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1 Article

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 **Sacnicté 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 ¹ Institute for Advanced Biosciences, Team Regulation and Pharmacology of the Cytoskeleton, INSERM
10 U1209, CNRS UMR5309, Université Grenoble Alpes, Grenoble, France.

11 ² Grenoble Institute of Neurosciences, INSERM U1216, Université Grenoble Alpes, CEA, Grenoble, France

12 ³ Cell Biology, Neurobiology and Biophysics, Department of Biology, Faculty of Science, Utrecht University,
13 Utrecht, Netherlands

14 ⁴ NORMANDIE UNIV, UNICAEN, CERMN, Caen, France

15 ⁵ Institute for Advanced Biosciences, Team Cancer targets and experimental therapeutics, INSERM U1209,
16 CNRS UMR5309, Université Grenoble Alpes, Grenoble, France.

17 ⁶ Structural and Chemical Biology Department. Centro de Investigaciones Biológicas, CSIC, Ramiro de
18 Maeztu 9, 28040 Madrid, Spain.

19 * Corresponding author: Laurence Lafanechère, Institute for Advanced Biosciences, Site Santé, Bâtiment
20 Albert Bonniot, Allée des Alpes 38700 La Tronche, France. Phone number +33(0)4 76 54 95 71

21 laurence.lafanechere@univ-grenoble-alpes.fr

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23 **Abstract:** Paclitaxel is a microtubule stabilizing agent and a successful drug for cancer
24 chemotherapy inducing, however, adverse effects. To reduce the effective dose of paclitaxel, we
25 searched for pharmaceuticals which could potentiate its therapeutic effect. We have screened a
26 chemical library and selected Carba1, a carbazole, which exerts synergistic cytotoxic effects on
27 tumor cells grown *in vitro*, when co-administrated with a low dose of paclitaxel. Carba1 targets the
28 colchicine binding-site of tubulin and is a microtubule-destabilizing agent. Catastrophe induction
29 by Carba1 promotes paclitaxel binding to microtubule ends, providing a mechanistic explanation
30 of the observed synergy. The synergistic effect of Carba1 with paclitaxel on tumor cell viability
31 was also observed *in vivo* in xenografted mice. Thus, a new mechanism favoring paclitaxel binding
32 to dynamic microtubules can be transposed to *in vivo* mouse cancer treatments, paving the way for
33 new therapeutic strategies combining low doses of microtubule targeting agents with opposite
34 mechanisms of action.

35 **Keywords:** Cancer therapy; microtubules; drug synergy; carbazole; paclitaxel

37 **1. Introduction**

38 Microtubules (MTs), dynamic polymeric filaments composed of α -tubulin and β -tubulin
39 heterodimers, are key components of the cytoskeleton of eukaryotic cells. Their crucial roles in cell
40 division and physiology mainly rely on their ability to rapidly polymerize or depolymerize.
41 Targeted perturbation of this finely tuned process constitutes a major therapeutic strategy. Drugs
42 interfering with MTs are major constituents of chemotherapies for the treatment of carcinomas. A
43 number of compounds bind to the tubulin-MT system. They can be roughly classified into MT-
44 stabilizers such as taxanes or epothilones, and MT-destabilizers such as vinca alkaloids,

45 combretastatin and colchicine [1]. It has been demonstrated that binding of vinca alkaloids or
46 colchicine prevents the curved-to-straight conformational change of tubulin at the tip of the
47 growing MT, necessary for proper incorporation of new tubulin dimers into the MT lattice (see
48 reviews [1,2]).

49 Paclitaxel (PTX) binds to the taxane-site of β -tubulin and stabilizes the MT lattice by
50 strengthening lateral and/or longitudinal tubulin contacts within the MT [1]. At stoichiometric
51 concentrations, it promotes MT assembly. At low and clinically relevant concentrations, PTX
52 primarily suppresses MT dynamics without significantly affecting the MT-polymer mass [3,4]. PTX
53 is one of the most successful chemotherapeutic drugs in history. It is currently used to treat patients
54 with a variety of cancers including lung, breast and ovarian cancers [5].

55 Several mechanisms have been proposed to explain the anti-tumor activity of PTX. It can
56 induce a mitosis dependent cell death, either by producing a mitotic arrest [6], when applied at high
57 concentrations, or by promoting chromosome mis-segregation at low concentrations [7].
58 Alternatively, PTX can act on interphase cells and drive autonomous cell death by perturbation of
59 intracellular trafficking [8]. It has also been recently proposed that post-mitotic formation of
60 micronuclei induced by PTX can promote inflammation and subsequent tumor regression *via*
61 vascular disruption and immune activation [9].

62 While PTX is a successful anti-cancer drug, its low solubility, its toxicity, and the fact that cells
63 become resistant to this drug, impose serious limits to its use. Cell resistance to PTX is due to the
64 high expression of P-glycoprotein or multidrug resistance-associated proteins, as well as to the
65 overexpression of some β -tubulin isoforms or mutations in β -tubulin that affect the MT polymer
66 mass and/or drug binding [10]. Another major drawback of PTX in clinical applications is the
67 development of peripheral neuropathies, primarily involving the sensory nervous system.
68 Although the molecular bases of these neuropathies are not completely understood, an inhibition of
69 MT-based axonal transport appears to be a possible mechanism [11]. It has been recently shown
70 that anterograde kinesin based-axonal transport is specifically affected by PTX, whereas MT
71 destabilizing drugs that bind preferentially to the ends of MTs have much less effect on axonal
72 transport [12].

73 An alternative therapeutic solution would be the use of pharmaceuticals which, when co-
74 administered with PTX, could potentiate its effect without significantly increasing its toxicity. Such
75 agents could allow the use of lower doses of PTX in cancer therapy, may limit the occurrence of
76 resistances and reduce MT-independent adverse effects.

77 To identify such agents, we have screened a collection of 8,000 original compounds using a
78 cytotoxicity assay and selected a derivative of the carbazole series (Carba1) able to sensitize cells to
79 a low, non-toxic dose of PTX. We demonstrated that Carba1 exerts synergistic cytotoxic effects with
80 PTX. In cells, Carba1 has no major effect on the total MT mass in interphase cells and shows
81 moderate cytotoxicity. We found that Carba1 targets the colchicine-binding site of tubulin, inhibits
82 *in vitro* tubulin polymerization and promotes catastrophes, similar to other MT-destabilizing agents.
83 A combination of Carba1 and PTX causes synergistic perturbation of MT growth *in vitro*. Carba1-
84 induced modulation of MT dynamics increases the binding of fluorescent taxane, Fchitax-3 to MTs,
85 similar to what we have described previously for vinblastine [13], providing a biochemical
86 explanation of the observed synergy between Carba1 and PTX.

87 Carba1 has no major anti-tumor effect when administered alone in animals and no detectable
88 toxicity. The administration of a combination of Carba1 and a low, ineffective, dose of PTX showed,
89 however, a significant effect on tumor growth, indicating that Carba1 and PTX act synergistically *in*
90 *vivo*. Our results pave the way for new therapeutic strategies, based on the combination of low
91 doses of MT targeting agents with opposite mechanisms of action. These combinations may have
92 reduced toxicity compared to high therapeutic PTX doses.

93 2. Results

94 2.1. A pairwise chemical genetic screen identifies a carbazolone derivative, Carba1, that sensitizes cells to
95 paclitaxel

96 We designed a screen to select compounds that sensitize cells to paclitaxel (PTX). In a first step,
97 we determined a minimal dose of PTX that is not toxic for cells. We found that 1 nM of PTX showed
98 no toxicity when applied on HeLa cells for 48 hours (**Figure S1**). Furthermore, such a dose has no
99 detectable impact on MT dynamics as assessed by EB3 tracking after time lapse fluorescence
100 microscopy using GFP-EB3-transfected HeLa cells and subsequent calculation of dynamic
101 instability parameters (**Table S1**).

102 We then screened a library of 8 000 compounds at a concentration of 5 μ M (**Figure 1A and Table**
103 **S2**) and compared their cytotoxicity on HeLa cells when administrated alone or in combination
104 with 1 nM PTX. We selected 76 compounds that show no or moderate cytotoxicity when applied
105 alone, and that were found cytotoxic when applied in combination with 1 nM PTX. We observed
106 that many carbazole derivatives were present in this selection (**Table S3**) and we decided to focus
107 our study on the 6-chloro-1,4-dimethyl-3-pyrrol-1-yl-9H-carbazole (Carba1, **Figure 1B**) because it
108 lacked reactive chemical groups that could interact non-specifically with protein targets, was
109 minimally cytotoxic when assayed alone on HeLa cells (**Table S3 and Figures 1C and 2A**) and
110 showed a synergistic activity with PTX (**Figure 1C**) when assayed using the sensitive PrestoBlue
111 assay. We further used the combination index (*CI*) method of Chou-Talalay [14] to quantify the
112 synergistic interaction between Carba1 and PTX. A *CI* value of less than 1 indicates synergism, a *CI*
113 value =1 indicates an additive effect and *CI*>1 indicates antagonism. Interestingly, we found that
114 the synergistic effect was closely dependent on PTX concentrations. Indeed, for all Carba1
115 concentrations tested, synergism was observed for PTX concentrations lower than 5 nM, additive
116 effects for a PTX concentration of 5 nM and antagonistic effects for all higher concentrations (7.5
117 and 10 nM PTX, **Figure S2**).

118 A clear synergism (*CI* = 0.57) was observed for PTX 1 nM plus Carba1 12 μ M. At these
119 concentrations, the compounds showed low cytotoxic activity when applied alone. Indeed, the
120 comparison of HeLa cell apoptosis induced by Carba1 (12 μ M), PTX (1 nM) to the apoptosis
121 induced by the combination of Carba1 and PTX (12 μ M/ 1 nM) confirmed the synergistic activity
122 (**Figure 1D**).

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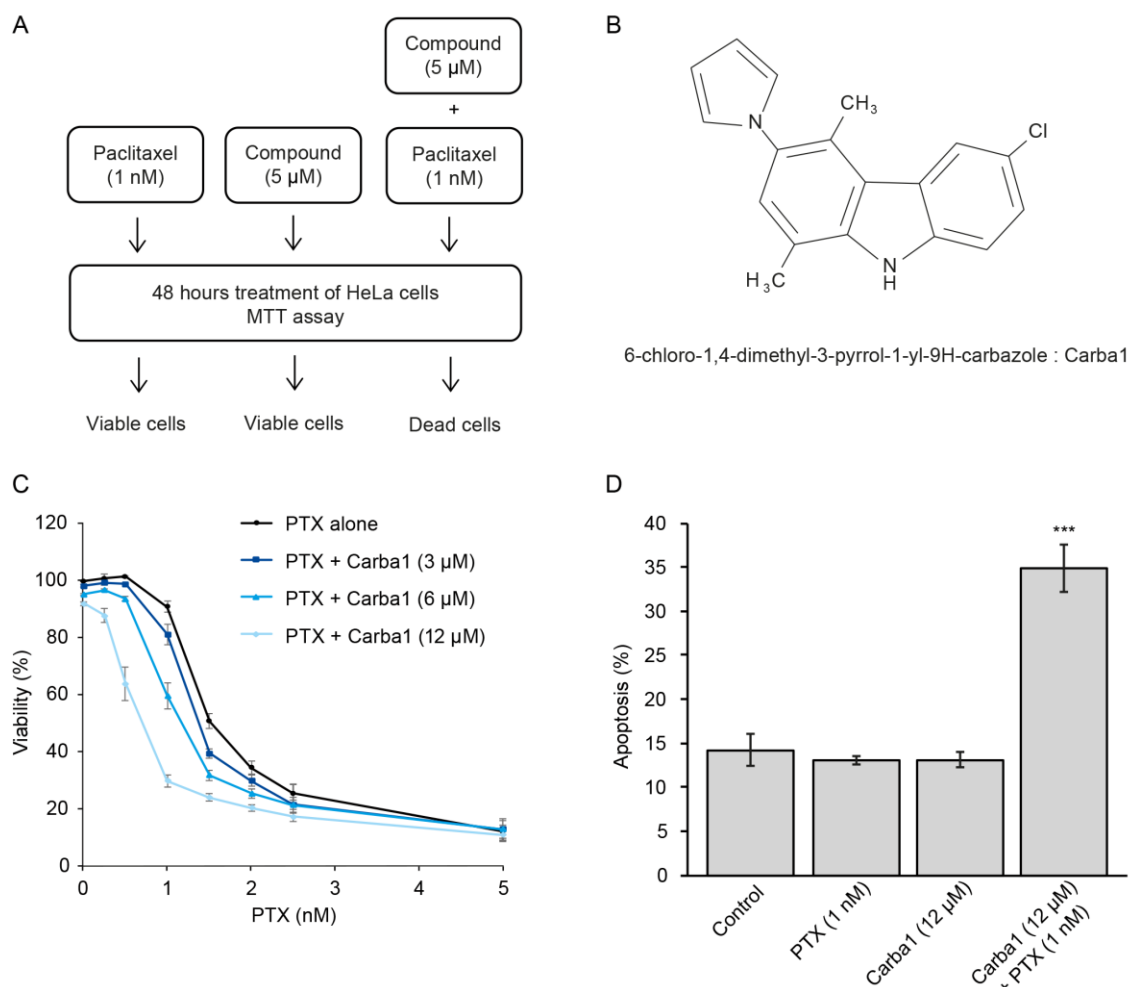
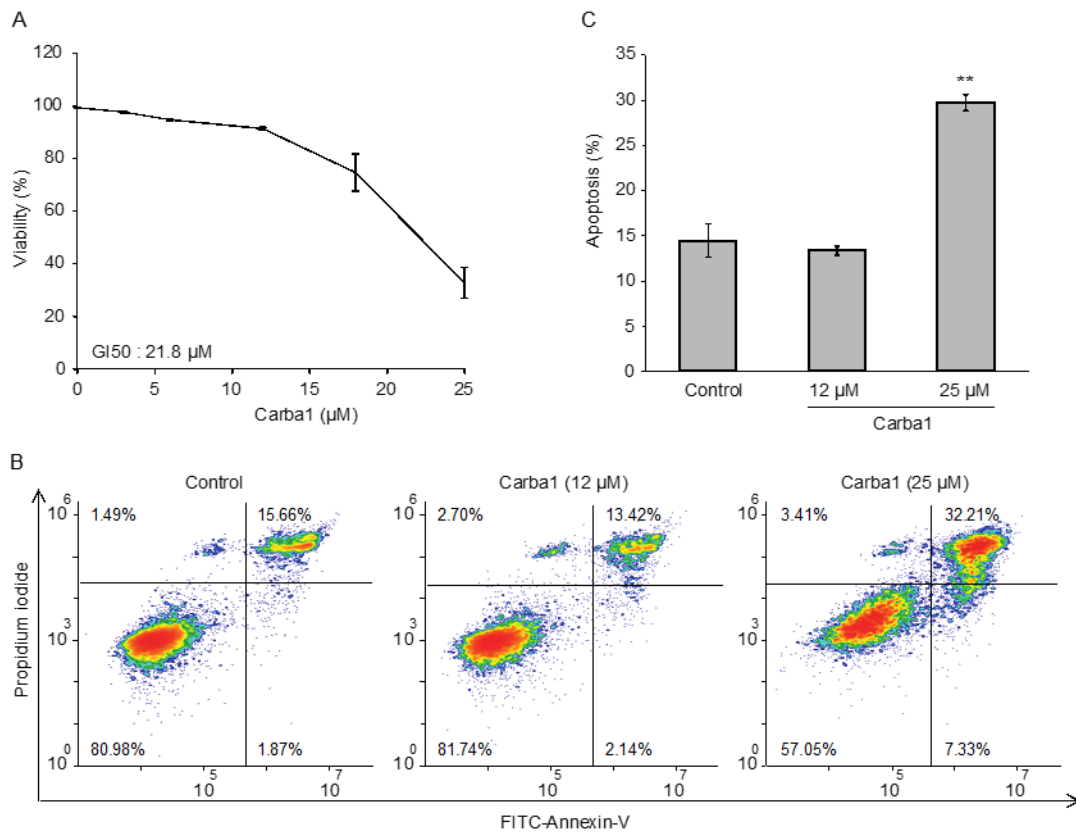


Figure 1. Selection of a compound that sensitizes cells to PTX. (A) Schematic illustration of the concept used to screen a chemical library for compounds that sensitize cells to PTX. Treatment of cells with compounds of the library alone (5 μ M) or PTX (1 nM) alone has no effect on cell viability, compounds (5 μ M) that have no effect when applied alone but induced cell death when applied in combination with PTX (1 nM) were selected. (B) Chemical structure of Carba1. (C) Effect of Carba1/PTX combinations on the viability of HeLa cells. Cells were incubated for 72 hours with the indicated combinations of Carba1/PTX. The percentage of viable cells was calculated following a Prestoblue assay. Data are presented as mean \pm SEM of 3 independent experiments. Note that the effect of the different doses of Carba1 alone on cell viability can be seen at the x-coordinate = 0, corresponding to 0 nM PTX. (D) Effect of Carba1 (12 μ M), PTX (1 nM) and the combination of Carba1 and PTX (12 μ M/1 nM) on HeLa cell apoptosis. HeLa cells, treated with the indicated concentrations of drugs, were stained with propidium iodide and annexin V and analyzed by flow cytometry. Results are expressed as mean \pm SEM of 3 separate experiments. The significance was determined by a Student's t-test (***) p <0.001, compared to the control).

2.2. Carba1 has a moderate cytotoxicity when applied at high concentrations

As our final aim was to test the therapeutic efficacy of Carba1 in combination with PTX, it was important to investigate its cellular effects and to check that this compound is not or moderately toxic by itself. We first analyzed the cytotoxicity of Carba1 on HeLa cells, using the PrestoBlue assay. As shown in **Figure 2A**, Carba1 has a moderate cytotoxicity with a calculated GI50 of 21.8 μ M after a 72-hour treatment.



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Figure 2. Carba1 has a low cytotoxicity. (A) Effect of Carba1 on HeLa cell viability. Cells were incubated for 72 hours with increasing concentrations of Carba1. The percentage of viable cells was calculated following the Prestoblue assay. The results are expressed as mean \pm SEM of three separate experiments. (B) Effect of Carba1 on HeLa cells apoptosis. HeLa cells, treated with the indicated concentrations of Carba1 for 72 hours, were stained with propidium iodide and FITC-annexin V and analyzed by flow cytometry. Apoptotic cells are observed in the upper right part of the graphs. (C) Results for apoptotic cell death (as shown in figure 2B) are expressed as mean \pm SEM of 3 separate experiments. The significance was determined by a Student's t-test (** $p < 0.01$, compared to the control).

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Since the Prestoblue assay is a metabolic test that indirectly measures cell viability, we directly detected cells in apoptosis using Annexin V staining, and quantified them by flow cytometry. We compared the effect of two concentrations of Carba1: a concentration (12 μ M) that has no detectable effect on cell viability and a cytotoxic concentration (25 μ M). No apoptosis was detected when Carba1 was applied for 72 hours at a concentration of 12 μ M whereas at 25 μ M, it induced apoptosis of 30% of the cells (**Figure 2B and 2C**).

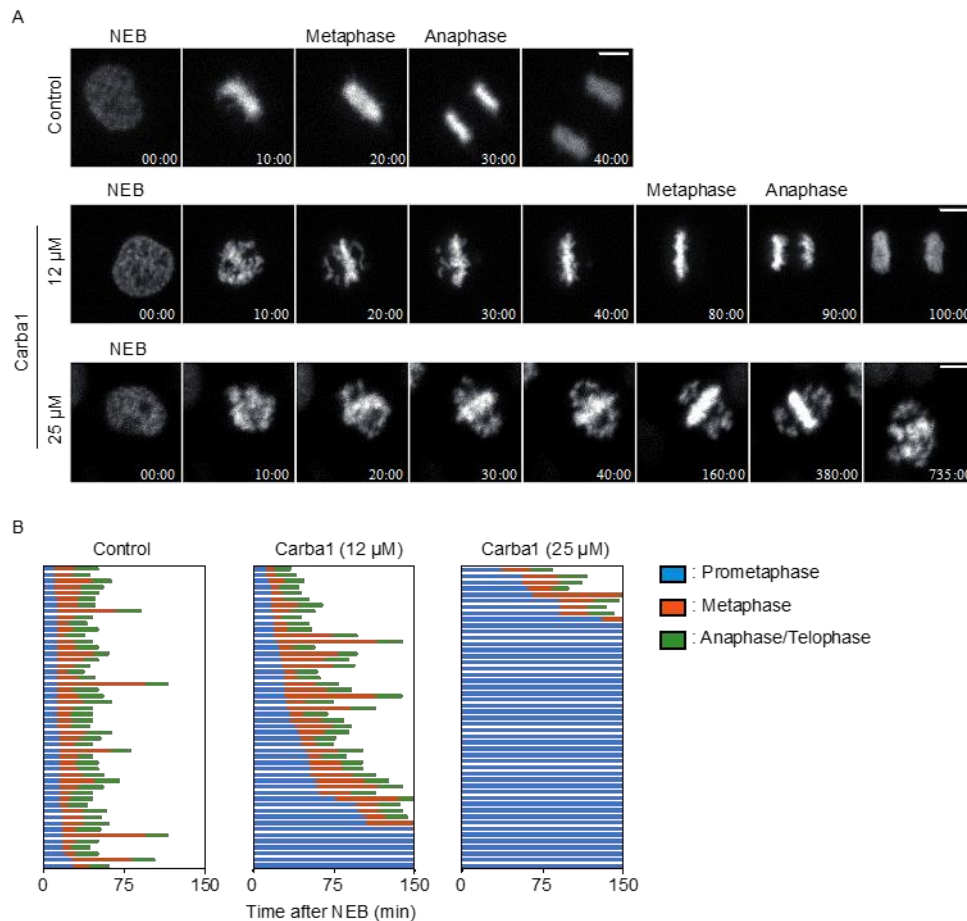
These results indicate that Carba1 is only weakly toxic, even when applied at a high concentration. A toxicity analysis of a single 10 μ M dose of Carba1 on a set of 60 human cancer cell lines (NCI-60 screen [15]) confirmed the low cytotoxic activity of Carba1 (**Table S4**).

Finally, we checked that Carba1 was not toxic on a normal cell line. We used immortalized RPE-1 human cells and compared the effect of Carba1, PTX and their combination on the viability of these cells, using a Prestoblue assay. We found that the GI50 for PTX (7 nM) was higher in this cell line than in HeLa cells, as expected [16] (**Figure S3**). When used in combination with PTX, Carba1 was able to synergistically affect cell viability, at high dose. Carba1 was not toxic for this cell line (**Figure S3**). These results indicate that Carba1 does not induce additional toxicity.

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2.3. Cell-cycle progression is blocked at mitosis by Carba1

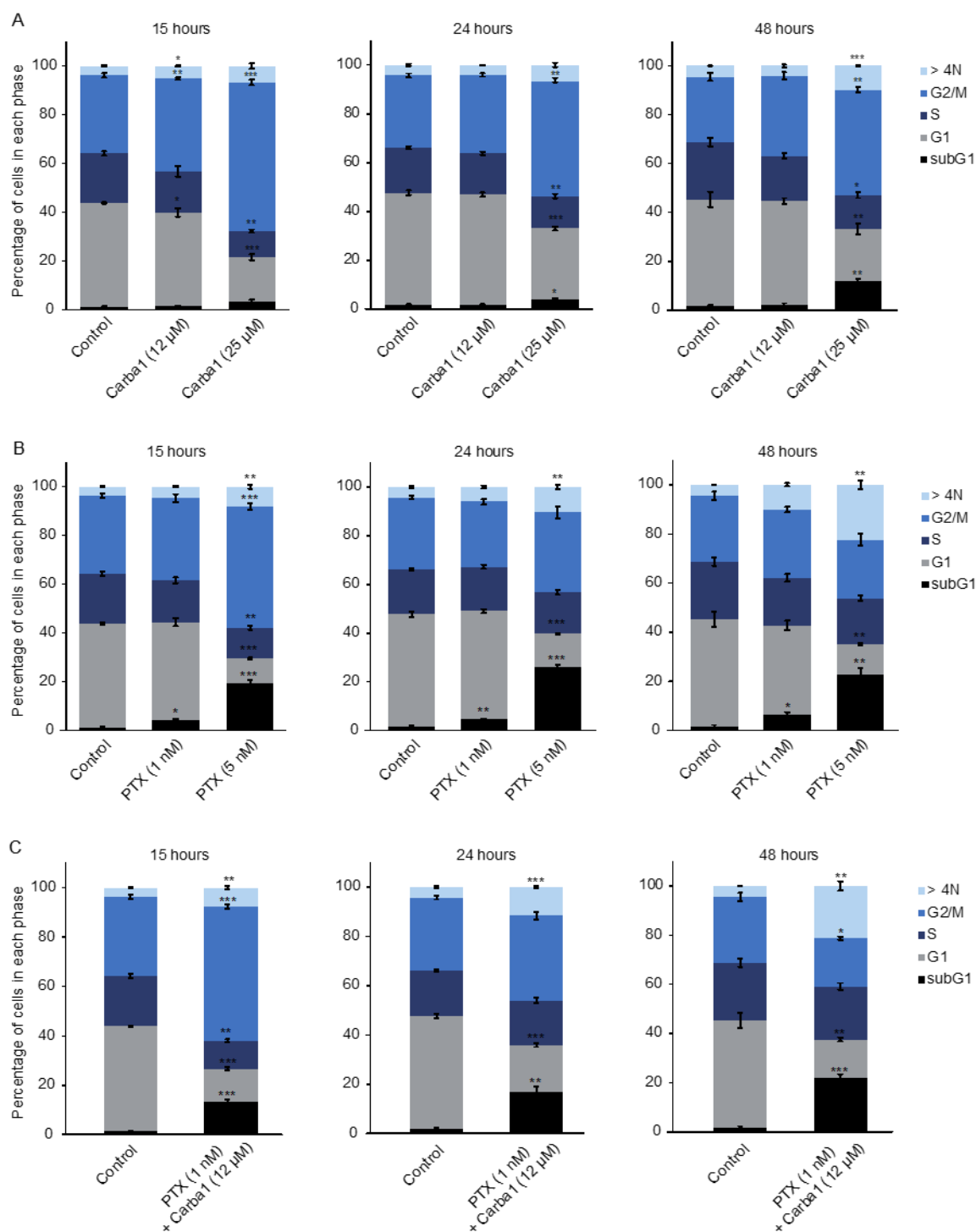
171 A videomicroscopy analysis, using different doses of Carba1, showed that the compound
 172 impacted mitosis. As compared to DMSO, Carba1 (12 μ M) induced a significant delay in the
 173 completion of metaphase and a slight increase of aberrant mitosis (**Figure 3A, B, Movie S1 and**
 174 **Table S5**). When Carba1 was applied at a concentration of 25 μ M, the majority of the cells stayed
 175 blocked in prometaphase (**Figure 3A, B and Movie S1, right**). We followed and quantified the fate
 176 of the cells treated with 25 μ M Carba1 in a 20-hour time lapse video (**Movie S2**) and noted that 61%
 177 of the mitotic cells eventually died during mitosis, 29% were still dividing abnormally, whereas
 178 only 10% underwent apparently normal mitosis (**Table S5**). We thus concluded that a cytotoxic
 179 dose of Carba1 induced a very long duration of mitotic arrest, followed by mitotic catastrophe.



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181 **Figure 3. Carba1 induces a mitotic arrest.** (A) Representative images, selected from movie S1 of
 182 HeLa Kyoto cells treated with DMSO (control) and the indicated concentrations of Carba1. Bar =10
 183 μ m. (B) Analysis of the duration of mitosis in HeLa Kyoto cells treated with DMSO (control) or with
 184 different doses of Carba1, as indicated. Duration of prometaphase (from nuclear envelope
 185 breakdown (NEBD) to chromosome alignment; blue), metaphase (from chromosome alignment to
 186 anaphase onset; orange) and anaphase/telophase (from anaphase onset to chromosome
 187 decondensation; green) were analyzed from movie S1. The data represent 50 cells for each
 188 treatment.

189 In accordance with the effect of Carba1 on mitosis, a flow cytometry analysis using propidium
 190 iodide staining indicated that a 15-hour exposure to 25 μ M Carba1 induced a dose-dependent cell-
 191 cycle arrest at the G2/M phase (**Figure 4A**). Prolonged exposure (24 and 48 hours) led to a reduction
 192 of the number of cells blocked in the G2/M phase and to an increase of aneuploid cells, as assessed
 193 by the increased number of cells in sub G1 and of cells containing more than 4N DNA (**Figure 4A**).



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Figure 4. Carba1 increases PTX effects on cell cycle. (A) HeLa cells treated with the indicated concentrations of Carba1 for 15, 24 and 48 hours, were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. (B) HeLa cells treated with the indicated concentrations of PTX for 15, 24 and 48 hours, were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. (C) HeLa cells treated with the combination of Carba1 and PTX (12 μM/1 nM) for 15, 24 and 48 hours, were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. The results are expressed as mean ± SEM of 3 separate experiments. The significance was determined by a Student's t-test (*p<0.05, **p<0.01, ***p<0.001, compared to the control).

204 2.4. Carba1 increases PTX effects on cell cycle and mitosis

205 We similarly analyzed the effect of PTX, using time-lapse microscopy. PTX at a concentration
206 of 1 nM induced a delay in chromosome congression during prometaphase and a moderate increase
207 of aberrant mitosis (**Movie S3, left** and **Table S5**). When treated with a cytotoxic concentration of
208 PTX (5 nM) 80% of HeLa cells underwent aberrant mitosis followed by a mitotic slippage, as shown
209 by a 12-hour time-lapse video (**Movie S3, middle** and **Table S5**). We conducted a flow cytometry
210 analysis to get further insight of the effect of 5 nM PTX on the fate of HeLa cells treated for longer
211 times (15, 24 or 48 hours). After a 15-hour treatment, half of the cell population was blocked in the
212 G2/M phase and nearly 20% of the cells were dead, as indicated by the increased proportion of cells
213 in subG1. Then, the proportion of cells in G2/M gradually decreased, in parallel with an increased
214 number of cells in subG1 and of multinucleated cells (**Figure 4B**). Because the effects of such a
215 cytotoxic concentration of PTX were different from those of a cytotoxic (25 μ M) concentration of
216 Carba1, we wondered which compound effect was predominant in the cytotoxicity of the
217 combination of Carba1 and PTX (12 μ M/1 nM). We thus compared the effects of this cytotoxic
218 combination to the effects of Carba1 25 μ M and PTX 5 nM administrated separately. As shown on
219 **Figure 4C**, the combination of Carba1 and PTX (12 μ M/1 nM) induced an arrest of the cell cycle
220 almost superimposable to the arrest observed when cells are treated with PTX 5 nM. Moreover the
221 videomicroscopy analysis of the cells treated with this combination showed that cell death occurred
222 after mitotic slippage (**Movie S3, right**). The similarity of the results obtained with the combination
223 Carba1 and PTX (12 μ M/1 nM) to those obtained with PTX 5 nM indicates that the overall effect of
224 the combination results from an increase of the PTX effect induced by Carba1.

225 2.5. Carba1 is a microtubule-destabilizing agent

226 In an attempt to understand the Carba1 mechanism of action, we first analyzed its effect on
227 cellular MTs, using immunofluorescence. Carba1 treatment (12-25 μ M) did not visibly perturb the
228 MT network in interphase cells, as compared to DMSO (control; **Figure 5A**). In mitosis,
229 chromosome congression defects were visible in several mitotic cells of the 12 μ M treated cell
230 population. The occurrence of such defects was increased at a higher dose (25 μ M) of Carba1
231 (**Figure 5A**). Such defects in chromosome congression are similar to those observed on cells treated
232 by some inhibitors of kinases involved in the mitotic process such as Aurora B or Plk1 kinases [17].
233 Moreover, compounds structurally related to Carba1 often target protein kinases [18,19]. We
234 therefore tested the ability of Carba1 to inhibit the activity of a panel of 64 protein kinases including
235 kinases known to be involved in the regulation of the cytoskeleton and/or the cell cycle. We found
236 that, when *in vitro* assayed at a 10 μ M concentration, Carba1 did not show any selective inhibitory
237 activity on the kinases tested (**Table S6**). It is therefore unlikely that Carba1 is a direct inhibitor of
238 these kinases.

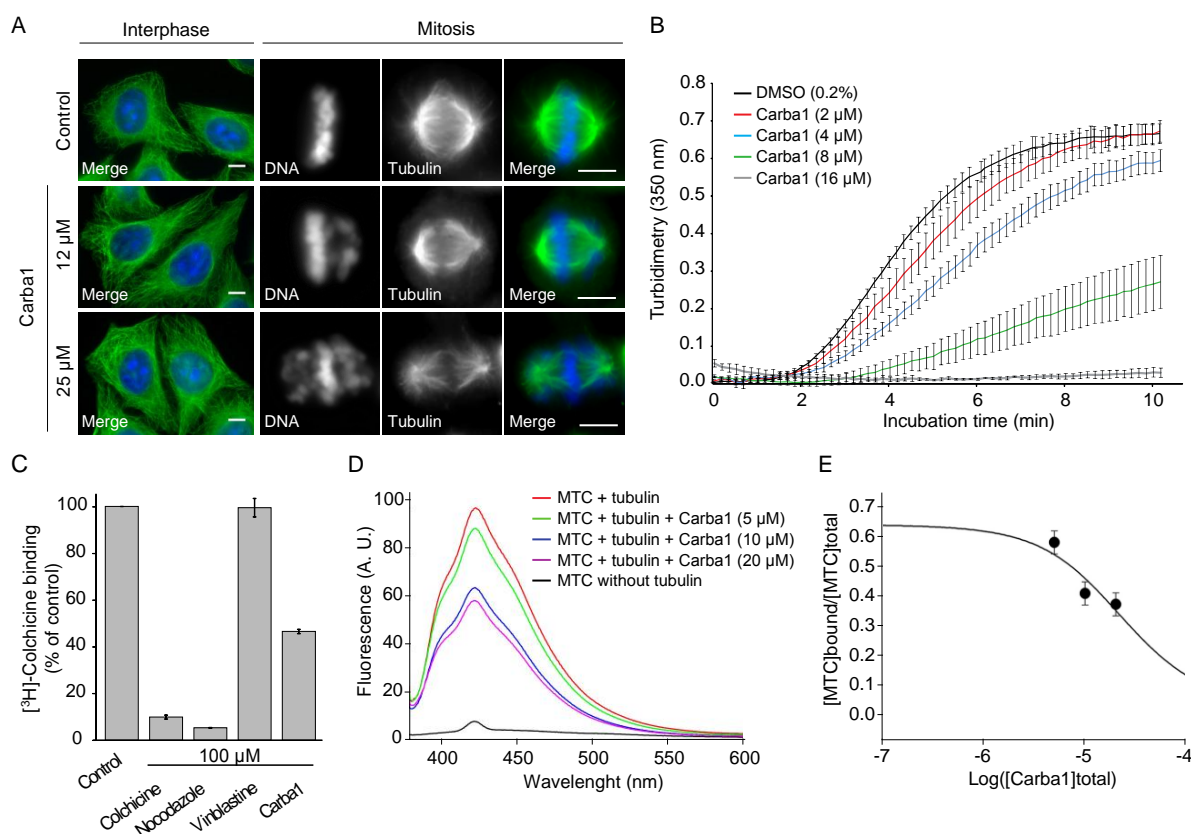


Figure 5. Carba1 is a microtubule-depolymerizing agent that competes with colchicine for tubulin binding. (A) Immunofluorescence analysis of the Carba1 effect on interphase and mitotic MTs. MTs in interphase (left panels) or in mitosis (right panels) were stained using an anti-tubulin antibody, as described in the methods section. DNA was stained using Hoechst reagent. Bars = 10 μm . (B) Time course of tubulin polymerization at 37°C in the presence of vehicle (DMSO, black line) and Carba1 at different concentrations (colored lines) as indicated, measured by turbidimetry at 350 nm. Purified tubulin: 30 μM in BRB80 buffer with 1 mM GTP. Each turbidity value represents the mean \pm SEM from 3 independent experiments. (C) Effect of Carba1 on the binding of [^3H]-colchicine to tubulin. Carba1 (100 μM) was used to compete with [^3H]-colchicine (50 nM) as described in the methods section. Each value represents the mean \pm SEM of 3 independent experiments. Colchicine and nocodazole were used as positive and vinblastine as negative control. (D) Displacement of MTC from the colchicine site. Fluorescence emission spectra of 10 μM MTC and 10 μM tubulin in 10 mM phosphate-0.1 mM GTP buffer pH 7.0, in the absence or presence of increasing concentrations of Carba1. (E) Displacement isotherm at 25°C of the fluorescent probe MTC (10 μM) bound to tubulin (10 μM) by Carba1 (black line and circles). The solid line is the best-fit value of the binding equilibrium constant of the competitors, assuming a one-to-one binding to the same site.

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The observed effects of Carba1 on the cellular MT network were reminiscent to those described for low doses of MT depolymerizing agents such as nocodazole or vinca alkaloids: a mitotic arrest with a similar aberrant chromosome organization, with no detectable effect on the total MT mass [20]. We thus wondered if Carba1 was, as nocodazole, able to directly impact MT assembly. The effect of a high dose (25 μM) of Carba1 on MT dynamic instability parameters was measured using time-lapse fluorescence microscopy on GFP-EB3 transfected cells (Table S1). Carba1 reduced the MT growth rate as well as the MT growth, as indicated by the increase of the distance-based catastrophe frequency, and increased time spent in pause, indicating that Carba1 suppresses MT dynamics.

We therefore tested the Carba1 effect on *in vitro* tubulin assembly. As shown in Figure 5B, Carba1 was able to inhibit polymerization of pure tubulin in a dose-dependent manner. Increasing

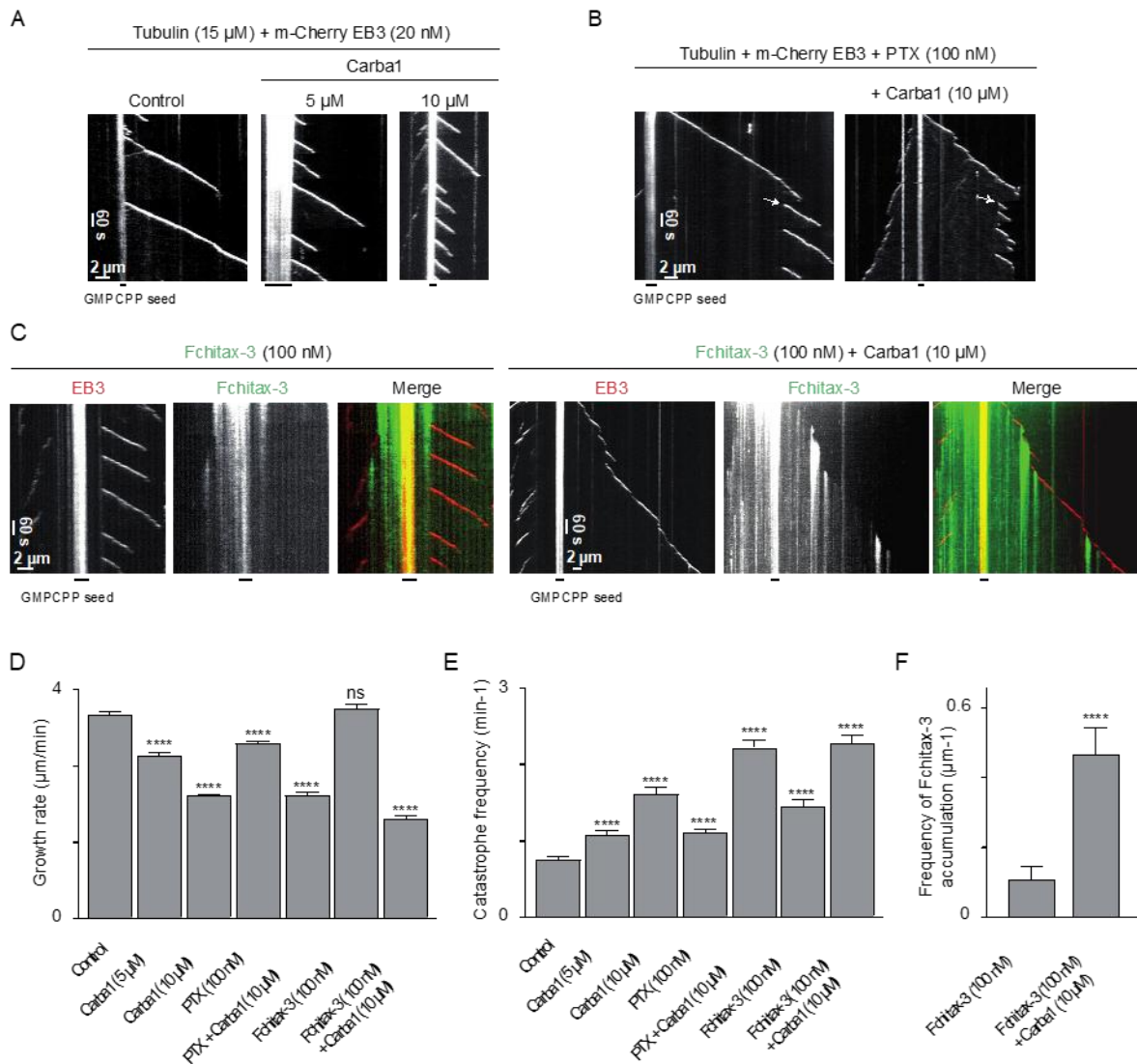
267 Carba1 doses induced a decrease in the rate of polymerization, as well as a delay in nucleation and
268 a reduction in the total quantity of assembled MTs, attested by the scaling down of the level of
269 assembly at equilibrium (**Figure 5B**). The concentration of Carba1, which inhibits 50% of tubulin (30
270 μM) assembly under these experimental conditions, was 6.9 μM .

271 We then looked for the binding site of Carba1 on tubulin. Among the four binding sites
272 described for MT depolymerizing agents, the most common binding site is the colchicine site [1].
273 We checked whether Carba1 could compete with [^3H]-colchicine for its tubulin binding-site (**Figure**
274 **5C**). Carba1 selectively inhibited colchicine binding to tubulin, indicating that it binds to tubulin at
275 or near the colchicine site. However, it did not completely prevent the binding of [^3H]-colchicine,
276 suggesting that its affinity for this site is lower than that of colchicine.

277 In order to measure the binding constant of the compounds, a competition assay with 2-
278 methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC), an analogue of colchicine
279 lacking the B ring that rapidly reaches an equilibrium ($K_b = 4.7 \times 10^5 \text{ M}^{-1}$, 25°C [21]) in its binding
280 reaction with tubulin, was designed. In the absence of tubulin the compound lacked fluorescence
281 (**Figure 5D**) while in the presence of tubulin an emission maxima at 423 nm was observed upon
282 excitation at 350 nm. As expected from its activity as an inhibitor of [^3H]-colchicine binding to
283 tubulin, Carba1 is able to displace MTC from the colchicine site, strongly supporting that Carba1
284 binds to the colchicine site of tubulin. The dissociation constant of Carba1, for the colchicine site is
285 $3.03 \pm 0.5 \times 10^{-6} \text{ mol L}^{-1}$ (**Figure 5E**). Altogether, these results show that Carba1 is a direct inhibitor of
286 MT polymerization.

287 2.6. Carba1 binding to tubulin enhances the tubulin binding capacity of PTX and its MT stabilizing activity

288 Recently, high resolution imaging of MT dynamics *in vitro* has demonstrated that fluorescent
289 PTX analogs strongly accumulate at MT ends that are transitioning from growth to
290 depolymerization, and, therefore, low non-saturating concentrations of a MT depolymerizing agent
291 such as vinblastine, which enhances catastrophes, promote taxane binding to growing MT tips [13].
292 Such a mechanism could explain the observed synergy between Carba1 and PTX. To test if this
293 mechanism is at work with Carba1, we first determined the Carba1 concentration able to induce
294 catastrophes. We used a Total Internal Reflection Fluorescence microscopy (TIRF)-based assay, in
295 which microtubules are grown from seeds stabilized with GMPCPP in the presence of microtubule
296 plus end marker EB3 (**Figure 6**) [22]. Similar to our previous findings with other MT-destabilizing
297 agents [22], we found that 10 μM Carba1 reduced MT growth rate and induced a two-fold increase
298 of the catastrophe frequency (**Figure 6A, D, E**). Catastrophe frequency increased even further when
299 Carba1 was combined with 100 nM PTX (**Figure 6B, D, E**), and moreover, we observed formation of
300 sites where MTs were repeatedly rescued, resulting in frequent switching between MT growth and
301 shortening (**Figure 6B**). As described recently, appearance of such “stable rescue sites” points to
302 formation of “hotspots” of enhanced taxane accumulation at MT ends transitioning to
303 catastrophe [13]. To confirm this idea, we visualized taxane binding to MTs by using a fluorescent
304 PTX derivative Fchitax-3. As expected, the combination of Carba1 with 100 nM Fchitax-3 induced
305 an increase of catastrophe frequency similar to the increase observed when Carba1 was combined
306 with PTX (**Figure 6C, E**). We found that Carba1 indeed increased the frequency of accumulations of
307 Fchitax-3, very similar to what we observed previously with vinblastine [13] (**Figure 6C, F**). This
308 result strongly suggests that the two drugs act synergistically because Carba1-induced catastrophe
309 induction leads to formation of “hotspots” of enhanced binding of PTX at growing MT ends during
310 their transition to catastrophe. The observed effect is thus not due to the binding of Carba1 and PTX
311 to the same tubulin dimer but rather due to the changes in MT lattice conformation which are
312 induced by Carba1 and promote local accumulation of PTX.



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Figure 6. Carba1 binding to tubulin enhances the tubulin binding capacity of PTX. (A, B, C) Representative kymographs illustrating MT plus end growth in the presence of 15 μ M tubulin and 20 nM m-Cherry EB3. (A) without (control) or with 5 and 10 μ M Carba1; (B) PTX (100 nM) without or in combination with 10 μ M Carba1. Arrows indicate repeatedly rescued sites; (C) Fchitax-3 (100 nM) without or with 10 μ M Carba1. (D, E) Parameters of MT plus end dynamics determined using acquisitions as shown in A, B and C (3 movies per condition, 2 independent experiments). The total number of analyzed growth events is 82 for 100 nM PTX, 72 for 100 nM PTX with 10 μ M Carba1 and 100 for all other conditions. Each value represents the mean \pm SD. Each condition was compared to the control condition using a Mann-Whitney analysis. (F) Quantification of Fchitax-3 accumulation frequencies per MT unit length in the presence of 15 μ M tubulin and 20 nM mCherry-EB3 without (n=11) or with 10 μ M Carba1 (n=13). Values represent the mean \pm SD. Both conditions were compared using a Mann-Whitney analysis. $p < 0.0001$ (****), not significantly different (ns).

2.7. Carba1 and PTX act synergistically to reduce tumor growth in vivo

330 Could the synergy between Carba1 and PTX, which we observed both at the level of individual
331 MTs and at the cellular level, be translated into a therapeutic anti-cancer effect? To address this
332 question, we compared the effects on tumor growth of Carba1 and PTX administrated separately to
333 the effect of administration of Carba1 in combination with PTX in two animal models with different
334 PTX sensitivity levels. We first evaluated the effect of the combination on the allogeneic 4T1-rvLuc2
335 mouse mammary carcinoma model. This model is known to be poorly sensitive to PTX and we
336 hypothesized that the co-administration of Carba1 could enhance PTX sensitivity. Before starting
337 the experiments on animals, we evaluated if Carba1 also exerts a synergy with PTX on the 4T1 cell
338 line *in vitro* (**Figures S4A and S5**). The IC₅₀ of PTX alone on the viability of these cells was 90 nM
339 and was decreased by 1.8 times in the presence of Carba1 at 12 μM and by 9 times in combination
340 with Carba1 at 25 μM.

341 Tumor allografts were then established by implantation of 4T1 tumor cells into the mammary
342 fat pad of nude mice. The mice were randomly assigned to five treatment groups: vehicle, Carba1
343 30 mg/kg, PTX 2 mg/kg, Carba1 30 mg/kg plus PTX 2 mg/kg, and PTX 8 mg/kg and the drugs were
344 then daily injected intraperitoneally.

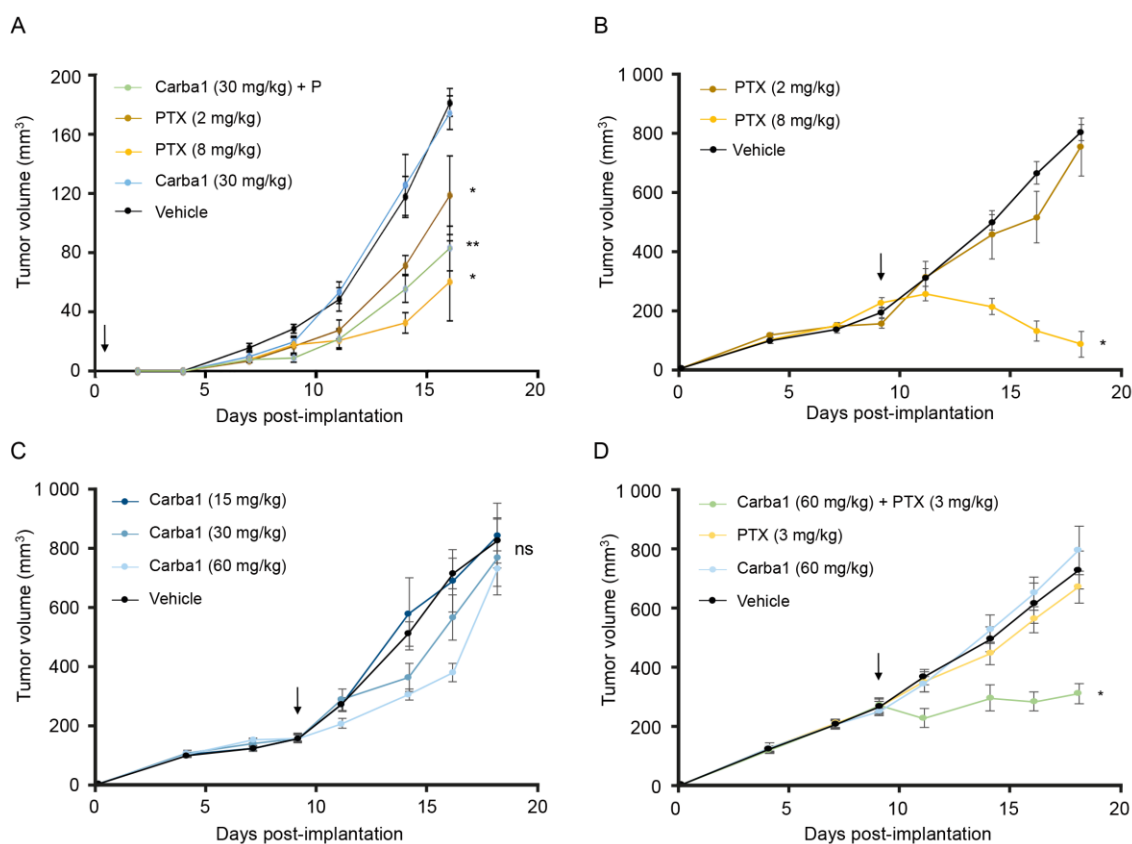
345 From 14 days of treatment, although the body weight of the animals was not significantly
346 altered (**Figure S6A**), some of the mice treated with PTX at 8 mg/kg (three out of eight) died or
347 presented signs of suffering (prostration and abdominal distension) that require their killing for
348 ethical reasons. Autopsy revealed that intestinal occlusion was the cause of those trouble and
349 deaths. Interestingly, no death or signs of suffering were observed in the Carba1 and Carba1 plus
350 PTX 2 mg/kg groups.

351 Tumor growth is presented in **Figure 7A**, and shows that whereas Carba1 30 mg/kg has no
352 effect when administrated alone, PTX (2 mg/kg), PTX (8 mg/kg) as well as the combination Carba1
353 30 mg/kg plus PTX 2 mg/kg induced a significant decrease of the tumor volumes, when compared
354 to vehicle-treated animals. However, although we observed a greater reduction in tumor growth
355 after the Carba1/PTX combination treatment than after the exposure to PTX alone, this difference
356 remains non-significant. We also analyzed the tumor cell viability using *in vivo* bioluminescence
357 imaging with similar results (**Figure S6B, C**).

358 Although these results suggest that Carba1 can synergize with PTX, neither the combination of
359 Carba1 (30 mg/kg) + PTX (2 mg/kg) nor the highest dose of PTX alone (8 mg/kg daily
360 administration) could break the tumor growth curve.

361 We thus concentrated our efforts on the fully PTX-sensitive HeLa tumor model whose
362 response to high-dose PTX treatment is complete but concomitant to adverse effects that would be
363 desirable to reduce without losing therapeutic effectiveness.

364 In a first series of experiments, we analyzed the effect of increasing doses of PTX or Carba1
365 when administered alone. To that aim, mice bearing already well-established tumors (>250 mm³)
366 received intravenous (i.v.) injections of PTX (from 2 or 8 mg/kg), every two days during 10 days
367 (**Figure 7B**). In the same experiment, we analyzed the effect of Carba1 (from 15 to 60 mg/kg, i.v.)
368 injected with the same schedule (**Figure 7C**). We found that PTX, when administered at 8 mg/kg,
369 induced a significant reduction of tumor size (**Figure 7B**). Carba1 did not induce a significant effect
370 on tumor size whatever the dose injected, although a tendency towards smaller tumors appears
371 with increasing Carba1 concentrations (**Figure 7C**). The results confirmed the anti-tumor effect of
372 high PTX concentrations in this model. They also indicate that Carba1, when applied alone, has no
373 significant anti-tumor activity, even at high concentrations. As shown in **Figure S6B** the weight of
374 PTX or Carba1 treated animals and vehicle-treated animals were not significantly different.
375 Moreover the animals did not show any sign of discomfort, suggesting a good tolerance of the
376 treatments.

Figure 7. Carba1 acts synergistically with PTX to reduce tumor growth *in vivo*

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Figure 7. Carba1 acts synergistically with PTX to reduce tumor growth *in vivo*. (A) Exploratory analysis of the effect of PTX, Carba1 and their combination on the growth of 4T1 cells allografted in mice. Mice were treated with PTX (2 and 8 mg/kg), Carba1 (30 mg/kg), the combination of PTX (2 mg/kg) and Carba1 (30 mg/kg) or the vehicle. Tumor growth was monitored with a sliding caliper. Data represent a median. Error bars = MAD, * $p < 0.05$, ** $p < 0.01$ compared to vehicle (Kruskal Wallis), $n = 8$ mice per group. (B) PTX inhibits the growth of HeLa cells xenografted in mice. When the tumors have reached a volume of about 200 mm³, mice were treated with PTX (2 and 8 mg/kg) or the vehicle. Tumor growth was monitored with a sliding caliper. Error bars = SEM, * $p < 0.01$ compared to vehicle (ANOVA), $n = 6$ mice per group. (C) Carba1 has no significant effect on the growth of HeLa cells xenografted in mice. When the tumors have reached a volume of about 200 mm³, mice were treated with Carba1 (15, 30 and 60 mg/kg) or the vehicle. Tumor growth was monitored with a sliding caliper. Error bars = SEM, ns = non-significant (ANOVA), $n = 6$ mice per group. (D) The combination of otherwise ineffective doses of Carba1 and PTX inhibits the growth of HeLa cells xenografted in mice. When the tumors have reached a volume of about 200 mm³, mice were treated with PTX (3 mg/kg), Carba1 (60 mg/kg), the vehicle or the combination of PTX (3 mg/kg) and Carba1 (60 mg/kg). Tumor growth was monitored with a sliding caliper. Error bars = SEM. * $p < 0.001$ compared vehicle (t-test), $n = 8$ mice per group. Arrows indicate the onset of the treatments.

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We then conducted a study of the effect on tumor size of a low PTX dose (3 mg/kg) in combination with Carba1 (60 mg/kg). No animal death and no modification of body weight were observed throughout the study (**Figure S6C**). In this experiment neither PTX (3 mg/kg), nor Carba1 (60 mg/kg) has an effect on tumor size (**Figure 7D**). As shown in **Figure 7D**, while the size of tumors still increases when each compound is administered separately, an arrest of tumor growth is observed with the combination of PTX and Carba1. These results indicate that the observed synergy

402 between PTX and Carba1 *in vitro* also occurs *in vivo*, leading to an enhanced therapeutic efficacy at a
403 low-dose of PTX treatments.

404 3. Discussion

405 Our initial aim was to discover an agent that would allow lowering the dose of PTX while
406 obtaining the same anti-tumor efficacy as the currently used therapeutic dose of PTX. We thus
407 screened a chemical library to detect compounds able to sensitize cells to a low, non-toxic dose of
408 PTX. The test we used was a cytotoxicity test, therefore probing all vital cell functions. Whereas
409 such a whole cell-based assay screens molecules having multiple potential targets and allows the
410 biology to dictate the best targets [23], it may not be insignificant to have selected Carba1, an agent
411 that targets tubulin and impairs MT dynamics. Indeed, this indicates that the most sensible target,
412 in this specific context, is tubulin.

413 Recently, a series of carbazole-based MT targeting agents has been reported [24]. These acyl-
414 substituted derivatives, conceived as analogs of nocodazole represent other examples of carbazole
415 scaffolds able to interact with the colchicine site of tubulin. Unlike Carba1, they exert potent killing
416 activities in human glioblastoma cells. A possible explanation for the difference in cytotoxicity
417 observed between Carba1 and the compounds described by Diaz et al. [24] may reside in a lower
418 affinity of Carba1 for the colchicine binding site of the tubulin dimer. Indeed, we found that the
419 affinity of Carba1 for the colchicine site is not very high, in the micromolar range. This difference in
420 affinity could be due to the presence of the pyrrol ring on Carba1, which could generate steric
421 hindrance and decrease the affinity of the compound for the colchicine site. In addition, the work of
422 Diaz et al. emphasizes the importance of substitutions at the level of the nitrogen atom of the
423 carbazole moiety. Such substitutions are absent from Carba1 and thus may impact binding of the
424 compound to the colchicine site.

425 The Carba1 scaffold is a versatile one and we are currently synthesizing modified analogs for
426 medicinal chemistry optimization.

427 The PTX binding site at the interior of the MT has been characterized at the atomic level: PTX
428 binds to a pocket in β -tubulin that faces the MT lumen and is near the lateral interface between
429 protofilaments (for review see [1]). The binding of PTX results in the expansion of the taxane
430 binding pocket [25] of the tubulin dimer. Moreover PTX binding inhibits, in the protofilament, the
431 compaction at the longitudinal interdimer interface, induced by GTP hydrolysis [26]. This allosteric
432 mechanism would strengthen the longitudinal tubulin contacts leading to a stabilization of the MTs
433 [1]. In this context, it is conceptually counterintuitive that an agent that depolymerizes MTs acts in
434 synergy with PTX, an agent that stabilizes them.

435 A possibility is that the binding of Carba1 to the tubulin dimer modifies its affinity for PTX.
436 However, although it has been shown that the covalent occupancy of the taxane site can affect the
437 structure of the colchicine site [27], the reverse has not yet been described. Moreover, in cells, due to
438 the low affinity of Carba1 for tubulin and the nanomolar concentration of PTX that was used, it can
439 be assumed that the probability that a single tubulin dimer has both a molecule of Carba1 and
440 another of PTX bound is very low. Thus an allosteric effect at the level of the tubulin dimer, due to
441 such a simultaneous binding, cannot be responsible for synergistic cytotoxicity.

442 Another possibility is that the binding of Carba1 can induce conformational changes of the
443 growing MT ends that can facilitate the subsequent binding of PTX to the MT lattice. Recently,
444 using TIRF analysis, it has been shown that non-saturating doses of vinblastine induce a switch to
445 catastrophe and convert the MT plus end to a state that allows more efficient taxane accumulation
446 [13]. Indeed, we have conducted the same type of experiment, replacing vinblastine with Carba1
447 and observed an increase in the rate of catastrophes associated with more frequent formation of
448 accumulation “hotspots” of fluorescent taxane. Moreover, we observed a synergistic effect of
449 Carba1 and PTX on MT growth inhibition *in vitro*. Although the underlying structural mechanism is
450 yet unknown, it is highly probable that Carba1 acts similarly to vinblastine to facilitate PTX

451 accumulation. Our experimental *in vitro* data thus strongly suggest that Carba1 acts by modifying
452 the growing tip of microtubules, favoring the accumulation of PTX.

453 It is known that PTX accumulates intracellularly [4], reaching intra-tumor concentrations that
454 are higher in the tumors than in the plasma [7]. It is thus remarkable that the synergistic effect is
455 observed not only at the MT level, but also at the cellular level, as well as when both drugs are
456 applied systemically in animals to exert their anti-tumor action. Although the most probable
457 hypothesis is that the same molecular mechanism is at work in these different contexts, it cannot be
458 excluded that Carba1 has other target(s). For instance Carba1 could also inhibit a drug export pump
459 and further facilitate PTX accumulation.

460 We observed a different anti-tumor response in the two models used. In one case (allogeneic
461 orthotopic 4T1 cell transplant model) the difference in tumor size observed between PTX alone and
462 Carba1/PTX combination therapy was not significant. In the other case (xenograft of HeLa cells), a
463 synergistic effect is clearly demonstrated.

464 We assume that these different tumor responses reflect the sensitivity of these cells to PTX *in*
465 *vitro* (PTX GI50 is 1.5 nM in HeLa cells and 90 nM in 4T1 cells). More trivially, differences in
466 response between the two models may also result from the protocols used, particularly with respect
467 to the injection route - i.p. (4T1) versus i.v. (HeLa) - and the frequency of injections - every day (4T1)
468 versus every other day (HeLa). Nor can we rule out the possibility that the combination is more
469 effective on certain tumor types. A thorough study of the effect of the combination on different cell
470 lines, such as the one performed for Carba 1 alone in the NCI60 screen, will probably answer this
471 question.

472
473 These last years, attention has been directed to the combined use of therapeutic agents to target
474 critical cellular pathways involved in carcinogenesis. Accordingly, several small molecules have
475 been reported to synergize with PTX to kill cancer cells. For instance, combined administration of
476 the src inhibitor dasatinib and PTX has a synergistic antiproliferative activity on different cancer cell
477 lines [28,29]. Similarly, aberrations in the PI3K/AKT/mTOR pathway are commonly described in
478 aggressive cancers [30] and inhibitors of this pathway can improve outcomes in some patients when
479 combined with paclitaxel [30–32]. We cannot completely exclude that Carba1 exerts its synergistic
480 effect by additionally targeting a pathway involved in cell survival. However, it is less probable that
481 it targets a survival pathway involving some kinases as we have tested Carba1 potential inhibitory
482 activity on a panel of 64 kinases, including src and mTOR, and found that Carba1 did not exhibit
483 any selective activity on the assayed kinases.

484 Because the combined administration of Carba1 and a low dose of PTX can have an anti-tumor
485 effect, one could imagine that this combination should reduce the unwanted side effects observed
486 with high doses of PTX. This has to be tested. For instance the effect of the combination should be
487 compared to the PTX effect on the kinesin-based anterograde transport, since perturbation upon
488 PTX treatment is thought to be part of the mechanism involved in peripheral neuropathy [12].
489 Given the mode of action we have described, with Carba1 facilitating the accumulation of PTX in
490 MTs, we can bet that the combination should diminish MT-independent adverse events.
491 Interestingly, it has been shown that a compound sharing the same carbazole scaffold protects
492 against PTX-induced peripheral neuropathy [33].

493 Anti-cancer strategies based on the concomitant administration of taxanes and depolymerizing
494 agents such as vinorelbine have been reported [34–36]. However, these approaches used high doses
495 of each of these drugs. Our results suggest that good therapeutic efficacy could be achieved with
496 the combined administration of each of these agents at low doses, which could improve patient
497 comfort.

498 In addition, reducing the doses of taxane administered to patients could delay the onset of
499 acquired resistances. This work thus paves the way to new therapeutic perspectives that are easy to
500 implement.

501 4. Materials and methods

502 4.1. Chemical Reagents and cells

503 The chemical library that was used for the initial screening is part of the collection assembled
504 by the CERMN (Centre d'Études et de Recherche sur le Médicament de Normandie, University of
505 Caen). This collection is composed of original compounds (> 19 000), synthesized within the frame
506 of the numerous drug design programs the unit has developed since forty years. These compounds
507 are mainly small molecules, often including heteroelements and heterocycles, they basically obey to
508 the Lipinski's rules of five and are considered as druggable. The 8 000 compounds screened have
509 been selected from this chemical library using a clustering method in order to adapt the size of the
510 panel to the capacity of the screening, while respecting the structural diversity of the collection.

511
512
513 Carba1 was re-synthesized at the CERMN and supplied as a powder. It was dissolved in
514 anhydrous dimethyl sulfoxide (DMSO, Sigma, #D4540) and stored at -20°C as 10 mM stock
515 solution. Paclitaxel (PTX) was purchased from Sigma (#T7402) and was dissolved in DMSO and
516 stored at -20°C as 1 mM stock solution.

517 The human HeLa and RPE-1 cell lines and the murine 4T1 cell line were obtained from the
518 American Type Culture Collection (ATCC), routinely tested and authenticated by the ATCC. HeLa
519 Kyoto cells expressing EGFP-alpha-tubulin and H2B-mcherry were from Cell Lines Service,
520 #300670. HeLa cells and 4T1 cells were grown in RPMI 1640 medium (Gibco, Invitrogen) and RPE-1
521 cells were grown in DMEM (Gibco, Invitrogen), supplemented with 1% penicillin/streptomycin and
522 10% Fetal Bovine Serum, and maintained in a humid incubator at 37°C in 5% CO₂.

523 4.2. Analysis of cell viability using MTT (screening of the chemical library)

524 The assay was performed in 96-well microplates. Cells were seeded at a density of 2 500 cells
525 per well and allowed to adhere for 24 hours before being treated for 48 hours with either DMSO
526 (0.1 % final concentration) or compounds at 5 µM, with or without 1 nM PTX. Viability was
527 evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric
528 assay (Sigma, #M5655).

529 4.3. Analysis of cell viability using Prestoblue assay

530 Cell viability was analyzed using the colorimetric Prestoblue assay (Invitrogen, #A13262). Cells
531 were seeded in 96-well microplates (Greiner, #655077) at a density of 2 500 cells per well and
532 allowed to adhere for 24 hours before being treated for 72 hours with either DMSO (0.1 % final
533 concentration) or drugs at indicated concentrations. After a 72-hour treatment, 11 µL Prestoblue
534 was added to each well and cells were incubated for another 45 minutes. The absorbance of each
535 well was measured using a FLUOstar Optima microplate reader (Excitation, 544 nm; Emission, 580
536 nm).

537 4.4. Apoptosis assay

538 The apoptosis assay was performed with FITC Annexin V Apoptosis Detection Kit I (BD
539 Biosciences, #556547) using flow cytometry and analyzed by FCS express software.

540 4.5. Drug Combination Analysis

541 The Chou–Talalay analysis on the basis of dose–response curves was used to evaluate the
542 synergism, additivity and antagonism of the combination drug treatment. Combination index (CI)
543 values were calculated using the CompuSyn software (ComboSyn Iw. combosyn software. 2014)
544 which uses the equation:

$$545 CI = CA_{,x} / SA_{,x} + CB_{,x} / SB_{,x}$$

546 Where CA_x and CB_x are the concentrations of drug A and drug B in the combination to
547 produce a certain effect x . SA_x and SB_x are the concentrations of drug A and drug B used as a
548 single agent to produce that same effect. CompuSyn also generates a plot of CI values at different
549 fraction affected (Fa) levels referred to as Fa- CI plot, which are widely used to interpret drug
550 combination effects [37]. A CI value of <0.1 indicates very strong synergism, $0.1-0.3$ strong
551 synergism, $0.3-0.7$ synergism, $0.7-0.9$ moderate to slight synergism, 1 , additivity, $1.1-1.45$ slight to
552 moderate antagonism, $1.45-3.3$ antagonism, and >3.3 strong to very strong antagonism.
553

554 4.6. Cell cycle analysis

555 Cells were harvested and washed by centrifugation in PBS. Then, 10^5 cells were fixed in 1 mL
556 of 70% ethanol at 4°C overnight. Following two washes with PBS the cells were incubated with 50
557 $\mu\text{g/mL}$ propidium iodide and 0.2 mg/mL RNase A (Sigma, #10109142001) / PBS for 30 minutes at
558 37°C before analysis. The percentage of cells in the specific cell-cycle phases (G0, G1, S, G2, and M)
559 was determined using an Accuri C6 flow cytometer (Becton Dickinson).

560 4.7. Analysis of Carba1 effect on kinases

561 The analysis of Carba1 effect was performed on a panel of 64 recombinant protein kinases. The
562 assays were performed at $10\ \mu\text{M}$ ATP in the presence of $10\ \mu\text{M}$ Carba1 using the Upstate Kinase
563 profiler panel service (Millipore). Inhibition, expressed as the percent of activity determined in the
564 absence of inhibitor, was calculated from the residual activity measured in the presence of $10\ \mu\text{M}$
565 Carba1.
566

567 4.8. Immunofluorescence microscopy and live cell imaging

568 HeLa cells at a density of 20 000 cells were grown for 48 hours on glass coverslips placed in a
569 24-well microplate. When cells reached 70% confluence the medium was replaced with a fresh one
570 supplemented with Carba1. After a 5-hour exposure to Carba1, cells were fixed and permeabilized
571 with -20°C absolute methanol for 6 minutes. After washing and saturation with 3% BSA (Bovine
572 Serum Albumin; Sigma, #A7906) / PBS (Phosphate Buffered Saline; Dutscher, #L0615-500), cells
573 were incubated for 45 minutes at room temperature (RT) with anti-alpha-tubulin antibody (clone
574 $\alpha 3A1$, 1:4000), produced by L. Lafanechère [38]. Cells were washed twice again and subsequently
575 incubated with Alexa 488 conjugated anti-mouse antibody (1:1 000, Jackson immunoresearch, #115-
576 545-4637) for 30 minutes at RT. DNA was stained with $20\ \mu\text{M}$ Hoechst 33342 (Sigma, #23491-52-3)
577 and coverslips were mounted on glass slides with Mowiol 4-88 (Calbiochem, #475904). Images were
578 captured with a Zeiss AxioimagerM2 microscope equipped with the acquisition software
579 AxioVision and analyzed using the Fiji software. For live-cell imaging, HeLa Kyoto cells expressing
580 EGFP-alpha-tubulin and H2B-mcherry were seeded on 2-well glass-slides (Ibidi, #80297) at a
581 density of 7 000 cells per well and allowed to grow for 24 hours prior to imaging. After treatment,
582 the slide was placed on a 37°C heated stage, at 5% CO_2 , and images were acquired every 2.5
583 minutes by a spinning disk confocal laser microscope (Andromeda iMIC) equipped with a Plan-
584 Apochromat $20\times/0.75\ \text{WD}610$ objective and an EMCCD camera (iXon 897). For each time point, a
585 stack of 7 planes (thickness: $1\ \mu\text{m}$) was recorded. Acquisition (LA), off-line analysis (OA) and Fiji
586 software programs were used.

587 4.9. Transfection of GFP-EB3

588 To label MT plus ends, GFP-EB3 plasmids were used because EB3 has a strong binding affinity
589 to MT plus ends. Cell transfection was performed using electroporation (AMAXA®, Köln,
590 Germany). $2\ \mu\text{g}$ of purified plasmid DNA were used for each transfection reaction.

591 4.10. Fluorescence time-lapse videomicroscopy of MT plus ends

592 Live imaging of MT plus ends was performed as described in Honoré et al. [39], on transiently
593 GFP-EB3 transfected-HeLa cells by using an inverted fluorescence microscope (ZEISS Axiovert
594 200M with a 63X objective). Time-lapse acquisition was performed with a COOLSNAP HQ (Roper
595 Scientific), driven by Metamorph software (Universal Imaging Corp.). Image acquisition was
596 performed at a temperature of $37 \pm 1^\circ\text{C}$ / 5% CO_2

597 Data are from 3 independent experiments. For each experiment, 6 MTs/cell in 6 cells per
598 condition were analyzed.

599 4.11. Dynamic instability parameter analysis

600 The dynamic instability parameter analysis was performed by tracking MT plus ends over
601 time using the imageJ software. The methods of calculation were as described in Honoré et al. [39].

602 4.12. Tubulin Polymerization Assay

603 Tubulin was prepared from bovine brain as previously described [40]. Tubulin polymerization
604 assays were carried out at 37°C in BRB80 buffer (80 mM Pipes, 0.5 mM MgCl_2 , 2 mM EGTA, 0.1 mM
605 EDTA, pH 6.8 with KOH) by mixing $7 \mu\text{M}$ of pure tubulin, 1 mM GTP, 5 mM MgCl_2 and indicated
606 concentrations of drugs (0.2% DMSO, final concentration) in a final volume of $100 \mu\text{L}$. The time
607 course of the self-assembly activity of tubulin was monitored as turbidity at 350 nm, 37°C , during
608 30 minutes, using a spectrophotometer (ThermoScientific, Evolution 201).

609 4.13. [^3H]-Colchicine Tubulin-Binding Assay

610 The tubulin was prepared from bovine brain as previously described [40]. Pure tubulin ($3 \mu\text{M}$
611 final concentration) in cold BRB80 buffer was mixed at 4°C with a mix of [^3H]-colchicine (82.6
612 Ci/mmol, Perkin-Elmer, #NET189250UC, 50 nM final concentration) and the competitor Carba1 (100
613 μM final concentration) in a final volume of $200 \mu\text{L}$. Following a 30-minute incubation at 30°C , the
614 samples were deposited onto $50 \mu\text{L}$ of presedimented DEAE Sephadex A25 in BRB80 buffer. All
615 subsequent steps were carried out at 4°C . Samples were incubated for 10 minutes with continuous
616 shaking to ensure quantitative binding of tubulin to the gel. Following centrifugation (2 400g, 4
617 minutes), supernatants were discarded and the pellets containing the bound molecule-tubulin
618 complexes were washed four times with 1 mL of BRB80 buffer. Pellets were incubated for 10
619 minutes with $500 \mu\text{L}$ of ethanol to solubilize the tubulin-bound tritiated colchicine and $400 \mu\text{L}$
620 aliquots of the ethanol solutions were transferred to 5 mL of Ultima Gold scintillant (Perkin-Elmer)
621 for determination of radioactivity.

622 4.14. Determination of the binding constant of Carba1 on tubulin using a competition assay

623 Calf brain tubulin was purified as described [41]. 2-Methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-
624 cycloheptatrien-1-one (MTC)[42] was a kind gift of Prof. T. J. Fitzgerald (School of Pharmacy,
625 Florida A & M University, Tallahassee, FL). The compounds were diluted in 99.8% DMSO-d₆
626 (Merck, Darmstadt, Germany) to a final concentration of 10 mM and stored at -80°C .

627 Competition of the compound with MTC was tested by the change in the intensity of
628 fluorescence of MTC upon binding to tubulin. The fluorescence emission spectra (excitation at 350
629 nm) of $10 \mu\text{M}$ tubulin and $10 \mu\text{M}$ MTC in 10 mM sodium phosphate, 0.1 mM GTP, pH 7.0, were
630 measured in the presence of different concentrations (0 to $20 \mu\text{M}$) of the desired ligand with 5 nm
631 excitation and emission slits using a Jobin-Yvon SPEX Fluoromax-2 (HORIBA, Ltd., Kyoto, Japan).
632 The decrease in the intensity of the fluorescence in the presence of the competitor ligand indicated
633 competition for the same binding site. The data were analyzed and the binding constants
634 determined using Equigra V5.0 as described in Díaz and Buey [43].

635 4.15. In vitro MT dynamics and analysis of MT dynamics parameters

636 *In vitro* assay for MT growth dynamics and analysis of MT dynamic parameters in the presence
637 of tubulin (Cytoskeleton Inc.) and EB3 with Carba1 and Fchitax-3 was performed as described
638 previously[13]. For statistical analysis, graphs were plotted in GraphPad Prism 7 and statistical
639 analysis was done using non-parametric Mann-Whitney U-test.

640 4.16. Tumor xenografts in mice

641 All animal studies were performed in accordance with the institutional guidelines of the
642 European Community (EU Directive 2010/63/EU) for the use of experimental animals and were
643 authorized by the French Ministry of Higher Education and Research under the reference:
644 apafis#8854-2017031314338357 v1.

645 In a first exploratory experiment, the effects of PTX and/or Carba1 were evaluated on the
646 allogeneic 4T1-rvLuc2 orthotopic mammary carcinoma model. Five-week-old female NMRI nude
647 mice (Janvier Labs, Le Genest-Saint Isle, France) were anesthetized (4% isoflurane/air for anesthesia
648 induction and 1.5% thereafter) and 20 000 4T1 cells in 1X PBS were implanted into the mammary fat
649 pad. Mice were then randomized in 5 groups of 8 mice each and drugs were administered
650 intraperitoneally every day. A first group received the vehicle (14% DMSO, 14% Tween 80 and 72%
651 PBS). Two groups received PTX at different doses (2 and 8 mg/kg) while one other group received
652 Carba1 at 30 mg/kg. A last group received a combination of Carba1 (30 mg/kg) and PTX (2 mg/kg).
653 Tumor size was measured every two days, using a caliper, and the tumor volume was calculated as
654 follows: length \times (width)² \times 0.4. At the end of experiment, 300 μ L of D-luciferin (Promega, 10 mg/mL
655 in PBS) was injected intraperitoneally and the tumor cell viability was evaluated using *in vivo*
656 bioluminescence imaging (IVIS Kinetic, Perkinelmer). Living Image software (Perkinelmer) was
657 used to analyze the results. Kruskal-Wallis test was used to compare the effects of the treatments on
658 tumor size.

659 In a second series of experiment, the effects of PTX and/or Carba1 were evaluated on a tumor
660 model based on HeLa cells transplantation. First, the effects of PTX or Carba1 when administrated
661 alone were evaluated. To that aim, five-week-old female NMRI nude mice (Janvier Labs, Le Genest-
662 Saint Isle, France) were anesthetized (4% isoflurane/air for anesthesia induction and 1.5% thereafter)
663 and were injected subcutaneously in the flank with 10⁷ exponentially dividing HeLa cells in 1X PBS.
664 Tumor size was measured three times a week using a caliper. When tumors have reached a volume
665 of about 250 mm³ i.e. nine days after cell injection, mice were randomized in 7 groups of 6 mice
666 each and drugs were injected intravenously every two days. A first group received the vehicle (14%
667 DMSO, 14% Tween 80 and 72% PBS). Three groups received PTX at different doses (2, 4 and 8
668 mg/kg) while three other groups received Carba1 at different doses (15, 30 and 60 mg/kg). Statistical
669 comparison between mice groups were determined using two-way ANOVA.

670 Then, the effect of a combination of PTX- Carba1 was evaluated, and compared to the effect of
671 the compounds alone. To that aim, five-week-old female NMRI nude mice were injected
672 subcutaneously with 10⁷ exponentially dividing HeLa cells into the right flank. When tumors have
673 reached a volume of about 200 mm³ i.e. nine days after cell injection, mice were randomized in 4
674 groups of 8 mice each and drugs were injected intravenously every two days. The first group
675 received PTX at 3 mg/kg, the second group Carba1 at 60 mg/kg, the third group received a
676 combination of Carba1 (60 mg/kg) and PTX (3 mg/kg), and the fourth group received the vehicle
677 (14% DMSO, 14% Tween 80 and 72% PBS). For statistical analysis, we verified the normality of the
678 data using a Shapiro test and the homogeneity of the variances using a Bartlett test. We then used a
679 t-test to compare the different groups to the control (i.e. vehicle). We found that the p values of the
680 comparisons of the group treated with PTX (3 mg/kg) and of the group treated with Carba1 (60
681 mg/kg) with the vehicle were respectively 0.5355 and 0.5139. These p values are greater than 0.05,
682 indicating that these groups are not different from the vehicle group at the risk beta calculated
683 greater than 90 % (power of the test lower than 10%). On the contrary, the comparison of the group
684 treated with the combination to the control group gave a p value of 0.00015, indicating that the
685 combination has an effect on the tumor size, at the risk alpha of 5 %.

686

687 **5. Conclusion**

688 A new mechanism favoring paclitaxel binding to dynamic microtubules can be transposed to
689 *in vivo* mouse cancer treatments, paving the way for new therapeutic strategies combining low
690 doses of microtubule targeting agents with opposite mechanisms of action.

691

692 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure S1: Effect of
693 PTX on the viability of HeLa cells, Figure S2: Combination index (CI) values as a function of Fraction affected
694 (Fa) in HeLa cells exposed to combination drug treatment, Figure S3: Effect of Carba1/PTX combination on the
695 viability of RPE-1 cells, Figure S4: Effect of Carba1/PTX combination on 4T1 cells *in vitro* and *in vivo*, Figure S5:
696 Figure S5: Combination index (CI) values as a function of Fraction affected (Fa) in 4T1 cells exposed to
697 combination drug treatment, Figure S6: Weight and survival curves of mice treated with PTX, Carba1 and the
698 Carba1/PTX combination, Table S1: Analysis of the effect of PTX (1 nM) and Carba1 (25 µM) on microtubule
699 dynamics in cells, Table S2: Small molecule screening data, Table S3: Carbazole-related compounds and their
700 effect on HeLa cell viability, Table S4: Carba1 moderately inhibits the proliferation of cancer cells included in
701 the NCI-60 panel, Table S5: Quantification of the fate of HeLa cells treated with Carba1 and/or PTX, Table S6:
702 Profile of kinase inhibitory activity of Carba1, Movie S1: Carba1 induces a delay in mitosis, Movie S2: Carba1
703 blocks cells in mitosis and induces mitotic catastrophe, Movie S3: Effects of PTX (1 nM and 5 nM) and of the
704 combination of Carba1 and PTX (12 µM/1 nM) on HeLa Kyoto cell mitosis.

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707 Lafanechère; Investigation, Lauralie Peronne, Ankit Rai, Audrey Vernet, Sacnicte Ramirez-Rios, Sophie
708 Michallet, Mélanie Guidetti, Julien Vollaire, Daniel Lucena-Agell and Anne-Sophie Ribba; Methodology, Eric
709 Denarier and Julien Vollaire; Resources, Peggy Suzanne, Patrick Dallemagne and Annie Andrieux;
710 Supervision, Eric Denarier, Véronique Jossierand, Jean-Luc Coll, Patrick Dallemagne, Jose-Fernando Diaz,
711 Maria Angela Oliva, Anna Akhmanova, Annie Andrieux and Laurence Lafanechère; Visualization, Lauralie
712 Peronne, Ankit Rai and Daniel Lucena-Agell; Writing – original draft, Lauralie Peronne, Eric Denarier, Karin
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