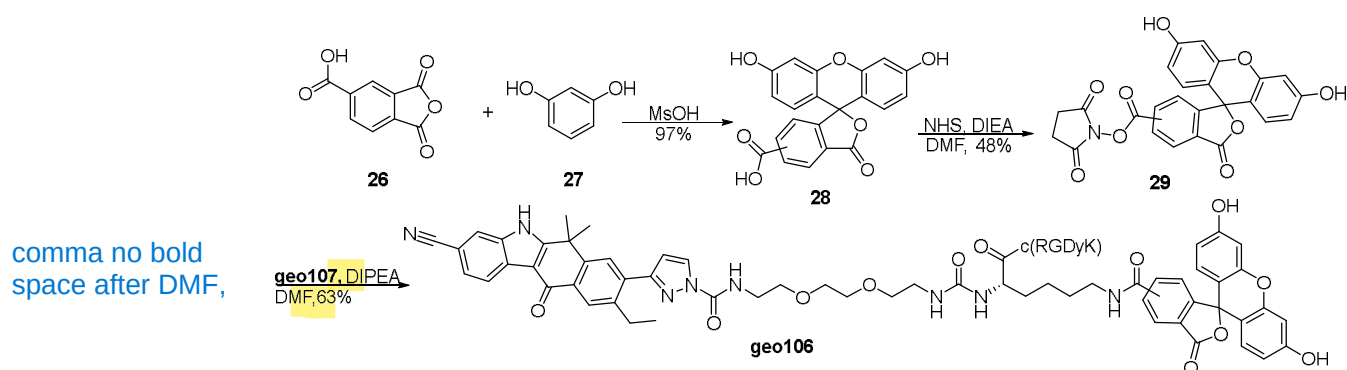


Scheme 5. Synthesis of conjugate **geo85** bearing urea linker.

Scheme 6. Synthesis of **geo107** conjugate.

3.1.3. Synthesis of fluorescein tagged (JH-VII-139-1)-c(RGDyK) conjugate

To gain insight into the cellular entry mechanism and the cellular biodistribution of (JH-VII-139-1)-c(RGDyK) conjugates, fluorescence labeling of **geo107** with fluorescein was undertaken (Scheme 7). Two molecules of resorcin reacted with trimellitic anhydride to produce a mixture of 5- and 6-carboxy-fluoresceins **28** in quantitative conversion [40]. Activation of 5(6) carboxyl group with NHS and DIPEA led to the formation of NHS ester **29** in 48% yield, [41] which subsequently reacted with **geo107** in basic conditions and formed the 5(6)-carboxyfluorescein tagged conjugates **geo106** in 63% yield. The crude photosensitive material was then subjected to semi-preparative HPLC and **geo106** were isolated in 96% purity.

Scheme 7. Synthesis of multifunctional **geo106** conjugates.

3.2 In Vitro Stability Studies

JH-VII-139-1 and its c(RGDyK) conjugates **geo75**, **geo77**, **geo85** and **geo107** were subjected to stability experiments in pH = 5.2 and 7.4 buffer solutions, cell medium and human plasma. All experiments were carried out at 37 °C and samples analyzed with LC-ESI/MS at predetermined times intervals. Starting with JH-VII-139-1, the compound was found very stable under all conditions tested as presented in Figure 2 (Figure S77, Figure S78). The c(RGDyK) conjugates exhibit different stability profiles as analyzed below. The hydrolysis and JH-VII-139-1 release was the main reaction with half-lives of the conjugates ranging from minutes to hours.

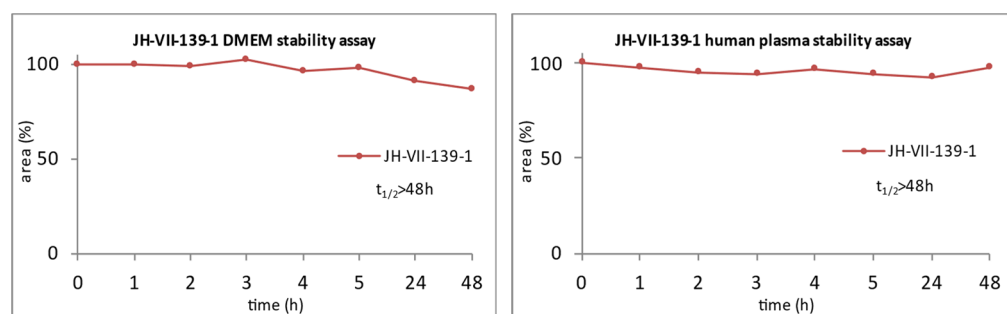


Figure 2. Chemostability of JH-VII-139-1.

3.2.1. Chemostability profile of conjugate **geo75**

at Dicarbamate **geo75** was tested for its stability to buffer solutions and it was found completely stable in pH = 5.2 for more than 48 hours, while after 24 hours, its residual concentration in pH = 7.4 retained more than 51% of its initial concentration. In addition, the conjugate had a declining stability pattern in Dulbecco's modified eagle medium (DMEM) and human plasma, where it was almost completely hydrolyzed after 9- and 1-hours respectively, exhibiting half-lives $t_{1/2}$ = 2h 36 min and $t_{1/2}$ = 11 min (Figure 3, S61-S64).

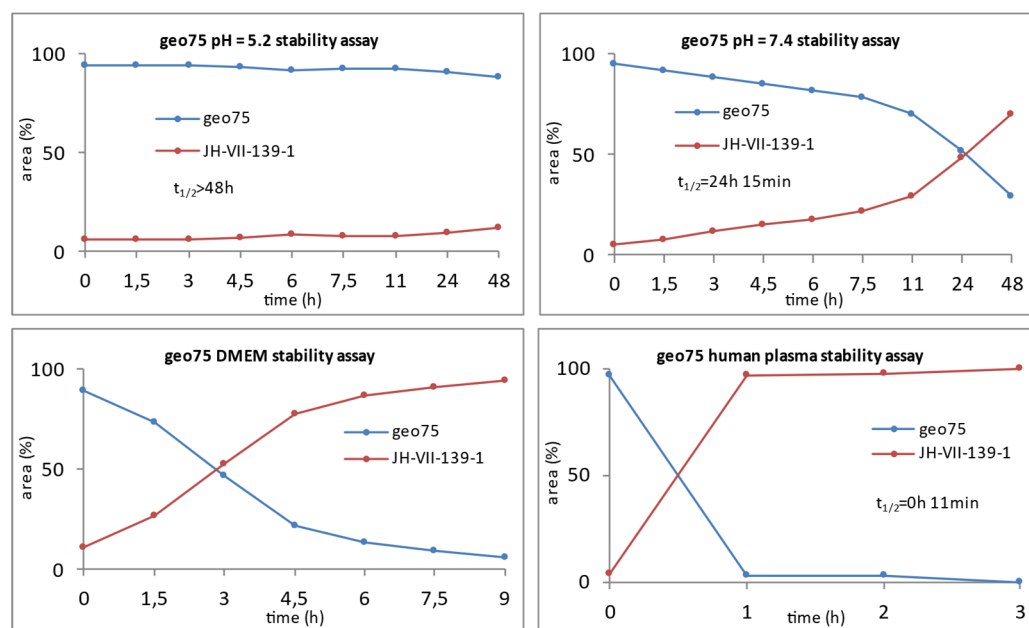


Figure 3. Chemostability of conjugate **geo75**.

3.2.2. Chemostability profile of conjugate **geo77**

at The stabilities of conjugate **geo77** were also examined in the same conditions. The conjugate exhibited similar hydrolysis patterns, but with different reaction ratios (Figure 4, S65-S68). In detail, **geo77** was less stable in pH = 5.2 buffer having half-life $t_{1/2}$ = 14h 42min compared to $t_{1/2}$ > 48h of **geo75**. In neutral pH and DMEM solutions, the conjugate was readily hydrolyzed leading to half-lives $t_{1/2}$ = 1h 44min and $t_{1/2}$ = 0h 50min, respectively. In human plasma, the conjugate was also unstable, and the half-live time was less than ten minutes.

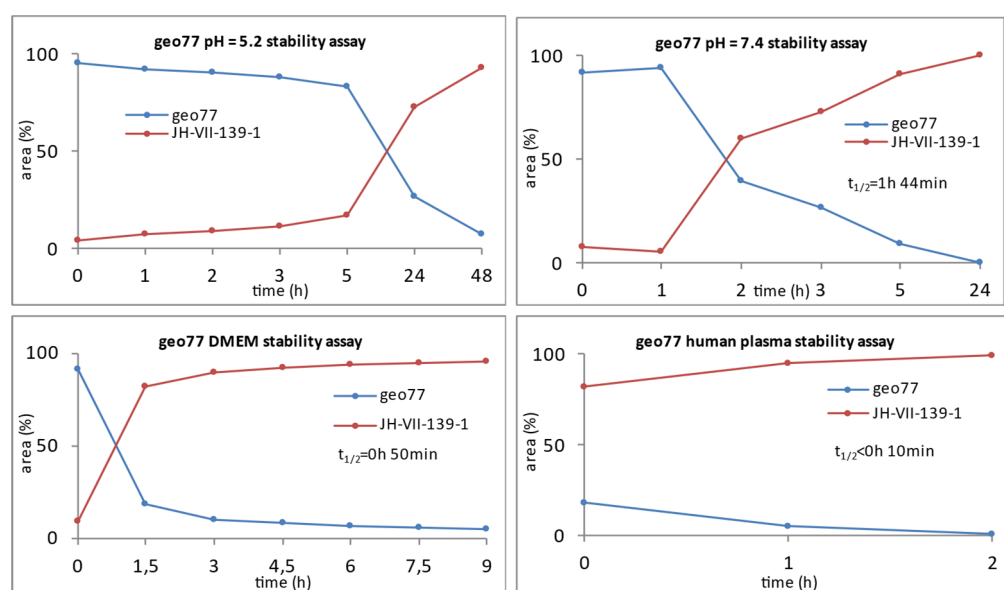


Figure 4. Chemostability of **geo77**.

3.2.3 Chemostability profile of conjugate **geo85**

Since both carbamate and amide conjugates, **geo75** and **geo77** exhibited short half-lives during their incubation in cell media and human plasma, our efforts were focused on the urea derivative **geo85** (Figure 5, S69-S72). Conjugate **geo85** proved to be completely stable in both buffer media having half-lives that exceeded two days. **Geo85** maintained its stability in DMEM and human plasma, although a 10% hydrolysis was observed after 24 hours. Therefore, **geo85** was the most stable conjugate and proved to be an excellent candidate for fluorescent tagging and mechanistic studies.

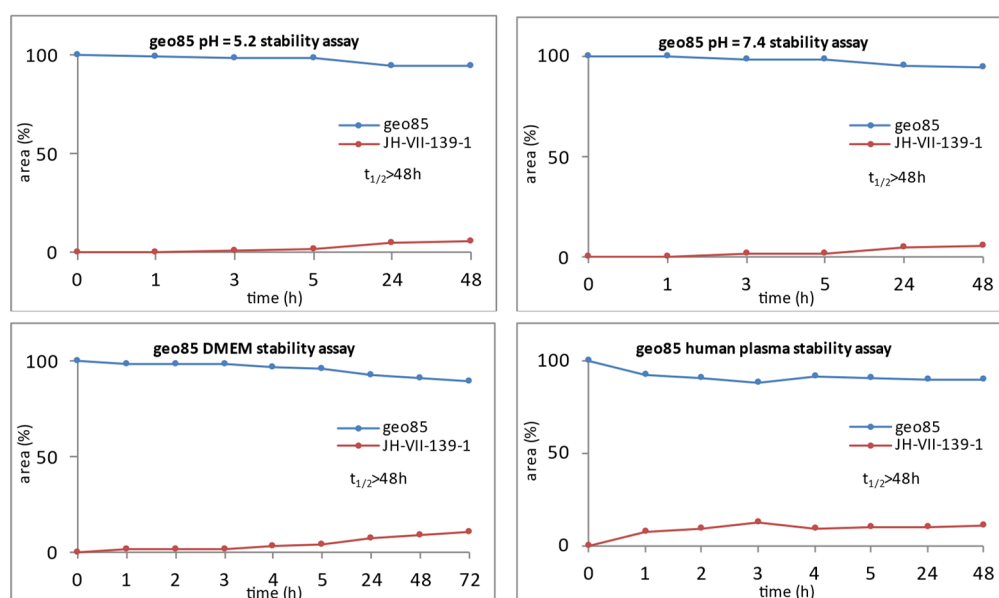


Figure 5. Chemostability of conjugate **geo85**.

3.2.4 Chemostability profile of **geo107**

The stability profile of **geo107** was analogous to **geo85**, as it maintained half-lives $t_{1/2} > 48$ hours in all stability experiments (Figure 6, S73-S76). Hydrolysis and JH-VII-139-1 was

also detected. Residual concentrations of the conjugate dropped no further than 80% of its initial concentration even in cell media or human plasma.

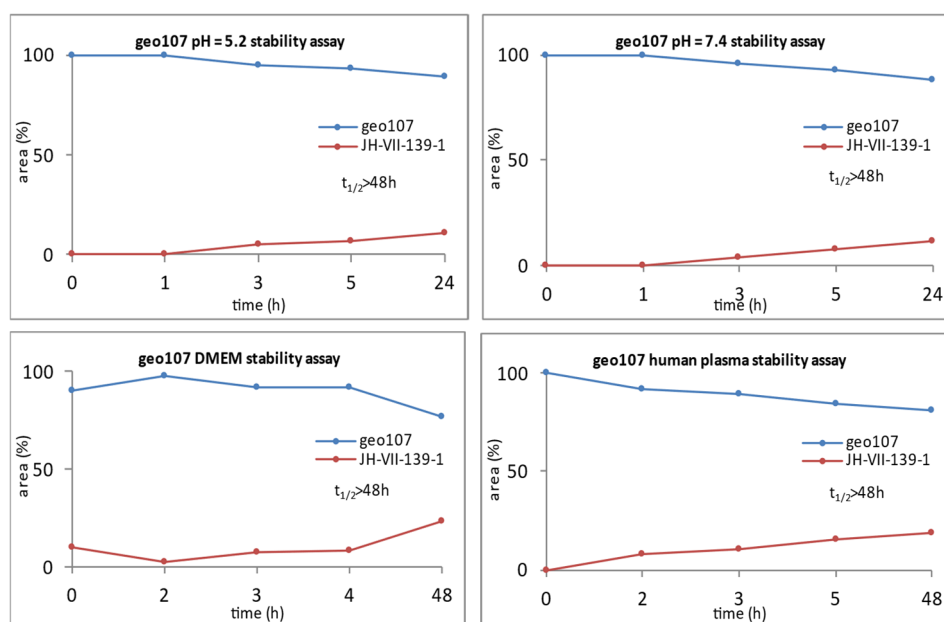


Figure 6. Chemostability of conjugate geo107.

3.3. Biological activity of the synthesized compounds

3.3.1. Inhibition of kinase activity by JH-VII-139-1-c(RGDyK) conjugates

The inhibitory activity of JH-VII-139-1 and RGD-conjugated compounds was evaluated by *in vitro* kinase assay. As shown in Table 2, both JH-VII-139-1 and the conjugated compounds, had a significant effect on SRPK1 activity, with IC_{50} values at the nM level (Figure S79). Therefore, the conjugation of JH-VII-139-1 to the peptide did not affect the inhibitory activity against SRPK1.

what is geo48?
see also FigS79

Table 2. Half-maximal inhibitory activity (IC_{50}) of JH-VII-139-1, geo48, geo75, geo77 and geo85 over SRPK1. Results are presented as mean $\pm$ SD of three independent experiments.

Compound	IC_{50} (nM) for SRPK1
JH-VII-139-1	32 ± 0.5
geo75	35 ± 1.1
geo77	30 ± 1.7
geo85	42 ± 1.9

3.3.2 Cytotoxicity of JH-VII-139-1-c(RGDyK) conjugates

The cytotoxic activity of JH-VII-139-1 and JH-VII-139-1-c(RGDyK) hybrid compounds was evaluated at different concentrations (0.5–50 μ M) over a panel of cell lines including HeLa cervical cancer, MCF7 mammary carcinoma, the triple-negative breast cancer MDA-MB-231 and K562 lymphoblast cells. Integrin receptors are highly overexpressed on the surface of many types of cancer [42]. The metastatic breast cancer cell lines MDA-MB-435 [43, 44] and MCF-7 [45, 46] as well as HeLa cells [47] express high levels of integrin $\alpha v \beta 3$ integrins. On the other hand, K562 express very low levels of integrins $\alpha v \beta 3$ [22].

insert time 48 h

Table 3. Growth inhibition/cytostatic (GI₅₀ and TGI) and cytotoxic/cytocidal (IC₅₀) anticancer effects induced by JH-VII-139-1, c(RGDyK), and JH-VII-139-1-c(RGDyK) hybrid compounds against a panel of cancer cell lines. Results are presented as mean ± SD of three independent experiments.

Compound (μM)		HeLa	K562	MDA-MB-231	MCF7
JH-VII-139-1	GI ₅₀	8 ± 0,2	8 ± 0,5	11 ± 0,3	9 ± 0,2
	TGI	19 ± 0,2	15 ± 0,5	21 ± 0,4	19 ± 0,3
	IC ₅₀	14 ± 0,8	13 ± 0,3	18 ± 0,5	8 ± 0,6
geo75	GI ₅₀	5 ± 0,4	5 ± 0,3	8 ± 0,2	5 ± 0,5
	TGI	11 ± 0,3	10 ± 0,3	16 ± 0,3	10 ± 0,5
	IC ₅₀	11 ± 0,3	12 ± 0,2	11 ± 0,4	4 ± 0,5
geo77	GI ₅₀	5 ± 0,2	12 ± 0,9	8 ± 0,5	4 ± 0,4
	TGI	11 ± 0,3	23 ± 0,5	15 ± 0,7	9 ± 0,4
	IC ₅₀	9 ± 0,2	17 ± 0,5	10 ± 0,3	3 ± 0,4
geo85	GI ₅₀	>100	>100	>100	33 ± 0,9
	TGI	>100	>100	>100	64 ± 0,9
	IC ₅₀	>100	>100	>100	45 ± 1,0
c(RGDyK)	GI ₅₀	79 ± 2,2	>100	80 ± 1,2	>100
	TGI	>100	>100	>100	>100
	IC ₅₀	>100	>100	>100	>100

The cytotoxic and cytostatic activities of the JH-VII-139-1-c(RGDyK) hybrid compounds were estimated by three concentration-dependent parameters: GI₅₀ (concentration that results in 50% growth inhibition), TGI (concentration that results in total growth inhibition or cytostatic effect), and IC₅₀ (concentration that results in 50% growth cytotoxic effect) (Table 3). JH-VII-139-1 and the hybrid compounds, **geo75** and **geo77**, showed a significant cytostatic (GI₅₀ = 4–12 μM) and cytotoxicity (IC₅₀ = 3–18 μM) effect against HeLa, MCF-7, MDA-MB-231 and K562 cancer cells. The most active compound was **geo77**, showing IC₅₀ and GI₅₀ values of 3 μM and 4 μM, respectively in the most sensitive cell line MCF-7. **Geo85** exhibited a slightly cytostatic effect (GI₅₀ = 33 μM), with a minor cytotoxicity (IC₅₀ = 45 μM) only against MCF-7 cells.

3.3.3 Cellular uptake of c(RGDyK)-conjugate **geo106**

The cellular uptake of **geo106**, a c(RGDyK)-conjugate carrying a fluorescent label, was examined by fluorescent microscopy in MCF7 and MDA-MB-231 cancer cell lines (Figure 7). Confocal image data showed efficient cellular uptake of **geo106** even after a short time of incubation (2 h) in MDA-MB-231 cells, with the most intense fluorescent signal obtained in 48 h in both cancer cell lines.

To demonstrate the role of the RGD moiety in targeting the conjugate to α_vβ₃ integrin-rich tumor cells, MDA-MB-231 cells were preincubated with okadaic acid (an inhibitor of endocytosis) prior to addition of **geo106** [48]. Confocal image data showed a decreased fluorescence signal, confirming that the cellular uptake of the conjugates was mediated by endocytosis (Figure 8).

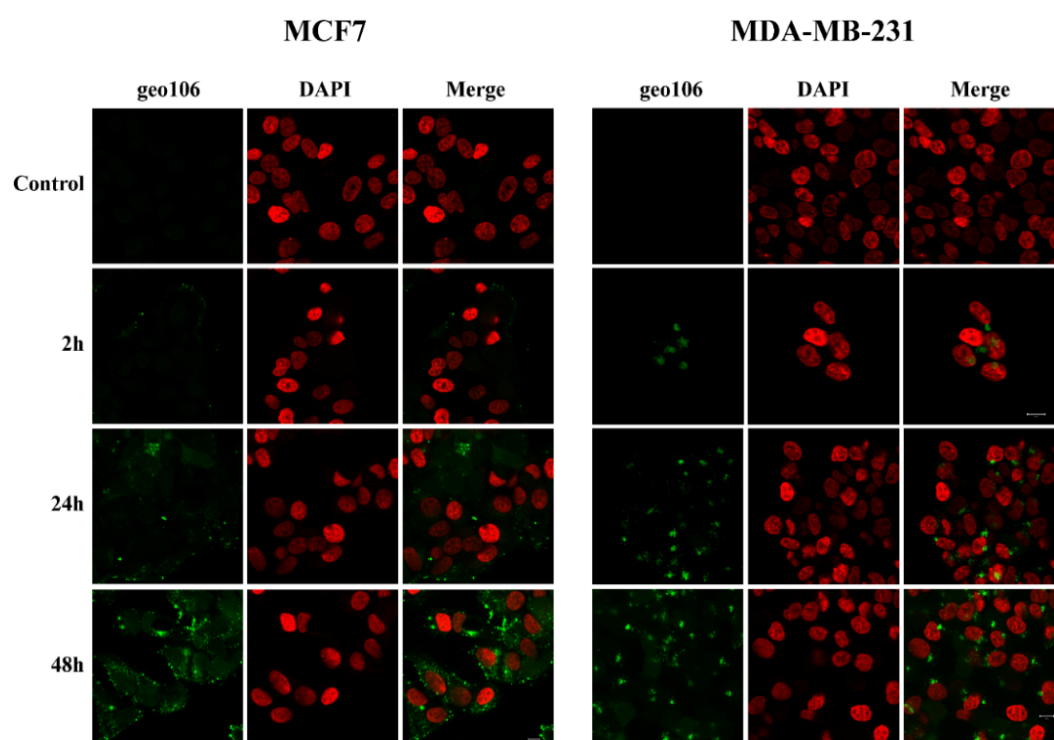


Figure 7. Confocal microscopy images of MCF7 and MDA-MB-231 cells after incubation with 5 μ M of **geo106** for 2, 24 and 48 h, respectively. Cells were fixed with 4% paraformaldehyde. DNA was stained with DAPI (pseudo color red). Cells were visualized with a Zeiss LSM 780 confocal microscope using the Zen 2011 software.

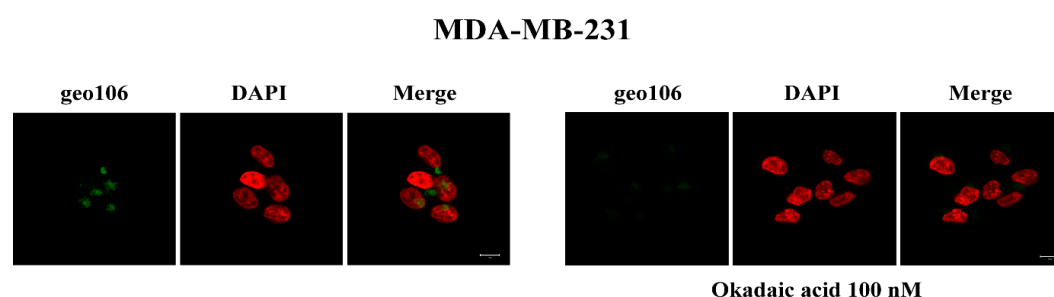


Figure 8. Confocal microscopy images of MDA-MB-231 cells after incubation with 5 μ M of **geo106** for 2h. Cells were preincubated, prior to addition of **geo106**, with okadaic acid (100 nM) for 30 min. Cells were fixed with 4% paraformaldehyde. DNA was stained with DAPI. Cells were visualized with a Zeiss LSM 780 confocal microscope using the Zen 2011 software.

3.3.4 *In Vivo* Zebrafish Angiogenesis Studies

To determine if compounds c(RGDyK), JH-VII-139-1, **geo75**, **geo77**, **geo85**, and **geo107** have any effect on angiogenesis, embryos from the transgenic zebrafish line *Tg(kdrl:gfp)* have been used. The compounds were added in the E3 medium 24h post fertilization (hpf) and scored angiogenesis in the intersegmental vessels (ISVs) at 72hpf. The embryos were examined under a fluorescence microscope, and the length as well as the width of the ISVs was used as a measurement to evaluate angiogenesis.

Our results show that the extracts significantly inhibit zebrafish width angiogenesis (Figure 9), while exert a milder effect in length angiogenesis (Figure 10). More specifically, **geo75**, **geo85**, **geo77**, and **geo107** could significantly inhibit width angiogenesis at concentrations of 1.3 μ M, while c(RGDyK) and JH-VII-139-1 at 10 μ M. On the other hand,

c(RGDyK), **geo75** and **geo85** did not significantly inhibit length angiogenesis, while JH-VII-139-1, **geo77** and **geo107** exhibit a stronger effect but not as significant as with the width inhibition angiogenesis. In this study, no other additional morphological phenotypic changes were observed.

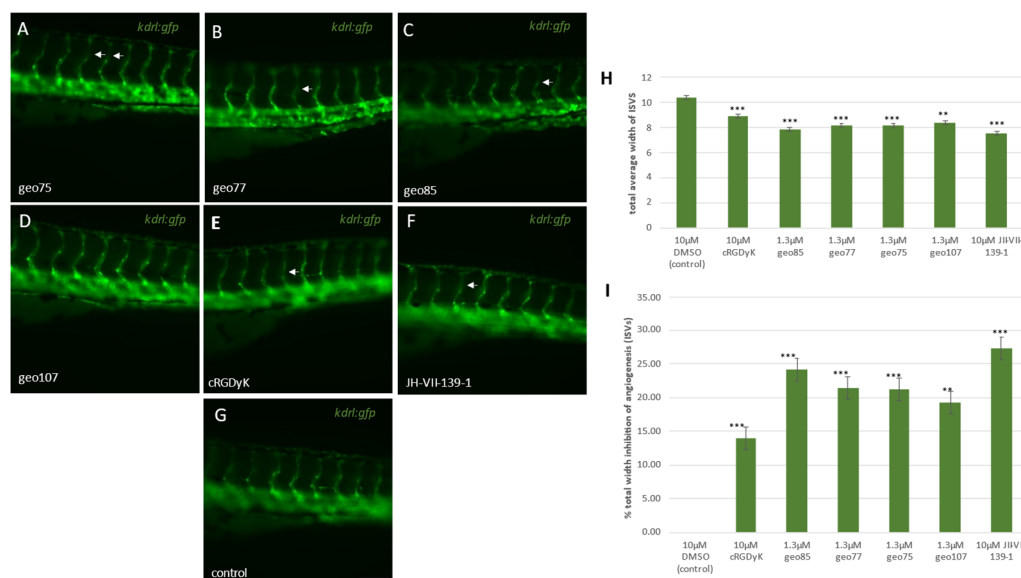


Figure 9. *In vivo* screening unravels five very strong and one milder inhibitors of width angiogenesis. (A–G) Fluorescence microscopy images of representative compounds treated from 24hpf, and DMSO-treated control 72hpf *Tg(kdr:GFP)* embryos. White arrows show sections of ISVs with decreased width angiogenesis. Scale bar, 100 µm. (H) The total width of 8 ISVs per embryo was estimated and presented. It was measured and averaged the width at three different points (top, middle and bottom point) of each ISV. Compounds c(RGDyK) (10µM), JH-VII-139-1 (10µM), **geo75** (1.3µM), **geo77** (1.3µM), **geo85** (1.3µM) show very high statistically significant inhibition on the total width of ISVs, while compound **geo107** (1.3µM), shows slightly milder effect at the tested concentrations. (I) The percentage total width inhibition of angiogenesis was calculated compared to DMSO-treated controls for each compound. Data are expressed as mean + SEM (n=20/group). $p^{**}<0.01$, $p^{***}<0.001$.

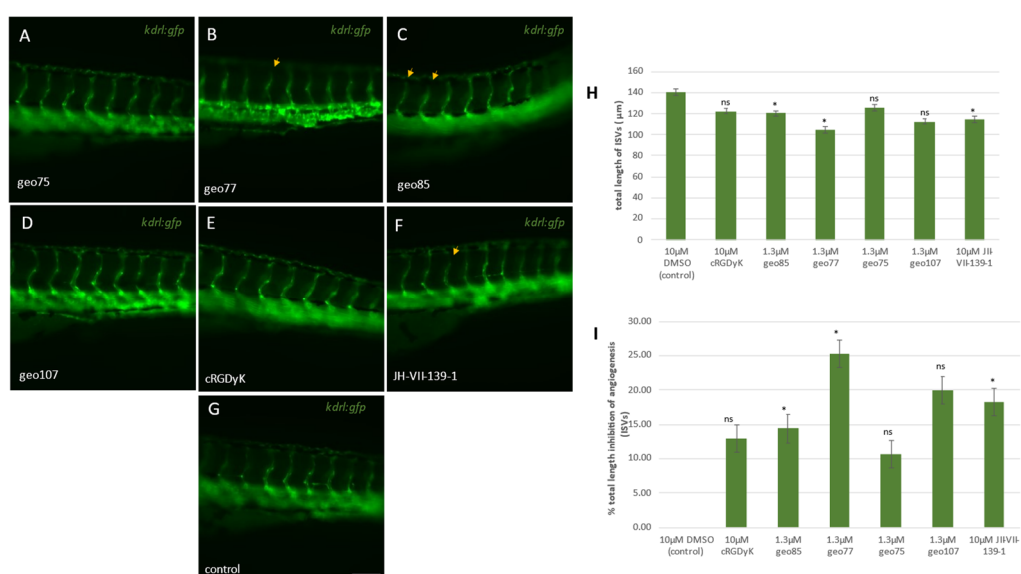


Figure 10. *In vivo* screening unravels 3 mild inhibitors of length angiogenesis (A–G) Fluorescence microscopy images of representative compounds treated from 24hpf, and DMSO-treated control

72hpf *Tg(kdrl:gfp)* embryos. Yellow arrows show sections of ISVs with decreased length angiogenesis. Scale bar, 100 μ m. (H) The total length of 8 ISVs per embryo was measured and presented. Compounds **geo77** (1.3 μ M), **geo107** (1.3 μ M), and JH-VII-139-1 (10 μ M) show mild statistically significant inhibition on the total length of ISVs, while the other tested compounds had no effect at the tested concentrations. (I) The percentage total length inhibition of angiogenesis was calculated compared to DMSO-treated controls for each compound. Data are expressed as mean + SEM (n=20/group). $p^* < 0.005$.

4. Discussion

In the present study, we disclose the synthesis and biological evaluation of several JH-VII-139-1-c(RGDyK) conjugates, which inhibit SRPK1 kinase. To develop novel anti-angiogenic compounds JH-VII-139-1, a potent SRPK1 inhibitor, was coupled to c(RGDyK) peptide using different linkers. Four c(RGDyK) conjugates **geo75**, **geo77**, **geo85** and **geo107** have been synthesized, which were examined for their stability in pH = 5.2 and 7.4 buffer solutions, cell medium and human plasma. Conjugates **geo75** and **geo77** demonstrated limited stability and were rapidly hydrolyzed releasing JH-VII-139-1. In human plasma, the conjugates had short half-lives of 10-50 minutes. On the other hand, **geo85** and **geo107** showed lasting stability in buffer solutions, cell medium and human plasma. *In vitro* kinase assay against SRPK1 showed that all the tested JH-VII-139-1-c(RGDyK) conjugates retained the inhibitory activity against SRPK1 in the nanomolar range (30-42 nM). The cytotoxic activity of the synthesized compounds was tested against the cancer cell lines HeLa, MCF7, MDA-MB-231 and K562. Between the tested cell lines, MCF-7 cells proved to be the most sensitive (lower IC₅₀ values) to JH-VII-139-1 and its conjugates. Overall, the conjugates **geo75** and **geo77** bearing the cleavable linkers exhibit a slight improvement of antiproliferative activity compared to JH-VII-139-1 against integrin $\alpha_v\beta_3$ overexpressing cells (HeLa, MDA-MB-231, MCF7). However, the observed activities are in part attributed to an early JH-VII-139-1 release from the conjugate as the stability studies indicate. The stable conjugate **geo85** induced selective cytotoxicity towards MCF7 cancer cells in the mid-micromolar range, whereas it was significantly less active against HeLa and MDA-MB-231 cells. The cellular uptake of the conjugate **geo106**, which carries a fluorescent label, was examined by fluorescent microscopy in MCF7 and MDA-MB-231 cancer cell lines. Confocal image data showed efficient cellular uptake of **geo106** even after a short time mediated by integrin mediated endocytosis. Then, the effect of (JH-VII-139-1)-c(RGDyK) conjugates on angiogenesis was examined *in vivo* in zebrafish embryos. Notably, the new conjugates significantly inhibit zebrafish width angiogenesis and exert a milder effect in length angiogenesis. Among the tested conjugates the stable **geo85** was the most potent inhibitor of zebrafish width angiogenesis.

5. Conclusions

The synthesized (JH-VII-139-1)-c(RGDyK) conjugates retained the inhibitory activity of JH-VII-139-1 against SRPK1, while exhibiting different cytotoxicity profile against cancer cells expressing integrins to various extend. The activities were strongly related to the stability of the linkers and the early release of JH-VII-139-1. The stable conjugate **geo85** induced increased targeting ability against MCF7 cancer cells, however it was mild cytotoxic with GI₅₀=33 $\pm$ 0,9 μ M, TGI=64 $\pm$ 0,9 μ M, IC₅₀=45 $\pm$ 1,0 μ M. Remarkably, all (JH-VII-139-1)-c(RGDyK) conjugates display *in vivo* antiangiogenic effects, although a more significant effect was observed for **geo85** against width angiogenesis in zebrafish embryos. Further experiments in particular *in vivo* are clearly necessary to exhibit the full potential of such peptide conjugates in cancer therapy and angiogenesis-related diseases.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1-78 ¹H-NMR, ¹³C-NMR, LC/ESI-MS data

Author Contributions: “Conceptualization, T.G., E.N. and V.S.; methodology, T.G., E.N., D.B. and V.S.; investigation, G.L., A.K., I.S., K.T.; resources, T.G., E.N., D.B. and V.S.; data curation, V.S., G.L., I.S.; writing—original draft preparation, G.L., T.G., E.N., D.B. and V.S.; writing—review and editing, T.G., E.N., V.S.; visualization, G.L., A.K., V.S.; supervision, T.G., E.N., D.B. and V.S.; funding acquisition, T.G., E.N., D.B., V.S. All authors have read and agreed to the published version of the manuscript.”

Funding: This study was supported by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the ‘First Call for H.F.R.I. Research Projects to support Faculty members and Researchers and the procurement of high-cost research equipment grant’ (Project Number:12), Title: Synthesis and biological evaluation of RGD-conjugated SRPK1 kinase inhibitors for integrin-targeted cancer therapy.

Conflicts of Interest: “The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results”.

References

- Yoo, S.Y.; Kwon, S.M. Angiogenesis and its therapeutic opportunities. *Mediators Inflamm.* **2013**, *2013*, pp. 127170.
- Potente, M.; Gerhardt, H.; Carmeliet, P. Basic and therapeutic aspects of angiogenesis. *Cell* **2011**, *146*(6), pp. 873–887.
- Garcea, G.; Lloyd, T.D.; Gescher, A.; Dennison, A.R.; Steward, W.P.; Berry, D.P. Angiogenesis of gastrointestinal tumours and their metastases—a target for intervention? *Eur. J. Cancer.* **2004**, *40*(9), pp. 1302–1313.
- Folkman, J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol.* **2002**, *29*(6 Suppl 16), pp. 15–18.
- Bielenberg, D.R.; Zetter, B.R. The Contribution of Angiogenesis to the Process of Metastasis. *Cancer J.* **2015**, *21*(4), pp. 267–273.
- Fallah, A.; Sadeghinia, A.; Kahroba, H.; Samadi, A.; Heidari, H.R.; Bradaran, B.; Zeinali, S.; Molavi, O. Therapeutic targeting of angiogenesis molecular pathways in angiogenesis-dependent diseases. *Biomed. Pharmacother.* **2019**, *110*, pp. 775–785.
- Adamis, A.P.; Aiello, L.P.; D’Amato, R.A. Angiogenesis and ophthalmic disease. *Angiogenesis.* **1999**, *3*(1), pp. 9–14.
- Girolamo, F.; Coppola, C.; Ribatti, D.; Trojano, M. Angiogenesis in multiple sclerosis and experimental autoimmune encephalomyelitis. *Acta Neuropathol. Commun.* **2014**, *2*, pp. 84.
- Elshabrawy, H.A.; Chen, Z.; Volin, M.V.; Ravella, S.; Virupannavar, S.; Shahrara, S. The pathogenic role of angiogenesis in rheumatoid arthritis. *Angiogenesis.* **2015**, *18*(4), pp. 433–448.
- Zimmerman, M.A.; Selzman, C.H.; Raeburn, C.D.; Calkins, C.M.; Barsness, K.; Harken, A.H. Clinical applications of cardiovascular angiogenesis. *J. Card. Surg.* **2001**, *16*(6), pp. 490–497.
- Jaipersad, A.S.; Lip, G.Y.; Silverman, S.; Shantsila, E. The role of monocytes in angiogenesis and atherosclerosis. *J. Am. Coll. Cardiol.* **2014**, *63*(1), pp. 1–11.
- Serini, G.; Valdembrì, D.; Bussolino, F. Integrins and angiogenesis: a sticky business. *Exp. Cell Res.* **2006**, *312*(5), pp. 651–658.
- Ahmed, Z.; Bicknell, R. Angiogenic signalling pathways. *Methods Mol. Biol.* **2009**, *467*, pp. 3–24.
- Thurston, G.; Kitajewski, J. VEGF and Delta-Notch: interacting signalling pathways in tumour angiogenesis. *Br. J. Cancer.* **2008**, *99*(8), pp. 1204–1209.
- Uemura, A.; Fruttiger, M.; D’Amore, P.A.; De Falco, S.; Joussen, A.M.; Sennlaub, F.; Brunck, L.R.; Johnson, K.T.; Lambrou, G.N.; Rittenhouse, K.D.; Langmann, T. VEGFR1 signaling in retinal angiogenesis and microinflammation. *Prog. Retin Eye Res.* **2021**, *84*, pp. 100954.
- Shibuya, M. Role of VEGF-flt receptor system in normal and tumor angiogenesis. *Adv. Cancer Res.* **1995**, *67*, pp. 281–316.
- Ferrara, N.; Kerbel, R.S. Angiogenesis as a therapeutic target. *Nature.* **2005**, *438*(7070), pp. 967–974.
- Simons, M.; Gordon, E.; Claesson-Welsh, L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat. Rev. Mol. Cell Biol.* **2016**, *17*(10), pp. 611–625.
- Gammons, M.V.; Dick, A.D.; Harper, S.J.; Bates, D.O. SRPK1 inhibition modulates VEGF splicing to reduce pathological neovascularization in a rat model of retinopathy of prematurity. *Invest. Ophthalmol. Vis. Sci.* **2013**, *54*(8), pp. 5797–5806.
- Gammons, M.V.; Lucas, R.; Dean, R.; Coupland, S.E.; Oltean, S.; Bates, D.O. Targeting SRPK1 to control VEGF-mediated tumour angiogenesis in metastatic melanoma. *Br. J. Cancer.* **2014**, *111*(3), pp. 477–485.
- Mavrou, A.; Brakspear, K.; Hamdollah-Zadeh, M.; Damodaran, G.; Babaei-Jadidi, R.; Oxley, J.; Gillatt, D.A.; Ladomery, M.R.; Harper, S.J.; Bates, D.O.; Oltean, S. Serine-arginine protein kinase 1 (SRPK1) inhibition as a potential novel targeted therapeutic strategy in prostate cancer. *Oncogene.* **2015**, *34*(33), pp. 4311–4319.
- Chatzisdieri, T.; Leonidis, G.; Karampelas, T.; Skavatsou, E.; Velentza-Almpani, A.; Bianchini, F.; Tamvakopoulos, C.; Sarli, V. Integrin-Mediated Targeted Cancer Therapy Using c(RGDyK)-Based Conjugates of Gemcitabine. *J. Med. Chem.* **2022**, *65*(1), pp. 271–284.
- Chatzisdieri, T.; Dalezis, P.; Leonidis, G.; Bousis, S.; Trafalis, D.; Bianchini, F.; Sarli, V. Synthesis and biological studies of c(RGDyK) conjugates of cucurbitacins. *Future Med. Chem.* **2021**, *13*(10), pp. 877–895.

24. Chatzisideri, T.; Thysiadis, S.; Katsamakas, S.; Dalezis, P.; Sigala, I.; Lazarides, T.; Nikolakaki, E.; Trafalis, D.; Gederaas, O.A.; Lindgren, M.; Sarli, V. Synthesis and biological evaluation of a Platinum(II)-c(RGDyK) conjugate for integrin-targeted photo-dynamic therapy. *Eur. J. Med. Chem.* **2017**, *141*, pp. 221–231. 922
25. Kapp, T.G.; Rechenmacher, F.; Neubauer, S.; Maltsev, O.V.; Cavalcanti-Adam, E.A.; Zarka, R.; Reuning, U.; Notni, J.; Wester, H.J.; Mas-Moruno, C.; Spatz, J.; Geiger, B.; Kessler, H. A Comprehensive Evaluation of the Activity and Selectivity Profile of Ligands for RGD-binding Integrins. *Sci. Rep.* **2017**, *7*, pp. 39805. 923
26. Leonidis, G.; Dalezis, P.; Trafalis, D.; Beis, D.; Giardoglou, P.; Koukiali, A.; Sigala, I.; Nikolakaki, E.; Sarli, V. Synthesis and Biological Evaluation of a c(RGDyK) Peptide Conjugate of SRPIN803. *ACS Omega*. **2021**, *6*(42), pp. 28379–28393. 924
27. Hatcher, J.M.; Wu, G.; Zeng, C.; Zhu, J.; Meng, F.; Patel, S.; Wang, W.; Ficarro, S.B.; Leggett, A.L.; Powell, C.E.; Marto, J.A.; Zhang, K.; Ki Ngo J.C.; Fu, X.D.; Zhang, T.; Gray, N.S. SRPKIN-1: A Covalent SRPK1/2 Inhibitor that Potently Converts VEGF from Pro-angiogenic to Anti-angiogenic Isoform. *Cell Chem. Biol.* **2018**, *25*(4), pp. 460–470. 925
28. Larkins, E.; Blumenthal, G.M.; Chen, H.; He, K.; Agarwal, R.; Gieser, G.; Stephens, O.; Zahalka, E.; Ringgold, K.; Helms, W.; Shord, S.; Yu, J.; Zhao, H.; Davis, G.; McKee, A.E.; Keegan, P.; Pazdur, R. FDA Approval: Alectinib for the Treatment of Meta-static, ALK-Positive Non-Small Cell Lung Cancer Following Crizotinib. *Clin. Cancer Res.* **2016**, *22*(21), pp. 5171–5176. 926
29. Song, D.; Lee, M.; Park, C.H.; Ahn, S.; Yun, C.S.; Lee, C.O.; Kim, H.R.; Hwang, J.Y. Novel 2,4-diaminopyrimidines bearing tetrahydronaphthalenyl moiety against anaplastic lymphoma kinase (ALK): Synthesis, in vitro, ex vivo, and in vivo efficacy studies. *Bioorg Med. Chem. Lett.* **2016**, *26*(7), pp. 1720–1725. 927
30. Kinoshita, K.; Kobayashi, T.; Asoh, K.; Furuichi, N.; Ito, T.; Kawada, H.; Hara, S.; Ohwada, J.; Hattori, K.; Miyagi, T.; Hong, W.S.; Park, M.J.; Takanashi, K.; Tsukaguchi, T.; Sakamoto, H.; Tsukuda, T.; Oikawa, N. 9-substituted 6,6-dimethyl-11-oxo-6,11-dihydro-5H-benzo[b]carbazoles as highly selective and potent anaplastic lymphoma kinase inhibitors. *J. Med. Chem.* **2011**, *54*(18), pp. 6286–94. 928
31. John, A.A.; Ramil, C.P.; Tian, Y.; Cheng, G.; Lin, Q. Synthesis and Site-Specific Incorporation of Red-Shifted Azobenzene Amino Acids into Proteins. *Org. Lett.* **2015**, *17*, 24, pp. 6258–6261. 929
32. Wan, T.; Capaldo, L.; Laudadio, G.; Nyuchev, A.V.; Rincón, J.A.; García-Losada, P.; Mateos, C.; Frederick, M.O.; Nuño, M.; Noël, T. Decatungstate-Mediated C(sp³)-H Heteroarylation via Radical-Polar Crossover in Batch and Flow. *Angew. Chem. Int. Ed.* **2021**, *60*, pp. 17893–17899. 930
33. Young, M.B.; Barrow, J.C.; Glass, K.L.; Lundell, G.F.; Newton, C.L.; Pellicore, J.M.; Rittle, K.E.; Selnick, H.G.; Stauffer, K.J.; Vacca, J.P.; Williams, P.D.; Bohn, D.; Clayton, F.C.; Cook, J.J.; Krueger, J.A.; Kuo, L.C.; Lewis, S.D.; Lucas, B.J.; McMasters, D.R.; Miller-Stein, C.; Pietrak, B.L.; Wallace, A.A.; White, R.B.; Wong, B.; Yan, Y.; Nantermet, P.G. Discovery and evaluation of potent P1 aryl heterocycle-based thrombin inhibitors. *J. Med. Chem.* **2004**, *47*(12), pp. 2995–3008. 931
34. Yeager, A.R.; Finney, N.S. The first direct evaluation of the two-active site mechanism for chitin synthase. *J. Org. Chem.* **2004**, *69*(3), pp. 613–618. 932
35. Lee, M.; Grissom, C.B. Design, Synthesis, and Characterization of Fluorescent Cobalamin Analogues with High Quantum Efficiencies. *Org. Lett.* **2009**, *11*, 12, pp. 2499–2502. 933
36. Voukkalis, N.; Koutroumani, M.; Zarkadas, C.; Nikolakaki, E.; Vlassi, M.; Giannakouros, T. SRPK1 and Akt Protein Kinases Phosphorylate the RS Domain of Lamin B Receptor with Distinct Specificity: A Combined Biochemical and In Silico Approach. *PLoS One* **2016**, *11*(4), pp. e0154198. 934
37. Sigala, I.; Tsamis, K. I.; Gousia, A.; Alexiou, G.; Voulgaris, S.; Giannakouros, T.; Kyritsis, A. P.; Nikolakaki, E. Expression of SRPK1 in gliomas and its role in glioma cell lines viability. *Tumour Biol.* **2016**, *37*, pp. 8699–8707. 935
38. Aleström, P.; D'Angelo, L.; Midtlyng, P. J.; Schorderet, D. F.; Schulte-Merker, S.; Sohm, F.; Warner, S. Zebrafish: Housing and husbandry recommendations. *Lab Anim.* **2020**, *54*, pp. 213–224. 936
39. Davis, R.A.; Lau, K.; Hausner, S.H.; Sutcliffe, J.L. Solid-phase synthesis and fluorine-18 radiolabeling of cycloRGDyK. *Org. Biomol. Chem.*, **2016**, *14*, pp. 8659–8663. 937
40. Hammershøj, P.; Kumar, E.K.P.; Harris, P.; Andresen, T.L.; Clausen, M.H. Facile Large-Scale Synthesis of 5- and 6-Carboxyfluoresceins: Application for the Preparation of New Fluorescent Dyes. *Eur. J. Org. Chem.* **2015**, pp. 7301–7309. 938
41. Lee, M.; Grissom, C.B. Design, Synthesis, and Characterization of Fluorescent Cobalamin Analogues with High Quantum Efficiencies. *Org. Lett.* **2009**, *11*, 12, pp. 2499–2502. 939
42. Desgrosellier, J.S.; Cheresch, D.A. Integrins in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer.* **2010**, *10*(1), pp. 9–22. 940
43. Czarnomysy, R.; Surazyński, A.; Popławska, B.; Rysiak, E.; Pawłowska, N.; Czajkowska, A.; Bielawski, K.; Bielawska, A. Synergistic action of cisplatin and echistatin in MDA-MB-231 breast cancer cells. *Mol. Cell Biochem.* **2017**, *27*(1–2), pp.13–22. 941
44. Kazmierczak, P.M.; Todica, A.; Gildehaus, F.J.; Hirner-Eppeneder, H.; Brendel, M.; Eschbach, R.S.; Hellmann, M.; Nikolaou, K.; Reiser, M.F.; Wester, H.J.; Kropf, S.; Rominger, A.; Cyran, C.C. 68Ga-TRAP-(RGD)3 Hybrid Imaging for the In Vivo Monitoring of αvβ3-Integrin Expression as Biomarker of Anti-Angiogenic Therapy Effects in Experimental Breast Cancer. *PLoS One.* **2016**, *11*(12), e0168248. 942
45. Braun, K.; Wiessler, M.; Pipkorn, R.; Ehemann, V.; Bäuerle, T.; Fleischhacker, H.; Müller, G.; Lorenz, P.; Waldeck, W. A cyclic-RGD-BioShuttle functionalized with TMZ by DARinv "Click Chemistry" targeted to αvβ3 integrin for therapy. *Int. J. Med. Sci.* **2010**, *7*(6), pp. 326–339. 943
46. Kim, H.A.; Nam, K.; Kim, S.W. Tumor targeting RGD conjugated bio-reducible polymer for VEGF siRNA expressing plasmid delivery. *Biomaterials.* **2014**, *35*(26), pp. 7543–7452. 944

-
47. Xiong, L.; Yu, M.; Cheng, M.; Zhang, M.; Zhang, X.; Xu, C.; Li, F. A photostable fluorescent probe for targeted imaging of tumour cells possessing integrin $\alpha(v)\beta(3)$. *Mol Biosyst.* **2009**, *5*(3), pp. 241-243. 982
983
48. Lee, M.H.; Kim, J.Y.; Han, J.H.; Bhuniya, S.; Sessler, J.L.; Kang, C.; Kim, J.S. Direct fluorescence monitoring of the delivery and cellular uptake of a cancer-targeted RGD peptide-appended naphthalimide theragnostic prodrug. *J. Am. Chem. Soc.* **2012**, *134*(30), pp. 12668-12674. 984
985
986