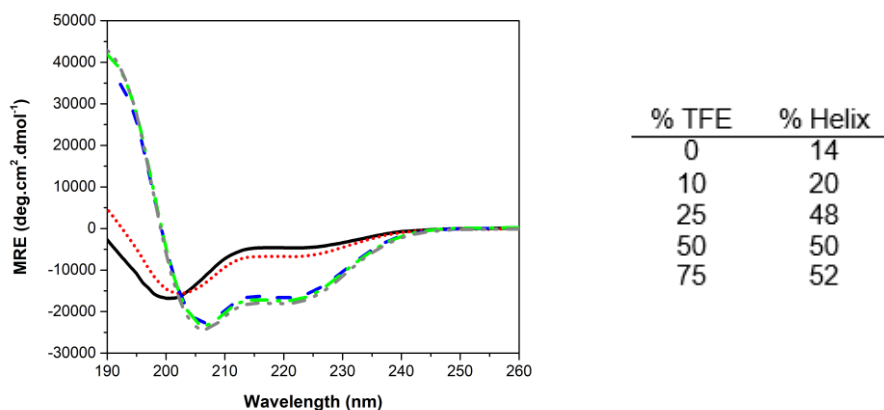


SUPPLEMENTARY INFORMATION

Figure S1

A



B

DrugScore PPI Analysis of CDK4

Residue	$\Delta\Delta G$ (Kcal/mol)	Residue	$\Delta\Delta G$ (Kcal/mol)	Residue	$\Delta\Delta G$ (Kcal/mol)
G ₄₈	≤ 0	R ₅₅	0.59	R ₆₂	0.34
L ₄₉	1.42	E ₅₆	≤ 0	L ₆₃	≤ 0
P ₅₀	≤ 0	V ₅₇	0.65	E ₆₄	0.22
I ₅₁	1.07	A ₅₈	≤ 0	A ₆₅	≤ 0
S ₅₂	≤ 0	L ₅₉	0.21	F ₆₆	0.23
T ₅₃	0.11	L ₆₀	≤ 0	E ₆₇	0.24
V ₅₄	1.04	R ₆₁	1.04		

Computational alanine scanning (Drugscore PPI) of the C-helix of CDK4 (G₄₈L₄₉P₅₀I₅₁S₅₂T₅₃V₅₄R₅₅E₅₆V₅₇A₅₈L₅₉L₆₀R₆₁R₆₂L₆₃E₆₄A₆₅F₆₆E₆₇) in interaction with cyclin D1 (PDB: 2W9Z). Free energy binding differences ($\Delta\Delta G$) for wildtype residue-to-Ala mutations. Positive free energy binding differences indicate a potential hot spot residue. In red $\Delta\Delta G > 0.5$ Kcal/mol, in green $\Delta\Delta G \leq 0$.

FIGURE S1 –

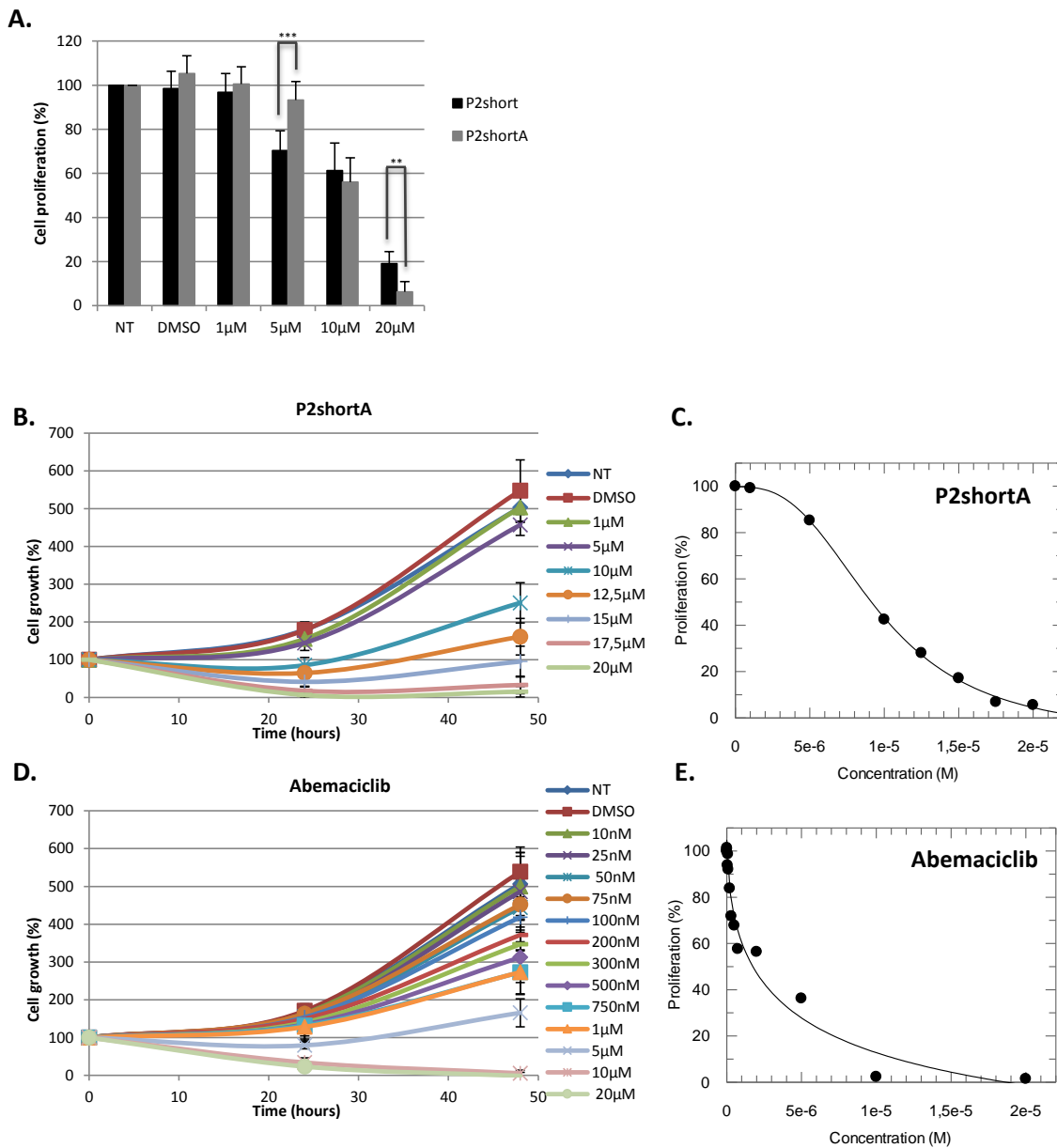
(A) Tendency of the peptide derived from CDK4 C helix to adopt a helical conformation

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100 μ M CDK4-Chelix peptide in NaP 50 mM, pH 7.0, 20°C displays a random conformation, whilst TFE induces helicity, with a maximum achieved with 25% TFE, indicating its propensity to form a helix in the appropriate environment. Overlay of far-UV CD spectra of CDK4-Chelix peptide short alone (black line) and in presence of 2,2,2-Trifluoroethanol (TFE), 10 % TFE (red dots), 25 % (blue dashed line), 50 % (green dashed-dotted line) and 75 % (grey dashed-dot-dot).

(B) Computational alanine scanning (Drugscore PPI) of the C-helix of CDK4 (G₄₈L₄₉P₅₀I₅₁S₅₂T₅₃V₅₄R₅₅E₅₆V₅₇A₅₈L₅₉L₆₀R₆₁R₆₂L₆₃E₆₄A₆₅F₆₆E₆₇) in interaction with cyclin D1 (PDB: 2W9Z). Free energy binding differences ($\Delta\Delta G$) for wildtype residue-to-Ala mutations. Positive free energy binding differences indicate a potential hot spot residue. In red $\Delta\Delta G > 0.5$ Kcal/mol, in green $\Delta\Delta G \leq 0$.

Figure S2



FIGURE

S2– Proliferation assays and determination of IC50s

Proliferation assays were performed by crystal violet staining every 24 hours.

(A) A549 cells were exposed to different concentrations of P2short and P2shortA peptides during 48 hours. Results are presented as average of nine independent experiments (n=4/experiment) ± SD. Means were compared with t test, * p<0,01, ** p<0,001, *** p<0,0001.

(B-G) A549 cells were treated with different concentrations of P2shortA (B,C) or Abemaciclib (D,E) for 48 hours. Curves represented means of at least two independent experiments (n=4/experiment) ± SD. IC₅₀ were determined with Grafit software.

Figure S3

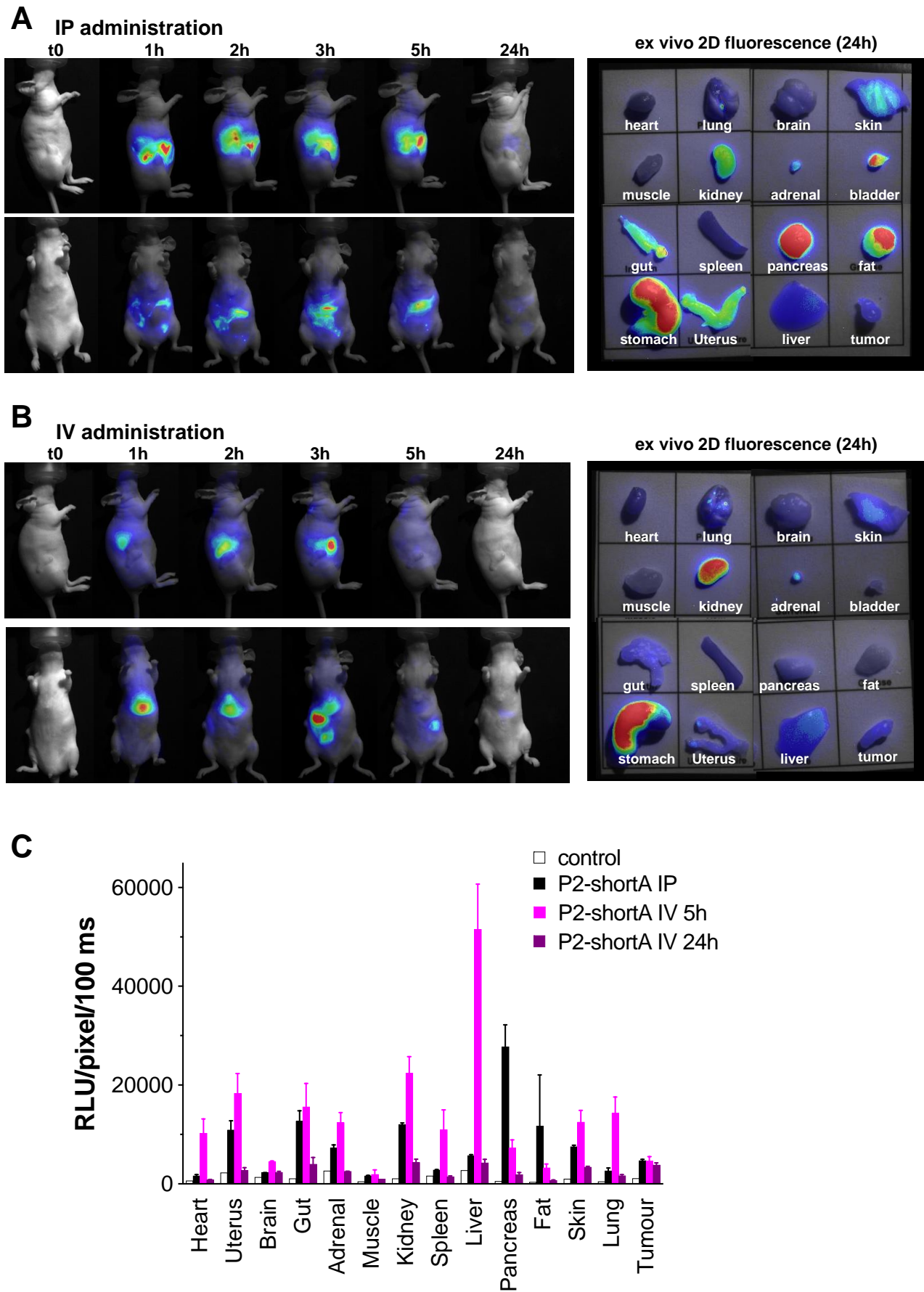


FIGURE S3 – Cy5.5-P2shortA circulates a few hours in the bloodstream after intravenous administration, but not after intraperitoneal administration, in mice with NSCLC subcutaneous tumour. 8 $\mu\text{mol.L}^{-1}$ Cy5.5-P2shortA were administered intraperitoneally (IP) or intravenously (IV) in mice with subcutaneous NSCLC A549 or H358 tumour. **(A)** Fluorescence images (20 ms integration time) were recorded at the indicated time after IP injection of Cy5.5-P2-short (min-max: side 4000-23093; frontal 4000-60775). Representative images performed on isolated organs 24h after IP are shown. **(B)** Fluorescence images (20 ms integration time) were recorded at the indicated time after IV injection of Cy5.5-P2-short (min-max: side 2121-40518; frontal 2121-37520). Representative images performed on isolated organs 24h after IV injection are shown. **(C)** Region of interest (ROI) are defined on extracted organs to semi-quantify the amount of photons detected per pixel after a 100 ms exposure. The results in each organ are expressed as the mean \pm SD in tumour-bearing mice (IP, $n \geq 2$; IV, $n=3$), or non-injected healthy mouse (control). IP injection showed biodistribution of Cy5.5-P2shortA mainly in the abdomen, whereas IV injection showed biodistribution of Cy5.5-P2shortA in several organs after 5 h and mainly in the kidneys after 24h.

Figure S4

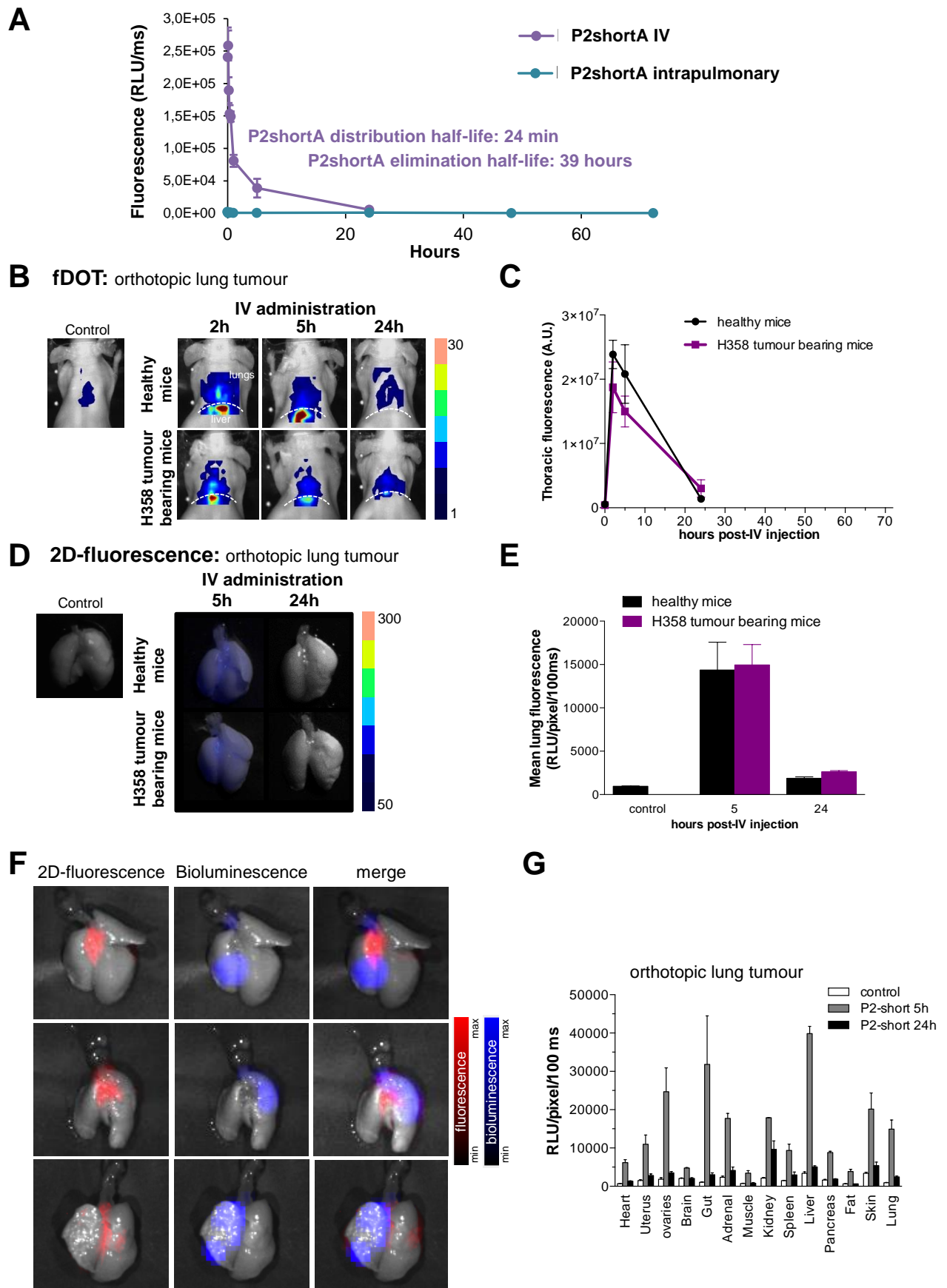


FIGURE S4 – Cy5.5-P2shortA circulates in the bloodstream after intravenous injection.

(A) Healthy mice were injected intravenously (200 μ L) or intrapulmonary (50 μ L) with 10 μ mol.L⁻¹ Cy5.5-P2shortA. The fluorescence intensity measurements of blood samples were performed over time. The results are expressed as the mean \pm SD (n=6). Intravenous injection showed Cy5.5-P2shortA blood distribution half-life of 24 min and Cy5.5-P2shortA blood elimination half-life of 39h, whereas intrapulmonary administration showed Cy5.5-P2shortA below sensitivity threshold.

(B-G) 10 μ mol.L⁻¹ Cy5.5-P2shortA were administered intravenously (200 μ L) in orthotopic H358 tumour-bearing mice. (B) 3D-fluorescence tomography imaging (fDOT) was performed at the indicated time after administration of Cy5.5-P2shortA. Dotted line: position of diaphragm. (C) Volumes of interest are defined on the thoracic region to semi-quantify the total amount of photons. The results are expressed as the mean \pm SEM in healthy (5h, n \geq 5; 24h, n \geq 3) or H358 tumour-bearing mice (5h, n \geq 6; 24h, n \geq 2). (D) Fluorescent images were performed on isolated lungs at the indicated time after Cy5.5-P2shortA injection. (E) Regions of interest (ROIs) are defined on the extracted lungs to semi-quantify the amount of photons detected per pixel after a 100 ms exposure. The results are expressed as the mean \pm SEM in healthy (n=4), or H358 tumour-bearing lungs (n \geq 3), or non-injected healthy mouse (control). (F) Bioluminescence, and 2D-fluorescence imaging were performed on isolated lungs 5h after intravenous injection of Cy5.5-P2shortA. Bioluminescent signal showed H358-Luc tumour cells in lungs (in blue); 2D-fluorescent signal showed Cy5.5-P2shortA location in the lungs (in red); fluorescent and bioluminescent signals were merge. No colocalization between Cy5.5-P2shortA signal and H358 tumours was observed in lungs after intravenous injection of P2-short. (G) ROIs are defined on the organs to semi-quantify the amount of photons detected per pixel after a 100 ms exposure. The results are expressed as the mean \pm SEM in healthy (n=4), or H358 tumour-bearing lungs (n \geq 3), or non-injected healthy mouse (control).

Figure S5

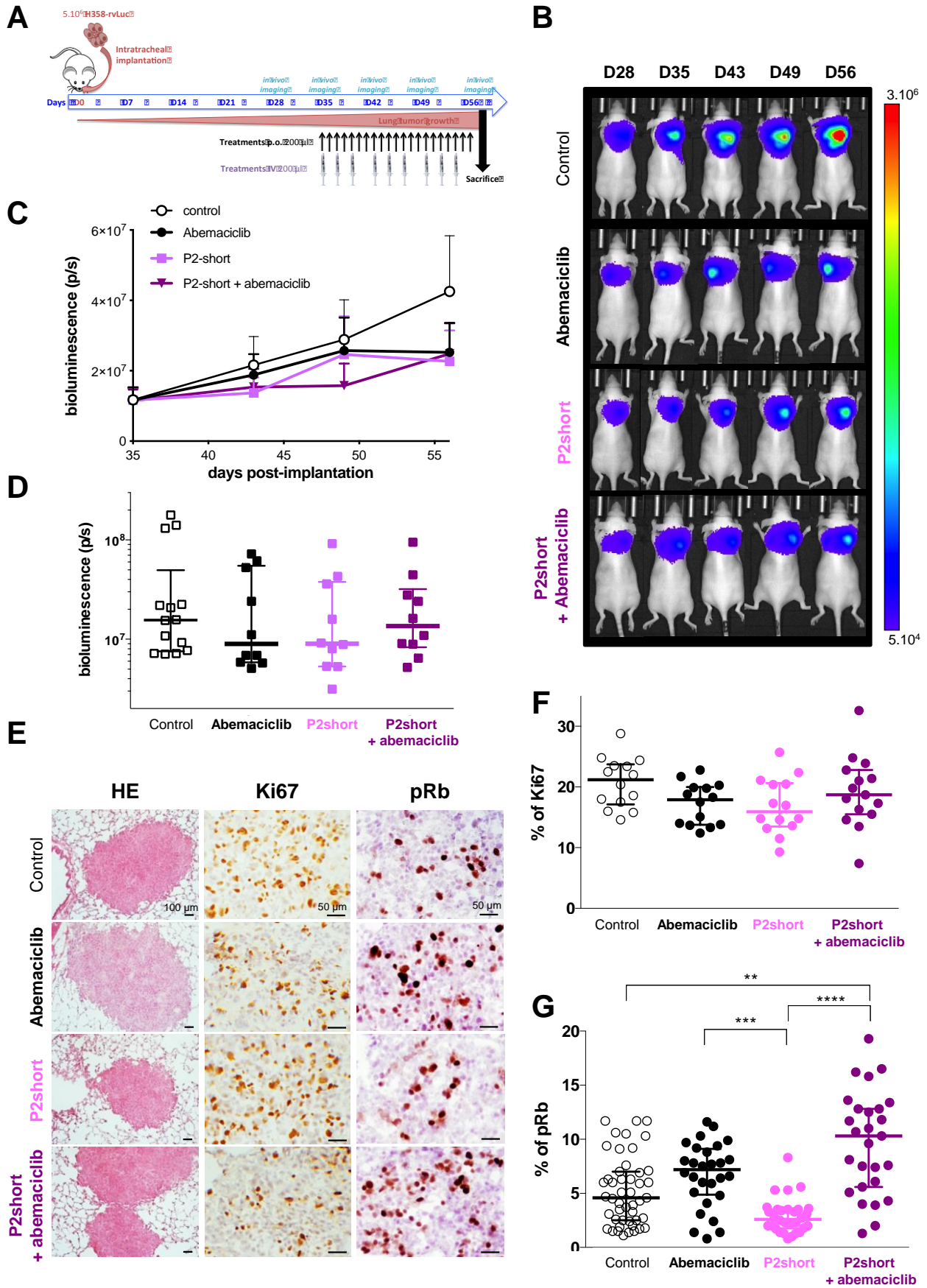


FIGURE S5 – Effect of P2shortA and Abemaciclib in orthotopic H358 tumour-bearing mice following intravenous injection

(A) Schematic representation of H358 orthotopic tumours growth and of the treatments plan. The mice were inoculated with H358-rvLuc cells and randomized after 5 weeks into 3 groups of 10 mice and one control group of 14 mice. Vehicle or Abemaciclib 10 mg.kg⁻¹ were administered *per os* every days for 3 weeks. 0.9 mg.kg⁻¹ P2shortA was injected intravenously 3 times a week for 3 weeks. (B) Overtime thoracic bioluminescence images of H358-Luc tumours. One representative mouse (dorsal view) per group is shown. (C) Treatments with abemaciclib and/or P2shortA trend to inhibit the growth of orthotopic H358 tumours. The results are expressed as the mean \pm SEM (control group, n=14; treated groups, n=10). (D) Thoracic bioluminescence level in each mouse at day 56. Bars, medians with interquartiles. (E) Histological (HE) and immunohistochemistry for Ki67 and phosphorylated-Rb (pRb) on lung tumour sections. Scale bars, 100 μ m (HE) and 50 μ m (Ki67 and pRb). (F) Ki67-positive cells were quantified on different fields (2-8) in three or four mice per group, and reported as % Ki67 positive cells. Bars, medians with interquartiles. (G) pRb-positive cells were quantified on different fields (2-29) in three or four mice per group, and reported as % pRb positive cells. Bars, medians with interquartiles. Kruskal-Wallis test with Dunn's multiple comparisons *posthoc* tests ($p < 0.0001$); **, treatment combination group compared to control group; ***, abemaciclib group compared to P2shortA group; ****, treatment combination group compared to P2shortA group.

Figure S6

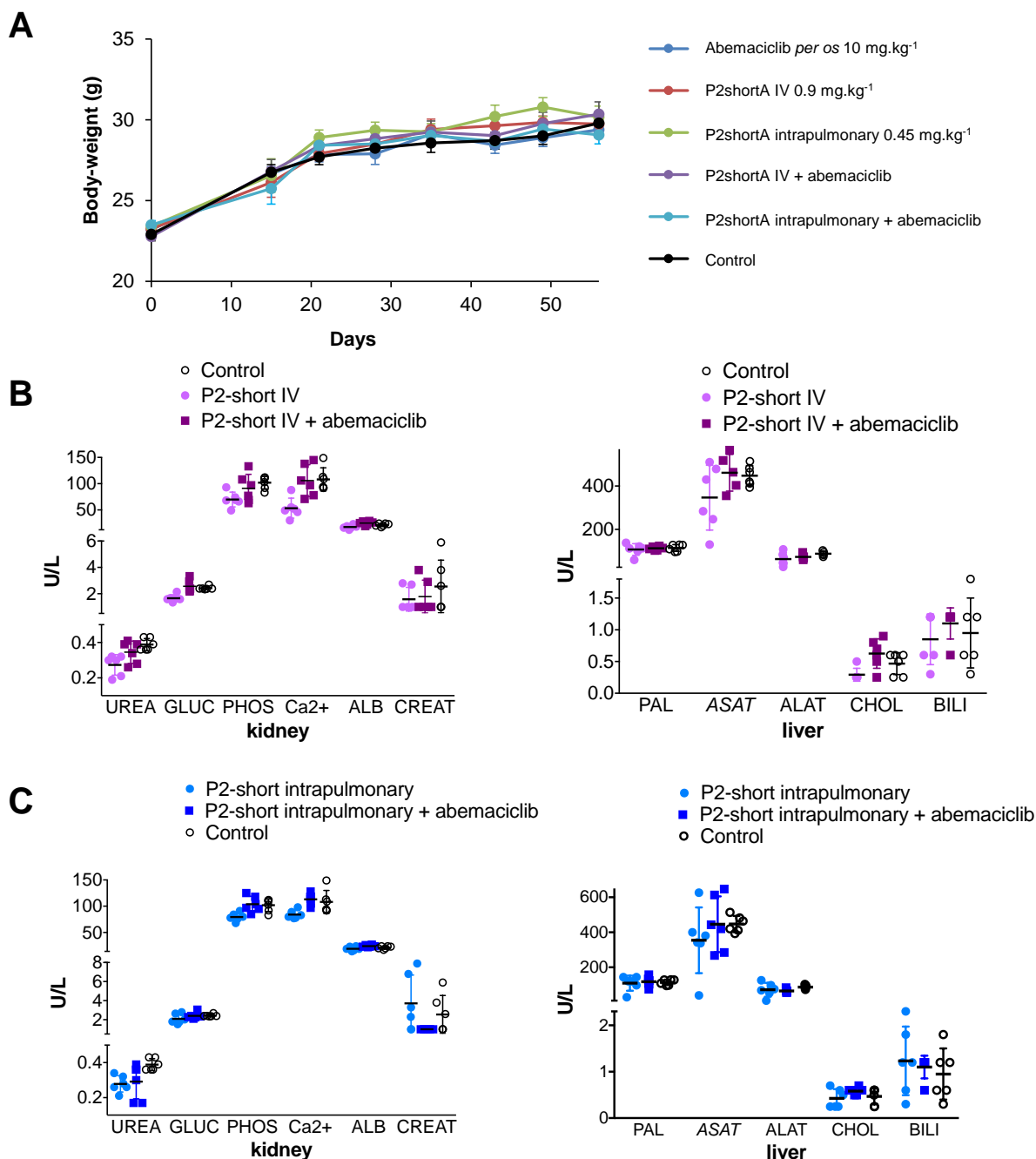


FIGURE S6 – P2shortA and Abemaciclib treatments were well-tolerated in mice with orthotopic H358 lung tumour.

(A) Mice body-weight in each group from the tumour implantation to sacrifice at day 56. Treatments began at day 35 (means \pm SD; control, n=14; treated groups, n=10). (B-C) Plasma biochemical values of mice after intravenous (B) or intrapulmonary (C) administration of P2shortA were assessed. Data represent the means \pm SD of 6 mice per group. UREA: blood urea nitrogen; GLUC, glucose; PHOS, phosphorus; Ca²⁺, calcium; ALB, albumin; CREAT, creatinine; PAL, alkaline phosphatase; ASAT, aspartate amino-transferase; ALAT, alanine amino-transferase; CHOL, cholesterol, BILI, bilirubin.

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Table S1. Clinical and biological characteristics of patients according to the histological subtype.

	All patients n=215 n (%)	ADC n=111 n (%)	SCC n=104 n (%)	p value
Gender				
Male	161 (75%)	71 (64%)	90 (88%)	0.0001
Female	54 (25%)	40 (36%)	14 (12%)	
Age, Median (range)	61.2 (47-70)	64 (40-82)	67.5 (41-85)	
Smoking status				
Current smokers	102 (48%)	56 (50%)	46 (44%)	0.0069
Former smokers	95 (44%)	40 (36%)	55 (53%)	
Passive smokers	2 (1%)	1 (1%)	1 (1%)	
Non -smokers	16 (7%)	14 (13%)	2 (2%)	
Professional exposures	50 (23%)	25 (23%)	25 (24%)	
Stage	n=211	n=108	n=103	0.02
I	79 (38%)	42 (39%)	37 (36%)	
II	57 (27%)	24 (22%)	33 (32%)	
III	60 (28%)	29 (27%)	31 (30%)	
IV	15 (7%)	13 (12%)	2 (2%)	
EGFR mutation*	n=112	n=109	n=3	1.00
No mutation	100 (89%)	97 (89%)	3 (100%)	
Mutation (exons 18-21)	12 (11%) [#]	12 (11%)	0	
KRAS mutation*	n=112	n=108	n=4	0.30
No mutation	73 (65%)	69 (64%)	4 (100%)	
Mutation (exon 2)	39 (35%)	39 (36%)	0	
HER2 mutation*	n=86	n=85	n=1	1.00
No mutation	83 (97%)	82 (96%)	1 (100%)	
Mutation	3 (3%)	3 (4%)	0	
PI3KCA mutation*	n=30	n=30	n=0	
No mutation	30 (100%)	30 (100%)		
Mutation	0	0		
BRAF mutation*	n=89	n=86	n=3	0.13
No mutation	85 (96%)	83 (97%)	2 (67%)	
Mutation	4 (4%)	3 (3%)	1 (33%)	
FGFR1 amplification**	n=5	n=0	n=5	
No amplification	5 (100%)	0	5 (100%)	
Amplification	0	0	0	
ALK rearrangement**	n=66	n=64	n=2	1.00
Negative	66 (100%)	64 (100%)	2 (100%)	
Positive	0	0	0	
ROS1 rearrangement**	n=30	n=27	n=3	1.00
Negative	29 (97%)	26 (96%)	3 (100%)	
Positive	1 (3%)	1 (4%)	0	
CMET amplification**	n=11	n=8	n=3	1.00
Negative	10 (91%)	7 (88%)	3 (100%)	
Positive	1 (9%)	1 (12%)	0	
Cyclin D1 expression	n=206	n=107	n=99	<0.0001
0-10%	87 (42%)	24 (22%)	63 (64%)	
>10%	119 (58%)	83 (78%)	36 (36%)	
CDK4 expression	n=215	n=111	n=104	0.67
0-10%	81 (38%)	40 (36%)	41 (39%)	
>10%	134 (62%)	71 (64%)	63 (61%)	
pRb expression	n=215	n=111	n=104	0.0003
0-10%	166 (77%)	97 (87%)	69 (66%)	
>10%	49 (23%)	14 (13%)	35 (34%)	

*Mutations were detected by next- generation sequencing (NGS). **Rearrangements and amplifications were detected by immunohistochemistry and/or fluorescent in situ hybridization (FISH). Statistical analysis was

Stapled Peptide targeting CDK4/Cyclin D

performed using Fisher's exact test. Missing data were excluded from the analysis. #One mutation of exon 18, five deletions of exon 19, one mutation of exon 20, and five substitution of exon 21 L858R.

Table S2: immunohistochemical and molecular characterization of NSCLC tumours

	Cyclin D1 expression				CDK4 expression				pRb expression			
	All patients n (%)	0-10% n=87 n (%)	>10% n=119 n (%)	p value	All patients n (%)	0-10% n=81 n (%)	>10% n=134 n (%)	p value	0-10% n=166 n (%)	>10% n=49 n (%)	p value	
Cyclin D1 expression	n=206				n=206			0.0001			0.9	
0-10%	87 (42%)				87 (42%)	47 (54%)	40 (46%)		66 (76%)	21 (34%)		
>10%	119 (58%)				119 (58%)	29 (24%)	90 (76%)		91 (76%)	28 (24%)		
CDK4 expression	n=206			0.0001	n=215						0.0014	
0-10%	76 (37%)	47 (54%)	29 (24%)		81 (38%)				72 (89%)	9 (11%)		
>10%	130 (63%)	40 (46%)	90 (76%)		134 (62%)				94 (70%)	40 (30%)		
pRb expression	n=206			0.9	n=215			0.0014				
0-10%	157 (76%)	66 (76%)	91 (76%)		166 (77%)	72 (89%)	94 (70%)					
>10%	49 (24%)	21 (34%)	28 (24%)		49 (23%)	9 (11%)	40 (30%)					
EGFR	n=108	n=26	n=82	1.00	n=112	n=41	n=71	0.53	n=96	n=16	0.20	
No mutation	97 (90%)	24 (72%)	73 (89%)		100 (89%)	38 (93%)	62 (87%)		84 (88%)	16 (100%)		
Mutation ^a	11 (10%)	2 (8%)	9 (11%)		12 (11%)	3 (7%)	9 (13%)		12 (12%)	0		
KRAS	n=107	n=26	n=81	0.0178	n=112	n=41	n=71	0.53	n=97	n=15	0.57	
No mutation	69 (64%)	22 (85%)	47 (58%)		73 (65%)	25 (61%)	48 (68%)		62 (64%)	11 (73%)		
Mutation ^b	38 (36%)	4 (15%)	34 (42%)		39 (35%)	16 (39%)	23 (32%)		35 (36%)	4 (27%)		
HER2	n=81	n=19	n=62	1.00	n=83	n=30	n=53	1.00	n=73	n=10	0.32	
No mutation	78 (96%)	19 (100%)	59 (95%)		80 (96%)	29 (97%)	51 (96%)		71 (97%)	9 (90%)		
Mutation	3 (4%)	0	3 (5%)		3 (4%)	1 (3%)	2 (4%)		2 (3%)	1 (10%)		
PI3KCA	n=28	n=7	n=21		n=31	n=9	n=22		n=28	n=3		
No mutation	28 (100%)	7 (100%)	21 (100%)		31 (100%)	9 (100%)	22 (100%)		28 (100%)	3 (100%)		
Mutation	0	0	0		0	0	0		0	0		
BRAF	n=85	n=20	n=65	1.00	n=88	n=32	n=56	0.0154	n=77	n=11	1.00	
No mutation	83 (98%)	20 (100%)	63 (97%)		84 (95%)	28 (88%)	56 (100%)		73 (95%)	11 (100%)		
Mutation	2 (2%)	0	2 (3%)		4 (5%)	4 (12%)	0		4 (5%)	0		
FGFR1	n=5	n=2	n=3		n=5	n=1	n=4		n=4	n=1		
No amplification	5 (100%)	2 (100%)	3 (100%)		5 (100%)	1 (100%)	4 (100%)		4 (100%)	1 (100%)		
Amplification	0	0	0		0	0	0		0	0		
ALK ^c	n=62	n=10	n=52		n=66	n=18	n=48		n=61	n=5		
Negative	62 (100%)	10 (100%)	52 (100%)		66 (100%)	18 (100%)	48 (100%)		61 (100%)	5 (100%)		
Positive	0	0	0		0	0	0		0	0		
ROS1 ^c	n=29	n=2	n=27	1.00	n=30	n=7	n=23	1.00	n=29	n=1	1.00	
Negative	28 (97%)	2 (100%)	26 (96%)		29 (97%)	7 (100%)	22 (96%)		28 (97%)	1 (100%)		
Positive	1 (3%)	0	1 (4%)		1 (3%)	0	1 (4%)		1 (3%)	0		
CMET	n=10	n=1	n=9	1.00	n=11	n=3	n=8	1.00	n=11	n=0	1.00	
No amplification	9 (90%)	1 (100%)	8 (89%)		10 (91%)	3 (100%)	7 (88%)		10 (91%)	0		
Amplification	1 (10%)	0	1 (11%)		1 (9%)	0	1 (12%)		1 (9%)	0		

Missing data were excluded from the analysis. ^a exons 18-21; ^b exon 2; ^c rearrangement. Statistical analysis was performed using Fisher's exact test

