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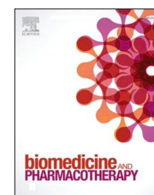

High levels of soluble CD73 unveil resistance to BRAF inhibitors in melanoma cells

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ABSTRACT

Melanoma cells express high levels of CD73 that produce extracellular immunosuppressive adenosine. Changes in the CD73 expression occur in response to tumor environmental factors, contributing to tumor phenotype plasticity and therapeutic resistance. Previously, we have observed that CD73 expression can be up-regulated on the surface of melanoma cells in response to nutritional stress. Here, we explore the mechanism by which melanoma cells release soluble CD73 under low nutrient availability and whether this might be affected by agents targeting the proto-oncogene B-Raf (BRAF). We found that starved melanoma cells can release high levels of CD73, able to convert AMP into adenosine, and this activity is abrogated by selective CD73 inhibitors, APCP or PSB-12489. The release of CD73 from melanoma cells is mediated by the matrix metalloproteinase MMP-9. Indeed, MMP-9 inhibitors significantly reduce the levels of CD73 released from the cells, while its surface levels increase. Of relevance, melanoma cells, harboring an activating *BRAF* mutation, upon treatment with dabrafenib or vemurafenib, show a strong reduction of CD73 cell expression and reduced levels of CD73 released into the extracellular space. Conversely, melanoma cells resistant to dabrafenib show high expression of membrane-bound CD73 and soluble CD73 released into the culture medium. In summary, our data indicate that CD73 is released from melanoma cells. The expression of CD73 is associated with response to BRAF inhibitors. Melanoma cells developing resistance to dabrafenib show increased expression of CD73, including soluble CD73 released from cells, suggesting that CD73 is involved in acquiring resistance to treatment.

1. Introduction

In the last decades, treatment of patients with metastatic melanoma with targeted and immunotherapies has substantially improved response and overall survival. Inhibitors of immune checkpoints, including agents blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis, can boost immune response against cancer cells inducing durable responses [1].

On the other side, patients with metastatic melanoma bearing activating *BRAF* mutations [2], that play a critical role in the RAS-RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cell signaling pathway in melanoma cells, can reach clinical benefits with inhibitors of mutated, activated BRAF [3], especially in combination with MEK inhibitors [4]. However, although the clinical benefits derived from these therapies in melanoma patients are unprecedented, a large part of patients develop resistance and tumour relapse. Therefore, the identification of new therapeutic targets as well as of signaling pathways

List of abbreviations: ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; APCP, adenosine 5'-α,β-methylene diphosphonate; ATP, adenosine 5'-triphosphate; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; MAPK, mitogen-activated protein kinase; MMPs, metalloproteinases; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PSB-12489, N^6-Benzyl-2-chloro-N^6-methyladenosine-5'-O-[phosphonomethylphosphonic acid]; S.d., standard deviation.

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involved in therapeutic resistance can be helpful to develop potential strategies to reach additional benefits.

Within the tumor microenvironment, the extracellular adenosine signaling pathway can enhance the melanoma cells' escape by limiting the activity and functions of T cells against cancer cells [5]. The ecto-nucleotidase CD73, expressed on many cell types, including tumor cells and immune cells, is a key component in the extracellular adenosine producing pathway, as it generates adenosine by hydrolysing AMP, derived from ATP/ADP in a canonical pathway [6], or from other substrates in an alternative pathway [7]. Inhibitors of CD73, alone or in combination with immune checkpoint inhibitors, have been tested in preclinical studies [8-10] or are under clinical investigation [11]. Inhibitors of the CD73/adenosine pathway have been tested also in combination with BRAF and MEK inhibitors in mouse models of *BRAF*-mutated melanoma, showing improved tumor control [12].

In melanoma, it has been demonstrated that the gene expression of *NT5E* encoding CD73 can be epigenetically regulated [13], and driven by MAPK signaling and inflammation-induced dedifferentiation in response to T-cell immunotherapy [14]. Accordingly, we have recently demonstrated that elevated CD73 protein expression is associated with an invasive-like phenotype within human melanoma metastases [15]. Thus, the expression of CD73 within tumor tissue can be affected by inflammatory signals, pharmacological treatments and activating MAPK mutations, and thus, changes in extracellular adenosine production might potentially influence the efficacy of therapies.

Notably, in melanoma patients, a soluble form of CD73 was found in the blood, whose levels change in response to PD-1 inhibitors [16,17]. Previous studies have demonstrated that the extracellular form of CD73 can be generated by cleavage from cell membranes, a mechanism also known as shedding, but CD73 can also be present on extracellular vesicles [18,19]. However, whether melanoma cells release CD73 has not been evaluated, and how therapeutic agents, that directly inhibit the activated MAPK pathway in melanoma cells, might affect the release of CD73 is not fully understood.

In this study, we explored the mechanism by which melanoma cells release CD73 into the extracellular space. In addition, expression profiles of soluble CD73 in *BRAF*-mutated melanoma cells sensitive or resistant to BRAF inhibitors, and upon BRAF inhibitor treatment have been studied.

2. Material and methods

2.1. Cell lines, treatments and reagents

Human A375 and A2058 melanoma cells were cultured in DMEM medium supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 10 % Fetal Bovine Serum (FBS) in a humidified incubator (5 % CO_2, 37 °C). Human SK-MEL28 melanoma cells were cultured in MEM medium supplemented as described above. All reagents for cell culture were purchased from Euroclone S.p. A. (Pero, Italy). Experiments were conducted in complete medium and in serum-free medium to mimic a condition of low nutrient availability as previously described [15]. A375 cells were treated with the broad-spectrum inhibitor of matrix metalloproteinases (MMPs) GM6001 (HY15768; 10–20 μM) (MedChemExpress, New Jersey; USA), the MMP-2 inhibitor SB-3CT (HY12354; 5–10 μM) (MedChemExpress, New Jersey; USA) or the MMP-9 inhibitor, MMP-9 inhibitor II (444293; 10–50 μM) (Sigma Aldrich; Missouri, USA), for 48 h. Cells and supernatants were collected for further analyses described below.

Dabrafenib resistant A375 cells (A375-R) were obtained by culturing the cells with increasing concentration of dabrafenib up to 100 nM (HY14660) (MedChemExpress; New Jersey; USA) for two months as previously described by others [20].

Sensitive and resistant cells to dabrafenib were treated with 10 nM of dabrafenib for 48 h. Vemurafenib (HY12057; 0.1 μM) (MedChemExpress; New Jersey; USA) was also used to treat sensitive and resistant cells to BRAF inhibitor. Cells and supernatants, upon clean up from the debris by centrifugation, were collected for further analyses described below.

2.2. Western blot analysis

Collected cells were lysed in RIPA lysis buffer and a total amount of 25 μg of proteins from whole-cell lysates for each sample, in Laemmli buffer, was loaded into a 10 % polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane by Bio-Rad Trans-Blot Turbo (Bio-Rad Laboratories; California, USA) The following antibodies were used: anti-CD73 antibody (1:1000, clone D7F9A) (Cell Signaling Technology; Massachusetts, USA); phospho ERK1/2 (1:5000; Thr202/Tyr204; clone 197G2) (Cell Signaling Technology; Massachusetts, USA); ERK1/2 (1:5000; clone 137F5) (Cell Signaling Technology; Massachusetts, USA). For the detection of housekeeping proteins, the following antibodies were used: GAPDH (1:1000; clone 6C5) (Santa Cruz Biotechnology; Texas, USA); β-actin (1:1000) (ELabScience, Texas, USA). Signals were detected by using the charge-couple device LAS4000 Imaging System (GE Healthcare Life Sciences; Illinois, USA).

For the detection of CD73 released in culture medium from cells by Western blotting, medium supernatants were collected, cleaned up from the debris by centrifugation, and concentrated. Then a total volume of 30 μL of each sample was used to perform Western blotting assay as described above.

2.3. ELISA assay

ELISA assays were used to measure CD73, MMP-9 and MMP-2 in culture medium supernatants collected from cells. Briefly, supernatants collected from cells were cleaned up from the debris by centrifugation, concentrated and used for ELISA assay. The assay was performed following the manufacturer's instruction using a commercially available ELISA kit detecting human CD73 (ELH-5NTE; RayBiotech, USA), human MMP-9 (E-EL-H6075; Elabscience, Texas, USA) or human MMP-2 (E-EL-H1445; Elabscience, Texas, USA). All the results are expressed as pg of analysed target per mg of protein producer cells.

2.4. Analysis of the enzymatic activity of CD73

Analysis of the AMPase activity in the cell supernatant samples was performed using multiple reaction monitoring mass spectrometry (MRM-MS), as described before [17]. Briefly, cell supernatants were collected and the protein concentration of each sample was determined by the bicinchoninic acid (BCA) assay. Then, a preliminary assay was conducted, testing different protein amounts: 3.75 μg – 15 μg – 37.5 μg – 75 μg (Supplementary file 1). A total amount of 75 μg of supernatant proteins for each sample was selected to evaluate the AMPase activity. Thus, proteins were incubated in 100 μL of reaction buffer of phosphate buffered saline (PBS) at 37 °C for 10 min. Afterwards, ^{15}N-adenosine 5'-monophosphate (^{15}N-AMP; 10 μM) (Sigma-Aldrich; Missouri, USA) was added to each sample. Experiments were also conducted in the presence of the selective CD73 inhibitors adenosine 5'-α,β-methylene diphosphate (APCP; 100 μM) (Sigma-Aldrich; Missouri, USA) or PSB-12489 (1 μM) that were added before adding the substrate ^{15}N-AMP. The novel potent CD73 inhibitor PSB-12489 was synthesized according to a described procedure [21]. Then, a total volume of 25 μL of the reaction mix was collected immediately after adding the substrate and after 60 min and 120 min of reaction. An equal volume of ice-cold trichloroacetic acid (TCA, 5 % final concentration) (Sigma-Aldrich; Missouri, USA) was added to each collected sample to quench the reaction. Then samples were centrifuged at 3000 g for 10 min at 4 °C, dried in Concentrator Plus (Eppendorf; Hamburg, Germany) and dissolved in 30 μL of an aqueous solution containing 10 mM ammonium acetate (AmAc, Sigma-Aldrich) and 0.1 % acetic acid (Sigma-Aldrich). To measure the amount of the ^{15}N-adenosine produced, samples were analysed by ultra-high

performance liquid chromatography-electrospray ionization (UHPLC-ESI)-MRM-MS, exploiting the 6500 Q-TRAP from AB Sciex equipped with Shimadzu LC-20A and Autosampler systems. UHPLC separation was performed on a Luna Omega Polar 1.6 μm C18 100 Å column (50 × 2.10 mm, Phenomenex, Torrance, California, USA) at a flow rate of 400 μL/min and using 10 mM AmAc/0.1 % acetic acid in H_2O (A) and 0.1 % acetic acid in methanol (B) as mobile phases. The gradient was set as follows: 0.5 min at 0 % B, 0.5 min to 3 min at 5 % B, 3 min to 5 min at 95 % B, 5 min to 7 min at 95 % B and then back to 0 % B for a 5 min re-equilibration step. ^{15}N-AMP was monitored through the 353.19/140.00 transition and ^{15}N-adenosine through the 273.00/141.00 one. Peak area quantification was performed through the Analyst Software (1.6.2 version) from AB Sciex.

2.5. Gelatin gel zymography

MMP-9 and MMP-2 gelatinolytic activity was detected by SDS-PAGE zymography as previously reported [22]. Briefly, A375 cells serum-free supernatants were analysed under non-reducing conditions and without boiling. Each sample was loaded onto a 8 % polyacrylamide gel, co-polymerized in presence of gelatine (0.1 % w/v) (Sigma-Aldrich; Missouri, USA). The gel was scanned by ImageScanner III LabScan 6.0 (GE Healthcare Life Sciences; Illinois, USA).

2.6. Flow cytometry analysis

Cells (1 × 10^6 cells/mL) were harvested and stained in staining buffer with the mouse anti-human CD73 allophycocyanin (APC)-conjugated antibody (0.125 μg/test) or the corresponding APC mouse IgG1K isotype control (0.125 μg/test). All antibodies were purchased form Invitrogen (Massachusetts, USA). Samples were acquired using a BD FACScalibur (Becton Dickinson; New Jersey, USA) and analyzed by the BD CellQuest Pro Software.

2.7. Cell cycle analysis

A375 and A375-R cell cycle profiles were evaluated by propidium iodide staining. Briefly, cells were harvested, washed and incubated with 50 μg/mL propidium iodide (PI) (Sigma Aldrich; Missouri, USA). Samples were acquired using a BD FACScalibur (Becton Dickinson; New Jersey, USA) and data were analysed using a ModFit LT (Becton Dickinson) cell cycle analysis programme.

2.8. Quantitative real-time PCR

Total RNA was extracted from treated and untreated A375 and A375-R cells, using EuroGOLD RNA Pure reagent (Euroclone S.p.A.; Pero, Italy) following the manufacturer's instructions. Reverse transcription was performed by using M-MLV Reverse Transcriptase (GeneSpin S.r.l., Milan, Italy) and the real-time PCR was conducted by using Luna Universal qPCR Master Mix (New England Biolabs; Massachusetts, USA).

The sequences of primers are reported as follows:

CD73 (NT5E) (Forward): 5'-AAAGGACACGAGAGAAAGGAAGG-3',

CD73 (NT5E) (Reverse): 5'-GAAGAAAGAGGACAGAGGCAGAG-3'.

β-actin (Forward): 5'-CTACAATGAGCTGCGTGTGGC-3',

β-actin (Reverse): 5'- CAGGTCCAGACGCAGGATGGC-3'.

Samples were run in technical duplicate using the QuantStudio™ 5 instrument (Thermo Fisher Scientific, Massachusetts, USA). Data were examined using the ΔΔCt method and β-actin was used as a reference gene.

2.9. Statistical analyses

Statistical analysis and plots were performed using GraphPad Prism V.9.0. Data results are expressed as mean ± s.d. The Mann-Whitney test was used to compare two groups. To compare more groups one- or two-way ANOVA test was used, followed by a Multiple Comparison test as appropriated. P values < 0.05 were considered statistically significant, as reported in the figures and in the figure legends.

3. Results

3.1. CD73 is released from melanoma cells

CD73 is expressed on the surface of melanoma cells and can be upregulated in a stressful condition of low nutrient availability, as we have recently demonstrated [15]. Of note, CD73 also exists in a soluble form (sCD73), identified in many biological fluids [18]. Therefore, we sought to examine whether, under a condition of low nutrient availability, CD73 could also be released from melanoma cells. As shown in Fig. 1A (left panel), CD73 was detected in supernatants of starved-A375 cells and accumulated in a time-dependent manner. The release of sCD73 from A375 melanoma cells was also evaluated by ELISA assay. The levels of sCD73 were significantly increased in the supernatants collected from cells cultured in a condition of starvation (-serum) compared to that from cells cultured in a complete medium (+ serum) (Fig. 1A, right panel) (data are expressed as pg CD73 in supernatant per mg protein producer cells). Similar results were obtained in SK-MEL-28 and A2058 melanoma cells supernatants (Fig. 1B). In addition, we observed that different cancer cells release CD73, including starved breast cancer MDA-MB-231 cells and starved colon cancer DLD-1 cells (Supplementary Fig. 2). These results suggest that sCD73 can be released from tumor cells and accumulated in a time-dependent manner in the supernatants in response to nutritional stress.

3.2. sCD73 is enzymatically active in producing adenosine

The main role of CD73 is to hydrolyse AMP into adenosine and inorganic phosphate, causing extracellular adenosine accumulation [23].

To evaluate whether the sCD73 had a functional significance, we measured the AMPase activity in medium samples collected from A375 melanoma cells. The AMPase activity was evaluated by multiple reaction monitoring mass spectrometry (MRM-MS), measuring the production of ^{15}N-adenosine by the hydrolysis of the substrate ^{15}N-AMP, as described in the material and method section. As shown in Fig. 1C, levels of ^{15}N-adenosine were significantly higher in conditioned medium collected from starved A375 cells (ctrl, -serum) compared to that measured in samples from cells cultured in complete medium (ctrl, + serum). Moreover, when we performed the experiments in the presence of selective CD73 inhibitors, APCP and PSB12489, we found that both inhibitors completely abrogated the levels of ^{15}N-adenosine produced by the substrate ^{15}N-AMP (Fig. 1C), confirming that the increased production of ^{15}N-adenosine was entirely dependent on the activity of CD73 present in the supernatant samples. This result indicates that high levels of sCD73 in the supernatants of starved cells are associated with high levels of adenosine.

3.3. Inhibition of metalloproteases reduces the levels of sCD73

CD73 is a glycosylphosphatidylinositol (GPI)-anchored enzyme that can be shed from the cell surface or released by proteolytic cleavage (reviewed in Schneider et al. [18]). Early reports indicate that CD73 could be released from the cell membrane by phospholipase C (PLC) [24-26] or, more recently, by matrix metalloproteinase 9 (MMP-9) [27, 28].

Based on such evidence, we next evaluated whether metalloproteinases prompted CD73 release from cells, under our experimental conditions. Following treatment of A375 melanoma cells with the metalloproteinase (MMP) inhibitor GM6001 [29], we observed that the levels of sCD73 were reduced compared with control cells as shown in the representative Western blotting image and by ELISA (Figs. 2A and

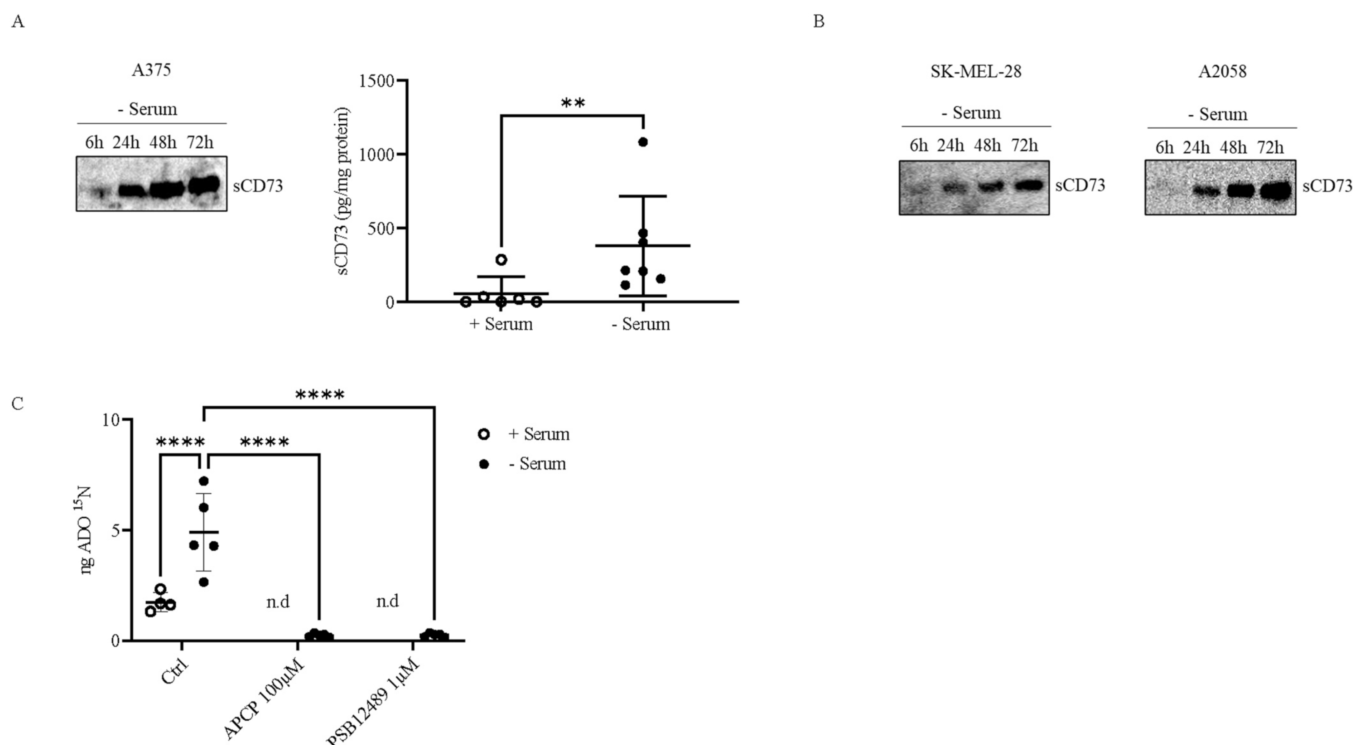

Fig. 1. Levels and AMPase activity of sCD73 in melanoma cells. A) Expression of sCD73 in medium supernatants of A375 cells cultured in a serum free condition (– Serum) analyzed by Western blot (left panel) and by ELISA assay (48 h; n = 6–7) (right panel). (+) Serum: control cells cultured in complete growth medium. Data are expressed as mean ± sd and indicate pg sCD73 per mg protein producer cells. P value is from a Mann-Whitney test. $^{**}p < 0.01$. B) Expression of sCD73 in medium supernatants of SK-MEL-28 and A2058 cells cultured in a serum free condition (- Serum) analyzed by Western blot at 6–24–48–72 h. C) [^{15}N] adenosine (ADO) levels (ng) measured by mass spectrometry in medium supernatants of A375 cells cultured in a serum free condition (– Serum) or in complete medium (+ Serum), after incubation with [^{15}N]AMP (10 μM) (Ctrl), in presence or not of CD73 enzymatic inhibitors APCP (100 μM) or PSB12489 (1 μM) (n = 5). n.d. = not detectable. Data are expressed as mean ± s.d. P value is obtained from a two-way ANOVA test, followed by Tukey's multiple comparison test. $^{****}p < 0.0001$.

2B, respectively). In contrast, treatment of cells with the PLC inhibitor U73122 [30,31] did not affect the level of sCD73 (data not shown). The results suggest that the release of CD73 in our system is mediated by metalloproteinases.

3.4. MMP-9 mediates the CD73 release from melanoma cells

Metalloproteases are expressed by melanoma cells (reviewed in Hofmann et al. [32]). MMP-9 and MMP-2 are expressed by A375 melanoma cells as expected (data not shown). In addition, MMP-9 and MMP-2 are present in the medium supernatants of cells and, in particular, we observed that the protein levels of both MMP-9 and MMP-2 significantly increased in the supernatants collected from cells in a condition of starvation (-serum) compared with that of cells cultured in normal condition (+ serum) (Figs. 3A and 3B, respectively). Moreover, we verified that both MMP-9 and MMP-2, present in the cell supernatants, are functionally active (Figs. 3C and 3D, respectively). These results suggest that the expression levels of MMP-9 and MMP-2 increased

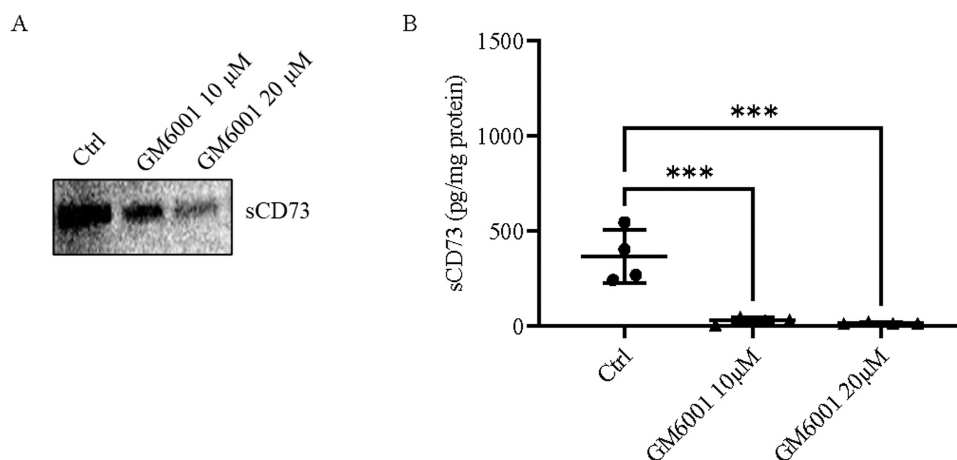

Fig. 2. Levels of sCD73 in A375 cells supernatants in presence of the inhibitor of matrix metalloproteinases. A) Expression of sCD73 detected in the supernatant of A375 cells treated or not with GM6001 (10–20 μM) by Western blot (48 h) and B) by ELISA assay (48 h; n = 4). Data on graphs are expressed as mean ± s.d. and indicate pg sCD73 per mg protein producer cells. P value is obtained from one-way ANOVA test, followed by Dunnett's multiple comparisons test. $^{***}p < 0.001$.

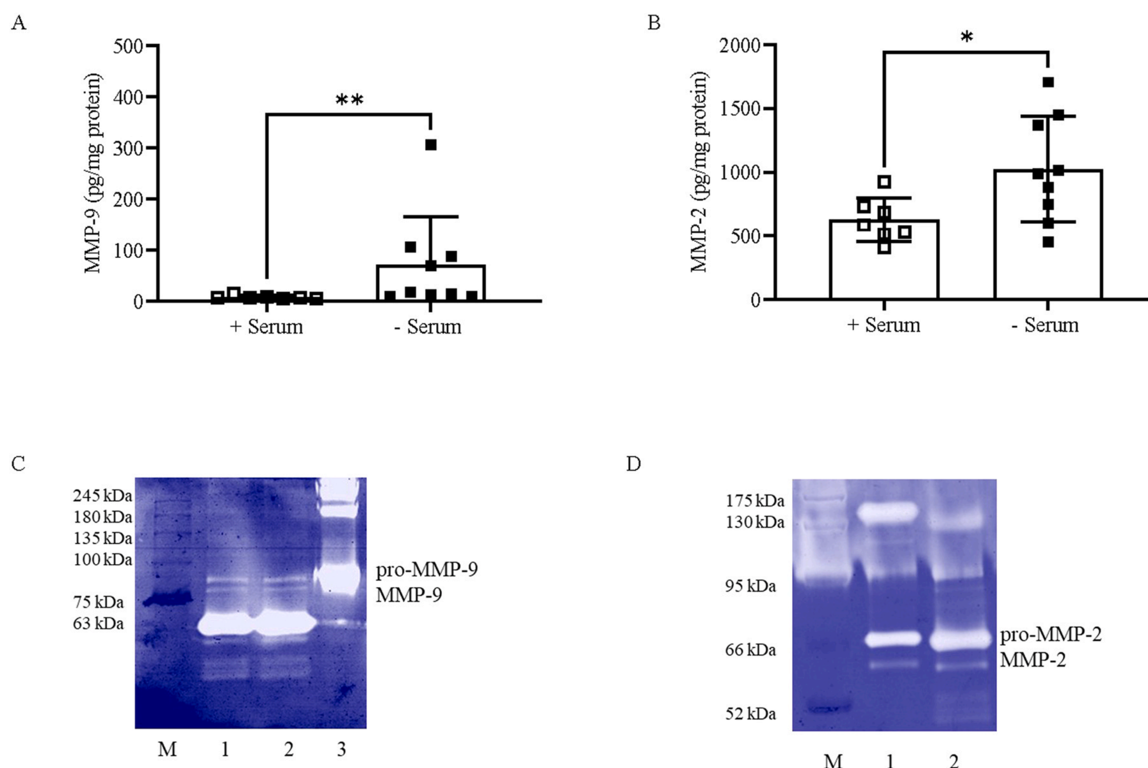

Fig. 3. Levels of extracellular MMP-9 and MMP-2 in melanoma cells. A) MMP-9 and B) MMP-2 protein levels were measured by ELISA assay in A375 cells supernatants cultured in complete growth medium (+ Serum) or in serum free medium (– Serum) (48 h; n = 7–9). Data are expressed as mean ± s.d. and represent pg MMP-9 (A) or MMP-2 (B) per mg protein producer cells p value is obtained from a Mann-Whitney test. *$p < 0.05$; **$p < 0.01$. C) Representative gelatin zymogram of MMP-9 MMP-2 (D) activity in A375 cells supernatants (48 h). Line 1 – 2: A375 cells conditioned medium samples; Line 3: purified human recombinant MMP-9. D) Representative gelatin zymogram of MMP-2 activity. Line 1: purified human recombinant MMP-2; Line 2: A375 cells conditioned medium sample.

in a condition of starvation and they are enzymatically active.

We, therefore, focused our attention on MMP-9 and MMP-2 as possible triggers for sCD73 release from melanoma cells. We evaluated the effects of the MMP-9 inhibitor or MMP-2 inhibitor on the release of sCD73 and on the cell surface expression of CD73. Interestingly, levels of sCD73 measured by ELISA in cell supernatants resulted significantly reduced upon treatment with the MMP-9 inhibitor MMP-9-In II [33] (Fig. 4A). Conversely, the MMP-2 inhibitor SB-3CT [34,35] did not significantly affect the levels of sCD73 (Fig. 4A). Moreover, we also found that in the presence of the inhibitor MMP-9-InII, but not of the

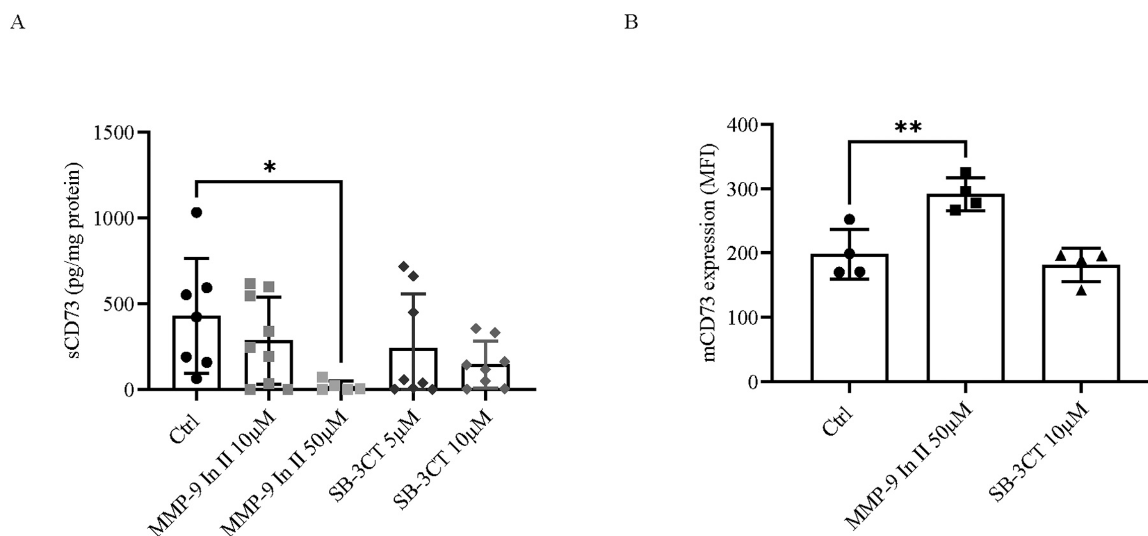

Fig. 4. Analysis of CD73 measured in cells treated with specific metalloproteinases inhibitors. A) sCD73 levels measured by ELISA assay in starved A375 cells supernatants in presence of the MMP-9 Inhibitor II (10–50 μM) or in presence of the MMP-2 inhibitor SB-3CT (5–10 μM) (48 h; n = 7–8). Ctrl=control cells. Data are expressed as mean ± s.d. and indicate pg sCD73 per mg protein producer cells. P value is obtained from a one-way ANOVA test followed by Dunnett's multiple comparison test. *$p < 0.05$. B) Membrane CD73 (mCD73) expression analyzed by flow cytometry in A375 cells treated with the MMP-9 Inhibitor II (50 μM) or SB-3CT (10 μM) (48 h; n = 4). Ctrl = control cells. MFI = mean fluorescence intensity. Data are expressed as mean ± s.d. P value is obtained from one-way ANOVA test, followed by Dunnett's multiple comparisons test. **$p < 0.01$.

inhibitor SB-3CT, the membrane expression of CD73 increased, as evaluated by flow cytometry (Fig. 4B). Thus, the MMP-9 inhibitor reduced the release of sCD73 from the cells, while the MMP-2 inhibitor neither affected the release nor the surface expression of CD73. In support of these results, we performed the same experiments also in the presence of two different MMP-9 or MMP-2 inhibitors (MMP-9 In I or MMP-2 In III, respectively) [33,36,37]. In the presence of MMP-9In I the levels of sCD73 in the cell supernatants were significantly reduced compared with control (Supplementary Fig. 3A), while the surface accumulation of CD73 on melanoma cells increased (Supplementary Fig. 3B). Conversely, the MMP-2 inhibitor, MMP-2 In III, did not influence the levels of sCD73 or the surface accumulation of CD73 (Supplementary Fig. 3A and 3B, respectively). These results indicate that MMP-9 prompts the release of sCD73 from the melanoma cells.

3.5. BRAF inhibition reduces the levels of sCD73 released from melanoma cells sensitive to dabrafenib

A375 melanoma cells are *BRAF* V600E mutated and sensitive to BRAF inhibitors such as dabrafenib [38]. As previous studies have demonstrated that the *NT5E* (*CD73*) gene expression is downregulated by MAPK inhibition [12,39], we next sought to assess whether BRAF inhibition could affect sCD73 release into the extracellular space from starved melanoma cells.

Treatment of cells with dabrafenib reduced the CD73 mRNA levels in cells sensitive to dabrafenib (Fig. 5A). Concomitantly, the protein surface levels of CD73 on melanoma cells were significantly reduced after treatment with dabrafenib (Fig. 5B).

The expression profile of CD73 was also evaluated in A375 cells resistant to dabrafenib. Dabrafenib-resistant A375 cells were obtained as reported in the material and method section, and the resistance to dabrafenib was evaluated by measuring the rate of cell cycle in resistant cells compared with sensitive parental cells (Fig. 5C), and the pERK1/2 levels (Fig. 5D). In melanoma cells resistant to dabrafenib, the CD73 mRNA levels remained unaltered following dabrafenib treatment (Fig. 5E). Moreover, in resistant cells, dabrafenib did not affect the surface level of CD73 (Fig. 5F). We noticed that the membrane levels of CD73 in cells resistant to dabrafenib were higher if compared with that observed in the cells sensitive to dabrafenib (A375 resistant CD73 MFI: 227.7 ± 29.69 mean $\pm$ s.d. versus A375 sensitive CD73 MFI: 171.8 ± 34.14 mean $\pm$ s.d.; $p = 0.0152$ Mann-Whitney test). These results indicate that the expression of CD73 can be inhibited by the MAPK signaling pathway inhibitor dabrafenib in melanoma cells, while it remains overexpressed and unaltered in cells resistant to dabrafenib.

So far, we have shown that melanoma cells release high amounts of sCD73 in response to low nutrient availability. We next tested whether dabrafenib could also affect the levels of sCD73 released from melanoma cells. Interestingly, we observed that dabrafenib reduced the levels of sCD73 in the culture supernatants of sensitive cells, but not in the supernatants of resistant cells (Fig. 6A). Levels of sCD73 in the supernatants of cells sensitive and resistant to dabrafenib were also assayed by ELISA (Fig. 6B). We found that dabrafenib reduced the expression levels of sCD73 released from sensitive melanoma cells (Fig. 6B, left panel). Conversely, in resistant cell supernatants, the levels of sCD73 remained unchanged upon dabrafenib treatment (Fig. 6B, right panel).

We also examined the levels of CD73 on the cell surface and in the supernatant of sensitive and resistant melanoma cells upon treatment with vemurafenib, which also targets BRAF V600E [3]. The effect of vemurafenib was tested by evaluating the level of pERK1/2, which was significantly reduced in sensitive cells, but not in resistant cells (Fig. 7A). When we analyzed the levels of membrane CD73 we found that vemurafenib reduced it on the cell surface of sensitive melanoma cells but not of resistant cells (Fig. 7B). The levels of sCD73 were reduced in supernatant collected from sensitive cells but not from resistant cells after vemurafenib treatment (Fig. 7C).

These results indicate that the expression of CD73 can be inhibited by MAPK signaling pathway inhibitors, dabrafenib and vemurafenib, in melanoma cells sensitive to BRAF inhibitors. Moreover, a reduced expression of the surface CD73 is associated with reduced release of sCD73 upon BRAF inhibitor treatment. Melanoma cells that are resistant to BRAF inhibitors show both high levels of cell surface CD73 and sCD73 released from cells.

4. Discussion

Having previously observed that the membrane levels of CD73 increased in response to nutritional stress, in this work we demonstrate for the first time that melanoma cells in parallel can also release CD73 in a soluble form. The soluble CD73 is fully active in producing adenosine by hydrolyzing its substrate AMP. The release of soluble CD73 into the culture medium from the melanoma cell surface is mediated by MMP-9. Of relevance, we provide evidence that BRAF inhibitors, that reduce the cell expression of CD73, can inhibit the release of the sCD73. On the contrary, melanoma cells resistant to BRAF inhibitors, that express high levels of CD73, release a high amount of sCD73, and in this case, both forms are not affected by inhibiting the MAPK signaling pathway.

Many studies have demonstrated that CD73 is overexpressed on the surface of different tumour cell types, and the high expression of CD73 in human tumor tissues, including melanoma, is correlated with poor prognosis [11,40]. Since CD73 is the main enzyme responsible for the generation of the immuno-suppressive adenosine within the tumor tissue, a blockade of CD73 has been demonstrated to improve the anti-tumor immune response by boosting the T cell-mediated activity [40-42]. In addition, since the expression of CD73 within tumor tissues is dynamic and subject to regulation by environmental factors, as well as by pharmacological treatments, a plethora of evidence supports a role of CD73 in contributing to phenotype plasticity in cancer and therapeutic resistance to many anticancer agents (reviewed in Yang et al. [43]). CD73 can also exist in a soluble form (sCD73), derived from the proteolytic cleavage of the cell-membrane anchored CD73, able to generate adenosine from AMP [18]. Increased levels of sCD73 have been found in the blood of patients with cancer [44-50], including melanoma [51,16,17], emerging as a potential biomarker of tumour progression and/or response to anti-cancer therapies [19]. However, the cellular source of sCD73, the mechanism/s regulating its cleavage from the cell surface or those stimuli that prompt its release are still poorly investigated. Here, we demonstrate that melanoma A375 cells are a critical source of the sCD73, and consistent with the well-known role of CD73 in generating adenosine from AMP, we observed that the sCD73 released into the cell supernatants has an AMPase activity, that is completely abrogated in the presence of selective CD73 inhibitors. Using the same approach, we observed similar results for SK-MEL-28 and A2058 melanoma cells. Therefore, these results indicate that melanoma cells, in response to a nutritional stress condition, not only overexpress CD73 on their surface [15], but also release sCD73 into the extracellular space, and both forms may contribute to increased immuno-suppression through the adenosine signaling pathway. We also investigated whether other tumor cell types could release sCD73 in response to nutritional stress. In fact, breast cancer cells and colon cancer cells can release sCD73 as well.

Previous studies have proposed that the shedding of CD73 can occur by phospholipases as observed in human placenta [25], cerebral cortex [26] or human endothelial cells [24], or by proteolytic cleavage as observed in animal seminal plasma [52], mouse retinal pigment epithelial cells [28] and astrocytes [27]. Here, we provide evidence that metalloproteinases are implicated in the release of CD73 from the melanoma cell membrane. In particular, focusing on MMP-2 and MMP-9, that are two gelatinases belonging to the matrix metalloproteinase family, we observed that, only in the presence of a MMP-9 specific inhibitor, the levels of sCD73 were significantly reduced, while its levels increased on the cell surface. Although we have not explored the molecular details of the MMP-9-mediated shedding of CD73, which deserve further in-depth investigation, it is conceivable that this effect

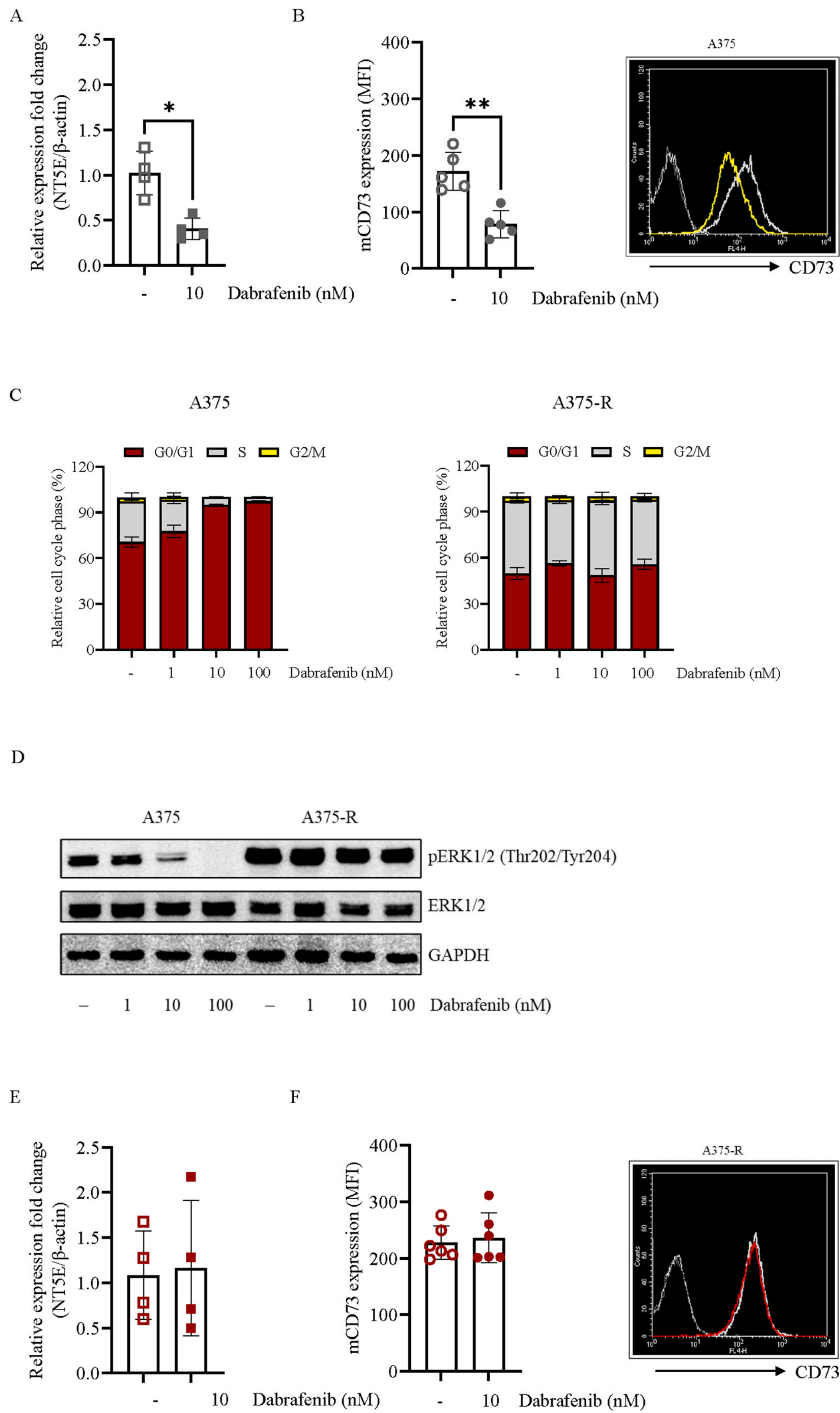

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Fig. 5. Analysis of the expression of CD73 on the cell membrane of A375 melanoma cells sensitive to dabrafenib or resistant to dabrafenib. A) CD73 (*NT5E*) mRNA levels in A375 sensitive cells treated or not with dabrafenib (10 nM) for 24 h (n = 4). Data are expressed as mean ± s.d. P value is obtained from a Mann-Whitney test. *p < 0.05. B) Membrane CD73 (mCD73) expression analyzed by flow cytometry on A375 cells treated or not with dabrafenib (10 nM) for 48 h and expressed as MFI values (n = 5). Left panel shows a representative FACS histogram of CD73 or isotype control (white, dark gray) in A375 cells treated with dabrafenib (yellow) or untreated (light gray). Data are expressed as mean ± s.d. P value is obtained from a Mann-Whitney test. **p < 0.01. C) Cell cycle analysis of A375 cells, sensitive to dabrafenib (A375, left panel) or resistant to dabrafenib (A375-R, right panel) after treatment with Dabrafenib (1–10–100 nM) for 48 h analysed by flow cytometry to assess the percentage of cells in the G0/G1, S and G2/M phases (n = 3). Data are expressed as mean ± sd. D) Expression of pERK1/2 (Thr202/Tyr204) in A375 and A375-R cells treated or not with dabrafenib (1–10–100 nM) (3 h). The expression of total ERK1/2 is also shown. GAPDH expression was evaluated as protein loading control. E) CD73 (*NT5E*) mRNA levels in A375 resistant cells treated or not with dabrafenib (10 nM) for 24 h (n = 4). Data are expressed as mean ± s.d. F) Membrane CD73 (mCD73) expression analyzed by flow cytometry on A375 cells resistant to dabrafenib (A375-R) and treated as described in A (n=6). Left panel shows a representative FACS histogram of CD73 or isotype control (white, dark gray) in A375-R cells treated with dabrafenib (red) or untreated (light gray). Data are expressed as mean ± s.d. MFI = mean fluorescence intensity.

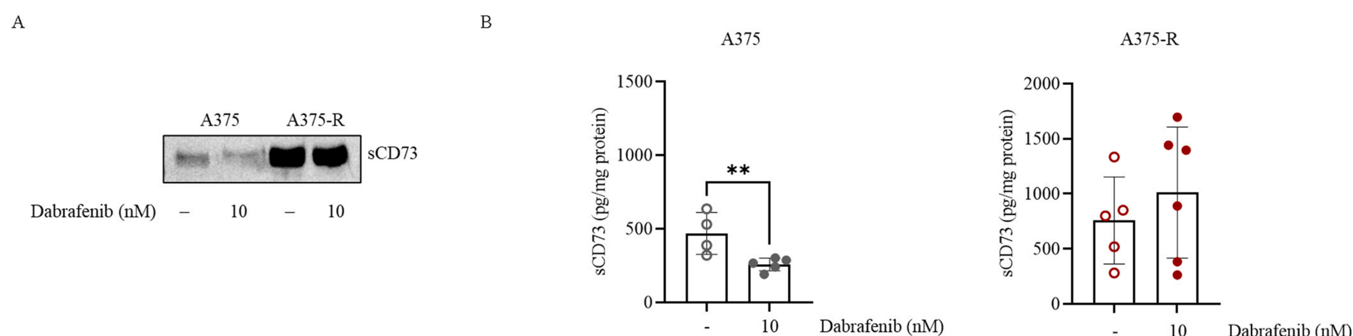

Fig. 6. Expression of sCD73 released from A375 melanoma cells sensitive to dabrafenib or resistant to dabrafenib. A) Expression of sCD73 in A375 and A375-R cells supernatants treated or not with Dabrafenib (10 nM) for 48 h by Western blot. B) Levels of sCD73 measured by ELISA in A375 (left panel) (n = 4–5) and A375-R (right panel) (n = 5–6) cells supernatants treated or not with Dabrafenib (10 nM) for 48 h. Data on graphs are expressed as mean ± s.d. P value is obtained from a Mann-Whitney test. **p < 0.01.

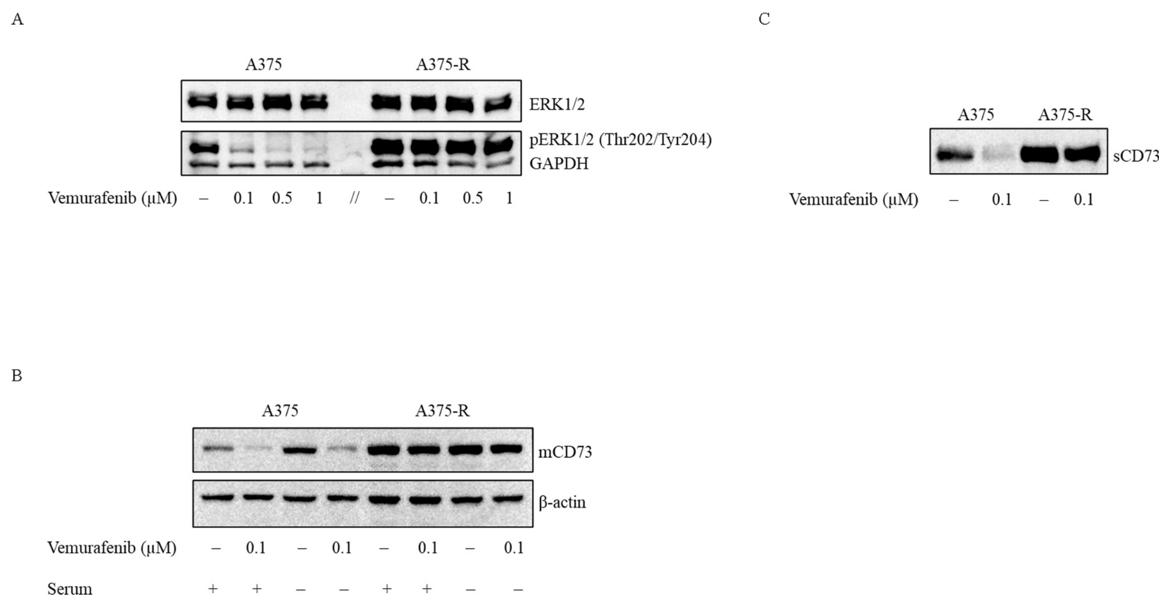

Fig. 7. Expression of CD73 on cells membrane and released from cells, sensitive or resistant to vemurafenib. A) Expression of pERK1/2 (Thr202/Tyr204) and total ERK1/2 in A375 and A375-R cells treated or not with Vemurafenib (0.1 μM-0.5 μM-1 μM) by Western blot (3 h). GAPDH expression was evaluated as protein loading control. B) Expression of membrane CD73 (mCD73) on A375 and A375-R cells treated or not with Vemurafenib (0.1 μM) for 48 h by Western blot. β-actin expression was evaluated as protein loading control. C) Expression of sCD73 in culture supernatants collected form A375 and A375-R cells treated or not with Vemurafenib (0.1 μM) for 48 h.

might be a consequence of preventing cleavage of the membrane-bound CD73. The MMP-9, together with the MMP-2, play a critical role in melanoma progression, being involved in many processes, including degradation of extracellular matrix, cell migration and invasion, and metastases formation (reviewed in Napoli et al. [53]). The expression of the MMPs can be regulated by genetic and epigenetic alterations or environmental factors (reviewed in Napoli et al. [53]). Under our experimental conditions of serum starvation, that influence the phenotype of melanoma cells [15], we observed increased extracellular levels of these metalloproteinases compared with levels found under normal culture conditions. Thus, likely the increased expression of MMP-9 in response to nutritional stress may contribute to the acquisition of an

aggressive behaviour of melanoma cells. In this scenario, in an MMP-9-dependent manner, the release of sCD73 into the extracellular space would be facilitated and, by causing adenosine accumulation, would contribute to a further immuno-suppression within tumor microenvironment.

Compelling evidence indicates that the expression of CD73 can be modulated by therapeutic anti-cancer agents, including inhibitors of immune checkpoints or BRAF inhibitors [39,54,14,55,56]. As previously reported, it has been observed that the cell expression of CD73 is transcriptionally regulated by c-Jun/AP-1 factor downstream MAPK signaling [14]. However, to the best of our knowledge, no studies have been reported on the modulation of sCD73, released by tumor cells, following inhibition of MAPK signaling or acquired resistance to BRAF inhibitors. Of relevance, we found that treatment of melanoma cells with dabrafenib or vemurafenib causes, in parallel with the decrease pERK, reduced levels of sCD73 released into the extracellular space, together with a significant reduction of CD73 expression. Therefore, we suppose that a reduction of the membrane levels of CD73 causes a consequent reduction in the release of CD73 from melanoma cells.

Previously, it has been shown that dabrafenib significantly reduces the expression of MMP-9 in A375 melanoma cells, while resistant cell clones overexpress MMP-9 [20]. As our experiments revealed that MMP-9 is involved in the release of sCD73 from cell membrane, in melanoma cells the expression profile of sCD73 together with that of MMP-9, shown by Salemi et al. [20], are associated with the activation of the MAPK pathway and correlate with the response to BRAF inhibitors. Therefore, our hypothesis is that the blockade of BRAF by dabrafenib in *BRAF*-mutant melanoma cells reduces the immuno-suppressive signaling pathway CD73/adenosine. This effect involves both CD73 expressed on cell membrane, in line with previous published data [14], and the sCD73 released by melanoma cells that, by producing adenosine, may critically contribute to impairing the immune cell function [43]. It is worth noting that melanoma cells resistant to dabrafenib show concomitantly high expression of CD73 and sCD73 released into the culture medium, that are not affected by dabrafenib treatment. Therefore, these data suggest that both forms of CD73 may be involved in the resistance mechanism to BRAF inhibitors; in this context, an increased expression and/or release of CD73/sCD73 may enhance the immune suppression within tumor lesions developing resistance to treatment. Previous evidence has indicated that the expression of CD73 enhances in cancer cells developing resistance to various therapies, such as chemotherapy, radiotherapy, immunotherapy and targeted therapy in breast cancer and non-small cell lung cancer (reviewed in Yang et al. [43]). To date, there are no data unrevealing in detail the regulation of CD73 in tumor cells resistant to BRAF inhibitors. The acquired resistance to BRAF inhibitors involves activation of MAPK/ERK signaling and/or activation of additional pro-survival pathways [57]. Therefore, in the context of acquired resistance to MAPK inhibitor the activation of compensatory survival signalling may contribute to up-regulate CD73. Further future studies are required to evaluate how mechanisms supporting BRAF inhibitor resistance in melanoma may regulate the expression of CD73.

Our recently published observations on circulating CD73 in melanoma patients undergoing anti-PD-1 monotherapy show that patients with high circulating CD73 and tumors with *BRAF* mutations presented an extremely poor prognosis [16]. However, there is limited evidence on the effect of BRAF inhibition in patients [14] and no one on sCD73 that requires further exploration. Therefore, these data support the rationale for investigating in future studies the expression profile of sCD73 in *BRAF*-mutant patients as well as in *BRAF*-resistant patients, and the use of CD73/adenosine signaling pathway inhibitors to potentially increase the immune response to treat tumors developing resistance to therapy.

5. Conclusions

Taken together our results demonstrate that a soluble form of CD73 is released from melanoma cells, and may contribute to enhance immunosuppression through adenosine production. The expression of CD73 in melanoma cells or its release are associated with a response to BRAF inhibitors.

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CRediT authorship contribution statement

Caterina Giraulo: Writing – original draft, Methodology, Formal analysis, Data curation. **Lavinia Orlando:** Methodology, Formal analysis. **Elva Morretta:** Methodology, Formal analysis. **Antonia Voli:** Methodology, Formal analysis. **Paola Plaitano:** Methodology, Formal analysis. **Carla Cicala:** Writing – review & editing. **Eugen Potaptschuk:** Validation, Resources. **Christa E. Müller:** Writing – review & editing, Resources. **Alessandra Tosco:** Writing – review & editing, Methodology. **Maria Chiara Monti:** Writing – review & editing, Formal analysis, Data curation. **Silvana Morello:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The graphical abstract was generated using images from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 4.0 Unported License (https://creativecommons.org/licenses/by/4.0/).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117033.

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