SUPPLEMENTARY APPENDIX

Differences and similarities in the effects of ibrutinib and acalabrutinib on platelet functions

Jennifer Series,[1,2] Cédric Garcia,[2] Marie Levade,[1,2] Julien Viaud,[1] Pierre Sié,[1,2] Loïc Ysebaert[3,§] and Bernard Payrastre[1,2,§]

[1]Inserm, U1048 and Université Toulouse 3, Toulouse Cedex 04; [2]Laboratoire d'Hématologie, CHU de Toulouse, Toulouse Cedex 04; [3]Service d'Hématologie IUCT-Oncopôle, Toulouse Cedex 09, France

[§]*These authors share senior authorship.*

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Correspondence: *BERNARD PAYRASTRE* - bernard.payrastre@inserm.fr

LOIC YSEBAERT - ysebaert.loic@iuct-oncopole.fr

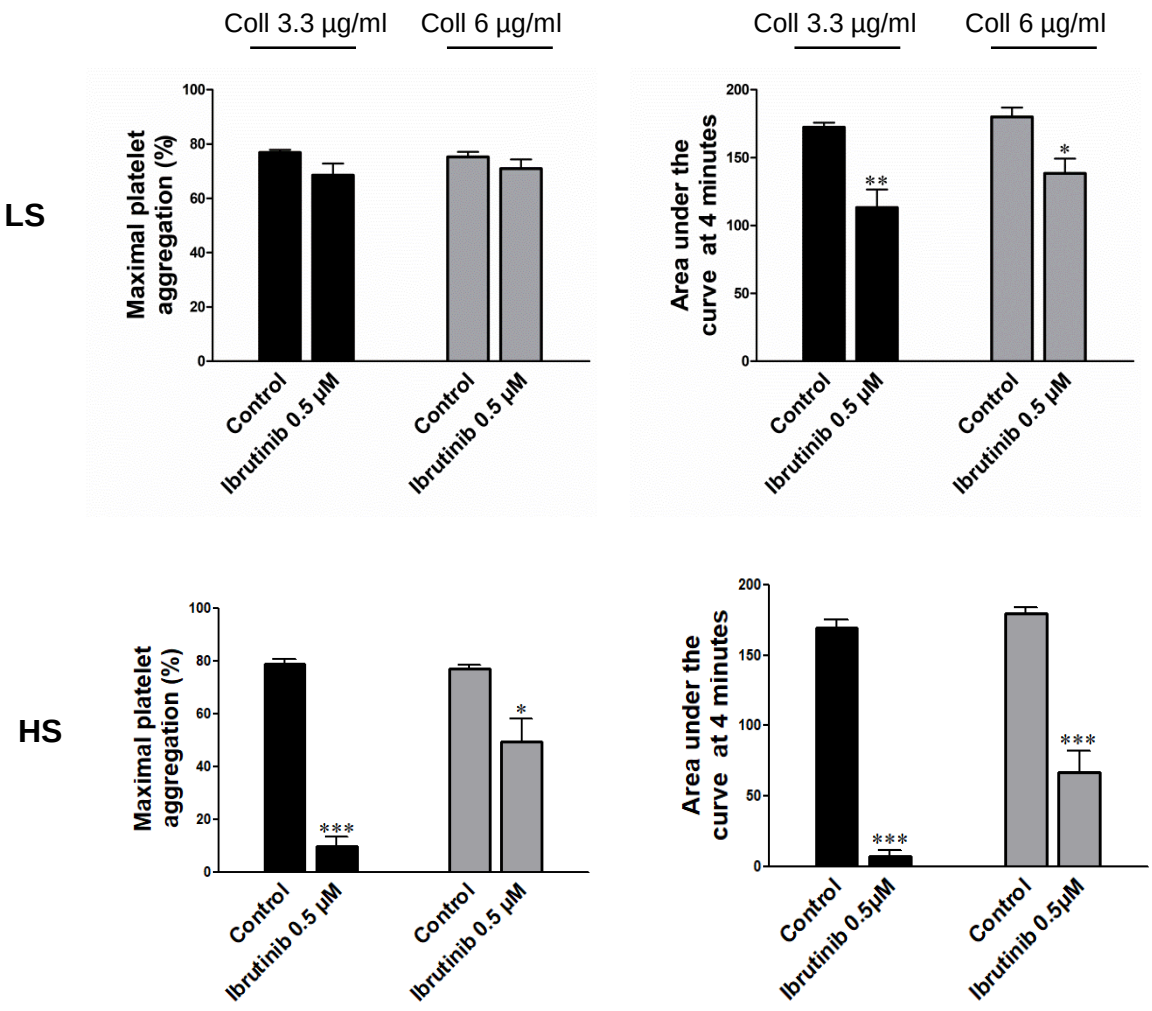
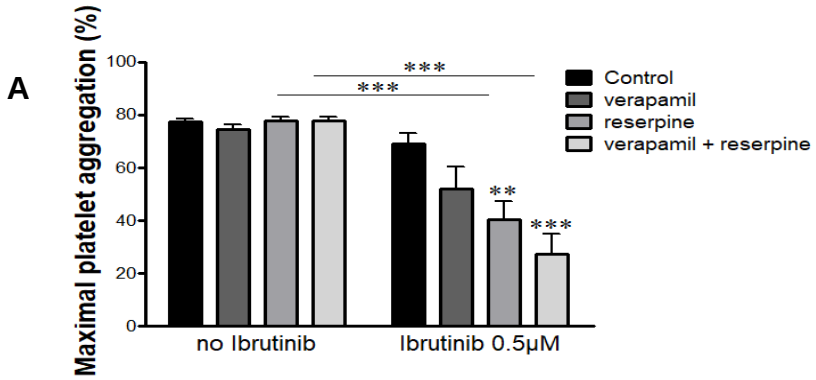
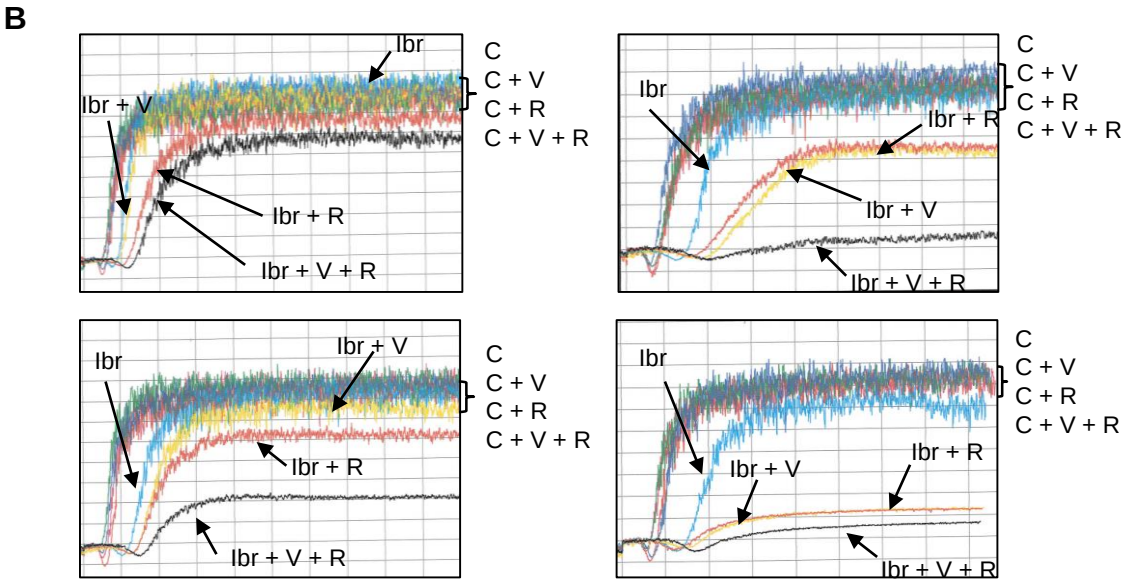


Figure S1: Effect of ibrutinib on platelet aggregation induced by collagen 3.3 µg/ml versus collagen 6 µg/ml. PRP from healthy donors were treated or not with 0.5 µM of ibrutinib during 1 hour at 37°C and stimulated with collagen 3.3 µg/ml or collagen 6 µg/ml. Platelet aggregation was assessed by turbidimetry during 10 min and results, expressed as percentage of maximal aggregation and area under the curve, are mean ± SEM (LS: n=5, HS: n=9, *p<0.05, **p<0.01, ***p<0.001 according to student's t-test).





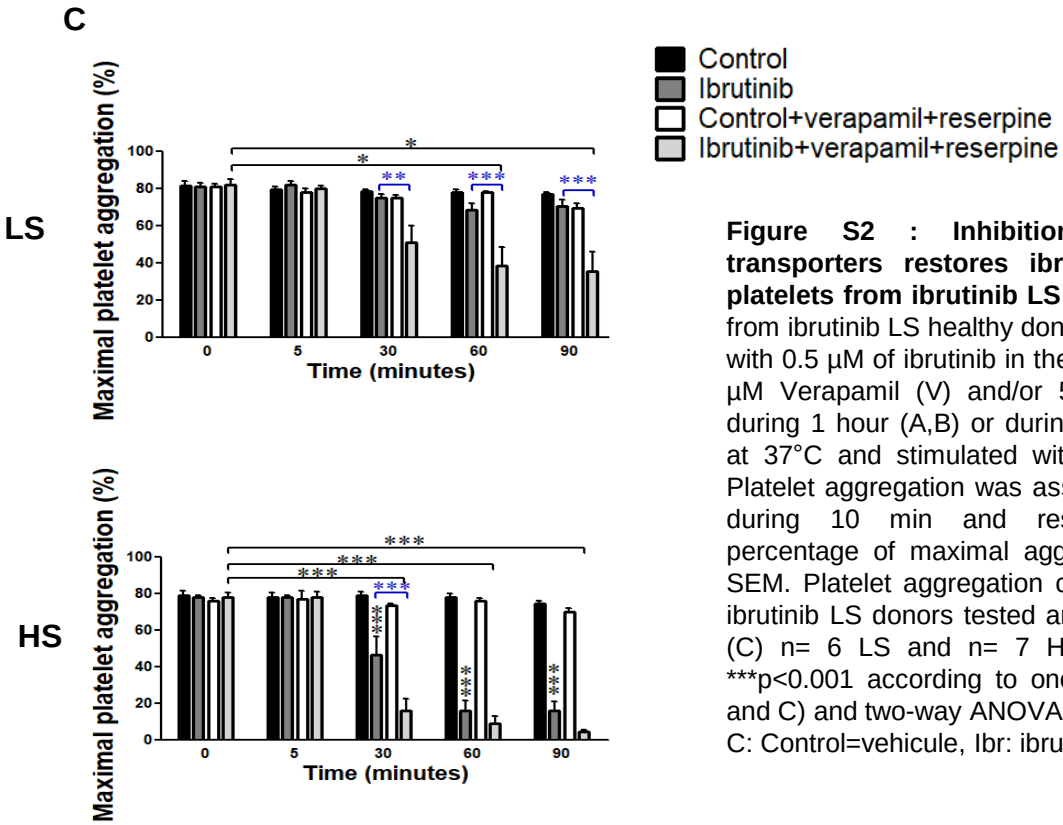


Figure S2 : Inhibition of drug efflux transporters restores ibrutinib sensitivity to platelets from ibrutinib LS healthy donors. PRP from ibrutinib LS healthy donors were treated or not with 0.5 µM of ibrutinib in the presence or not of 40 µM Verapamil (V) and/or 50 µM Reserpine (R) during 1 hour (A,B) or during increasing times (C) at 37°C and stimulated with collagen 3.3 µg/ml. Platelet aggregation was assessed by turbidimetry during 10 min and results, expressed as percentage of maximal aggregation, are mean ± SEM. Platelet aggregation curves from 4 different ibrutinib LS donors tested are shown (B). (A) n=9, (C) n= 6 LS and n= 7 HS. *p<0.05, **p<0.01, ***p<0.001 according to one-way ANOVA test (A and C) and two-way ANOVA test (C).
C: Control=vehicule, Ibr: ibrutinib.

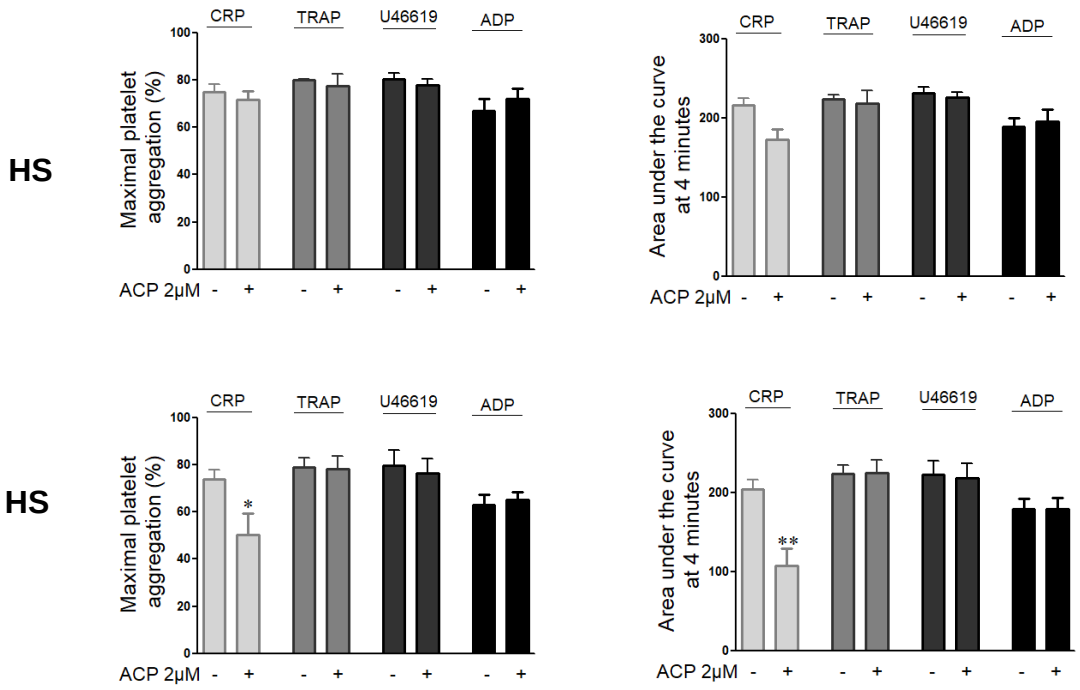


Figure S3: Effect of acalabrutinib on platelet aggregation induced by different agonists. PRP from healthy donors were treated or not with 2 µM of acalabrutinib (ACP) during 1 hour at 37°C and stimulated with different agonists (CRP 10 µg/ml, TRAP 25 µM, U46619 5 µM and ADP 10 µM). Platelet aggregation was assessed by turbidimetry during 10 min and results, expressed as percentage of maximal aggregation and area under the curve, are mean ± SEM (LS: n=5, HS: n=5, *p<0.05 , *p<0.01 according to student's t-test).

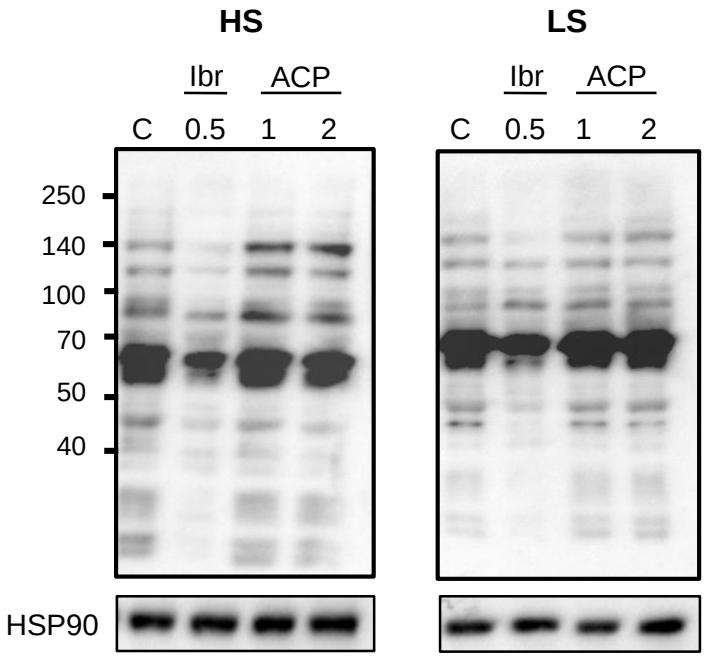


Figure S4: Effect of acalabrutinib and ibrutinib on tyrosine phosphorylations induced by collagen. Washed platelets from ibrutinib HS and LS healthy donors were treated with indicated doses of ibrutinib (Ibr), acalabrutinib (ACP) or vehicle (C) during 1 hour at 37°C and stimulated for 1 min with collagen 3.3 µg/ml. Platelet tyrosine phosphorylations were assessed by Western Blotting using the 4G10 anti-phosphotyrosine antibody. HSP90 was used as a loading control.

Table 1: Clinical and biological data of the CLL patients

Patients number	Gender	Age	Platelets (G/L)	Leucocytes (G/L)	Binet stage	Active disease	Previous lines of therapy	IgHV mutational status	Maximal aggregation (%)-collagen 3.3µg/ml		
									Control	Ibrutinib 0.5µM	ACP 2µM
#1	F	64	332	168	B	yes	2	UM	50.25	6.56	8.88
#2	M	72	108	118	C	yes	1	UM	68.56	0	0.1
#3	F	46	162	86	A	no	1	UM	63.87	30.34	30.83
#4	F	50	167	46	A	no	0	M	76.11	51.64	70.39
#5	F	69	260	204	B	no	0	UM	71.22	93.47	70.48
#6	M	47	271	32	A	no	1	UM	68.14	8.05	66.69
#7	F	79	119	135	A	no	0	M	30.4	0.07	4.03
#8	M	63	148	28	B	no	2	UM	44.72	13.87	19.35
#9	M	69	139	56	A	no	0	M	51.53	6.88	9.05
#10	M	65	177	146	A	no	0	M	57.67	7.22	7.82
#11	M	70	189	87	B	no	0	UM	37.43	7.6	14.53
#12	F	54	165	29	A	no	0	M	37.08	2.52	0.27
#13	F	66	121	80	B	yes	1	UM	34.03	5.85	
#14	M	76	77	99	C	yes	0	M	54.87	46.51	60.02
#15	F	73	188	105	B	no	0	M	63.97	36.04	26.48
#16	F	72	145	90	B	no	0	M	61.82	10.37	13.92

UM: un-mutated, M: mutated

Supplemental information

Reagents

Horm collagen from equine tendon was from Takeda (Linz, Austria), collagen-related peptide (CRP) was from Pr. Richard Farndale laboratory (Cambridge, UK), thrombin receptor agonist peptide (TRAP 14 mer), ADP and U46619 were from Sigma Aldrich (St. Quentin Fallavier, France) and DiOC6 was from Thermo Fisher (Courtaboeuf, France). The anti-phospho-PLCγ2 (Tyr^{753}) was from Santa Cruz Biotechnology (Santa Cruz, USA) and the other anti-phospho-antibodies were from Cell Signalling Technology (Saint Quentin Yvelines, France). All other reagents were from Sigma-Aldrich (St. Quentin Fallavier, France).

Preparation of human platelets

Platelet rich plasma (PRP) was obtained by centrifugating whole blood at 190g for 10 minutes. Washed platelets were prepared after washing in a buffer (pH 6.8) containing 140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose and 0.35 % bovine serum albumin (BSA) (w/v). The same buffer containing 1 mM $CaCl_2$ was used to prepare the final platelet suspension and pH was adjusted to 7.4.

Whole blood, platelet rich plasma (PRP) or washed platelets (2×10^8 cells/ml) were preincubated with the indicated concentrations of ibrutinib (PCI-32765 from Selleckchem), acalabrutinib (ACP-196 from Selleckchem) or vehicle (DMSO) for 60 minutes at 37°C.

For signaling studies, platelets (4×10^8 cells/ml) were lysed with Laemmli buffer and analyzed by Western blot as previously described (Gratacap et al. Blood 2009; 114(9):1884-1892).