Electronical supporting information

First *in vivo* evaluation of the radiolabelled Hsp90 inhibitor [^{11}C]Onalespib for cancer and brain PET

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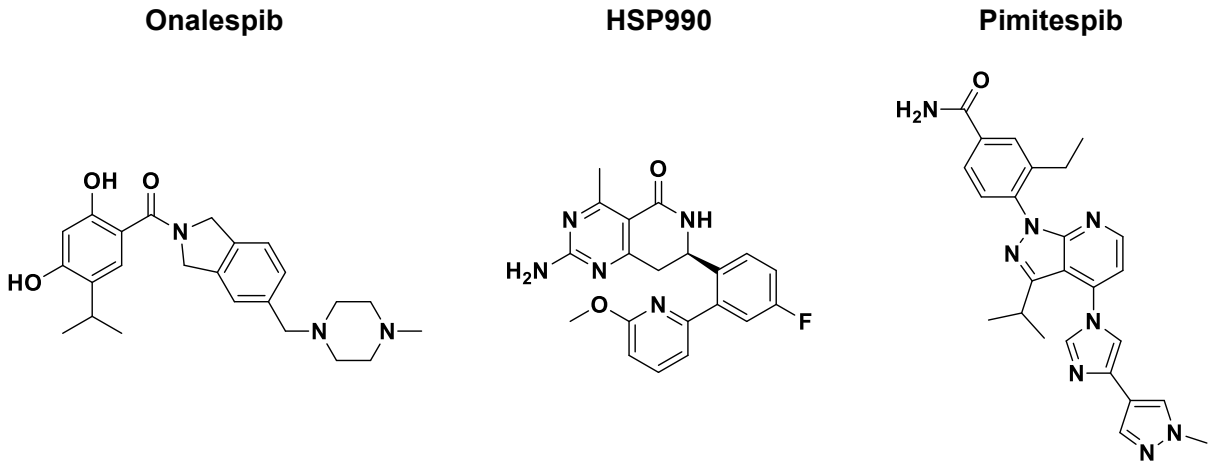
I- General information

Reagents and solvents used in synthesis were acquired from commercial suppliers (Sigma-Aldrich, Fisher Scientific, TCI, Fluorochem, Ambeed) and used without further purification. Reactions were monitored by analytical thin layer chromatography (TLC) using commercial pre-coated aluminium foils (silica gel matrix 60 F254, Merck, Darmstadt, Germany), visualised by UV light at 254 nm. Purifications were performed by manual column chromatography on silica gel.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Avance 300 MHz or 600 MHz instrument (Brüker, Fällanden, Switzerland). Chemical shifts (δ) are reported in parts per million (ppm) relative to deuterated solvents (DMSO-d_6: 2.50 ppm) and coupling constant (*J*) are given in Hertz (Hz). Multiplicities are described by symbols s (singlet), d (doublet), t (triplet), p (pentuplet) and m (multiplet). MNova software (Bruker) was employed for data analysis.

Intermediate reaction products were analysed using a Waters Alliance e2695 HPLC system equipped with a C_{18} XBridge column (3.5 µm, 3.0 × 100 mm, Waters). UV-Vis detection was carried out using a Waters 2489 PDA detector set at 254 nm. Mass spectrometric detection was performed on a Waters single quadrupole mass spectrometer operated in positive ionization mode.

High resolution mass spectrometry (HRMS) was performed on a quadrupole time-of-flight system (Synapt G2 HDMS, Waters, Milford, Massachusetts, USA) by the use of an electrospray ionisation (ESI) source in positive ionization mode.



Supplementary Fig. 1 Chemical structures of inhibitors used in this study.

Supplementary Table 1 Overview of Hsp90 inhibitors used in this study.

Compound	MW[a]	K_d[b] (nM)	Hsp90α IC_{50}[c] or K_i[d] (nM)	Hsp90β IC_{50} or K_i (nM)	Grp94 IC_{50} or K_i (nM)	TRAP1 IC_{50} or K_i (nM)
Onalespib	409.52	0.71 [1]	-	-	-	-
HSP990	379.39	-	IC_{50} = 0.6 [2]	IC_{50} = 0.8 [2]	IC_{50} = 8.5 [2]	IC_{50} = 320 [2]
Pimitespib	454.53	-	Ki = 34.7 [3]	Ki = 21.3 [3]	Ki > 50 000 [3]	Ki > 50 000 [3]

[a] MW = molecular weight

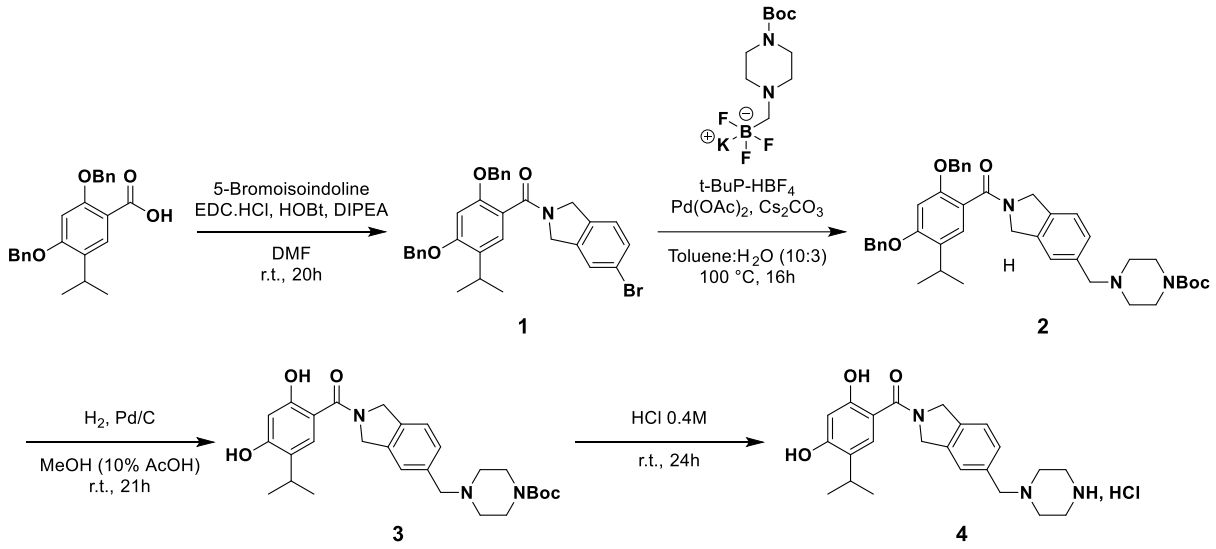
[b] K_d = dissociation constant

[c] IC_{50} = half maximal inhibitory concentration

[d] K_i = inhibitory constant

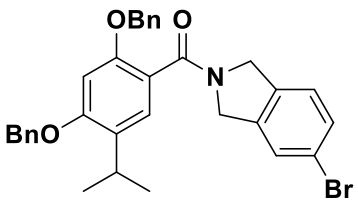
II-Chemistry

II-1. Synthesis of the onalespib precursor



Supplementary Fig. 2 Synthesis of precursor compound **4**.

(2,4-bis(benzyloxy)-5-isopropylphenyl)(5-bromoisoindolin-2-yl)methanone (1)



$C_{32}H_{30}BrNO_3$

MW: 556.50 g.mol^{-1}

Yield: 87%

Yellow solid

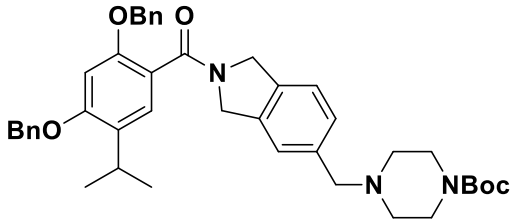
To a stirred solution of 2,4-Bis(benzyloxy)-5-isopropylbenzoic acid (463.4 mg, 1.23 mmol) in DMF (6.2 mL) was added HOBt (200.0 mg, 1.48 mmol) and EDC.HCl (283.2 mg, 1.48 mmol). After 30 min of

stirring at room temperature, 5-bromoisoindoline (268.2 mg, 1.35 mmol) and DIPEA (0.62 mL) were added. The solution was stirred at room temperature for 20 h. After adding water (12 mL) to quench the reaction, aqueous layer was extracted 3 times with EtOAc (3×85 mL). The combined organic layers were then dried over magnesium sulphate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using Heptane/EtOAc (8/2) as eluent to afford compound **1** as a yellow solid (592.8 mg, 87%).

^{1}H NMR (300 MHz, DMSO-d_6): δ 7.67 – 7.16 (m, 13H), 7.10 (s, 1H), 6.96 (s, 1H), 5.18 (s, 4H), 4.76 (d, J = 13.8 Hz, 2H), 4.51 (d, J = 16.3 Hz, 2H), 3.22 (p, J = 7.0 Hz, 1H), 1.15 (d, J = 6.9 Hz, 6H).

HPLC-MS: calculated for $C_{32}H_{30}BrNO_3$ $[M+H]^+$ 556.15; found 556.45.

***Tert*-butyl-4-((2-(2,4-bis(benzyloxy)-5-isopropylbenzoyl)isoindolin-5-yl)methyl)piperazine-1-carboxylate (2)**



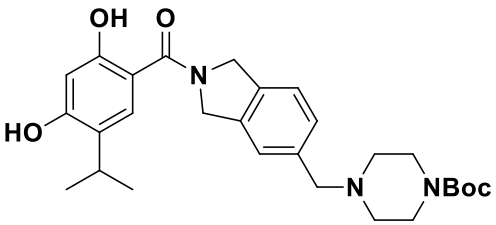
$C_{42}H_{49}N_3O_5$
MW: 675.87 g.mol^{-1}
Yield: 71%
Light yellow solid

A mixture of compound **1** (662.7 mg, 1.19 mmol), (4-(tert-butoxycarbonyl)piperazin-1-yl) potassium trifluoroborate (401.1 mg, 1.31 mmol), Cs_2CO_3 (1.16 g mg, 3.57 mmol) and tri-tert-butyl tetrafluoroborate phosphine (69.1 mg, 0.24 mmol) was prepared. After conducting the reaction under a nitrogen atmosphere, a toluene/water mixture (10:3, 34.1 mL) and $Pd(OAc)_2$ (26.8 mg, 0.12 mmol) were added. The resulting mixture was stirred at 100 °C for 16 h. After the reaction, the mixture was cooled to room temperature and filtered to remove the insoluble components. Then, water (55 mL) was added, and aqueous layer was extracted 3 times with EtOAc (3×130 mL). The combined organic layers were then dried over magnesium sulphate and concentrated under vacuum. The crude product was purified on silica gel column chromatography (gradient Heptane/EtOAc) to give the desired piperazine **2** as a light yellow solid (568.3 mg, 71%).

^{1}H NMR (300 MHz, DMSO-d_6): δ 7.53 – 7.12 (m, 13H), 7.10 (s, 1H), 6.96 (s, 1H), 5.19 (s, 4H), 4.77 (s, 2H), 4.50 (s, 2H), 3.46 (d, J = 11.5 Hz, 4H), 3.21 (d, J = 7.0 Hz, 1H), 2.37 – 2.19 (m, 4H), 1.38 (d, J = 3.8 Hz, 9H), 1.15 (d, J = 7.0 Hz, 6H).

HPLC-MS: calculated for $C_{42}H_{50}N_3O_5$ $[M+H]^+$ 676.38; found 676.79.

***Tert*-butyl-4-((2-(2,4-dihydroxy-5-isopropylbenzoyl)isoindolin-5-yl)methyl)piperazine-1-carboxylate (3)**



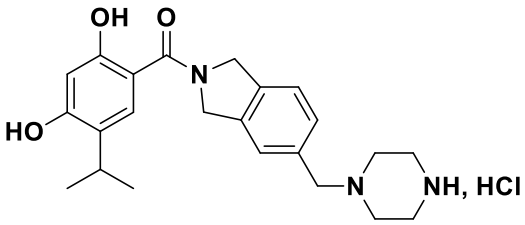
$C_{42}H_{49}N_3O_5$
MW: 675.87 g.mol^{-1}
Yield: 83%
White solid

To a solution of **2** (166.1 mg, 0.25 mmol) in MeOH (17 mL) containing 10% of AcOH was added Pd/C 10% (43.3 mg, 0.41 mmol). The reaction mixture was stirred for 6 h under hydrogen atmosphere. The reaction mixture was then filtered through a pad of Celite and rinsed with methanol. Solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography using DCM/MeOH (96/4) as eluent, to give compound **3** as a white solid (101.5 mg, 83%).

^{1}H NMR (300 MHz, DMSO-d$_6$): δ 10.06 (s, 1H), 9.60 (s, 1H), 7.27-7.19 (m, 3H), 7.04 (s, 1H), 6.39 (s, 1H), 4.76 (s, 4H), 3.46 (s, 2H), 3.29 (s, 4H), 3.09 (p, *J* = 6.9 Hz, 1H), 2.29 (t, *J* = 4.9 Hz, 4H), 1.38 (s, 9H), 1.15 (s, 3H), 1.12 (s, 3H).

HPLC-MS: calculated for $C_{28}H_{38}N_3O_5$ $[M+H]^+$ 496.28; found 496.43.

(2,4-dihydroxy-5-isopropylphenyl)(5-(piperazin-1-ylmethyl)isoindolin-2-yl)methanone hydrochloride (4)



$C_{23}H_{29}N_3O_3$
MW: 395.50 g.mol^{-1}
Yield: 70%
White solid

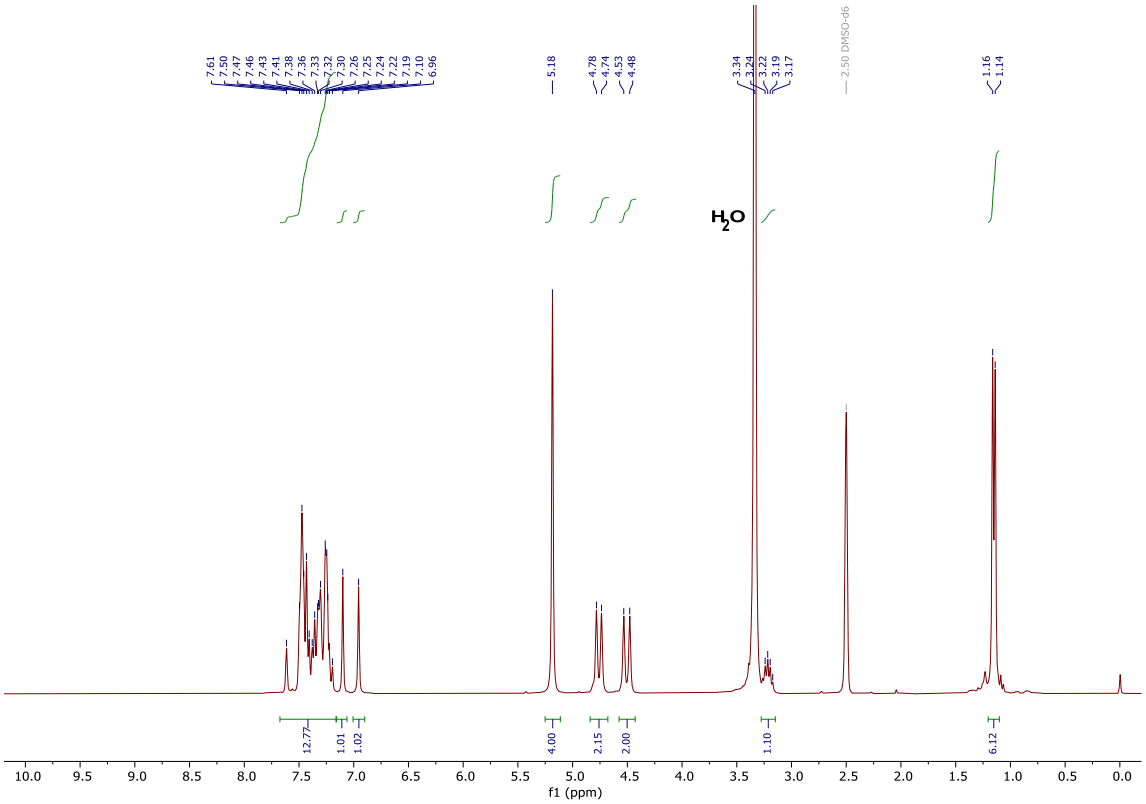
Methanone **3** (85.8 mg, 0.17 mmol) was dissolved in a solution of 0.5M HCl (1.5 mL, 0.75 mmol) in 1,4-dioxane (2 mL). The reaction mixture was stirred at room temperature for 24 h. The resulting precipitate was filtrated, washed with cold MeOH and dried under *vacuum* to afford hydrochloride salt precursor **4** as a white solid (52.2 mg, 70%).

^{1}H NMR (600 MHz, DMSO-d$_6$) δ 10.03 (s, 1H), 9.67 (s, 1H), 9.58 (s, 1H), 7.69 – 7.33 (m, 3H), 7.02 (s, 1H), 6.44 (s, 1H), 4.78 (d, *J* = 27.8 Hz, 4H), 4.39 (s, 1H), 3.56 (s, 4H), 3.48 – 3.31 (m, 4H), 3.18 (d, *J* = 18.9 Hz, 1H), 3.09 (p, *J* = 6.9 Hz, 1H), 1.13 (d, *J* = 6.8 Hz, 6H).

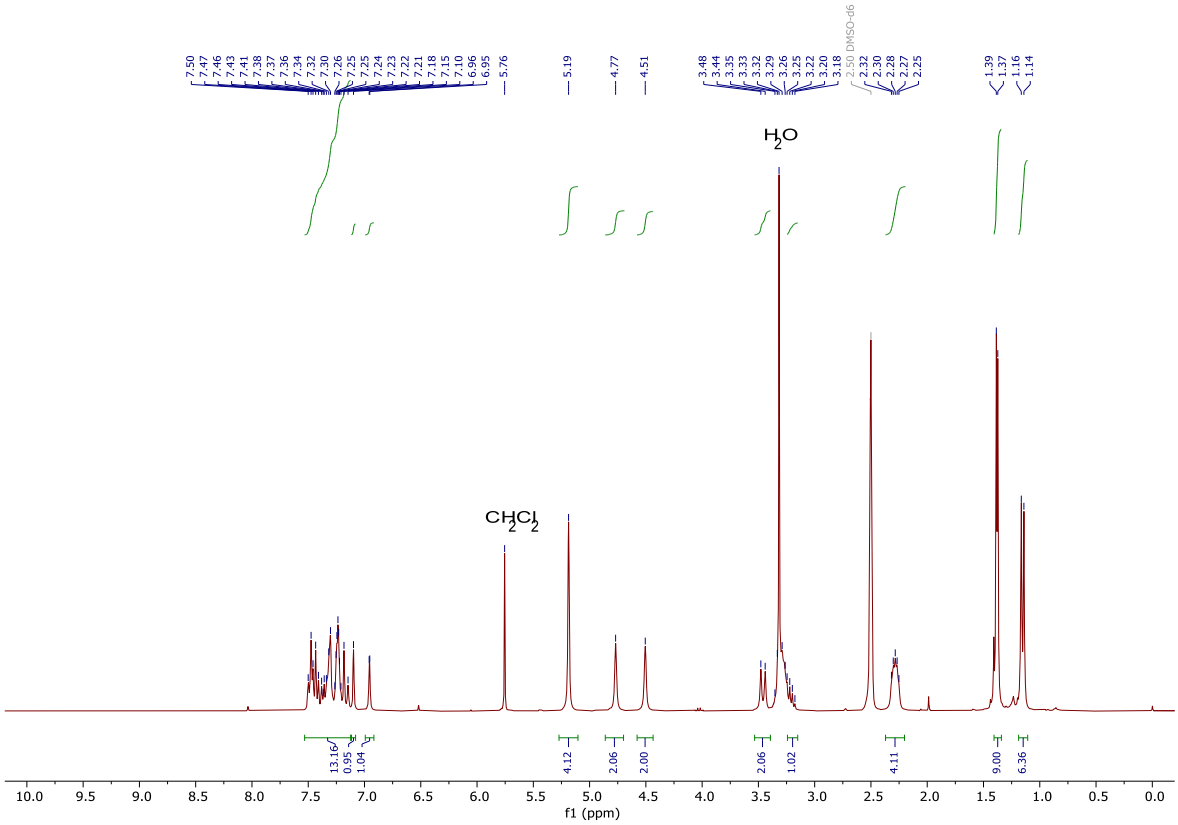
^{13}C NMR (150 MHz, DMSO-d$_6$) δ 168.76, 156.82, 153.54, 137.81, 136.83, 130.77, 128.01, 126.07, 125.61, 125.46, 123.30, 114.05, 102.50, 58.36, 52.77, 51.69, 47.27, 25.91, 22.69.

HRMS (ESI): calculated for $C_{23}H_{30}N_3O_3$ $[M+H]^+$ 396.2281; found 396.2277 (1.0 ppm).

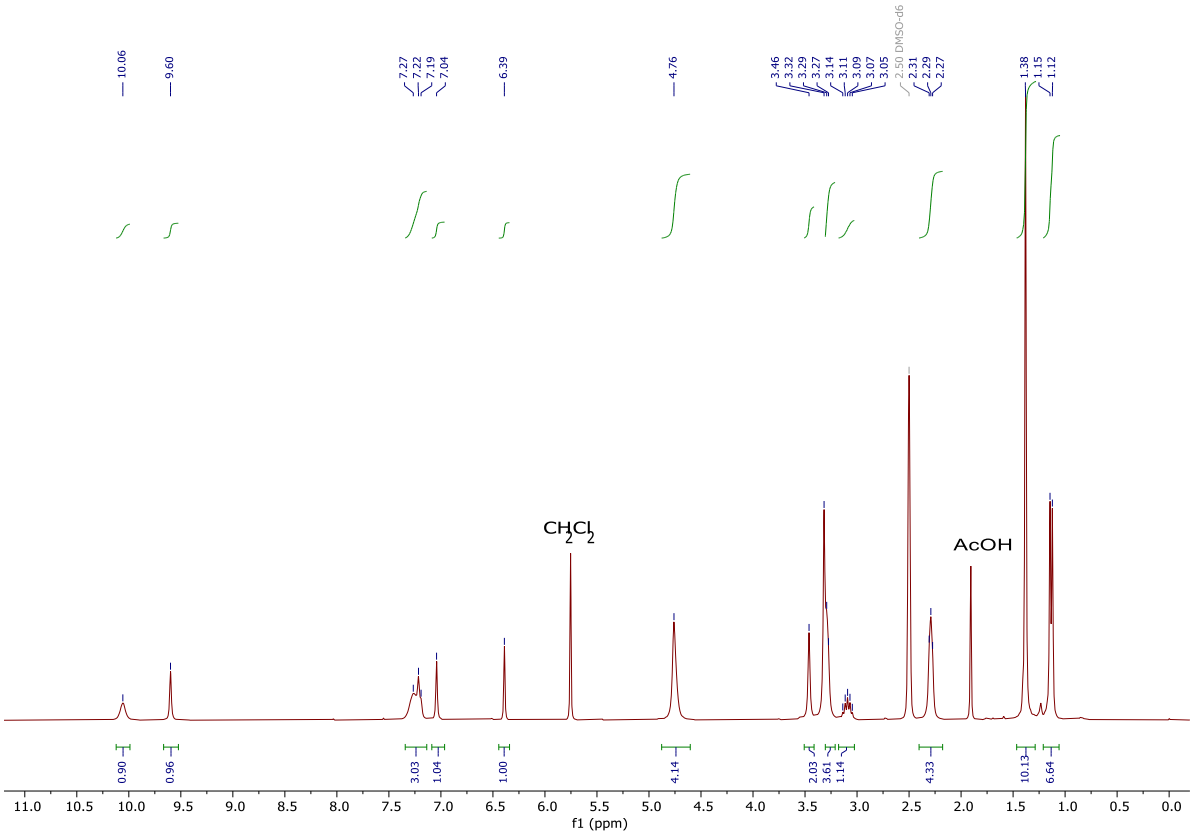
II-2. NMR spectra



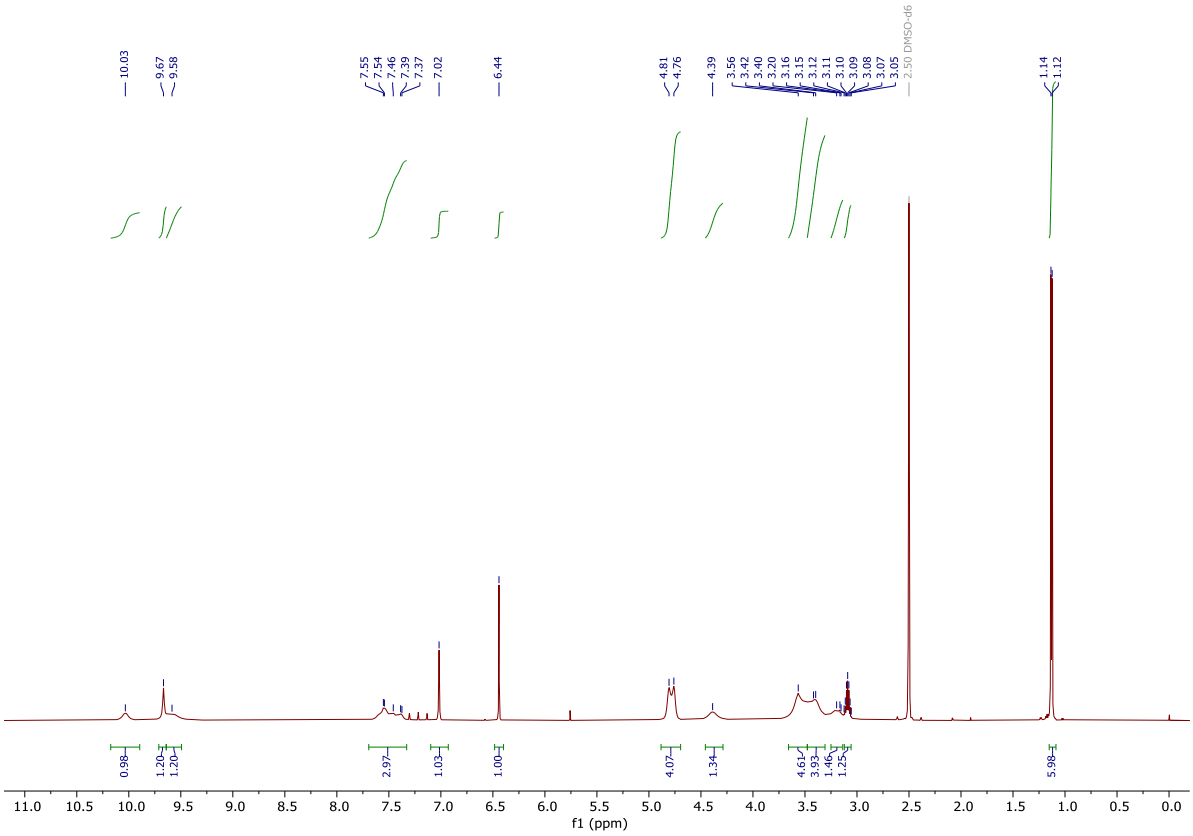
Supplementary Fig. 3 ^{1}H NMR spectrum of compound **1**.



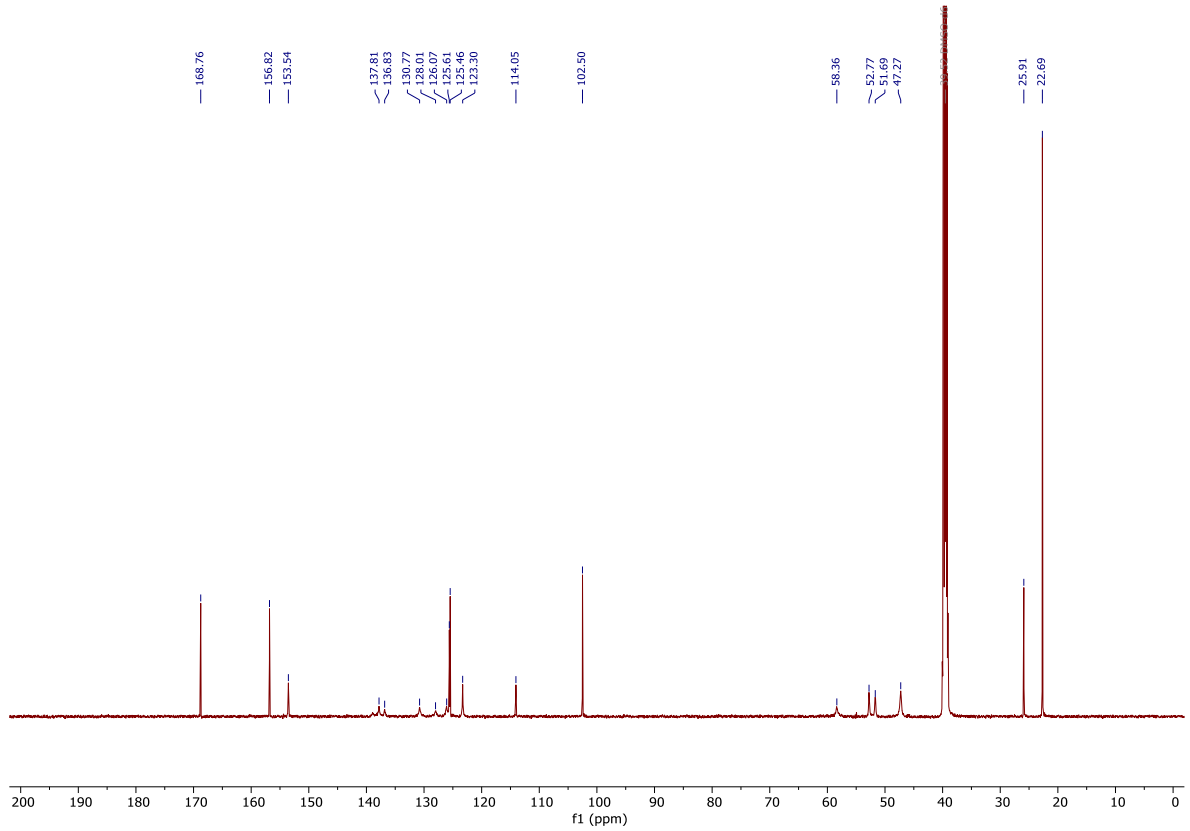
Supplementary Fig. 4 ^{1}H NMR spectrum of compound **2**.



Supplementary Fig. 5 ^{1}H NMR spectrum of compound **3**.



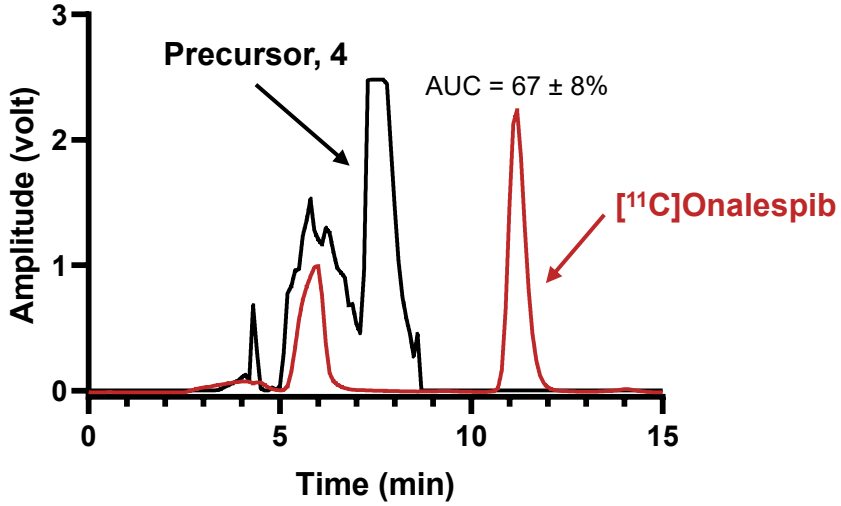
Supplementary Fig. 6 ^{1}H NMR spectrum of compound **4**.



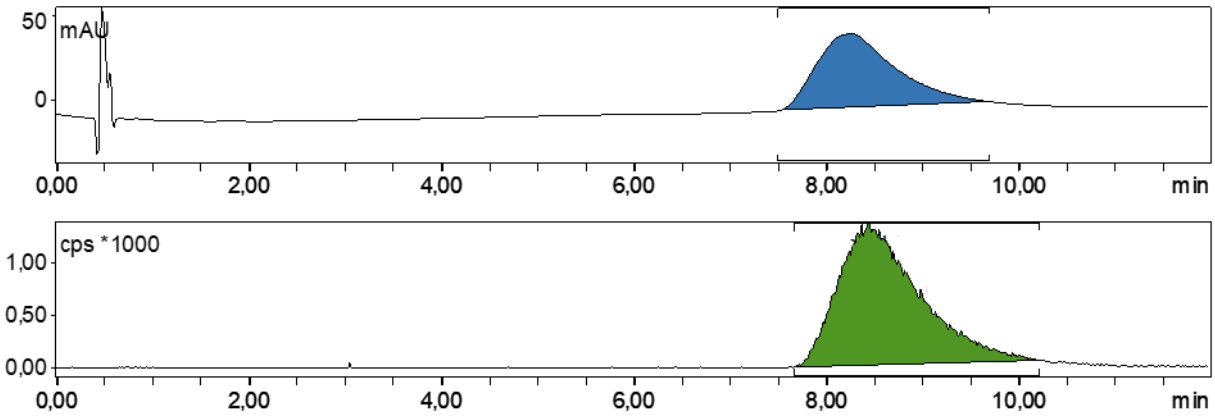
Supplementary Fig. 7 ^{13}C NMR spectrum of compound **4**.

III- Radiochemistry

III-1. Radiosynthesis



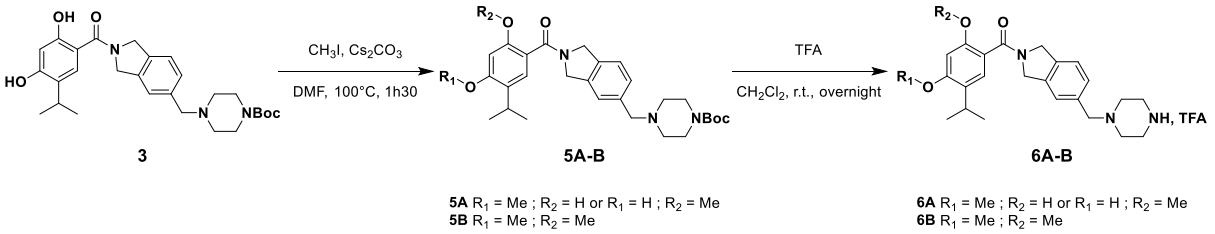
Supplementary Fig. 8 Preparative HPLC radiochromatogram of [^{11}C]Onalespib. Semi-preparative HPLC using an XBridge RP-C_{18} column (5 µm, 4.6 mm × 150 mm). Area under the curve (AUC) peak integration is indicated, as the radiochemical conversion relative to total recovered carbon-11 radioactivity on HPLC. Black: UV-trace (254 nm) ; Red: Radioactive signal.



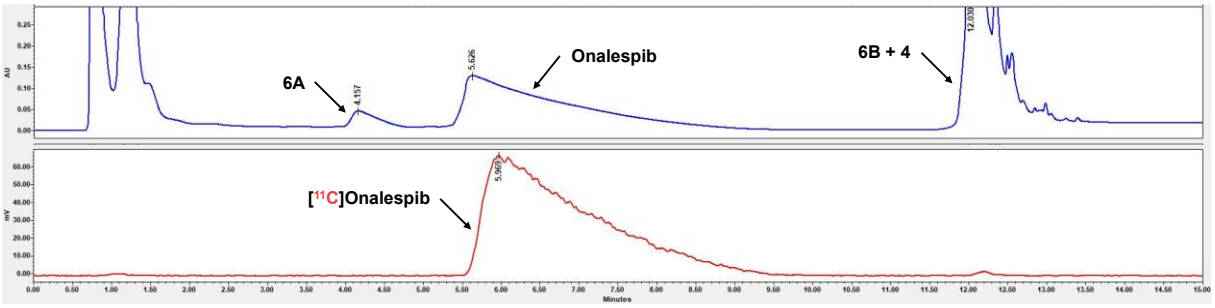
Supplementary Fig. 9 QC HPLC chromatogram of [^{11}C]Onalespib spiked with authentic reference compound onalespib. The upper channel represents the UV signal at 254 nm with indicated peak integration (blue), corresponding to the authentic reference compound. The lower channel represents the radioactive signal with indicated peak integration (green), corresponding to the carbon-11 labeled compound.

III-2. Confirmation of *N*-methylation

To a solution of resorcinol **3** (15.7 mg, 0.032 mmol) in dry DMF (1 mL), was added Cs_2CO_3 (17.2 mg, 0.053 mmol) and 16.4 µL (0.026 mmol) of a diluted CH_3I solution (prepared by mixing 75 µL of CH_3I with 675 µL of anhydrous DMF). The reaction mixture was stirred at 100 °C for 1h30. The crude mixture was dried under reduced pressure and treated with 2 mL of TFA in DCM (1:1) at room temperature.



Supplementary Fig. 10 Synthesis of *O*-methylated compound **6A-B**.



Supplementary Fig. 11 HPLC analysis – chromatograms. Injection of the crude reaction mixture (green); co-injection of crude reaction mixture with Onalespib at 254 nm (blue); injection of [^{11}C]Onalespib (red). Samples were run on an LC-MS system, described in the general information, with the flow rate set at 0.80 mL/min with the following gradient conditions: 0-10 min, 15% B; 10-11 min, 15 to 95% B; 11-15 min, 95% B. A = H_2O + 0.1% formic acid and B = CH_3CN + 0.1% formic acid. The column effluent was monitored using an in-line UV detector (254 nm) in series with a 3-inch NaI(Tl) scintillation detector connected to a single-channel analyser.

IV- Radiotracer evaluation

IV-1. *Ex vivo* biodistribution

Supplementary Table 2 *Ex vivo* [^{11}C]Onalespib biodistribution in MDA-MB-231 and U-87 MG tumour-bearing mice. Mice were sacrificed 30 min post tracer injection. Data are expressed as %ID/g[a] and SUV[b].

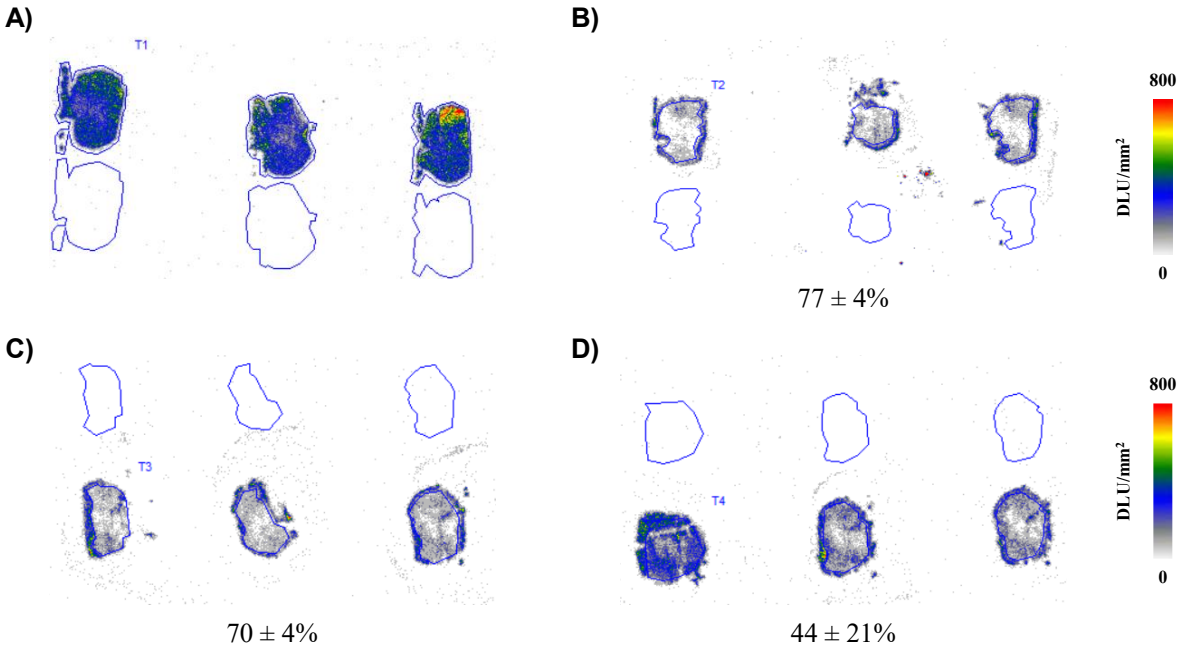
	%ID/g[a] 30 min *p.i*		SUV[b] 30 min *p.i*	
	MDA-MB-231	**U-87 MG**	**MDA-MB-231**	**U-87 MG**
Kidneys	23.4 ± 3.5	23.6 ± 1.9	4.2 ± 0.6	4.4 ± 0.3
Liver	11.7 ± 1.7	11.4 ± 0.4	2.1 ± 0.3	2.1 ± 0.1
Spleen	13.1 ± 2.0	10.3 ± 0.8	2.3 ± 0.4	1.9 ± 0.1
Pancreas	14.1 ± 2.0	10.5 ± 1.4	2.5 ± 0.4	2.0 ± 0.3
Lungs	37.8 ± 10.8	23.7 ± 2.2	6.8 ± 1.9	4.4 ± 0.4
Heart	5.3 ± 0.3	3.3 ± 0.3	1.0 ± 0.1	0.6 ± 0.1
Brain	0.5 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Blood	2.5 ± 0.3	3.2 ± 0.8	0.4 ± 0.1	0.6 ± 0.1
Bone	2.5 ± 0.6	3.0 ± 0.4	0.5 ± 0.1	0.6 ± 0.1
Muscle	3.0 ± 0.6	2.6 ± 0.8	0.5 ± 0.1	0.5 ± 0.1
Tumour	1.6 ± 0.3	4.9 ± 0.9	0.3 ± 0.1	0.9 ± 0.2

[a]Percentage of injected dose per gram (%ID/g) is calculated as (CPM in organ/total CPM recovered) × 100

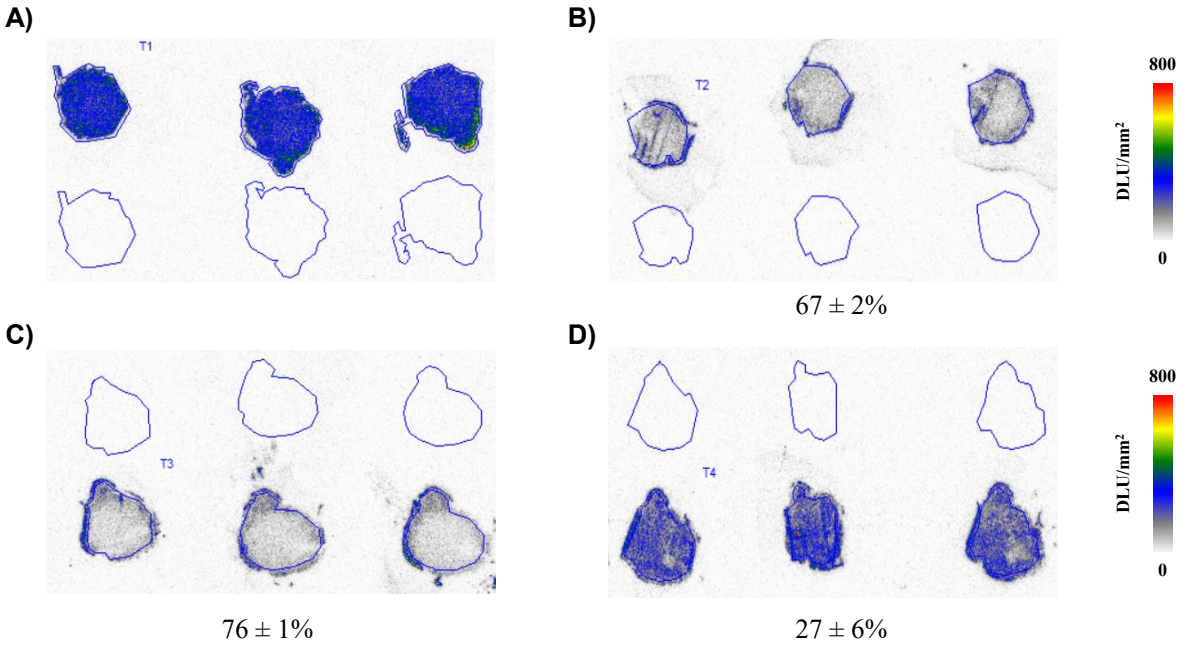
[b]SUV calculated as (radioactivity in CPM in organ/weight of organ in grams) / (total CPM recovered/body weight). Data expressed as mean ± SD; n = 4.

IV-2. *In vitro* autoradiography

Supplementary Fig. 12 *In vitro* [^{11}C]Onalespib autoradiography images of MDA-MB-231 tumour sections without artefacts. Tissue slices were pre-incubated with **A)** vehicle, **B)** onalespib, **C)** HSP990, and **D)** pimitespib. Signal intensity is depicted as DLU/mm^2. Blocking% was calculated as (1-((average DLU/mm^2 in tissue section in the presence of blocking agent))/(average DLU/mm^2 in tissue section tracer only))*100 and presented as mean ± SD.



Supplementary Fig. 13 *In vitro* [^{11}C]Onalespib autoradiography images of U-87 MG tumour sections without artefacts. Tissue slices were pre-incubated with A) vehicle, B) onalespib, C) HSP990, and D) pimitespib. Signal intensity is depicted as DLU/mm^2. Blocking% was calculated as (1-(average DLU/mm^2 in tissue section in the presence of blocking agent))/(average DLU/mm^2 in tissue section tracer only)*100 and presented as mean ± SD.



V-References

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