# Preventing Calpain Externalization by Reducing ABCA1 Activity with Probenecid Limits Melanoma Angiogenesis and Development

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Short title: ABCA1 Inhibition Prevents Melanoma Angiogenesis

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Keywords: calpain, calpastatin, angiogenesis, melanoma, ABCA1

Abbreviations;

ABCA1: ATP-binding cassette transporter A1

CRP: C Reactive Protein

CRP/Cast: mouse calpastatin gene under the control of the CRP gene promoter/signal peptide

IL: Interleukin

TLR-2: Toll Like Receptor-2

VEGF: vascular Endothelial Growth Factor

WT: Wild Type

Word count: 3491 (without references)

Figure number: 6

**Abstract**

Calpains, intracellular proteases specifically inhibited by calpastatin, play a major role in neo-angiogenesis involved in tumor invasiveness and metastasis. They are partly exteriorized via the ABCA1 transporter, but the importance of this process in tumor growth is still unknown. The aim of our study was to investigate the role of extracellular calpains in a model of melanoma, by blocking their extracellular activity or their exteriorization. In the first approach, a B16F10 model of melanoma was developed in transgenic mice expressing high extracellular levels of calpastatin. In these mice as compared to wild type animals, tumor growth was inhibited by ~3-fold. In vitro cytotoxicity assays and in vivo tumor studies demonstrated that this protection was associated with a defect in tumor neo-angiogenesis. Similarly, in wild type animals given probenecid to blunt ABCA1 activity, melanoma tumor growth was inhibited by ~3-fold. Again, this response was associated with a defect in neo-angiogenesis. In vitro studies confirmed that probenecid limited endothelial cell migration and capillary formation from vascular explants. The observed reduction in fibronectin cleavage under these conditions is potentially involved in the response. Collectively, these studies demonstrate that probenecid, by blunting ABCA1 activity and thereby calpain exteriorization, limits melanoma tumor neo-angiogenesis and invasiveness.

## Introduction

Calpains are calcium-activated neutral cysteine proteases (Goll et al. 2003; Zatz and Starling 2005). Two major isoforms, calpain μ and m are ubiquitously expressed, whereas the other isoforms are tissue-specific forms. Calpastatin, a specific endogenous inhibitor which contains four equivalent inhibitory domains, is responsible for the limited proteolysis of specific calpain substrates (Goll et al. 2003). As they are expressed in the cytosol, these targets are thought to be intracellular. Structural determinants of calpain substrates are still not well defined. Several of them contain a sequence rich in proline, glutamic acid, serine, and threonine (PEST domain) enhancing both calpain binding and calpain-dependent proteolysis (Shumway et al. 1999; Wang et al. 2003). Using mice with calpastatin transgene overexpression (since calpain μ and m knock-out mice are not viable), we and others have demonstrated that calpains are involved in a great diversity of inflammatory diseases (Howatt et al. 2016; Letavernier et al. 2012; Letavernier et al. 2011; Letavernier et al. 2008; Wan et al. 2015; Zafrani et al. 2012).

The contribution of calpains in tumor processes has been also questioned (Leloup and Wells 2011; Moretti et al. 2014; Ono et al. 2016; Storr et al. 2011). Their expression and activity appear increased in numerous cancer types, in response to different oncoproteins and epidermal growth factor. As a consequence, calpains are involved in lessened adhesiveness and increased invasiveness of transformed cells, mainly through the cleavage of cytoskeletal proteins (amoeboid mechanism) and the MMP-dependent degradation of extracellular matrix components (mesenchymal mechanism). Tumor invasiveness and dissemination also depends on tumor neovascularization in response to angiogenic factors, including vascular endothelial growth factors (e.g. VEGF) which stimulate the endothelial cell migration through the m-calpain activity. The function of calpains in tumor cell death and survival appears ambiguous

(Leloup and Wells 2011; Storr et al. 2011). In a recent study using a mouse model of melanoma, we observed that calpain inhibition restricted to melanoma cells amplifies their dissemination by accelerating migration process (Raimbourg et al. 2013). Meanwhile, calpain inhibition restricted to host cells blunts tumor infiltration by immune cells, allowing tumor cells to escape tumor niche and disseminate (Raimbourg et al. 2013).

Recent studies demonstrated that calpains are also partially externalized. In extracellular localization, they limit paradoxically inflammatory processes and promote tissue repair (Letavernier and Baud 2016; Abe et al. 1998; Yoshimura and Oppenheim 2008). They decrease IL-17A expression as well, by causing Toll like receptor 2 (TLR2) cleavage and thus inhibition of TLR2-induced transcription of molecules essential for IL-17A induction (Perez et al. 2016). In addition, we demonstrated that extracellular calpains participate in both epithelium regeneration in a model of acute kidney injury and angiogenesis in models of kidney inflammation (Frangié et al. 2006; Letavernier et al. 2012). Calpain exteriorization involves the ABCA1 transporter (Perez et al. 2016). Given the involvement of intracellular calpains in cancer development and the opposite functions of intra- and extra-cellular calpains in inflammatory processes, we sought to assess the modulatory role of extracellular calpains in melanoma development. To this aim, we first used a model of transgenic mice expressing the entire coding sequence of the mouse calpastatin gene Cast-201 under the control of the human CRP promoter and a signal peptide (CRP/Calpast) (Perez et al. 2016). This construction induces both liver expression and secretion of calpastatin and, hence, allows to blunt selectively the extracellular activity of calpains. We then used a pharmacological approach to limit calpain exteriorization through the ABCA1 transporter. Both studies demonstrate that blocking the extracellular activity of calpains reduces melanoma growth in mice mainly by limiting angiogenesis process.

## Results

### Limiting the activity of exteriorized calpains with extracellular calpastatin is protective against melanoma development in mouse.

To address the role of extracellular calpains in tumor development, we used transgenic mice expressing the mouse calpastatin gene under the control of the CRP gene promoter/signal peptide (CRP/Cast) (Perez et al. 2016). Such a construct induces the liver expression of calpastatin which then reaches tissue extracellular medium via the blood stream. We subcutaneously implanted these CRP/Cast mice and wild type (WT) mice with melanoma cell line B16-F10 ($10^6$ cells/flank) (Raimbourg et al. 2013). Tumor growth monitored by caliper measurements was significantly reduced in CRP/Cast relative to WT mice (Figure 1a and b).

We next investigated the mechanisms whereby extracellular calpastatin limits melanoma development. The first possibility was that inhibition of the extracellular activity of calpains would slow down tumor cell proliferation but BrdU incorporation assays showed that the proliferation of B16-F10 cells was not affected by extracellular calpain activity (Figure 2a).

The second hypothesis was that a decrease of extracellular calpain activity would alter immune polarization or the production of specific cytokines by tumor cells and/or infiltrating immune cells. In particular, B16 melanoma expansion has been shown to be increased by IL-6/IL-17 (through an IL-6-Stat3 pathway involving ROR γt) and conversely decreased by IFN-γ (Wang et al. 2009). However, our previous study demonstrated clearly that inhibition of extracellular calpains increases IL-17 expression dramatically and IL-6 to a lesser extent while it does not affect IFN-γ production (Perez et al. 2016). In CRP/Cast as compared with WT mice, gene expression analysis in melanoma tumors showed no significant change of IL-2, IL-6, ROR γt and Foxp3, (Figure 2b). Nevertheless, flow cytometry analysis of tumoral

cells revealed a mild but significant increased number of CD8 positive cells infiltrating CRP/CAST tumors (Figure 2c-f)

The third hypothesis was that a decrease of extracellular calpain activity would limit melanoma angiogenesis, which plays a crucial role in melanoma growth (Jour et al. 2016; Meierjohann 2015; Pastushenko et al. 2014). To this aim, capillary density has been measured in whole melanoma harvested at day 10 and actually, extracellular calpastatin limited dramatically tumoral neoangiogenesis in CRP/CAST mice (Figure 2g-i).

**Limiting the exteriorization of calpains by reducing ABCA1 activity is protective against melanoma development and tumoral neoangiogenesis in mouse**

Earlier studies suggested that the ABCA1 transporter is involved in non-classical export of calpains (Perez et al. 2016). Since drugs such as glyburide and probenecid are thought to be ABCA1 inhibitors, we first determined *in vitro* the effect of probenecid on calpain exteriorization from melanoma cell line B16-F10 (Flieger et al. 2003). Extracellular calpain activity was reduced in a dose-dependent manner by probenecid (Figure 3a). Conversely, intracellular calpain activity increased when melanoma cells were exposed to probenecid, confirming that probenecid limits calpain efflux from melanoma cells (Figure 3b). Similar results were obtained with glyburide (Figure 3c-d). At last, ABCA1 silencing by SiRNA limited significantly calpain externalization (Figure 3e-f). SiRNA inhibited 52 to 65% of *ABCA1* mRNA expression in each experiment.

Probenecid did not affect melanoma cell proliferation as shown by BrdU incorporation assays (Figure 4a). We next analyzed *in vivo* the effect of probenecid on melanoma development in WT mice. Tumor volume and weight were significantly reduced in mice given probenecid (50 mg per kg BW at days 0, 4 and 8) as compared with untreated mice (Figure 4b-c). Tumor growth and tumor weight at day 16 were significantly reduced by probenecid administration

(Figure 4b-c). Another set of experiments was conducted to quantify angiogenesis at day 10: tumor volume was significantly decreased (101±28 $\mu m^3$ vs 314±83 $\mu m^3$, p=0.02) and capillary density was reduced significantly in whole melanoma from mice treated with probenecid, evidencing the antiangiogenic role of probenecid in this model (Figure 4d).

**Limiting the exteriorization of calpains by probenecid inhibits neo-angiogenesis ex-vivo and in vitro**

To confirm that probenecid exerts antiangiogenic properties, the outgrowth of vessels from aortic rings exposed to probenecid has been measured at day 7. Probenecid decreased significantly ex vivo angiogenesis, even at low concentrations (Figure 5a). The exposure of aortic ring to calpains (4µM every 2 days) did not increase significantly the outgrowth of vessels, but the addition of calpains (4 µM every 2 days) to a medium containing 500 µM probenecid inhibited partly the antiangiogenic effect of probenecid, suggesting that probenecid inhibits angiogenesis by inhibiting calpain externalization from cells (Figure 5b-f). To confirm that probenecid exerted antiangiogenic properties on endothelial cells through a decrease in calpain extracellular activity, the formation of capillaries in matrigel has been assessed in presence of probenecid and/or calpain and calpastatin. Probenecid (500 µM) reduced significantly capillary lenght (Figure 5g). Extracellular calpain inhibition by calpastatin inhibited capillary formation in a similar manner and the addition of probenecid (500 µM) to calpastatin did not limit further angiogenesis (Figure 5g). In a medium containing low amounts of fetal calf serum, extracellular calpain (4µg/mL) increased capillary formation (Figure 5h-j). Probenecid (500µM) decreased capillary formation significantly in similar conditions (Figure 5h,i,k). The addition of 4 µg/mL calpain to medium containing 500 µM probenecid restored capillary formation (Figure 5h,k,l).

**Limiting the exteriorization of calpains by probenecid limits fibronectin cleavage**

We investigated the mechanisms whereby limitation of calpain externalization by probenecid would limit (neo-)angiogenesis. Probenecid did not induce endothelial cell death and did not reduce endothelial cell proliferation in vitro (Figure 6a,b). It has been shown previously that externalized calpains can degrade specifically fibronectin, an extracellular matrix protein, and by this way increase epithelial cell migration properties (Frangié et al. 2006; Letavernier et al. 2012). In vivo, intact fibronectin was increased significantly in melanoma in the presence of probenecid, suggesting that probenecid actually limited significantly fibronectin cleavage due to calpain activity (Figure 6c-d).

**Limiting the exteriorization of calpains by probenecid inhibits endothelial cell migration**

Probenecid inhibited the migration of endothelial cells in a dose-dependent manner (Figure 6e). Conversely, extracellular calpain (4 µg/mL) increased endothelial cell migration (Figure 6f-h) and the addition of 4 µg/mL calpain to a medium containing 500 µM probenecid restored endothelial cell migration properties (Figure 6f,i,j).

## Discussion

In this report, we show that calpains exteriorized via the ABCA1 transporter play a key role in melanoma neo-angiogenesis. The importance of neo-angiogenesis in melanoma maintenance, local growth and metastasis has been well demonstrated. Hypoxia-dependent processes lead to the activation of hypoxia-inducible factor-1 (HIF1), a potent inducer of pro-angiogenic factors, including VEGF, angiopoietin-2, matrix metalloprotease 14 (MMP 14), and angiogenin (Meierjohann 2015). Metalloproteinases expression by melanoma cells is responsible for the degradation of extracellular matrix proteins such as collagens and fibronectin which bind integrins, αvβ3 and α5β1, respectively, thereby amplifying tumor invasiveness (Jour et al. 2016). Extracellular calpains also cleave matrix fibronectin, inducing a decrease in the linkage between endothelial cells and matrix with a switch from α5β1 to αvβ3 integrin-based adhesion. In turn, αvβ3 engagement promotes angiogenesis (Cseh et al. 2010; Lee et al. 2017). In addition, we have shown that truncated forms of fibronectin resulting from extracellular calpain activity contain both the cell binding domain $III_9$ and the VEGF binding domain $III_{13}$ (Letavernier et al. 2012). These fragments of fibronectin, when released by calpain activity, increase the angiogenic response to VEGF. Thus, limiting the extracellular activity of calpains in WT mice given probenecid or CRP/Cast mice could decrease angiogenic response by preventing those processes. A significant increase in CD8+ cells was observed in CRP/CAST melanoma. Interestingly, VEGF/VEGFR signalling reduces adhesion molecule expression in melanoma intratumoral vessels, reducing CD8+ cells recruitment (Tan et al. 2017; Georganaki et al, 2018). Whether extracellular calpain and calpastatin may influence tumor-induced endothelial cell anergy is unknown.

We have previously identified that calpains are exteriorized from lymphocytes through an ABCA1-dependent process involving the shedding of microvesicles (Perez et al. 2016; Zafrani et al. 2012). Interestingly, mouse B16-F10 melanoma cell lines generate microvesicles that support tumor neo-angiogenesis, invasion and metastasis (Bland et al. 2018; Peinado et al. 2012). The expression of ABCA1 appears high in melanoma cells, especially in pro-angiogenic stem cells, a specificity which could explain their sensitivity to the inhibitory effect of probenecid in neo-angiogenesis process (Bachmeier et al. 2009; Lin et al. 2016). ABCA1 could promote angiogenesis by increasing cholesterol efflux from the plasma membrane, what determines the formation of lipid rafts, positively affecting VEGFR2 dimerization and activation (Zecchin et al. 2017). It also facilitates the generation of HDL which carry sphingosine-1-phosphate (S1P), a bioactive lipid involved in neo-angiogenesis, including in murine melanoma (B16-F10) allograft model (Visentin et al. 2006). ABCA1 transporter involved in melanoma growth is also expressed by tumor infiltrating immune cells. Deletion of myeloid *Abca1* limits melanoma growth, by decreasing the number of infiltrating myeloid derived suppressor cells (MDSCs), which promote tumor angiogenesis (Sorrentino et al. 2015; Zamanian-Daryoush et al. 2017). Thus, it appears that calpain exteriorization could involve the ABCA1 expression by both tumor cells and tumor infiltrating immune cells.

Our therapeutic approach to limit ABCA1 activity and, hence, melanoma neo-angiogenesis and growth has involved the use of probenecid, a uricosuric drug and an inhibitor of ABCA1 (Ahmed et al. 2016; Freeman 2017). We have confirmed ABCA1 blunts calpain exteriorization from melanoma (Fig. 3), endothelial and myeloid cells (data not shown), while promoting its cell retention. Similar results were obtained by using glyburide, another inhibitor of ABCA1 and ABCA1 silencing by SiRNA (Fig. 3). This response is associated

with a defect in capillary formation in vitro and neo-angiogenesis and melanoma growth in vivo. Interestingly, the addition of calpain to the extracellular milieu blunted totally the inhibition of angiogenesis induced by probenecid, supporting the hypothesis that probenecid anti-angiogenic properties imply calpain externalization. Nevertheless, it is possible that probenecid limits tumor angiogenesis also by additional mechanisms. For instance, this drug has been shown to block pannexin 1 (PANX1), a channel-forming glycoprotein responsible for the exteriorization of ATP (Freeman 2017). Its expression is upregulated in B16 melanoma cells as compared to normal melanocytes and its knockdown in B16 melanoma reverts tumor cells into a melanocytic-like phenotype and decreases tumor vascularization and growth (Penuela et al. 2012; Wu et al. 2016).

In conclusion, our present study suggests that ABCA1 pharmacological inhibition, using probenecid or any other specific inhibitory drug, is potentially a novel therapeutic approach in the prevention of melanoma growth. This protection is probably mainly explained by a slowing down of tumor neo-angiogenesis. Further studies exploring supplementary mechanisms involved in the response to ABCA1 inhibition and different tumor targets are warranted. Considering the complex and pleiotropic role of calpains in cell metabolism, synthetic calpain inhibitors may induce severe adverse events but a selective intervention on calpain externalization process could represent a reasonable therapeutic option.

## Materials and methods

**Mice and induction of melanoma**. C57BL/6 male mice expressing the mouse calpastatin gene Cast-201 under the control of the human CRP promoter and a signal peptide (CRP/Calpast; European Mouse Mutant Archive ID EM/ 05917) and wild type C57BL/6 mice bred and housed in similar conditions (WT) were used (Perez et al. 2016). Two-3 months WT and CRP/Calpast mice were inoculated subcutaneously under isoflurane anesthesia at the upper right part of the back with $10^6$ B16-F10 cells (ATCC, USA). Tumor size was measured between day 9 and 15, 3 times a week, as described previously (Raimbourg et al. 2013). The sacrifice was performed at day 16 (tumor growth) or at day 10 (angiogenesis) under sodium pentobarbital anesthesia and tumor volume was calculated from radius of the 3 axis: Volume = 4/3x(π)x(r1)x(r2)x(r3). Similar experiments were performed in C57BL/6 mice (WT) receiving either probenecid intraperitoneally (50 mg/Kg BW at day 0, 4 and 8) or saline (controls). All animal procedures were performed in accordance with the European Union Guidelines for the Care and Use of laboratory animals and approved by the local ethical committee and by the French Ministry of Research (Authorization for the document reference 00515.01).

**Cell cultures**. B16-F10 cells were cultured in DMEM medium (Gibco, France), containing 10% fetal bovine serum (Biowest, France) and supplemented with Hepes 10mM (Gibco) and penicillin/streptomycin 50 U/ml (Gibco). Human umbilical vein endothelial cells (HUVECs) were cultured in Endothelial Cell Basal Medium 2 (ECBM2; PromoCell) and used for experiments between passage 2 and 10.

**Calpain activity assay**. Intracellular calpain activity was determined in melanoma cells and endothelial cells, as previously described (Letavernier et al. 2012; Raimbourg et al. 2013). For measuring calpain activity in the extracellular milieu, these cells were cultured in 24-well tissue culture dishes in RPMI 1640 medium ($3 \times 10^6$ cells in 500 µl). After the indicated culture period, cell-conditioned medium was diluted in Krebs–Ringer HEPES (KRH) solution (pH 7.4) containing 4 mM $CaCl_2$, with or without 100 µM calpain inhibitor-1, and incubated for 10 min before the addition of 50 µM calpain substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC) (Sigma-Aldrich). After a 90-min incubation period, calpain activity was determined as the difference between fluorescence (measured at 360 nm excitation and 430 nm emission) with and without calpain inhibitor 1 (Letavernier et al. 2012; Perez et al. 2016; Raimbourg et al. 2013).

**SiRNA**. To modulate ABCA1 expression, B16-F10 cells were transfected with small interfering RNA targeting *ABCA1*murine gene, 4 siRNAs for Entrez gene 11303: SI02652692; SI02652685; SI02652678 and SI02652671 (FlexiTube siRNA) or 3'-Alexa Fluor 488 AllStars Negative control siRNA (QIAGEN). B16-F10 cells were seeded in a 24 wells plate at a density of 40 000 cells/ well in DMEM 10% FBS. Twenty-four hours after, cells were transfected with 5nM siRNA through the use of 3 µl HiPerFect transfection reagent (QIAGEN). Two days after transfection, cells were incubated with or without 100µM Probenecid for 4 h. Cells were lysed and RNA was isolated with an EZ-10 Spin Column Kit (Proteogenix, Schiltigheim, France) and reverse-transcribed with a First-Strand cDNA Synthesis Kit (Thermo Scientific,Illkirch, France). cDNA was amplified on a Light-Cycler 480 system (Roche) using SYBR Green (Roche) and specific primers for *ABCA1* and *Gusb* as housekeeping gene.

**Quantitative RT-PCR**. Total cellular RNA was collected from melanoma tumors using a RNA Miniprep Super Kit (Bio Basic), checked for quality by measuring the ratio of optical densities at 260 and 280 nm, reverse transcribed into cDNA with Superscript II (Life Technologies BRL), and amplified by PCR using a LightCycler 480 (Roche Diagnostic) with SYBR Green (Fast Start DNA Master SYBR Green I; Roche Applied Science, Roche Diagnostic) and specific primers for mouse IL-2, IL-6, FOXP3, and RORγt and housekeeping genes (β-actin and Gusb).

**Immunohistochemical analyzes**. Melanoma tumors embedded in paraffin were cut into 3 μm sections. Endothelial cells were immunostained with rat anti-mouse panendothelial cell antigen (MECA-32; BD Biosciences) and MAX-PO (rat) Histofine (Nichirei Biosciences). Two independent investigators (JP and ET) counted the number of capillaries in 10 fields at 200x magnification by using ImageJ grids. The mean number of capillaries/field was taken into consideration for each melanoma tumor.

**Melanoma flow cytometry**. Melanoma from 7 CRP/CAST and 7 WT mice were collected at day 16 and dissociated by using a gentleMACS Dissociator (Miltenyi-Biotec). Tissue was then passed through a 30μm sieve. Cells were platted during 30 mn at +4°C with mouse Fc Block (Miltenyi-Biotec). Antibodies were incubated during one hour at +4°C before flow cytometry analysis on a MACSquant analyser (Miltenyi-Biotec). Antibodies used were: mouse anti-CD45 PERCP (130-102-469), mouse anti-F4/80 PE (130-102-422), anti-CD4 APC (17-0042-82) and anti-CD8 FITC (130-102-490) (Miltenyi-Biotec).

**Cell proliferation and death**. To perform BrdU incorporation assays, B16-F10 cells or endothelial cells ($500\times10^3$ cells/well) were cultured in medium supplemented with probenecid

(0-0.5 mM). BrdU was added for 30 min after 4 hours incubation (B16-F10 cells) or for 2 hours after 2 hours incubation (endothelial cells). Thereafter, cells were fixed and immunostained with the anti-BrdU antibody (Cell Proliferation ELISA, BrdU, Roche) before flow cytometry analysis.

To quantify cell death, B16-F10 cells ($500\times10^3$ cells/well) were cultured in medium supplemented without or with probenecid (10-500 µM). After 24h, cell necrosis was quantified using the Cell Death Detection ELISA (Roche).

**Endothelial cell migration**. For endothelial monolayer repair assay, $7x10^4$ HUVEC were cultured to confluence in Culture-Insert (Ibidi). After 24h, the Culture Insert was removed and the cell monolayer including a central cell-free gap of 0.5 mm was covered with fresh medium ±probenecid (Fischer Scientific). Gap surface area was analyzed at 9h by phase contrast microscopy.

**In vitro angiogenesis assay**. $6\ x10^4$ endothelial cells in suspension were seeded in 24-wells on 150 µL Matrigel and incubated for 20h with or without calpastatin (20 µg/mL in the presence of 2.0% FCS) or human µ-calpain (4 µg/mL in the presence of 0.5% FCS) with or without probenecid (0.5 mM). The density of formed tubes was analyzed by measuring total capillary length by hpf, as previously described (Letavernier et al. 2012).

**Aortic ring outgrowth assay**. The thoracic aorta from WT mice was cut into 1mm segments that were placed on Matrigel and covered with culture medium supplemented with or without probenecid (0-0.5 mM) and/or µ-calpain (4 µg/mL every 2 days). After 7 days, 6 mean total vessel outgrowths were measured for each ring at 200x magnification, by using Analysis software.

**Western blots**. Total melanoma tumors were isolated from mice at day 10 and cell lysates were extracted using a RIPA buffer. Equivalent amounts of proteins (5 µg/lane) were separated on an SDS-PAGE gel and transferred to nitrocellulose membrane incubated with primary anti-fibronectin antibody (Santa Cruz 9098). Intact human fibronectin appeared with an apparent molecular weight >250 kD. Intact fibronectin expression was indexed to GAPDH expression (optical density, Image J).

**Statistical analysis**. Data are presented as mean (SEM) and percentages, and were compared with non-parametric (Mann-Whitney) test and $\chi^2$ test. A p value <0.05 was considered significant for all statistics.

**Data availability statement**: No datasets were generated or analyzed during the current study

**Credit statement:**

Guillaume Hanouna (GH), Ellie Tang(ET) and Joëlle Perez(JP) contributed equally to this paper

Laurent Baud (LB) and Emmanuel Letavernier (EL) contributed equally to this paper

Conceptualization: EL, LB

Investigation and experiments: GH, ET, JP, SV, JPH, LB, EL

Formal analysis: GH, ET, JP, SV, LB, EL

Manuscript revision and drafting: GH, ET, JP, SV, JPH, LB, EL

Conflicts of interest: the authors declare no conflict of interest

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**Figure legends**

**Figure 1: Inhibition of extracellular calpain activity limits melanoma growth in vivo**. Tumor volume was significantly lower in CRP/CAST mice than in control (WT) mice 15 days after the subcutaneous injection of 1 million B16F10 cells (p=0.037, n=9 animals/group at day 0-9 and n=7 and 8 mice/group at day 15, Figure 1a). Melanoma weight was lower in CRP/CAST than in controls at day 16 (p= 0.02, n=7 and 8 mice/group, Figure 1b).

**Figure 2: Extracellular calpain activity does not affect melanoma cell proliferation but promotes CD8+ immune infiltrate and tumoral neo-angiogenesis.** In vitro, exposure of B16F10 melanoma cells to calpains at various concentrations or inhibition of extracellular calpain activity by calpastatin did not affect tumoral cell proliferation (n=4 experiments, p=NS, Figure 2a). In vivo, there was no impact of extracellular calpain inhibition in CRP/CAST mice on the expression of the main nuclear transcription factors and cytokines involved in antitumoral immune response (n=7 tumors analysed/group, p=NS, Figure 2b). IL-2: interleukin 2; IL-6: interleukin 6. Immune cell infiltrate was quantified by flow cytometry to assess the percentage of leucocytes (CD45+), macrophages (F4/80), and lymphocytes (CD4+ and CD8+) among tumoral cells (n=7 tumors analysed/group, p= 0.03 for CD8+ cells, Figure 2c-f). Tumoral neoangiogenesis was quantified by MECA32 immunostaining in whole melanoma of animals sacrificed at day 10, CRP/CAST mice had significantly less capillaries/field than WT mice (n=16 and 15 animals/group respectively, p=0.0067, Figure 2 g-i).

**Figure 3: Probenecid, glyburide and ABCA1 SiRNA inhibit extracellular calpain activity.** In vitro, exposure of B16F10 melanoma cells to probenecid decreased extracellular calpain activity in a dose-dependent manner (p=0.016 probenecid 500µM vs control, n=5 experiments, Figure 3a). In parallel, probenecid increased intracellular calpain activity in a dose dependent manner (p=0.016 probenecid 100 and 500µM vs control, n=5 experiments, Figure 3b). Exposure of B16F10 melanoma cells to glyburide decreased extracellular calpain activity in a dose-dependent manner (p=0.03 glyburide 50 and 100µM vs control, n=4 experiments, Figure 3c). In parallel, there was a significant increase in intracellular calpain activity in cells exposed to 100 µM glyburide (p=NS, n=4 experiments, Figure 3d). Transfection of B16F10 with SiRNA targeting ABCA1 decreased significantly extracellular calpain activity (p= 0.009 SiRNA vs control, n=6 experiments, Figure 3e) and increased intracellular calpain activity (p=0.002 SiRNA vs control, n=6 experiments, Figure 3f).

**Figure 4: Probenecid limits tumor growth and tumoral neoangiogenesis in vivo.**
Exposure of B16F10 melanoma cells to probenecid at various concentrations did not affect tumoral cell proliferation (p=NS, n=5 experiments, Figure 4a).
In vivo, Probenecid administration reduced significantly tumor volume in comparison to control mice 15 days after the subcutaneous injection of 1 million B16F10 cells (p=0.002, n=6 animals/group, Figure 4b). Melanoma weight was lower in mice receiving probenecid than in controls at day 16 (p= 0.008, n=6 mice/group, Figure 4c). Tumoral neoangiogenesis was quantified by MECA32 immunostaining in whole melanoma of animals sacrificed at day 10, probenecid-treated mice had significantly less capillaries/field than control mice (p=0.0079, n=5 animals/group respectively, p=0.01, Figure 4d).

**Figure 5: Probenecid inhibits ex-vivo and in vitro angiogenesis by limiting extracellular calpain activity.**
Ex vivo, probenecid inhibited the outgrowth of vessels from aortic ring in a dose dependent manner (p=0.003 at 100µM and p=0.001 at 500µM vs control, n=7 experiments, Figure 5a). The exposure of aortic ring to calpains (4µM every 2 days) did not increase significantly the

outgrowth of vessels, but the addition of calpains to a medium containing 500µM probenecid inhibited partly the antiangiogenic effect of probenecid) (p=0.05, n=5 experiments, Figure 5b-f).
In vitro, probenecid inhibited the formation of capillaries by endothelial cells (HUVECs) in matrigel (p=0.0079, n= 5 experiments, Figure 5g). Extracellular calpain inhibition by calpastatin inhibited capillary formation in a similar manner, alone or in addition to probenecid (p=0.0079, n=5 experiments, Figure 5g). In a medium containing low amounts of fetal calf serum, extracellular calpain (4µg/mL) increased capillary formation (p=0.011, n=7 experiments, Figure 5h-j). Probenecid (500µM) decreased capillary formation significantly in similar conditions (p=0.0006, n=7 experiments, Figure 5h,i,k). The addition of 4 µg/mL calpain to medium containing 500 µM probenecid restored capillary formation (p=0.0041, n=7 experiments, Figure 5h,k,l). Scale bar=200 µM.

**Figure 6: Probenecid does not affect tumoral cell proliferation or death but limits fibronectin cleavage by extracellular calpains and inhibits endothelial cell migration.** In vitro, exposure of HUVECs to probenecid at various concentrations did not increase cell death (p=NS, n=4 experiments, Figure 6a). Probenecid did not decrease HUVECs proliferation (p=NS, n=5 experiments, Figure 6b). In vivo, intact fibronectin was increased in melanoma in the presence of probenecid, suggesting that probenecid actually limited fibronectin cleavage due to calpain activity (p=0.0079, n=6 experiments, Figure 6c, Figure 6d: representative western-blot).
In vitro, probenecid inhibited the migration of endothelial cells in a dose-dependent manner (p=0.05 at 100µM and p=0.037 at 500µM vs control, n= 5 experiments, Figure 6e). Extracellular calpain (4 µg/mL) increased endothelial cell migration (p=0.026, n=6 experiments, Figure 6f-h). The addition of 4 µg/mL calpain to a medium containing 500 µM probenecid restored endothelial cell migration properties (p=0.007, n=6 experiments, Figure 6f,i,j).