

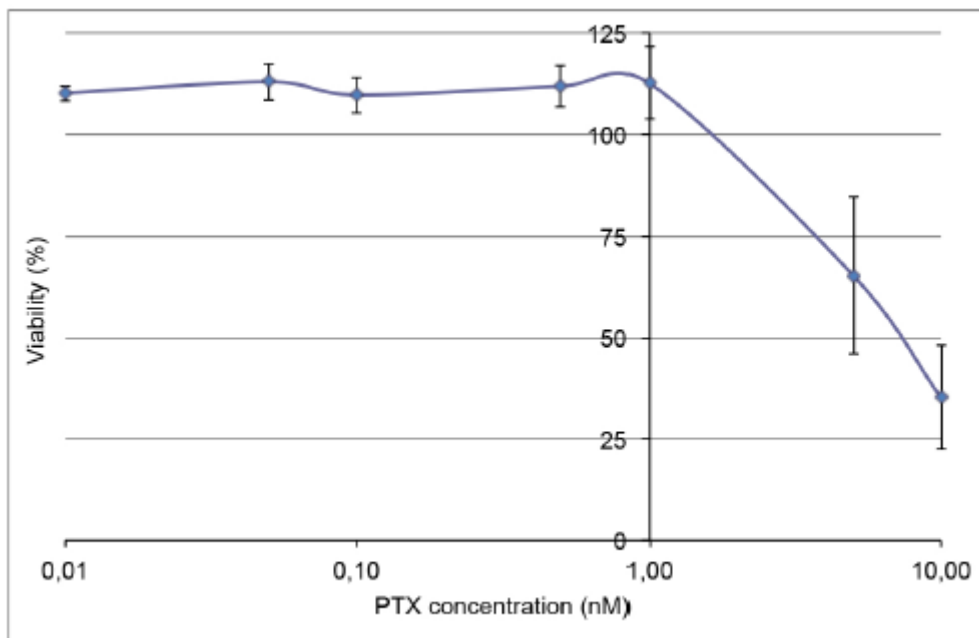
1 *Supplementary material*

2 **Two antagonistic microtubule targeting drugs act** 3 **synergistically to kill cancer cells**

4 **Lauralie Peronne¹, Eric Denarier², Ankit Rai³, Renaud Prudent¹, Audrey Vernet¹, Peggy Suzanne⁴,**
5 **Sacnité Ramirez-Rios¹, Sophie Michallet¹, Mélanie Guidetti⁵, Julien Vollaire⁵, Daniel Lucena-**
6 **Agell⁶, Anne-Sophie Ribba¹, Véronique Josserand⁵, Jean-Luc Coll⁵, Patrick Dallemagne⁴, J.**
7 **Fernando Díaz⁶, María Ángela Oliva⁶, Karin Sadoul¹, Anna Akhmanova³, Annie Andrieux² and**
8 **Laurence Lafanechère^{1*}**

9

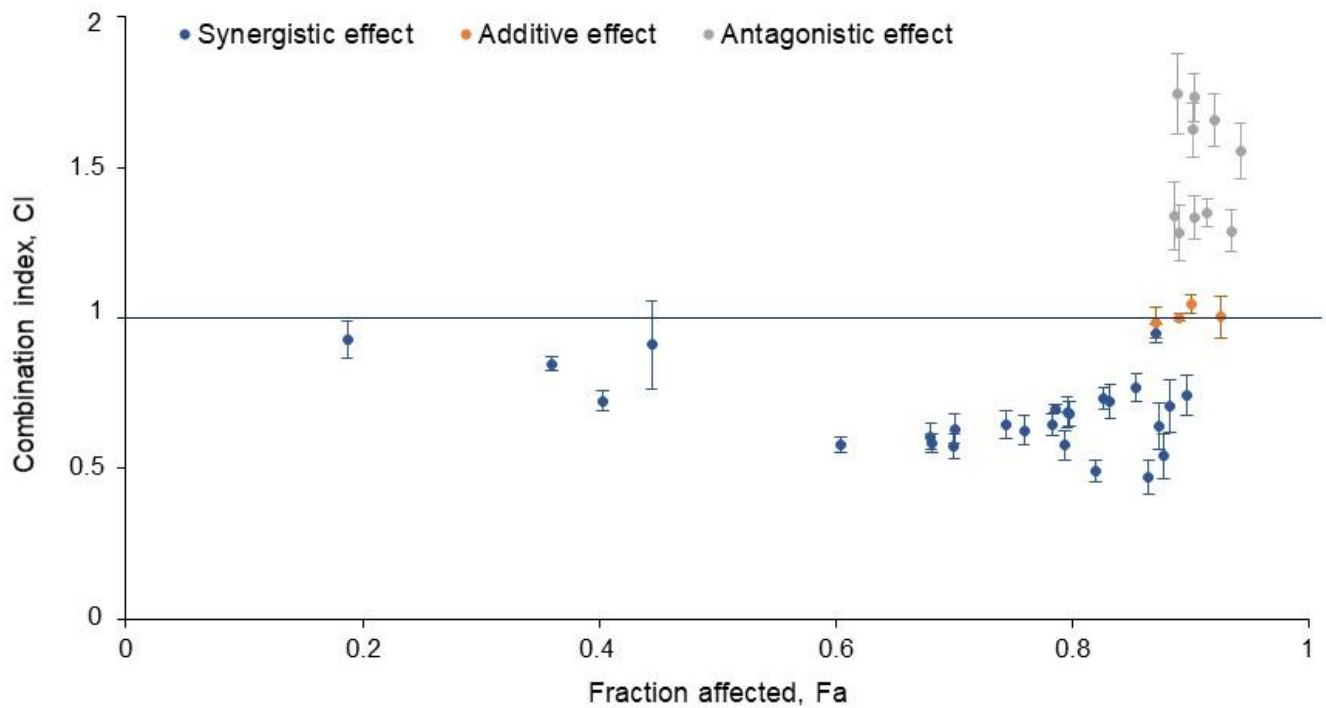
10



11

12 **Figure S1. Effect of PTX on the viability of HeLa cells.** Cells were incubated for 48 hours with
13 increasing concentrations of PTX. The percentage of viable cells was calculated following a MTT
14 assay. Data are presented as mean \pm SD of three independent experiments.

15



Synergistic effect

Carba1 (μM)	PTX (nM)	Fractional effect	CI
25	0,5	0.87	0.47 ± 0.06
25	0,25	0.82	0.49 ± 0.04
25	1	0.88	0.54 ± 0.07
12	1	0.70	0.57 ± 0.04
18	1	0.79	0.58 ± 0.05
3	1,5	0.60	0.58 ± 0.03
6	1,5	0.68	0.58 ± 0.03
18	0,5	0.68	0.61 ± 0.04
12	1,5	0.76	0.63 ± 0.05
3	2	0.70	0.63 ± 0.05
25	1,5	0.87	0.64 ± 0.08
6	2	0.74	0.65 ± 0.04
3	2,5	0.78	0.65 ± 0.03
18	1,5	0.80	0.68 ± 0.04
12	2	0.80	0.69 ± 0.05
6	2,5	0.79	0.70 ± 0.01
25	2	0.88	0.71 ± 0.09
18	2	0.83	0.72 ± 0.06
6	1	0.40	0.73 ± 0.03
12	2,5	0.83	0.73 ± 0.04
25	2,5	0.90	0.74 ± 0.07
18	2,5	0.86	0.77 ± 0.05
12	0,5	0.36	0.85 ± 0.02
18	0,25	0.45	0.91 ± 0.15
3	1	0.19	0.93 ± 0.06
3	5	0.87	0.95 ± 0.03

Additive effect

Carba1 (μM)	PTX (nM)	Fractional effect	CI
6	5	0.87	0.98 ± 0.05
12	5	0.89	1.00 ± 0.02
25	5	0.93	1.00 ± 0.07
18	5	0.90	1.05 ± 0.03

Antagonistic effect

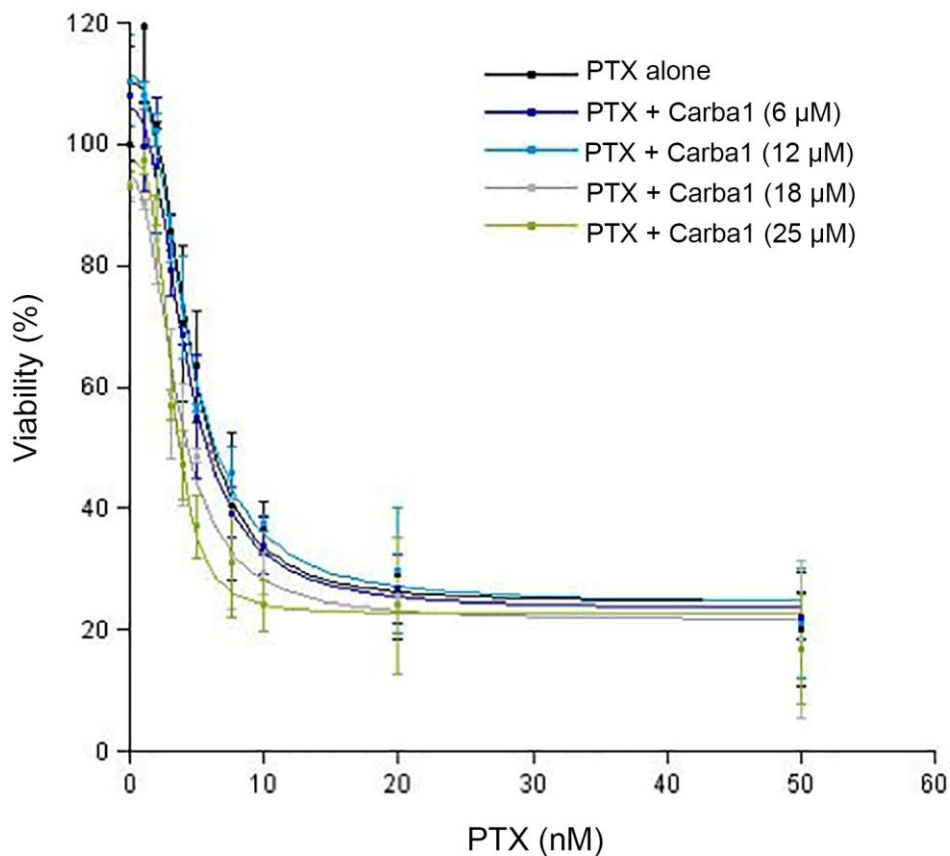
Carba1 (μM)	PTX (nM)	Fractional effect	CI
3	7,5	0.89	1.28 ± 0.09
25	7,5	0.93	1.29 ± 0.07
12	7,5	0.90	1.33 ± 0.07
6	7,5	0.89	1.34 ± 0.11
18	7,5	0.92	1.35 ± 0.05
25	10	0.94	1.56 ± 0.09
3	10	0.90	1.62 ± 0.09
18	10	0.92	1.66 ± 0.09
12	10	0.90	1.73 ± 0.08
6	10	0.89	1.74 ± 0.13

17 **Figure S2. Combination index (CI) values as a function of Fraction affected (Fa) in HeLa cells**
18 **exposed to combination drug treatment.** A) The Fa-CI plot. Cells were incubated for 72 hours with
19 the indicated combinations of Carba1/PTX. The percentage of viable cells was calculated following a
20 Prestoblue assay. The Chou-Talalay method of synergy determination was used, along with
21 CompuSyn software. Fa indicates the fractional inhibition for each combinational index (i.e. Fa = 0.5
22 corresponds to 50% of cell proliferation inhibition). The horizontal line at 1 indicates the line of
23 additive effect. CI values less than, equal to, or greater than 1 indicate synergy, additivity, or
24 antagonism, respectively. B) Combination Index for each combination. CI were ranked in ascending
25 order. The combination of Carba1 and PTX (12 μ M/1 nM) is indicated in blue. Data are presented as
26 mean \pm SEM of 3 independent experiments.

27

28

29



30

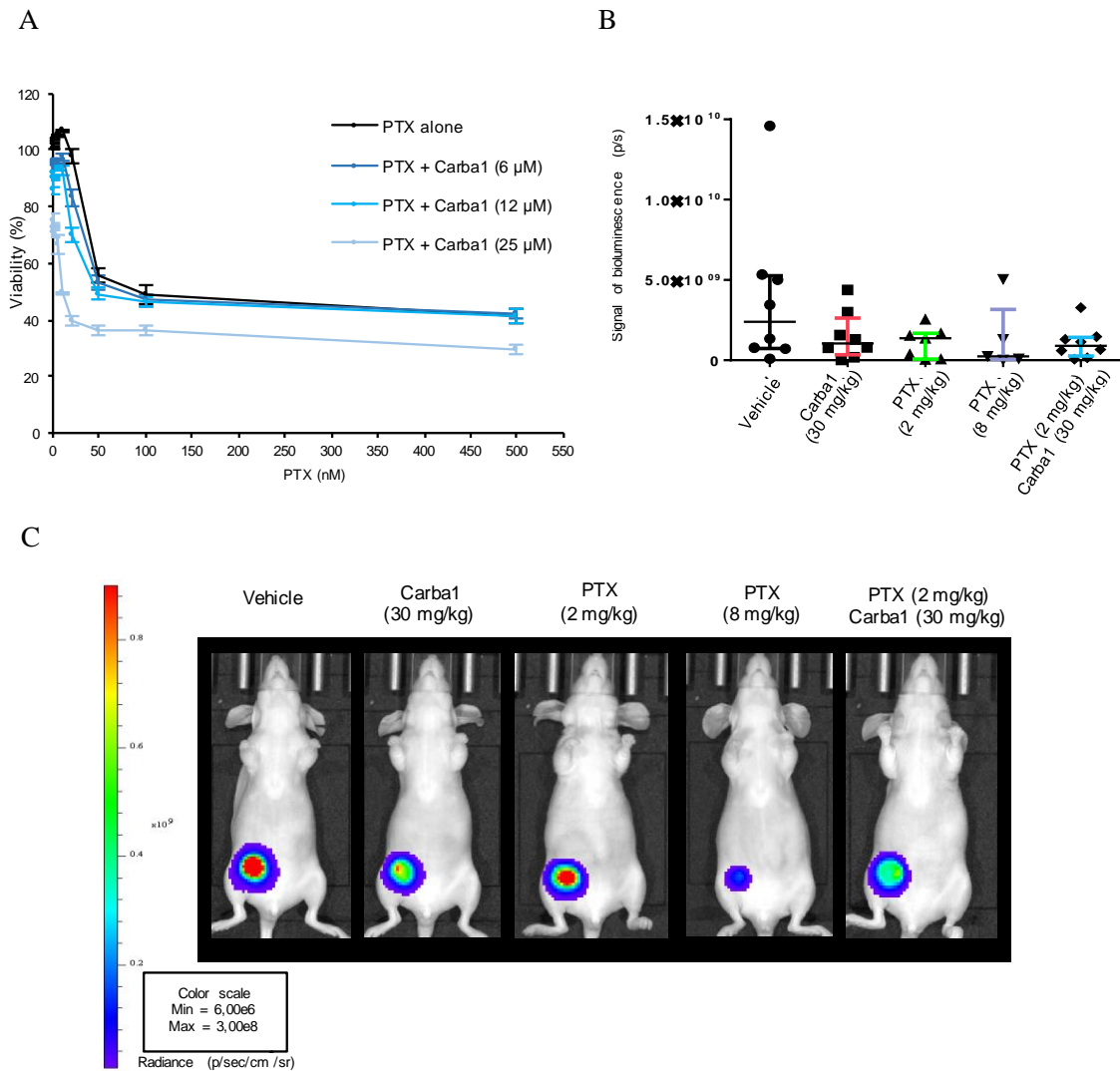
31 **Figure S3. Effect of Carba1/PTX combination on the viability of RPE-1 cells.** Cells were incubated
32 for 72 hours with the indicated combinations of Carba1/PTX. The percentage of viable cells was
33 calculated following a Prestoblue assay. Data are presented as mean \pm SEM of 3 independent
34 experiments. Note that the effect on cell viability of the different doses of Carba1 alone can be seen
35 at the x-coordinate = 0, corresponding to 0 nM PTX.

36

37

38

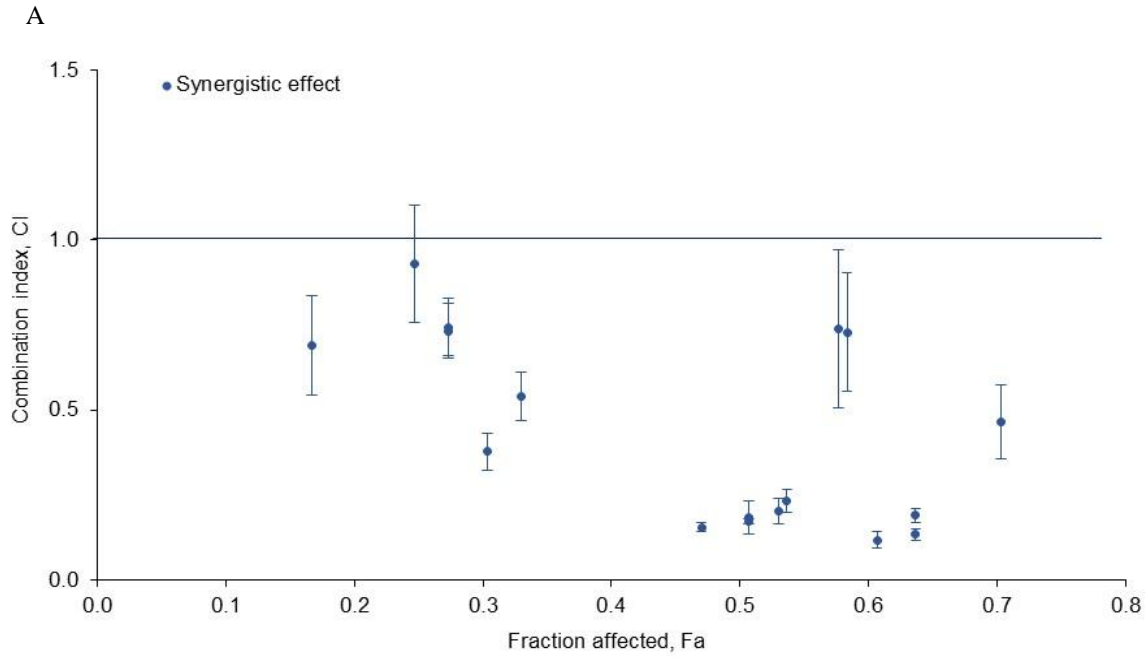
39



40

41 **Figure S4. Effect of Carba1/PTX combination on 4T1 cells *in vitro* and *in vivo*.** A) Effect of
42 Carba1/PTX combination on the viability of 4T1 cells. Cells were incubated for 72 hours with the
43 indicated combinations of Carba1/PTX. The percentage of viable cells was calculated following a
44 Prestoblue assay. Data are presented as mean ± SEM of 3 independent experiments. Note that the
45 effect on cell viability of the different doses of Carba1 alone can be seen at the x-coordinate = 0,
46 corresponding to 0 nM PTX, B) Bioluminescence signals from orthotopic 4T1rvluc2 mammary
47 tumors after 16 days of treatment by PTX, Carba1 and their combination. Error bars = SEM. n=8 for
48 vehicle, Carba1 alone, Carba1/PTX groups, n=7 for PTX (2 mg/kg) group and n=5 for PTX (8 mg/kg)
49 group, C) Bioluminescence images of orthotopic 4T1rvluc2 mammary tumor bearing mice after 16
50 days of treatment (one representative mouse per condition).

51



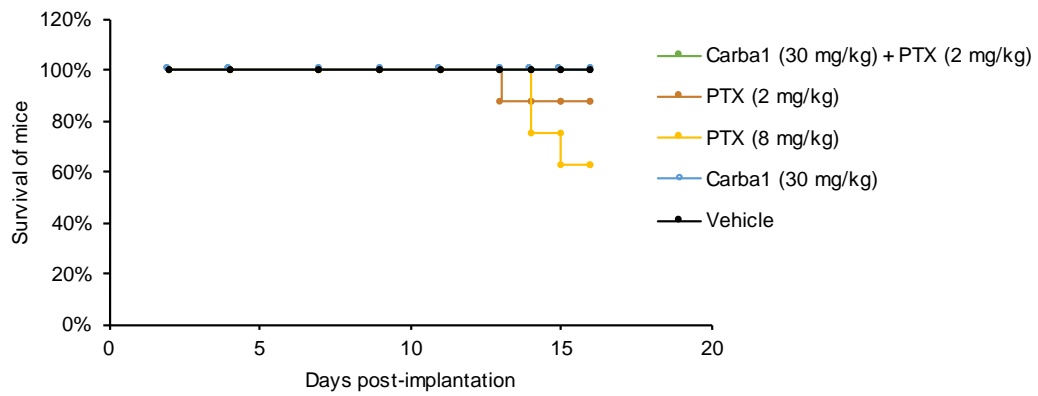
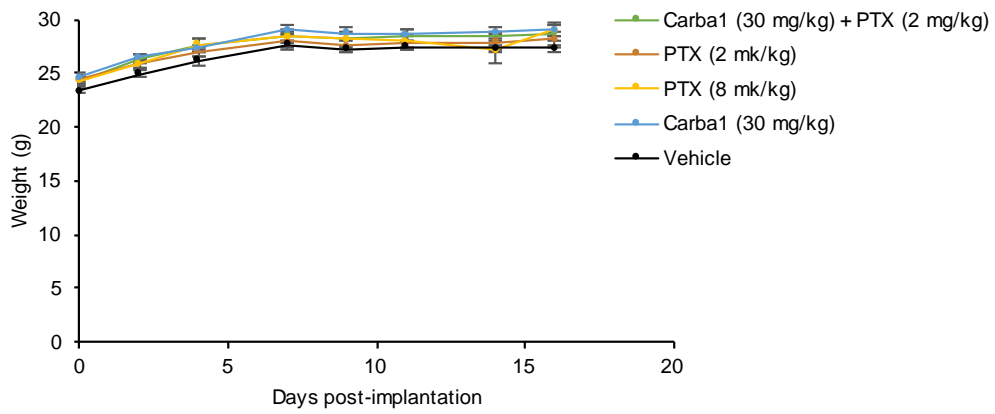
B

Synergistic effect			
Carba1 (μM)	PTX (nM)	Fractional effect	CI
25	20	0.61	0.12 ± 0.02
25	50	0.64	0.13 ± 0.02
6	50	0.47	0.16 ± 0.01
12	50	0.51	0.17 ± 0.01
25	10	0.51	0.18 ± 0.05
25	100	0.64	0.19 ± 0.02
6	100	0.53	0.20 ± 0.04
12	100	0.54	0.23 ± 0.03
12	20	0.30	0.38 ± 0.06
25	500	0.70	0.46 ± 0.11
25	5	0.33	0.54 ± 0.07
6	20	0.17	0.69 ± 0.15
12	500	0.58	0.73 ± 0.17
25	0.5	0.27	0.73 ± 0.08
6	500	0.58	0.74 ± 0.23
25	0.1	0.27	0.74 ± 0.08
25	1	0.25	0.93 ± 0.17

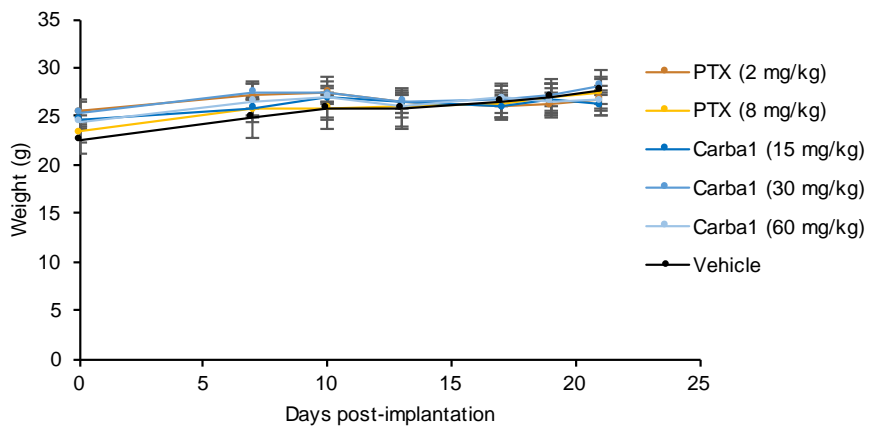
52 **Figure S5. Combination index (CI) values as a function of Fraction affected (Fa) in 4T1 cells**
 53 **exposed to combination drug treatment.** A) The Fa-CI plot. Cells were incubated for 72 hours with
 54 the indicated combinations of Carba1/PTX. The percentage of viable cells was calculated following a
 55 Prestoblu assay. The Chou-Talalay method of synergy determination was used, along with
 56 CompuSyn software. Fa indicates the fractional inhibition for each combinational index (i.e. Fa = 0.5
 57 corresponds to 50% of cell proliferation inhibition). The horizontal line at 1 indicates the line of
 58 additive effect. CI values less than, equal to, or greater than 1 indicate synergy, additivity, or
 59 antagonism, respectively. B) Combination Index for each combination. CI were ranked in ascending
 60 order. Data are presented as mean ± SEM of 3 independent experiments.

61

A



B



62

63

64

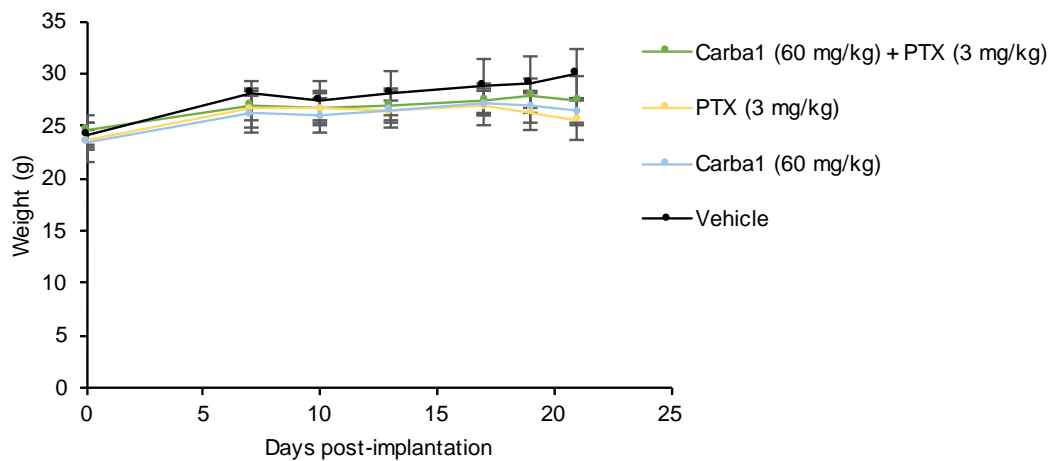
65

66

67

68

C



69

70

71

72

73

74

75

76

77

78

Figure S6. Weight and survival curves of mice treated with PTX, Carba1 and the Carba1/PTX combination. A) Orthotopic 4T1 allografts model: weight and survival curves of mice treated with Carba1 (30 mg/kg), PTX (2 mg/kg), PTX (8 mg/kg) and the combination Carba1 (30 mg/kg) / PTX (2 mg/kg) B) HeLa cells xenografts: weight of PTX and Carba1 treated mice C) HeLa cells xenografts : Weight of mice treated with Carba1 (60 mg/kg), PTX (3 mg/kg) and the combination Carba1 (60 mg/kg) / PTX (3 mg/kg)

Table S1. Analysis of the effect of PTX (1 nM) and Carba1 (25 μ M) on microtubule dynamics in cells.

Parameters	DMSO 0.25%	PTX 1 nM	Carba1 25 μ M
% time spent growing	84.19 \pm 0.20	80.08 \pm 0.25	60.40 \pm 0.25***
% time spent in pause	15.81 \pm 0.47	19.92 \pm 0.50	39.60 \pm 0.31***
Growth rate (μ m/min \pm SE)	14.65 \pm 0.10	13.62 \pm 0.14	10.20 \pm 0.05***
Catastrophe frequency (μ m ⁻¹ \pm SE)	0.23 \pm 0.03	0.27 \pm 0.04	0.46 \pm 0.05***
Catastrophe frequency (min ⁻¹ \pm SE)	2.80 \pm 0.61	2.81 \pm 0.70	2.74 \pm 0.70

79

80

81

82

HeLa cells were transfected with EB3-GFP, treated with or without indicated concentrations of drugs and followed by videomicroscopy. Data are from 3 independent experiments. For each experiment, 6 MTs/cell in 6 cells per condition were analyzed. ***p < 0.001, significantly different from control values (DMSO) using a Tukey test.

83

84

85

86

87

88

89

90

91

Table S2. Small molecule screening data.

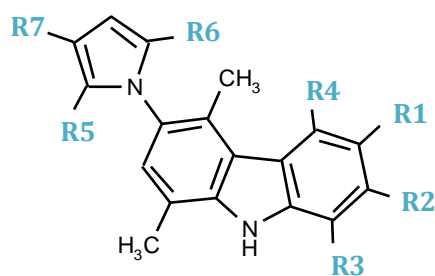
Category	Parameter	Description
Assay	Type of assay	Cytotoxicity (MTT) assay on HeLa cells
	Primary measurement	Measurement of absorbance using a microplate reader
	Key reagents	HeLa cells, paclitaxel (PTX), DMSO
	Assay protocol	Seed cells (2 500 cells/well). The day after, incubate cells for 48 hours with the compounds at a final concentration of 5 μ M, with or without 1 nM PTX. Then, carry out MTT assay
	Additional comments	We observed that the composition of the serum in the cell culture medium could have an effect on the growth of the cells and on their response to the drugs. It is thus important to keep the same batch of serum throughout the screening and the subsequent analyses
Library	Library size	
	Library composition	8 000 molecules
		Small, original and druggable molecules representative of the structural diversity of the CERMN's Chemical Library (> 19,000 compounds issued from numerous medicinal chemistry works).
	Source	Library from the Centre d'Etudes et de Recherche sur le M \acute{e} dicament de Normandie (part of the French CNRS chemical library, http://chimiotheque-nationale.cn.cnrs.fr)
	Additional comments	Compounds arrayed in 96-well plates as single compounds (5mM) in DMSO
Screening process	Format	
	Concentration tested	96-well plates, clear, flat bottoms (Greiner)
	Plate controls	5 μ M, 0.1% DMSO
		DMSO 0.1% was used as proliferation control and SDS 0.1% was used as a control of total cell death. These controls were included in each microplate, on the outer columns.
	Reagent/compound dispensing system	8-channel dispensers
	Detection instrument and software	FLUOstar OPTIMA microplate reader (BMG Labtechnologies)
	Normalization	Raw data converted into percentage of cell viability, based on plate control averages

92

93

94
95
96
97
98
99

Table S3: Carbazole-related compounds and their effect on HeLa cell viability

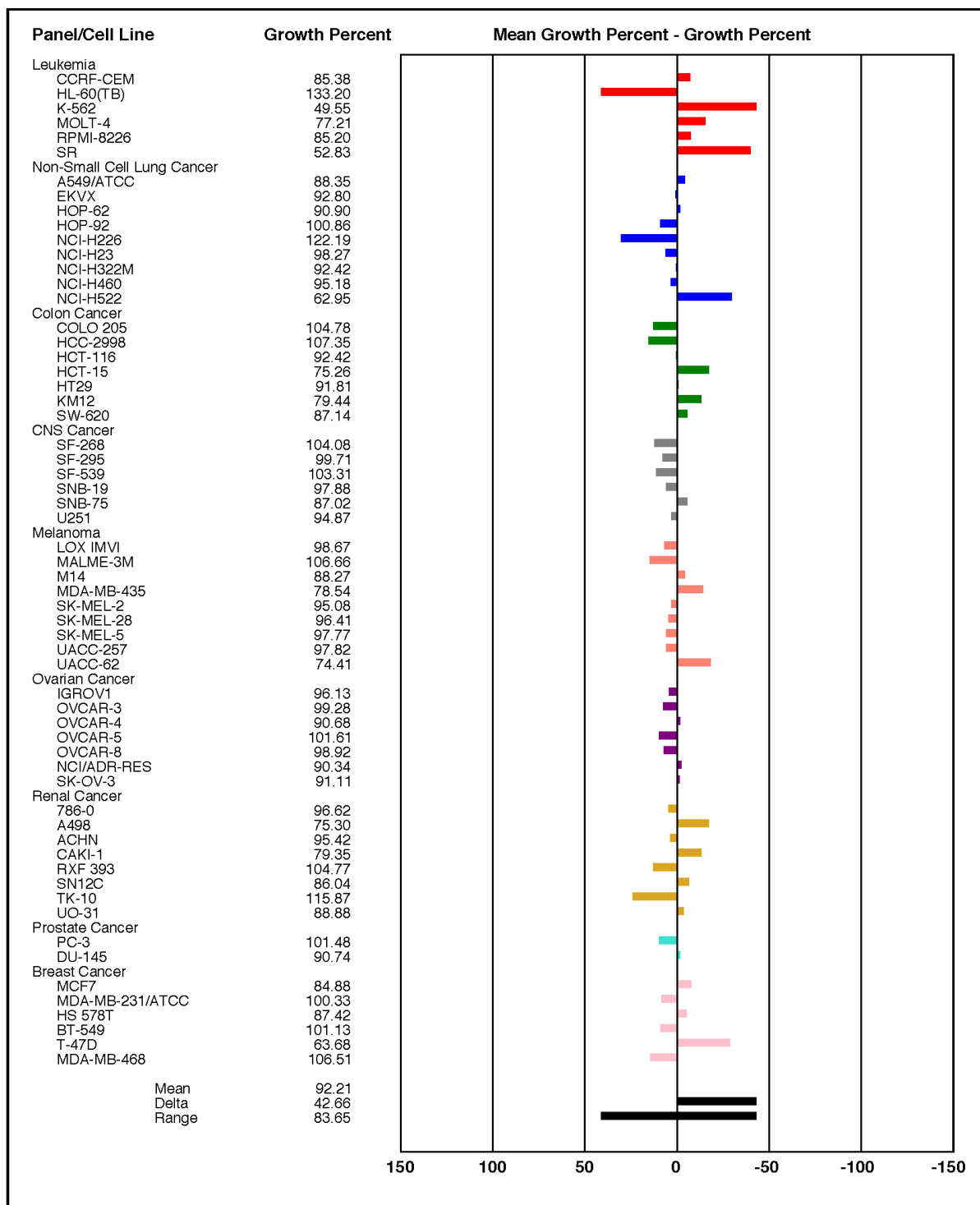


Compound	Substituents at position							HeLa cells viability
	R1	R2	R3	R4	R5	R6	R7	GI50 (μM)
Carba1	Cl	H	H	H	H	H	H	21.8 ± 1.2
T21	Br	H	H	H	H	H	H	16.2 ± 1.3
T22	Br	H	H	H	CH ₃	CH ₃	H	-
T23	OH	H	H	H	H	H	H	-
T25	F	H	H	H	CH ₃	CH ₃	H	-
T26	EtOCOO	H	H	H	CH ₃	CH ₃	H	-
T27	EtOCOO	H	H	H	H	H	H	-
T28	Cl	H	H	H	CH ₃	CH ₃	H	-
T29	F	H	H	H	H	H	CHO	14.2 ± 1.8
T30	Br	H	H	H	H	H	CHO	1.06 ± 0.04
T31	H	H	Et	H	H	H	H	-
T32	H	Cl	H	H	H	H	H	14.8 ± 1.6
T33	H	H	H	Cl	H	H	H	-

100
101
102
103
104
105
106
107
108

109
110

Table S4. Carba1 moderately inhibits the proliferation of cancer cells included in the NCI-60 panel.



111
112
113
114
115
116
117
118
119
120

The drug's efficacy (Carba1, 10 μ M) against the NCI-60 cancer cell lines was examined. The panel of cell lines analyzed included: six leukemia cell lines, nine non-small cell lung cancer cell lines, seven colorectal cancer cell lines, six CNS cancer cell lines, nine melanoma cell lines, seven ovarian cancer cell lines, seven renal cancer cell lines, two prostate cancer cell lines and six breast cancer cell lines. The screening was carried out by the US National Cancer Institute (NCI) using a sulforhodamine B (SRB) colorimetric assay. The data are reported as the percent growth of treated cells. The number reported is growth relative to the no-drug control, and relative to the time zero number of cells. This allows detection of both growth inhibition (values between 0 and 100) and lethality (values less than 0), as indicated by the color bar chart.

Table S5. Quantification of the fate of HeLa cells treated with Carba1 and/or PTX.

	12 hours of observation					20 hours of observation	
	Control	Carba1 (12 μ M)	Carba1 (25 μ M)	PTX (1 nM)	PTX (5 nM)	Carba1 (12 μ M) + PTX (1 nM)	Carba1 (25 μ M)
Mitotic catastrophe	0 %	0 %	The majority of cells stay blocked in pro-metaphase	0 %	0 %	5 %	61 %
Aberrant mitosis	3 %	11 %		25 %	93 %	83 %	29 %
Normal mitosis	97 %	89 %		75 %	7 %	12 %	10 %
	n = 73	n = 68		n = 100	n = 43	n = 44	n = 105

122
123
124
125
126
127

HeLa Kyoto cells were treated with the indicated concentrations of drugs and followed during 12 or 20 hours using time-lapse videomicroscopy. n represents the number of mitotic cells analyzed. Carba1 (12 μ M) and PTX (1 nM) induced a slight increase of aberrant mitosis as compared to control (DMSO). At a higher concentration (25 μ M), Carba1 induced cell prometaphase arrest followed by apoptotic death. PTX at 5 nM and the Carba1/PTX combination induced a mitotic delay followed by aberrant division.

128

Table S6. Profile of kinase inhibitory activity of Carba1.

Protein kinase	Activity	Protein kinase	Activity
Abl(h)	141	LIMK1(h)	117
ALK(h)	110	LKB1(h)	110
AMPK α 1(h)	99	MAPK1(h)	106
AMPK α 2(h)	104	MARK1(h)	102
ARK5(h)	87	Met(h)	86
Aurora-A(h)	105	MLCK(h)	82
Aurora-B(h)	72	MLK1(h)	83
BrSK1(h)	101	MRCK α (h)	140
BrSK2(h)	134	MRCK β (h)	136
c-RAF(h)	100	mTOR(h)	98
CaMKI(h)	102	NEK2(h)	111
CaMKII β (h)	67	p70S6K(h)	110
CaMKII γ (h)	104	PAK2(h)	103
CaMKII δ (h)	74	PAR-1B α (h)	99
CaMKIV(h)	85	PDGFR α (h)	112
CaMKI δ (h)	127	PDGFR β (h)	126
CDK2/cyclinA(h)	110	PKA(h)	141
CDK7/cyclinH/MAT1(h)	115	PKB α (h)	116
cKit(h)	97	PKB β (h)	116
cSRC(h)	100	PKC ζ (h)	113
eEF-2K(h)	145	PKC ι (h)	71
EGFR(h)	125	Plk1(h)	112
EphA2(h)	102	Plk3(h)	99
FAK(h)	104	Pyk2(h)	107
FGFR1(h)	72	Ret(h)	90
FGFR2(h)	94	ROCK-I(h)	107
FGFR3(h)	99	ROCK-II(h)	109
Flt3(h)	134	SIK(h)	124
GSK3 β (h)	73	Src(1-530)(h)	100
IGF-1R(h), activated	92	Syk(h)	103
IR(h), activated	81	TAK1(h)	94
JAK2(h)	106	TGFBR1(h)	98

129
130

The kinase activity determined in the presence of 10 μ M Carba1 is expressed as percentage of the control activity without inhibitor. Final concentration of ATP in the experiment was 100 μ M.

131 **Movie S1. Carba1 induces a delay in mitosis.**

132 Time-lapse microscopy was performed during 12 hours, on HeLa Kyoto cells treated with
133 DMSO (control), 12 μ M or 25 μ M Carba1. Images were acquired every 2.5 minutes. Bar = 10 μ m.

134 **Movie S2. Carba1 blocks cells in mitosis and induces mitotic catastrophe.**

135 Time-lapse microscopy was performed during 20 hours, on HeLa Kyoto cells treated with
136 Carba1 (25 μ M). Images were acquired every 2.5 minutes. Bar = 10 μ m.

137 **Movie S3. Effects of PTX (1 nM and 5 nM) and of the combination of Carba1 and PTX (12 μ M/1
138 nM) on HeLa Kyoto cell mitosis.**

139 Time-lapse microscopy was performed, during 12 hours on HeLa Kyoto cells treated with 1
140 nM or 5 nM PTX and the Carba1 PTX (12 μ M/1 nM) combination, as indicated. Images were
141 acquired every 2.5 minutes. Bar = 10 μ m.
142



© 2020 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).