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A New Quantitative Cell-Based Assay Reveals Unexpected Microtubule Stabilizing Activity of Certain Kinase Inhibitors, Clinically Approved or in the Process of Approval

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1 **A new quantitative cell-based assay reveals unexpected**
2 **microtubule stabilizing activity of certain kinase inhibitors,**
3 **clinically approved or in the process of approval**

4
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33 **Abstract**

34 Agents able to modify microtubule dynamics are important anticancer drugs. The absence of
35 microtubules resulting from drug-induced depolymerization is easy to detect. However the
36 detection of a stabilized microtubule network needs specific assays since there is not a
37 significant visual difference between normal and stabilized microtubule networks. Here, we
38 describe a quantitative cell-based assay, suitable for automation, which allows the detection of
39 stabilized microtubules without the need of microscopic examination. The rationale of this
40 assay is based on the drug-induced resistance of the microtubule network to the
41 depolymerizing agent combretastatin A4 and the subsequent detection of the residual
42 microtubules by immunoluminescence. Using this assay to screen a kinase inhibitor library
43 allowed the selection of seven known kinase inhibitors: selonsertib, masatinib, intedanib,
44 PF0477736, SNS-314 mesylate, MPI0479605, and ponatinib. The yet undescribed ability of
45 these inhibitors to stabilize cellular microtubules was confirmed using additional markers of
46 stable microtubules and time-lapse video-microscopy to track individual microtubules in
47 living cells. None of the compounds interacted, however, directly with tubulin. By employing
48 other inhibitors of the same kinases, which have structurally unrelated scaffolds, we
49 determined if the microtubule stabilizing effect was due to the inhibition of the targeted
50 kinase, or to an off-target effect. Many of these inhibitors are clinically approved or currently
51 assayed in phase 2 or phase 3 clinical trials. Their microtubule-stabilizing effect may account
52 for their therapeutic effect as well as for some of their adverse side effects. These results
53 indicate also a possible repurposing of some of these drugs.

54
55 **Keywords:** Cell-based assay; Microtubule stabilization; Kinase inhibitors;
56 Polypharmacology, Microtubule dynamics

57 58 **Introduction**

59 Pharmacological treatments used in cancer chemotherapy generally fall into two main classes:
60 cytotoxic drugs and targeted therapies (Winkler et al., 2014). Among drugs targeting specific
61 pathways, kinase inhibitors have played an increasingly prominent role. Currently, more than
62 25 oncology drugs that target kinases have been approved, and several additional kinase
63 inhibitors are in various stages of clinical evaluation. However, such drugs often
64 serendipitously display widely varying target profiles beyond their intended targets. While
65 these "off" targets are often either unknown or disregarded, they confer an inherent potential
66 for polypharmacology applications. Notably, phenotypic screening approaches have found
67 that some kinase inhibitors show antitumor activity unrelated to inhibition of their cognate
68 targets, for which the underlying mechanism of action is therefore not apparent but likely
69 involves one or more non-canonical targets (Remsing Rix et al., 2014).

70 Among cytotoxic drugs, microtubule-targeting agents belong to one of the oldest and most
71 diverse family of anticancer agents. Microtubules (MTs) are cylindrical polymers of α - and β -
72 tubulin heterodimers. These polymers are highly dynamic and their polymerization dynamics
73 is tightly regulated through the binding of microtubule-associated proteins (MAPs). Both
74 MAPs and tubulin are further subjected to regulatory post-translational modifications.
75 Microtubule-targeting agents act primarily by altering MT dynamics. They are roughly
76 classified into microtubule-stabilizing agents, such as taxanes or epothilones, and
77 microtubule-destabilizing agents, such as vinca alkaloids, [combretastatin](#) and [colchicine](#). The
78 [vinca alkaloids bind to a site of \$\beta\$ -tubulin located at the interface between two tubulin dimers](#)
79 [\(Gigant et al., 2005\)](#), whereas [colchicine binds to a site of \$\beta\$ -tubulin located at the intra-dimer](#)
80 [interface, between \$\alpha\$ - and \$\beta\$ - subunits \(Ravelli et al., 2004\)](#). More recently, it has been shown
81 [that the combretastatin binding-site is the same as the colchicine binding-site \(Zhou et al.,](#)
82 [2016\)](#). Although [vinca alkaloids](#) are powerful anticancer drugs, they also have severe side

83 effects such as myelosuppression or neurotoxicity. Moreover, many cancers are or become
84 resistant to these drugs (Dumontet and Jordan, 2010). This resistance often results from the
85 overexpression of β III-tubulin (an isoform of β -tubulin (Kavallaris, 2010)) and ATP-binding
86 cassette transporters (ABC transporters), which function as drug-efflux pumps (Haimeur et al.,
87 2004). A strategy to circumvent these limitations is to identify new chemical scaffolds that are
88 not substrates of resistance mechanisms or drugs that target regulators of MT-dynamics rather
89 than compounds interacting directly with the MT network. The selection of such drugs can be
90 achieved by phenotypic screening strategies using cell-based assays that probe MT dynamics
91 (Mayer et al., 1999; Roberge et al., 2000; Vassal et al., 2006; Prudent et al., 2012, 2013;
92 Martinez et al., 2015). High-content phenotypic screening, based on microscopic analysis of
93 the cellular MT cytoskeleton or on the cell morphology can easily detect an agent that
94 depolymerizes MTs (Ross-Macdonald et al., 2008; Munoz, 2017; Hoque et al., 2018).
95 Distinguishing a stabilized MT network based on its morphology is generally delicate, except
96 for the stabilization by paclitaxel (PTX), which at high doses induces the formation of
97 characteristic MT bundles. Assays based on the detection of indirect markers of stabilized
98 MTs, such as the immunodetection of post-translationally modified MTs (i.e., detyrosinated
99 or acetylated MTs) (Vassal et al., 2006; Hammond et al., 2008; Peris et al., 2009), are thus
100 often required. Here, we describe a new sensitive and quantitative cell-based assay, suitable
101 for scaling up for high throughput screening (HTS), which allows the identification of
102 microtubule-stabilizing agents without the need of microscopic image analysis. We used this
103 assay to screen a chemical library of kinase inhibitors. *We have chosen to focus on kinase
104 inhibitors with two goals. First, we aim at identifying kinases regulating directly or indirectly
105 the tubulin network. Second, kinase inhibitors are now commonly used in chemotherapy but
106 could be suspected to possess off-targets that may well be involved in the stabilization of the
107 microtubule network known to efficiently prevent cancerous cell proliferation. Tubulin itself
108 could be an off-target of some kinase inhibitors. Indeed, structural analyzes show that the
109 binding site of taxanes, like that of colchicine, is mainly hydrophobic in nature, large in size
110 and fairly deep (Steinmetz and Prota, 2018). These characteristics are similar to those of the
111 catalytic site of kinases. Accordingly, it has been shown that several kinase inhibitors can
112 bind to the colchicine site of tubulin and inhibit its polymerization (Bohnacker et al., 2017;
113 Tanabe, 2017). Conversely, nocodazole, a drug known to inhibit the polymerization of tubulin
114 is a high-affinity ligand of the kinases ABL, c-KIT, BRAF, and MEK (Park et al., 2012).
115 This approach led us to discover that some of those inhibitors, which are already in clinics or
116 currently assayed in phase 2 or phase 3 clinical trials, have potent and unknown cell MT
117 stabilizing properties, *without targeting tubulin directly*. Their newly discovered ability to
118 stabilize MTs is likely contributing to the cytotoxicity of these drugs and may be responsible
119 for adverse side effects.*

120

121 **Materials and methods**

122 **Cells and reagents**

123 Human HeLa cells were obtained from the American Type Culture Collection and cultured at
124 37°C in 5% CO₂ in the recommended media supplemented with 10% fetal bovine serum
125 (51810-500, Dutscher). The SYNlibrary Kinome Kinase Inhibitor Library, containing 187
126 kinase inhibitors, was purchased from SYNkinase (Australia) in a ready-to-screen format (2
127 μ L at 2 mM in 100% DMSO, one sample per compound). The following molecules were
128 further purchased and prepared at a 10 mM stock solution in DMSO, aliquoted and stored at -
129 20°C; Masatinib, Selonsertib hydrochloride, Intedanib, and PF-0477736 were from COGER.
130 MPI 0479605, SNS-314 mesylate and Ponatinib were from Selleckchem. Paclitaxel,
131 nocodazole and combretastatin A4 were from Sigma. GST-cofilin was prepared as previously

132 described (Martinez et al., 2015) and purified brain bovine tubulin was purchased at the
133 Centro de Investigaciones Biológicas, CSIC, Madrid, Spain.
134 SR series LIMK inhibitors: SR10847 (1-(2-aminoethyl)-1-(4-chlorophenyl)-3-(4-(5-methyl-
135 7H-pyrrolo[2,3-d]pyrimidin-4-yl)phenyl)urea), SR10854 (1-(2-aminoethyl)-1-(4-
136 methoxyphenyl)-3-(4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)phenyl)urea), SR10905(1-
137 (2-aminoethyl)-1-(4-chlorophenyl)-3-(2-fluoro-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-
138 yl)phenyl)urea). All three compounds were synthesized and characterized using similar
139 procedures as described in Yin et al. (Yin et al., 2015). In a cell-free LIMK1 biochemical
140 assay, the IC₅₀ values of the three compounds are 21, 27, and 15 nM, respectively.

141 **Antibodies**

142 The primary antibodies used were anti- α -tubulin (clone α 3A1 (Peris et al., 2006)), anti-
143 detyrosinated tubulin (L4(Peris et al., 2006)), anti-cofilin (Cell Signaling, 5175), anti-
144 phosphocofilin (ser3) (Cell Signaling, 3313). For immunofluorescence assays secondary
145 antibodies were coupled to Alexa 488 (Jackson Immuno-Research Laboratories, 115-545-
146 003) or Cy3 (Jackson Immuno-Research Laboratories, 111-165-003). For Western blots,
147 secondary antibodies coupled to HRP were used (Jackson Immuno-Research Laboratories,
148 711-039-152).

149 **HTS cell-based assay and procedure for the library screening**

150 7,500 HeLa cells were seeded in 96-well microplates (Greiner #655083) in 100 μ L of
151 complete medium per well and then incubated at 37°C in 5% CO₂. 24 hours after seeding,
152 cells were treated with compounds (1 compound per well) at 10 μ M final concentration for 90
153 minutes, Pyr1 at 25 μ M, 0.25% DMSO and DMSO alone at 0.25% were used as positive and
154 negative controls (8 wells per microplate and per control), respectively. Then combretastatin
155 A4 was added to each well at 0.5 μ M final concentration for 30 minutes. After medium
156 aspiration, treated cells were permeabilized for 10 minutes using 100 μ L per well of OPT
157 buffer (80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 0.5% Triton X-100, and 10% glycerol, pH
158 6.8) pre-warmed to 37°C. After buffer aspiration, cells were fixed overnight at room
159 temperature using 100 μ L per well of 4% formaldehyde in PBS pH 7.2. Cells were washed 3
160 times in PBS pH 7.2, 0.1% Tween-20 (150 μ L per well), then 50 μ L of α 3A1 anti-tubulin
161 antibody (1/5,000 in PBS pH 7.4, 0.3% BSA, 0.03% NaN₃) were added for 45 minutes. After
162 washing of cells as described above, 50 μ L of anti-mouse antibody coupled to HRP (1/2,000 in
163 PBS pH 7.4, 0.3% BSA, 0.03% NaN₃) were added for 45 minutes. Then cells were washed
164 again and, 50 μ L of ECL Western blotting substrate (Pierce #32106) were added to each well
165 and the luminescent signal was read after 5 minutes of incubation.

166 For the robotic execution of the assay at the HTS facility, the pipetting, washing and reading
167 steps were performed using MCA96 pipetting arm, HydroSpeed™ plate washer and Infinite®
168 M1000 plate reader (Tecan, Switzerland), respectively. The Z' factor was calculated according
169 to Zhang et al. (Zhang et al., 1999).

170 Because chemiluminescence is typically about 2 orders of magnitude more sensitive than
171 fluorescence, we have chosen this type of detection. The alternative use of a fluorescent
172 secondary antibody is also possible (Vassal et al., 2006).

173 **Immunofluorescence**

174 Cells were fixed and processed for immunofluorescence analysis of the MT network as
175 previously described (Prudent et al., 2012).

176 **Western Blots**

177 HeLa cells were treated with various concentrations of the kinase inhibitors for 2 hours or 48
178 hours, as indicated. DMSO was used as control. After the treatment, cells were washed with
179 cold PBS and then scraped in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1%
180 Triton X-100, 0.1% SDS, 20 mM *p*-glycerol phosphate, 1 mM sodium vanadate, 10 mM
181 sodium fluoride, and protease inhibitor cocktail (Roche). Samples were sonicated and

182 centrifugated (20 min, 15,000 rpm, 4°C) and protein concentration was quantified using the
183 Lowry method (Biorad). Equal amounts (5-10 µg) of proteins were loaded onto 4-20%
184 gradient polyacrylamide gels (Biorad) and separated by electrophoresis. Proteins were
185 transferred to PVDF membranes and probed with antibodies as previously described (Prudent
186 et al., 2012). The ECL Plus Chemiluminescent Kit (Biorad) was used for detection.

187 ***In vitro* microtubule assembly assay**

188 Pure tubulin was equilibrated in 80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8 buffer
189 (BRB80) and centrifuged at 75,000 rpm for 10 minutes in an TLA-100 rotor in a Beckman
190 Optima TLX centrifuge. The tubulin concentration was measured spectrophotometrically at
191 275 nm ($\epsilon=107.000 \text{ M}^{-1} \text{ cm}^{-1}$) and then diluted to 10 µM. The samples were supplemented
192 with 1 mM GTP and the desired drug at 10 µM (or the vehicle, i.e. DMSO). The time course
193 of assembly at 37°C was detected as turbidity by measuring the absorbance of the samples at
194 350 nm using a VISIONlite spectrophotometer (Thermo scientific).

195 **Microtubule sedimentation assay**

196 In a first step, MTs were polymerized for 30 min at 35°C using 60 µM of tubulin in BRB80
197 buffer with 1 mM of GTP in a total volume of 20 µl. After MT polymerization, the different
198 kinase inhibitors were added at 10 µM final concentration and again incubated for 30 min at
199 35°C. MTs were then subjected to depolymerization by cold treatment at 4°C for 30 min and
200 carefully loaded on 40 µl cushion of 60% (w/v) sucrose in BRB80 buffer before
201 centrifugation at 70000 rpm (Beckman rotor TLA-100) for 45 min at 35°C. After
202 centrifugation, pellets were dissolved in an equal volume as supernatants and proteins were
203 analyzed by SDS-PAGE and revealed by Coomassie blue stain.

204 **Kinase assay**

205 *In vitro* phosphorylation assay of GST-cofilin by LIMK1 was carried out as described
206 (Martinez et al., 2015). Briefly, 2.9 µM of GST-cofilin was incubated for 10 min at 30°C in
207 the presence of 100 nM human LIMK1 (Millipore #14-656) and 10 µM of different kinase
208 inhibitors in 50 mM MOPs pH 7.0, 200 µM EDTA, 25 mM MgOAc, 1 mM DTT and 200 µM
209 ATP, in a final volume of 30 µl. Kinase reactions were stopped by the addition of SDS
210 sample buffer, boiled and then subjected to SDS-PAGE and Western blotting.

211 **Lentivirus production and cell infection**

212 The mRuby3 construct that binds MTs +TIPS was a generous gift of Marina Mikhaylova's lab
213 (Honnappa et al., 2009). Briefly, a short peptide motif, Ser-x-Ile-Pro (SxIP), of the EB1
214 interacting protein MACF2 was tagged with mRuby3 and cloned at the place of mCherry into
215 a pLV-mCherry Plasmid (#36084, Addgene). 1×10^7 HeLa cells were infected with 10^8 IU of
216 mRuby3-MT+tips lentivirus and maintained in RPMI supplemented with 10% FBS. After
217 amplification, half of them were frozen and the rest was used for experiments. Every two
218 weeks, new cells were thawed to perform the experiments.

219 **Measurements of microtubule dynamics using videomicroscopy**

220 Fluorescence imaging of mRuby3-MT+tips of MTs in live HeLa cells maintained at 37°C,
221 5% CO₂ was performed using an inverted microscope (Axio Observer, Zeiss) coupled to a
222 spinning-disk confocal system (CSU-W1-T3, Yokogawa) connected to a wide-field electron-
223 multiplying CCD camera (ProEM+ 1024, Princeton Instrument). The microscope setup was
224 controlled by MetaMorph software. Time-lapse microscopy imaging was performed after 2
225 hours of treatment with 0.2% DMSO (control) or drugs at a concentration of 10-20 µM.
226 Acquisitions were performed every 1.5 sec with a 100 msec exposure time during 2 min using
227 a 63x NA 1.46 oil-immersion objective. Kymographs of MT plus end dynamics were done
228 using ImageJ software and a homemade KymoTool (Ramirez-Rios et al., 2016). For
229 quantification of plus end dynamics the time-lapse movies were visually inspected and only
230 mRuby3-MT+tip comets that were visible for at least three consecutive frames were included
231 in the analysis. The velocities of mRuby3-MT+tip comets were calculated by dividing the

232 distance travelled by the time spent travelling. The average length of MT growth events was
233 also measured. The number of comets within a cell was quantified in the first image of each
234 stack, by measuring the number of comets using "find maxima" in image J and dividing by
235 the cell surface.

236

237 **Results**

238 **Establishing a cell-based assay for the detection of microtubule stabilizing agents**

239 The assay probes a drug-induced resistance of the MT network to a depolymerization
240 provoked by agents such as combretastatin A4 (CA4) or nocodazole. CA4 or nocodazole
241 binds free tubulin dimers and prevents their incorporation into MTs, leading to a progressive
242 loss of the polymerized MT network. Stabilized MTs with slow dynamics have reduced
243 exchanges of their tubulin content with the free tubulin pool, and are thus less sensitive to
244 nocodazole- or CA4- induced depolymerization (Saoudi et al., 1998; Prudent et al., 2012).
245 Nocodazole appears, [however](#), to be less tubulin specific as it has been reported to be a high
246 affinity ligand for cancer-related kinases including Abl, cKit, BRAF and MEK (Park et al.,
247 2012). [We thus decided to choose CA4 as MT depolymerizing agent to establish the assay.](#)

248 The main steps of the assay are illustrated in **Figure 1A** and described in the methods section.
249 To set up the assay for HTS, a positive control - i.e. a known stabilizing agent- and a control
250 with no activity were defined. Because the PTX stabilizing activity is uniquely high we
251 decided to use a less potent positive control, to better adapt the dynamic window of the assay
252 and to increase its sensitivity. We thus used Pyr1, which induces a partial stabilization of
253 cellular MTs by LIM kinase (LIMK) inhibition (Prudent et al., 2012), as positive control for a
254 microtubule stabilizing agent. Using Pyr1 and the vehicle DMSO, as positive and negative
255 control respectively, we set up a high quality, reproducible assay as assessed by calculating
256 signal-to-background ratios and Z' factor according to Zhang and others (Zhang et al., 1999).
257 We obtained a Pyr1/DMSO ratio of 5.7 and a Z' value of 0.80, indicating that these assay
258 conditions are excellent for HTS (**Figure 1B**). To evaluate the sensitivity of the assay we
259 tested the effect of different concentrations of Pyr1 on the luminescence intensity measured
260 by the microplate reader. As shown in **Figure 1C**, a dose-dependent MT stabilization could
261 be detected for Pyr1. These results demonstrate that the assay is statistically robust,
262 quantitative and sensitive enough for HTS

263 **Identifying microtubule-stabilizing compounds in a library of kinase inhibitors**

264 We performed a pilot screen of a commercially available chemical library of 187 known
265 kinase inhibitors. In addition, the library was supplemented with the known microtubule
266 stabilizing agents, PTX and epothilone (Steinmetz and Prota, 2018). We also added a
267 collection of 12 bis-aryl urea derivatives for which a LIM kinase (LIMK) inhibitory activity
268 has been previously described *in vitro* in a cell-free system (Yin et al., 2015). Compounds
269 were screened at a final concentration of 10 μ M. Compounds that showed an activity superior
270 to 50% of Pyr1 activity were considered as hits. **Table 1** shows that different known kinase
271 inhibitors - masatinib, selonsertib, intedanib, PF0477736, SNS-314 mesylate, MPI0479605,
272 and ponatinib - were found positive using this screening assay. [Regarding compounds
273 structures \(Figure 2\), except for the fairly common N-methylpiperazine moiety \(present
274 in intedanib, masatinib and ponatinib\), no recurrent patterns are observed](#)

275 . PTX and epothilone B, known MT stabilizers, were the most active compounds. Several
276 compounds (SR10905, SR10854 and SR10847) from the added bis-aryl urea derivatives with
277 LIMK inhibitory activities were also detected as active compounds, revealing that they were
278 cell permeant and able to induce a stabilization of MTs, as it has been described for the LIMK
279 inhibitor Pyr1 (Prudent et al., 2012). These results indicate that the assay is indeed able to
280 select cell-permeant MT stabilizing agents.

281

282 **Comparative analysis of the microtubule stabilizing activity of the selected kinase**
283 **inhibitors**

284 The original screen was performed with only one data point and with molecules already
285 dissolved and stored in microplate wells. These results needed thus to be confirmed with a
286 new batch of freshly dissolved pure compounds. We first confirmed the results of the screen
287 by microscopic examination. As expected, immunofluorescence of the cellular MT network
288 showed that MTs are resistant to CA4-induced depolymerization after treatment with these
289 compounds (**Figure 3A**).

290 Stable MTs are specific substrates of some enzymes responsible for tubulin post-translational
291 modifications (Lafanechere and Job, 2000). For instance, the protein complex VASH/SVBP,
292 catalyzing the detyrosination of tubulin, acts preferentially on MTs rather than on the free
293 tubulin dimer (Aillaud et al., 2017). Immunofluorescence (**Figure 3B**) showed that the
294 amount of detyrosinated MTs was indeed clearly increased in cells treated with SNS-314
295 mesylate, masatinib and selonsertib, and moderately increased in cells treated with intedanib
296 and ponatinib, when assayed at a concentration of 20 μM . A few scattered cells with
297 detyrosinated MTs were found in the PF0477736 and MPI0479605 treated cell populations.
298 As noticed in previous studies (Prudent et al., 2012; Martinez et al., 2015) the generation of
299 detyrosinated MTs after treatment with MT stabilizing drugs was somehow heterogeneous
300 among cells. The analysis of the detyrosination level of tubulin in whole cell lysates using
301 Western blot confirmed the important detyrosination induced by SNS-314 mesylate and
302 masatinib treatment (**Figure 3C**).

303 Then, in an attempt to rank these compounds, we tested the MT stabilizing effect of different
304 concentrations of these compounds, using the above-described quantitative luminescence
305 assay. As shown in **Figure 4A**, SNS-314 mesylate, masatinib and intedanib were found more
306 active than Pyr1. Selonsertib was as active as Pyr1, whereas MPI0479605, ponatinib and
307 PF0477736 showed a lower stabilizing activity.

308 Finally, we also analyzed the effect of the four most potent compounds - i.e. selonsertib,
309 intedanib, masatinib and SNS-314 mesylate on MT dynamics, by following the fast growing
310 plus ends of MTs using time-lapse fluorescence microscopy. To this end, we infected HeLa
311 cells with a Ruby- construct that presents the Ser-x-Ile-Pro (SxIP) motif, which binds to the
312 endogenous MT plus-end binding protein EB1 (Honnappa et al., 2009) (**Movie S1**). In a
313 preliminary experiment, we found that masatinib has the most potent effect on MT dynamics
314 and its concentration had to be lowered to 10 μM to measure dynamic instability parameters,
315 as a 20 μM concentration completely hampered microtubule dynamics. We observed for all
316 these compounds a decrease of the number of comets per area (**Figure 4B**), indicative of a
317 stabilization of the MTs (Prudent et al., 2012). In addition, the total distance traveled by the
318 plus ends during the observation period is decreased (**Figure 4C**) as well as their rate of
319 growth (**Figure 4D**), indicating that these kinase inhibitors are able to slow down MT
320 dynamics.

321 To test if a longer exposure of the cells to lower doses of compounds could also induce a
322 stabilization of the MTs, we exposed the cells for 48 hours to a 100 nM concentration of the
323 compounds and then examined the content in detyrosinated tubulin, as an indicator of MT
324 stabilization. As shown on **Figure 4E**, all the compounds except masatinib were able to
325 generate detyrosinated tubulin, indicating that a prolonged incubation of cells with nanomolar
326 doses of these compounds can lead to MT stabilization.

327 **Cellular microtubule stabilization does not result from a direct interaction of the**
328 **compounds with tubulin**

329 The MT stabilizing activity of these compounds has never been described, to our knowledge.
330 MT stabilization can result from the direct binding of the compounds to tubulin. Otherwise it
331 can be the consequence of the inhibition of kinases, either the originally targeted kinase or a

332 secondary targeted kinase. It can also result from the binding of the compound to another, yet
333 un-described target.

334 To test whether the compounds directly interact with tubulin, we examined their ability to
335 interfere with *in vitro* pure tubulin self-assembly. The well-characterized MT-stabilizing
336 agent PTX can induce MT assembly *in vitro* at low tubulin concentrations that would not
337 induce spontaneous MT assembly (Tang et al., 2017). We found that, unlike PTX, none of the
338 compounds were able to induce MT assembly *in vitro* (**Figure 5A**). We also wondered if the
339 compounds could stabilize preformed MTs by binding to the MT lattice for instance. To test
340 this hypothesis, we prepared purified MTs that were subsequently incubated with the
341 compounds before assaying their stability to cold-induced depolymerization. At the end of
342 this experiment, stable MTs were separated from depolymerized tubulin by a centrifugation
343 through a warm sucrose cushion. As shown in **Figure 5B**, none of the compounds was able to
344 stabilize MTs, indicating that they do not directly bind to tubulin, in contrast to PTX.

345 LIM kinases (LIMK) are among the kinases reported to control MT dynamics during the
346 interphase of the cell cycle. Upon their inhibition, a stabilization of the interphase MT
347 network has been described (Gorovoy et al., 2005; Prudent et al., 2012; Mardilovich et al.,
348 2015; Prunier et al., 2016). We thus assayed the effect of the compounds on cellular LIMK
349 activity by analyzing the phosphorylation of its major substrate cofilin. We found that upon
350 cell treatment with SNS-314 mesylate and PF0477736, the intracellular cofilin was less
351 phosphorylated, indicating that these compounds likely target upstream components of the
352 pathway leading to cofilin phosphorylation (**Figure 6A**). An *in vitro* analysis of the direct
353 inhibitory effect of the compounds on recombinant LIMK activity, using also recombinant
354 cofilin as substrate (**Figure 6B**) showed that PF0477736 is indeed able to directly inhibit
355 LIMK. PF0477736 was originally described as a Chk1/Chk2 inhibitor. The MT stabilizing
356 effect of this compound could thus be ascribed to a combination of its inhibitory action on
357 LIMK and on Chk1/Chk2, which have been shown to be able to phosphorylate and inactivate
358 the MT-associated protein Tau (Mendoza et al., 2013).

359 SNS-414 mesylate is a potent, but non- selective Aurora Kinase inhibitor. It has been reported
360 that Aurora Kinase A - but not Aurora Kinase B- inhibition, can control MT dynamics in
361 interphase cells (Lorenzo et al., 2009). Thus the MT stabilization that we observed when cells
362 were treated with this compound could result from its inhibitory effect on Aurora Kinase A in
363 conjunction with its indirect inhibitory effect on LIMK activity. Indeed a positive regulation
364 of the LIMK2 isoform by Aurora A has been described, that could explain this indirect effect
365 (Johnson et al., 2012).

366 For the other most potent selected kinase inhibitors, i.e. selonsertib, intedanib and masatinib,
367 we conducted additional experiments, using other known available inhibitors of these kinases
368 with different scaffolds, to investigate if the MT stabilizing effect is due to the inhibition of
369 the targeted kinase, or an off-target effect of these compounds.

370 Selonsertib is an apoptosis signal-regulating kinase 1 (ASK1) inhibitor (Loomba et al., 2018).
371 Among the different described functions of ASK1, its involvement in cell division has been
372 reported, by regulating spindle orientation and positioning through phosphorylation of the
373 microtubule-binding protein EB1 (Luo et al., 2016). However, no ASK1-dependent regulation
374 of interphase MT dynamics has been described, to our knowledge. In order to determine if the
375 selonsertib-induced stabilization of the MT network is due to ASK1 inhibition, we tested the
376 MT stabilizing effect of a structurally unrelated ASK1 inhibitor, MSC2032964A (Guo et al.,
377 2010). We found that MSC2032964A does not induce a MT resistance to CA4 induced
378 depolymerization (**Figure 7**), indicating that the interphase MT stabilization by selonsertib
379 treatment is most probably not due to ASK1 inhibition.

380 Masatinib and intedanib (also known as nintedanib or BIBF 1120) are inhibitors of tyrosine
381 kinase receptors. They both inhibit platelet-derived growth factor receptor (PDGFR)

382 (Dubreuil et al., 2009; Papadopoulos and Lennartsson, 2018) and fibroblast growth factor
383 receptor (FGFR) (Papadopoulos and Lennartsson, 2018). In addition, masatinib targets c-Kit
384 and the colony stimulating factor-1 receptor (CSF-1R) whereas intedanib targets the vascular
385 endothelial growth factor receptor (VEGFR) and FLT3. Interestingly, imatinib, another
386 tyrosine kinase receptor inhibitor, that targets BCR-Abl as well as cKIT or CSF-1R, was
387 present in the library but not selected as a hit in the initial screen. Similarly, ponatinib, which
388 also inhibits BCR-Abl, cKit, PDGFR, FGFR, VEGFR and Flt3 presented only a moderate MT
389 stabilizing activity (**Figure 4A**). We also tested KI20227, an inhibitor that targets CSF-1R,
390 VEGFR, c-Kit and PDGFR, and we observed no significant effect on MT stability as assessed
391 by the resistance of cellular MTs to a CA4-induced depolymerization (**Figure 7**). Altogether,
392 these data suggest that the observed MT stabilizing effect is due to an off-target effect of
393 masatinib and intedanib.

394

395 **Discussion**

396 A full awareness of a drug's mechanism of action is a challenge in the development of target-
397 specific drugs with improved safety profiles. Non-kinase targets of protein kinase inhibitors
398 are regularly reported (Munoz, 2017). Several kinase inhibitors have been reported to bind
399 tubulin and inhibit its polymerization (Aoyama et al., 2014; Bohnacker et al., 2017; Steinmetz
400 and Prota, 2018). Their effect on tubulin polymerization, rather than on kinase activity, is
401 often responsible for their anticancer activity (Munoz, 2017). Kinase inhibitors targeting
402 tubulin cannot be recognized in the commonly used kinome-wide screens. Moreover, as both
403 kinase and tubulin anticancer drugs promote cell-cycle arrest and have antiproliferative
404 activities, it is not easy to identify their exact modes of action by overall toxicity and cell
405 cycle assays.

406 So far, besides the LIMK inhibitor Pyr1 (Prudent et al., 2012), no kinase inhibitor capable of
407 stabilizing the MT network has been described. However, as a stabilization of the MT
408 network must be sought to be seen, the stabilizing effect of inhibitory compounds can remain
409 completely unnoticed if it has not been specifically looked for it.

410 Here, we have screened a library of kinase inhibitors for their ability to stabilize the MT
411 network. Interestingly, when tested at 10 μ M, only 7 compounds out of 190 compounds of the
412 original library were found positive, yielding a 3.7% hit rate similar to commonly reported
413 HTS hit rates. The stabilizing activity was confirmed using two other independent assays: the
414 MT detyrosination status and, for the most potent compounds, the analysis of the modification
415 of MT dynamic behavior using videomicroscopy. These assays report different aspects of MT
416 dynamics and their regulation and may give different results. For instance, enhanced MT
417 detyrosination can be the indirect result of an enhanced microtubule stability (Lafanechere
418 and Job, 2000) as well as an activation of the enzyme that catalyzes tubulin detyrosination
419 (Aillaud et al., 2017) or an inhibition of the enzyme that catalyzes tubulin re-tyrosination
420 (Ersfeld et al., 1993). Moreover, the sensibility of these assays is different. The quantitative
421 analysis of the MT dynamics using videomicroscopy analysis is more sensitive, but less
422 parallelizable. Nevertheless, all these different assays give convergent results, which
423 reinforces the finding of the MT stabilizing activity of the compounds with masatinib,
424 selonsertib, intedanib and SNS-314 mesylate being the most active drugs.

425 In cell-free systems, these compounds have been reported to inhibit their targeted kinase when
426 applied in the nanomolar range. However, often higher concentrations are necessary to
427 observe their effect on target dependent cellular processes, like apoptosis, inhibition of
428 proliferation, etc. (1-30 μ M for selonsertib (Ji et al., 2019; Mukherjee et al., 2019), 0.1-1 μ M
429 for intedanib (Lehtonen et al., 2016; Knüppel et al., 2017), 0.1-10 μ M for masatinib (Dubreuil
430 et al., 2009; Trias et al., 2016), 1-10 nM for SNS-314 mesylate (Oslob et al., 2008)). Our
431 results show that the stabilizing effect on cellular MTs is observed when compounds are used

432 in the 5-10 μ M range, i.e. often at similar concentrations than those previously reported to
433 obtain a cellular effect. This indicates that the doses necessary to inhibit these kinases in cells
434 may cause a stabilization of MTs.

435 Moreover, the intracellular concentration of the compounds depends on the duration of
436 exposure of the cell to the compounds, of the equilibrium between the entry and exit of the
437 compounds through the plasma membrane and of their metabolism within the cell. We have
438 shown here that longer exposure of cells to lower doses of some of these compounds can also
439 induce a stabilization of the MTs. This is to be taken into account, especially for those of
440 these inhibitors, which are tested or used clinically and therefore often delivered at repeated
441 doses and which may reach high intracellular concentrations.

442 Among the hits we have found, the effect of some compounds on MTs can be explained
443 directly by the inhibition of the targeted kinase. This is the case for instance for MPI047960,
444 an inhibitor of the Monopolar spindle 1 (Mps1) kinase, whose activity plays a primary role in
445 the mitotic checkpoint (Abrieu et al., 2001) by regulating the kinetochore-microtubule
446 attachment stability (Maciejowski et al., 2017). Mps1 kinase is also reported to reside
447 predominantly in the cytoplasm where it is an important MT interphase regulator (Mattison et
448 al., 2011; Jia et al., 2015). Moreover, it has been shown that Mps1 kinase can bind to MTs *in*
449 *vitro* (Stucke et al., 2004). Thus, an attractive explanation of the observed MPI047960 partial
450 stabilizing effect on interphase MTs is that it is consecutive to Mps1 kinase inhibition.

451 We have also found that the MT stabilizing effect of two of the selected compounds, SNS-314
452 mesylate and PF0477736, whose primary targets are Aurora Kinase and Chk1/Chk2,
453 respectively, could be due to their inhibitory activity on LIMK, which has not been previously
454 described. The important MT stabilizing effect that we have observed for SNS-314 mesylate
455 can be the source of the *in vivo* synergy with paclitaxel that has been described for this
456 compound in a colon carcinoma model (VanderPorten et al., 2009).

457 Surprisingly, we have discovered a strong MT stabilizing activity for selonsertib, masatinib
458 and intedanib and a moderate one for ponatinib.

459 Selonsertib is currently evaluated in Phase 3 clinical studies for the treatment of non-alcoholic
460 steatohepatitis (<https://www.clinicaltrials.gov>). The rationale is that ASK1 inhibition can
461 reduce hepatic inflammation, apoptosis, and fibrosis through inhibition of stress response
462 pathways. Some reported side effects such as numbness of the extremities (Loomba et al.,
463 2018) suggest the occurrence of peripheral neuropathies. Similar undesirable side effects have
464 been reported in patients treated with taxanes. Thus, in the light of our observations, these
465 adverse effects of selonsertib could result from its effect on the MT network.

466 Masatinib is currently sold as a veterinary drug (Masivet®) to treat canine mastocytosis with
467 mutated cKit receptors. Because it inhibits enzymes involved in inflammation, and as nerve
468 inflammation is a significant aspect of amyotrophic lateral sclerosis (ALS), masatinib is also
469 promising for the treatment of ALS (Scott, 2017; Trias et al., 2018). A phase 2/3 study to
470 compare the efficacy and safety of masatinib with other existing ALS treatments has been
471 completed with encouraging positive results (Mora et al., 2019). In oncology, masatinib is
472 currently tested for the treatment of pancreas and prostate cancer and the results of these two
473 phase 3 trials are expected in 2020. Knowledge of the unexpected effects of masatinib on
474 MTs may be useful in interpreting the results of these studies, as well as possible side effects
475 and to design further best drug combinations.

476 Intedanib is clinically indicated to treat idiopathic pulmonary fibrosis. It is also currently
477 approved for use in combination with docetaxel for treating advanced lung cancer that has
478 progressed after first-line chemotherapy. Indeed, in a phase 3 study, progression free survival
479 was found significantly improved in the docetaxel plus intedanib group compared with the
480 docetaxel plus placebo group (Reck et al., 2014). In addition to the original rationale for
481 combining the anti-angiogenic effect of intedanib, which is due to its inhibitory effect on

482 VEGFR, with the anti-tumor effect of docetaxel, our finding provides new mechanistic
483 insights. Indeed, the underlying mechanism could be an additive effect of two microtubule-
484 stabilizing compounds. Moreover, when administrated alone (Ofev®, Vargatef®), intedanib
485 induces frequently described adverse effects such as neutropenia or peripheral neuropathies
486 that could be ascribed to its effect on MTs.

487 Ponatinib is clinically approved for the treatment of chronic myeloid leukemia and
488 Philadelphia chromosome-positive acute lymphoblastic leukemia, specifically targeting the
489 BCR-ABL gene mutation, T315I (Tan et al., 2019). Ponatinib is also pre-clinically evaluated
490 in other tumor types, with encouraging results. Our data encourage a re-analysis of the tumor
491 cell effect of ponatinib, with a focus on its effect on the MT network.

492 Overall, our results show that several kinase inhibitors, initially thought to exert their
493 therapeutic effect through a defined mechanism on the diverse kinase pathways can also
494 behave as more general cytotoxic drugs, due to the alteration of MT dynamics. In light of
495 these data, a possible repurposing of some of these drugs is conceivable.

496 Finally, although the binding site of taxanes has some structural characteristics similar to
497 those of the kinase catalytic site (Steinmetz and Prota, 2018), none of the inhibitors that our
498 assay has selected directly binds to tubulin. Understanding precisely how these inhibitors
499 induce MT stabilization will allow the identification of new regulators of MT dynamics. This
500 is of interest not only in terms of knowledge, but also for the identification of new targets for
501 anticancer therapies.

502

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513

514 **Author Contributions**

515 SRR and LP designed, performed and analysed the experiments characterising the stabilizing
516 effect of the selected compounds and searched for the compound mechanism of action. SM
517 and CR set up and miniaturized the assay and conducted dose-effect analysis of the selected
518 compounds. CB automatized the assay and screened the kinase inhibitor chemical library. YF
519 synthesized and provided LIM Kinase inhibitors. MOF, AA, KS and LL supervised the
520 researches. LL directed the project and wrote the manuscript. All authors provided critical
521 analysis of the manuscript.

522

523 **The authors declare no potential conflicts of interest**

524

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880

881 **Table 1: Hits selected by screening**

882

<i>Compound</i>	<i>Target</i>	<i>% Pyl1-like activity</i>
Paclitaxel	Tubulin	265
Epothilone B	Tubulin	253
Masatinib	Tyrosine kinase inhibitor	141
Selonsertib	Apoptosis signal-regulating kinase 1 (ASK1)	205
Intedanib	Receptor tyrosine kinase (RTK)	165
PF0477736	Chk1/Chk2	112
SNS-314 mesylate	Aurora kinases A, B, and C	107
MPI0479605	Monopolar spindle 1 (Mps1) kinase	114
Ponatinib	Receptor tyrosine kinase (RTK)	73
SR10905	LIM Kinase (Yin et al., 2015)	82
SR10854	LIM Kinase	147
SR10847	LIM Kinase	99

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884

885

886 **Figure Legends**

887

888 **Figure 1. A cell-based assay for detecting microtubule stabilizing agents.** A) Schematic
889 representation of the different steps of the assay. B) Evaluation of the intra-assay repeatability.
890 HeLa cells were seeded on a microplate. After 24 h, they were treated for 90 min with DMSO
891 (negative control, blue diamonds) or Pyr1 25 μ M (positive control, red squares). Then CA4
892 was added to each well at a final concentration of 0.5 μ M for 30 minutes. Cells were then
893 processed for chemiluminescence, as described in the “Materials and Methods” section. Each
894 point represents the luminescence intensity (expressed as Relative Luminescence Units)
895 obtained for each well of the 96-well microplate. C) Evaluation of the assay sensitivity. HeLa
896 cells were seeded on microplates. After 24 h, they were treated for 90 min with DMSO
897 (negative control) or with different concentrations of Pyr1 as indicated. CA4 was added to
898 each well at a final concentration of 0.5 μ M for 30 minutes. Cells were then processed for
899 chemiluminescence, as described in the “Materials and Methods” section. Results are
900 expressed as % of MTs resistant to CA4 induced depolymerization, with 100% corresponding
901 to cells treated with DMSO without CA4 and 0% corresponding to cells treated with DMSO
902 and CA4; mean +/- SEM.

903

904 **Figure 2. Structure of the selected kinase inhibitors**

905

906 **Figure 3. Confirmation of potent microtubule stabilization after treatment of cells with**
907 **the selected protein kinase inhibitors.** A) **Kinase inhibitors protect MTs from CA4-**
908 **induced depolymerization.** HeLa cells were incubated for 90 min with 0.25% DMSO, 10 μ M
909 paclitaxel or 10 μ M of the indicated kinase inhibitors. CA4 (0.5 μ M) was then added for 30
910 min. Cells were permeabilized and processed for immunofluorescence using an anti-
911 α -tubulin antibody. Scale bar, 10 μ m. B) Treatment with the kinase inhibitors increases the
912 detyrosinated MT content of cells. HeLa cells were treated for 2 h with 0.25% DMSO, 10 μ M
913 paclitaxel or 20 μ M of the indicated kinase inhibitors. Cells were then permeabilized and
914 processed for immunofluorescence, using an antibody against detyrosinated-tubulin. Scale
915 bar, 10 μ m. C) Western blot to quantify the effect of kinase inhibitors on the tubulin
916 detyrosination status. HeLa cells were treated for 2 h with 0.25% DMSO, 10 μ M paclitaxel or
917 20 μ M of the indicated kinase inhibitors. Cellular protein extracts were separated by SDS-
918 PAGE and Western blotted to detect detyrosinated-tubulin and β -actin. A representative
919 Western blot of three independent experiments is shown. The intensity of the bands was
920 quantified using ImageJ software and the calculated ratios are shown as mean +/- SEM on the
921 lower panel.

922

923 **Figure 4. Comparative analysis of the effect of the selected kinase inhibitors on**
924 **microtubule dynamics in HeLa cells.** A) Comparison of the MT stabilizing effect of the
925 different kinase inhibitors. Different doses of the kinase inhibitors were applied to HeLa cells
926 in microplates and their MT stabilizing effect was assessed after a 30 min CA4 (0.5 μ M)
927 treatment, using the luminescent assay, as described in the materiel and methods section. Pyr1
928 was used as positive control. Results are expressed as % of MTs resistant to CA4 induced
929 depolymerization, with 100% corresponding to cells treated with DMSO without CA4 and
930 0% corresponding to cells treated with DMSO and CA4; mean +/- SEM. B,C,D) Analyses of
931 the dynamic instability parameters of MTs in HeLa cells treated with the different kinase
932 inhibitors. The analyses were performed on the time-lapse movies (Movie S1) as described in
933 materials and methods, using DMSO as control. Results are expressed as mean +/- SEM of 3
934 independent experiments. The samples were compared to DMSO using Kruskal-Wallis test

935 with Dunn's multi comparison test. ****: $p < 0.0001$. E) Effect of longer exposition of cells to
936 inhibitors on tubulin dephosphorylation. HeLa cells were treated for 48 h with 0.25% DMSO or
937 100 nM of the indicated kinase inhibitors. Cellular protein extracts were separated by SDS-
938 PAGE and Western blotted to detect dephosphorylated-tubulin and tubulin. A representative
939 Western blot of three independent experiments is shown. The intensity of the bands was
940 quantified using ImageJ software and the calculated ratios are indicated as mean \pm SEM in
941 the lower panel.

942
943 **Figure 5. Effect of the kinase inhibitors on microtubule assembly in vitro.** A) Kinase
944 inhibitors do not promote tubulin polymerization. Time course of polymerization of purified
945 tubulin (10 μ M in BRB80 buffer with 1 mM GTP, 37°C) in the presence of vehicle (DMSO,
946 blue line), paclitaxel (10 μ M, red line) and the different kinase inhibitors (10 μ M), as
947 indicated, measured by turbidimetry at 350 nm. B) Kinase inhibitors do not protect MTs from
948 cold-induced depolymerization. Pre-polymerized MTs were incubated in the presence of 10
949 μ M of kinase inhibitors, 10 μ M paclitaxel or DMSO for 30 min at 37°C and then incubated at
950 4°C for 30 min. Free depolymerized tubulin (supernatant, S) and MTs (pellet, P) were
951 separated by centrifugation on a sucrose cushion, as described in the materials and methods
952 section. Equal volumes of the S and P fractions were subjected to SDS-PAGE separation and
953 proteins were stained by coomassie blue.

954
955 **Figure 6. Some kinase inhibitors target the cofilin pathway.** A) Cellular LIMK activity is
956 reduced when cells are treated with some kinase inhibitors. HeLa cells were treated for 2 h
957 with 10 μ M of kinase inhibitors. Cell lysates were separated by SDS-PAGE and
958 immunoblotted using anti-phosphocofilin (ser3) or anti-cofilin antibodies as indicated. B) *In*
959 *vitro* phosphorylation of recombinant GST-cofilin. GST-cofilin (2.9 μ M) was incubated with
960 LIMK (100 nM) in kinase buffer in the presence of the different kinase inhibitors as described
961 in the material and methods section. DMSO was used as negative control. Proteins were
962 separated by SDS-PAGE and cofilin phosphorylation was analyzed after immunoblotting
963 using the anti-phosphocofilin (ser3) antibody. A coomassie-stained SDS-PAGE gel shows the
964 corresponding content of GST-cofilin in each sample.

965
966 **Figure 7: Comparative analysis of the effect of structurally different inhibitors of ASK1**
967 **(Selonsertib and MSC203296A) and of tyrosine-kinase receptors (Masatinib and**
968 **KI20227) on microtubule stability, as assessed by the resistance of the microtubule**
969 **network to a CA4-induced depolymerization.** HeLa cells were incubated for 90 min with
970 0.25% DMSO (control) or 10 μ M of the indicated kinase inhibitors. CA4 (0.5 μ M) was then
971 added for 30 min. Cells were permeabilized and processed for immunofluorescence using the
972 anti- α -tubulin antibody. Scale bar, 10 μ m.

973
974 **Supplementary Movie 1:** Time-lapse microscopy was performed using cells infected with
975 the mRuby3-MT+tips construct and treated for 2 h with DMSO (control), 20 μ M selonsertib,
976 intedanib and SNS-314 mesylate or 10 μ M of masatinib, as indicated. Images were collected
977 every 1.5 sec for 2 min and films are 11x accelerated.

978
979