1 **Supplementary information**

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3 **A novel mechanism of mTOR inhibition displacing phosphatidic acid induces**

4 **enhanced cytotoxicity specifically in cancer cells**

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11

12 **Supplementary Material and Methods**

13 ***In vitro* kinase assays**

14 *mTOR assay*

15 This assay is based on TR-FRET (time-resolved fluorescence resonance energy transfer).

16 The LANCE® Ultrakinase assay uses ULight-FLGFTYVAP peptide, ATP and a human

17 recombinant mTOR kinase. After 30 min of incubation at room temperature, phospho-

18 FLGTYVAP was measured. PI-103 was used as internal control with an IC50 of 71 nM.

19 ICSN3250 test concentration ranged from 10^{-10} M to 10^{-5} M.

20 *Akt1 assay*

21 This assay is based on TR-FRET (time-resolved fluorescence resonance energy transfer).

22 The LANCE® Ultrakinase assay uses CREBtide-CKRREILSRRPSYRK peptide, ATP and a

23 human recombinant Akt1 kinase. After 60 min of incubation at room temperature, phospho-

24 CREBtide CKRREILSRRPSYRK was measured. Staurosporine was used as internal control

25 with an IC50 of 35 nM. ICSN3250 test concentration was at 500 nM.

26 *EGFR assay*

27 This assay is based on TR-FRET (time-resolved fluorescence resonance energy transfer).

28 The LANCE® Ultrakinase assay uses ULight-CAGAGAIETDKEYYTVKD peptide, ATP and a

29 human recombinant EGFR kinase. After 15 min of incubation at room temperature, phospho-
30 ULight -CAGAGAIETDKEYYTVKD was measured. PD153035 was used as internal control
31 with an IC50 of 0.13nM. ICSN3250 test concentration was at 500 nM.
32 *PDK1 assay*
33 This assay is based on TR-FRET (time-resolved fluorescence resonance energy transfer).
34 The LANCE® Ultrakinase assay uses ULight-FLGFTYVAP peptide, ATP and a human
35 recombinant FDK1 kinase. After 90 min of incubation at room temperature, phospho-ULight -
36 FLGFTYVAP was measured. Staurosporine was used as internal control with an IC50 of 200
37 nM. ICSN3250 test concentration was 500 nM.
38 *PKCα and PKCε assay*
39 This assay is based on HTRF (Homogeneous time-resolved fluorescence). For PKCα, the
40 assay uses biotinyl-βAβAβAKIQASFRGHMARKK peptide (60 nM), ATP and a human
41 recombinant PKCα kinase. After 15 min of incubation at room temperature, phosphor-
42 biotinyl-βAβAβAKIQASFRGHMARKK was measured. Bis10 was used as internal control
43 with an IC50 of 3.4 nM. ICSN3250 test concentration ranged from 10^{-9} M to 10^{-6} M.
44 For PKCβ, the assay uses biotinyl-βAβAβAKIQASFRGHMARKK peptide (400nM), ATP and
45 a human recombinant PKCβ kinase. After 60 min of incubation at room temperature,
46 phospho biotinyl-βAβAβAKIQASFRGHMARKK was measured. Bis10 was used as internal
47 standard with an IC50 of 10 nM. ICSN3250 test concentration ranged from 10^{-9} M to 10^{-6} M.
48 *SRC assay*
49 This assay is based on TR-FRET (time-resolved fluorescence resonance energy transfer).
50 The LANCE® detection method use the substrate Ulight-Poly GAT[EAY(1:1:1)]n, ATP, and a
51 human recombinant SRC kinase expressed in insect cells. After 60 min of incubation the
52 fluorescence transfer was measured at λ_{ex}=337 nm, λ_{em}=620 nm and λ_{em}=665 nm using a
53 microplate reader (Envision, Perkin Elmer). Staurosporine was used as internal standard
54 with an IC50 of 7.6 nM. ICSN3250 test concentration ranged from 10^{-9} M to 10^{-6} M
55 *PI3K assay*

56 The inhibition of PI3Ks (PI3Kα, PI3Kβ, PI3Kγ, PI3Kδ) activity was determined using the PI3
57 Kinase Activity/Inhibitor ELISA assay from Merck-Millipore (USA). The recombinant GRP-1
58 protein capture PIP3 generated as part of the kinase reaction or the biotinylated PIP3 tracer.
59 The captured PIP3 tracer was detected using streptavidin-HRP conjugates and a
60 colorimetric read out at 450 nm, following a kinase reaction (for 30 minutes). This
61 colorimetric signal negatively correlates with PI3 kinase activity. Assay were carried out in 96
62 well assay plates in the presence or absence of the ICSN3250 compound. Wortmannin
63 (100nM) was used as internal standard with an IC50 of 10 nM. Absorbance was recorded at
64 450 nm with a spectrophotometric plate reader PolarStar Omega (BMG Labtech, Germany).
65

66 ***Radical intermediates formation in presence of superoxide anion (O_2°-) assay***

67 After incubation of compound ICSN3250 (40µM) in presence of KO_2 (0.5mM), electron spin
68 resonance (ESR) spectra were recorded during 3 min at room temperature using a MiniScope
69 MS100 (Magnettech). ICSN3219 (40µM), a compound previously known to react with KO_2
70 (data not shown) was used as a positive control.

71

72 ***Reaction with superoxide anion (O_2°-) assay***

73 The superoxide anion radical scavenging ability was assessed according to the method of
74 Podczasy & Wei[1]. The used method is based on competitive equilibrium between transition of
75 electrons/radicals from O2°- generated by the complex (Xanthine /xanthine oxidase) to 2-(4-
76 Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and transition of
77 electrons/radicals to the measured compound. Transition of electrons to INT leads to formation
78 of formazan. Active compounds are able to inhibit the formation of the blue formazan, detected
79 as light absorption at 505 nm. A serial ½ dilution starting from 13,33µg/ml concentrations were
80 tested for each compound dissolved in DMSO. The mixture solution contained buffer

[1] Podczasy JJ & Wei R. Reduction of iodonitrotetrazolium violet by superoxide radicals. *Biochem Biophys Res Commun.* **1988**, 150, 1294-301.

phosphate 50mM, pH 7,8; 0,1mM EDTA, 0,5mM Xanthine (Sigma Aldrich) and 50µM INT (Sigma Aldrich). 0,1U/ml xanthine oxidase (Sigma Aldrich) was added to initiate the reaction. The plate was read for 3 min at 505 nm on a microplate reader spectrophometer (Spectramax). The inhibitory percentage of each compound was calculated. Each assay was performed in triplicate and superoxide dismutase 1 (Sigma Aldrich) was used as positive control.

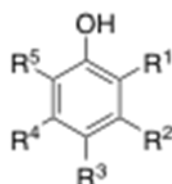
Measurements of intracellular H_2O_2

Generation of intracellular H2O2 was measured using H2DCFDA (Thermo Fisher) upon oxidation to the fluorescent derivative 2'-7'- dichlorofluorescin (DCF) by reaction with H_2O_2. Following treatments, HCT116 cells were collected and resuspended in 500µl RPMI medium containing 10%FCS and 10µM H2DCFDA for 15 min at 37°C. Subsequently, cells were washed with PBS and analyzed with flow cytometry (Becton Dickinson).

Measurements of mitochondrial H_2O_2

HCT116 cells were cultured either in 10% or 1% FCS with 50nM of compound ICSN3250 for 6h. Generation of mitochondrial H_2O_2 was measured using MitoSOX Red mitochondrial superoxide indicator (Thermo Fisher) upon oxidation to the fluorescent derivative by reaction with H_2O_2. Following treatment with ICSN3250, HCT116 cells were collected and resuspended in 500µl RPMI containing 10%FCS and 5 µM MitoSOX for 10 min at 37°C. Subsequently, cells were washed with PBS and analyzed with flow cytometry (Becton Dickinson).

Supplementary Tables



105 **Supplementary Table 1.** DFT-based prediction of aqueous pK_a value for **P1**, a simplified
106 analogue of **ICSN3250**, using the protocol described in Muckerman *et al.*[2] The linear fit (lfit)
107 coefficients obtained from our dataset were a_0=10.512 and a_1=2.596, with R^2=0.93705.

	R^1	R^2	R^3	R^4	R^5	$pK_{a,exp}$	$pK_{a,calc}$	$pK_{a,lfit}$	$\Delta pK_{a,exp-lfit}$
T1	H	H	H	H	H	9.95	3.58	9.94	0.01
T2	H	H	NO_2	H	H	7.14	1.97	7.00	0.14
T3	H	NO_2	H	H	H	8.35	2.81	8.53	-0.18
T4	NO_2	H	H	H	H	7.23	1.86	6.79	0.44
T5	H	H	OH	H	H	9.96	4.23	11.14	-1.18
T6	H	OH	H	H	H	9.44	3.54	9.88	-0.44
T7	OH	H	H	H	H	9.48	3.35	9.51	-0.03
T8	H	H	Me	H	H	10.19	3.72	10.19	0.00
T9	H	Me	H	H	H	10.08	3.66	10.10	-0.02
T10	Me	H	H	H	H	10.28	3.38	9.58	0.70
T11	H	H	Ph	H	H	9.51	3.53	9.85	-0.34
T12	H	Ph	H	H	H	9.59	3.57	9.92	-0.33
T13	Ph	H	H	H	H	9.93	3.49	9.78	0.15
T14	NO_2	H	H	H	OH	6.66	1.39	5.93	0.73
T15	NO_2	NO_2	H	H	OH	4.39	0.23	3.80	0.59
T16	H	NO_2	H	H	OH	6.89	1.71	6.52	0.37
T17	NO_2	H	NO_2	H	H	4.11	0.65	4.58	-0.47
T18	NO_2	H	H	NO_2	H	5.22	1.13	5.44	-0.22
T19	NO_2	H	H	H	NO_2	5.23	0.33	3.99	1.24
T20	H	NO_2	NO_2	H	H	5.42	1.22	5.61	-0.19
T21	NO_2	H	NO_2	H	NO_2	0.96	-0.79	1.92	-0.96
Standard deviation									**0.56**
Root mean square error									**0.55**
P1	NO_2	H	Pyrr	H	OH		1.39	**5.93**	

108 Pyrr = *N*-methyl-3-pyrrolyl

109

110

111

[2] Muckerman JT, Skone JH, Ning M, Wasada-Tsutsui Y. Toward the accurate calculation of pKa values in water and acetonitrile. *Biochim Biophys Acta*. **2013**, *1827*, 882-891.

112 **Supplementary figure legends**

113

114 **Supplementary Figure S1. ICSN3250 specifically inhibited mTORC1 pathway**. (**a**) U2OS
115 cells were treated with the indicated concentration of ICSN3250 during 24 h. Cell extracts
116 were analysed by western blot to determine the activation of the following pathways:
117 mTORC1 pathway (determined by the phosphorylation of S6K at residue Thr389); mTORC2
118 (determined by the phosphorylation of AKT at residue Ser473); AMPK pathway (determined
119 by the phosphorylation of AMPK at residue Thr172); p53 pathway (determined by the
120 phosphorylation of p53 at residue Ser15); PI3K pathway (determined by the phosphorylation
121 of AKT at residue Thr308); ERK pathway (determined by the phosphorylation of p44/42
122 MAPK at residue Thr202/Tyr204 and the phosphorylation of RSK at residue Thr359/Ser363);
123 NF-κB pathway (determined by the phosphorylation of p65 at residue Ser536). (**b-c**)
124 HCT116 cells (b) and U2OS cells (c) were incubated in the presence or absence of
125 ICSN3250 100 nM for 24h, 48h and 72h as indicated. Cell extracts were analysed by
126 western blot to determine the phosphorylation of S6, AKT, p44 and p65. (**d**) U2OS cells were
127 treated with the indicated concentration of ICSN3250 during 24 h. Cell extracts were
128 analysed by western blot to determine the activation of the mTORC1 pathway through the
129 phosphorylation of its downstream targets S6K, S6, and 4EBP1. (**e**) U2OS cells were treated
130 with 100 nM of ICSN3250 during the indicated time. Cell extracts were analysed as in d. (**f**)
131 U2OS cells were treated as in d. Cell extracts were analysed by western blot to determine
132 the activation of autophagy through the levels of LC3-II and p62. (**g**) U2OS cells were
133 treated as in e. Cell extracts were analysed as in f. (**h**) U2OS cells were treated with the
134 indicated concentration of ICSN3250 during 24 h. Cell cycle distribution was analysed by
135 flow cytometry.

136

137 **Supplementary Figure S2. ICSN3250 did not act through mechanisms previously**
138 **described for other mTOR inhibitors**. (**a-b**) Relative *in vitro* kinase activity of different
139 protein kinases as indicated in the presence of 100 nM ICSN3250 (100% of activity was

140 estimated as the activity of each protein kinase in the absence of ICSN3250). (**c-d**) mTOR
141 localization in U2OS cells treated with or without 100 nM of ICSN3250 during 24h, as
142 indicated. mTOR localization was determined (c) and quantified (d) by immunodetection
143 using confocal microscopy. CD63 was used as a lysosomal marker. (**e**) U2OS cells were
144 treated with 100 nM of ICSN3250 either in the presence or the absence of amino acids (AA)
145 during 24 h. Cell extracts were analysed by western blot to determine the activation of the
146 mTORC1 pathway through the phosphorylation of its downstream targets S6 and 4EBP1. (**f-**
147 **g**) HCT116 (f) or U2OS (g) cells were transfected with either an empty vector or with a
148 vector expressing Flag-Rheb as indicated. 24 hours later, cells were incubated either in the
149 presence or the absence of amino acids (AA). Cell extracts were analysed by western blot to
150 determine the activation of the mTORC1 pathway through the phosphorylation of its
151 downstream target S6 and 4EBP1. (**h**) U2OS cells were transfected with either an empty
152 vector or with a vector expressing Flag-Rheb as indicated. 24 hours later, cells were treated
153 with or without 100 nM of ICSN3250. Cell extracts were analysed by western blot to
154 determine the activation of the mTORC1 pathway through the phosphorylation of its
155 downstream target S6 and 4EBP1. Graphs show mean values ± s.e.m. (n=3). NS: $P>0.05$ (*t*
156 test).

157

158 **Supplementary Figure S3. ICSN3250 antagonized with phosphatidic acid to inhibit**
159 **mTORC1**. (**a**) U2OS cells were transfected either with a non-targeting siRNA or with a
160 siRNA against TSC2, as indicated. 48h later, cells where treated with ICSN3250 for 24h.
161 Cell extracts were analysed by western blot to determine the activation of the mTORC1
162 pathway through the phosphorylation of its downstream targets S6 and 4EBP1. The efficient
163 silencing of TSC2 was also determined by western blot. (**b-c**) U2OS cells were treated with
164 increasing concentrations of PA in the presence of 100 nM ICSN3250. Cell extracts were
165 analysed by western blot to determine the activation of the mTORC1 pathway through the
166 phosphorylation of its downstream targets S6K, S6, and 4EBP1 (b), and to analyse the

167 inhibition of autophagy by determining the levels of LC3-II and p62 (c). (**d-e**) U2OS cells
168 were treated with increasing concentrations of ICSN3250 in the presence or absence of 100
169 µM PA, as indicated. Cell extracts were analysed by western blot to determine the activation
170 of the mTORC1 pathway through the phosphorylation of its downstream targets S6K, S6,
171 and 4EBP1 (d), and to analyse the inhibition of autophagy by determining the levels of LC3-II
172 and p62 (e).
173
174 **Supplementary Figure S4. FRB domain of mTOR adopts different conformations in the**
175 **apo form and in complex with ICSN3250 and PA**. Root-mean-square deviation (RMSD) of
176 mTOR protein during molecular dynamics simulations: apo form (a), complex with ICSN3250
177 (b) and complex with phosphatidic acid (c).
178
179 **Supplementary Figure S5. ICSN3250 is redox inactive**. (**a**) ESR analysis of ICSN3250
180 (red line) in the presence of KO_2, recorded at room temperature. A positive compound
181 (ICSN3219) previously known to react with KO_2 was included as positive control (black line).
182 (**b**) Reactivity of ICSN3250 with superoxide anion at the indicated concentrations. SOD was
183 included as a positive control. (**c**) Cytosolic ROS formation in HCT116 cells incubated with
184 increasing concentrations of ICSN3250. (**d**) Mitochondrial ROS formation in HCT116 cells
185 incubated with or without ICSN3250 (50 nM) in the presence of either 10% or 1% of FCS. (**e**)
186 Cell viability of HCT116 cells incubated either in the presence or the absence of ICSN3250
187 (50 nM) and/or NAC (2mM).
188
189 **Supplementary Figure S6. Inhibition of mTORC1 by ICSN3250 is responsible for its**
190 **cytotoxicity in cancer cells**. (**a**) Microscopy photography of $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs
191 treated with or without ICSN3250 100 nM for 72h. (**b**) U2OS cells were transfected either
192 with a non-targeting siRNA or with a siRNA against TSC2, as indicated. 48h later,
193 transfected cells were treated with or without ICSN3250 100 nM for 24 additional hours, and
194 cell viability was determined. (**c-d**) HCT116 (c) or U2OS cells (d) were treated with several

195 mTOR inhibitors, as indicated. Cell extracts were analysed by western blot to determine the
196 activation of the mTORC1 pathway through the phosphorylation of its downstream targets
197 S6K, S6, and 4EBP1. (**e**) IC50 values of different mTOR inhibitors in U2OS cells. Graphs
198 show mean values ± s.e.m. (n=3). *P<0.05 (Anova post hoc Bonferroni).
199
200 **Supplementary Figure S7. ICSN3250 specifically targets cancer cells both *in vitro* and**
201 ***ex vivo*.** (**a**-**b**) Cell proliferation curves of HUVEC (a) and U2OS (b) cells treated with
202 increasing concentrations of ICSN3250, as indicated. (**c**) HUVEC cells where treated with
203 increasing concentrations of ICSN3250 for 24h as indicated. Cell extracts were analysed by
204 western blot to determine the activation of the mTORC1 pathway through the
205 phosphorylation of its downstream target S6. (**d**-**f**) GFP-positive U2OS cells and GFP-
206 negative NHDF or HUVEC cells were co-cultured for 72h in the presence of increasing
207 concentrations of ICSN3250 as indicated. The populations of GFP-positive and GFP-
208 negative cells were determined by flow cytometry (d), and the percentage of cancer cells
209 (U2OS, GFP-positive) *versus* non-cancer cells (NHDF or HUVEC, GFP-negative) was
210 determined (e-f).
211