Targeting p53 with SLMP53-2 for melanoma treatment: counteracting tumour proliferation, dissemination and therapeutic resistance

Running title: Pharmacological targeting of p53 for melanoma therapy

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Author contribution statement:

JBL performed experiments, analysed and interpreted the data, and wrote the manuscript; VB performed the synthesis of SLMP53-2; JC, MIA and MG performed *in vitro* experiments; JLC, NRL, LR and CG performed the *in vivo* experiments; MMMS conceived the design and synthesis of SLMP53-2; LS conceived the study, analysed and interpreted the data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Word counts (excluding figure legends and references): 4025

Number of figures: 6

Supporting information: 1

Acknowledgements: This work received financial support from PT national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through LAQV/REQUIMTE (UID/QUI/50006/2020), iMed.ULisboa (UIDB/04138/2020), PTDC/QUI-QOR/29664/2017, PTDC/MEC-ONC/32018/2017. We thank FCT for the fellowships SFRH/BD/128673/2017 (J. Loureiro), 2020.04613.BD (J. Calheiros), PD/BD/143126/2019 (V. Barcherini).

Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration of transparency and scientific rigour:

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies and other organizations engaged with supporting research.

Conflict of interests:

One international patent protecting the compound disclosed in this manuscript has been filed by M.M.M. Santos and L. Saraiva. The other authors declare no conflict of interest.

Abstract

Background and purpose: Melanoma is the deadliest form of skin cancer mostly due to its high metastatic propensity and therapeutic resistance in advanced stages. The frequent inactivation of the p53 tumour suppressor protein in melanomagenesis may predict promising outcomes for p53 activators in melanoma therapy. Herein, we aimed to investigate the anti-tumour potential of the p53-activating agent tryptophanol-derived oxazoloisoindolinone SLMP53-2 against melanoma.

Experimental Approach: 2D/3D cell cultures and xenograft mouse models were used to unveil the anti-tumour activity and the underlying molecular mechanism of SLMP53-2 in melanoma.

Key results: SLMP53-2 inhibited the growth of human melanoma cells in a p53-dependent manner through induction of cell cycle arrest and apoptosis. Notably, SLMP53-2 induced p53 stabilization by disruption of the p53-MDM2 interaction, with subsequent enhancement of p53 transcriptional activity. It also promoted the expression of p53-regulated microRNAs (miRNAs), including the tumour suppressors miR-145 and miR-23a. Moreover, it displayed anti-invasive and anti-migratory properties in melanoma cells, by inhibiting epithelial-to-mesenchymal transition (EMT), angiogenesis and extracellular lactate production. Importantly, SLMP53-2 did not induce resistance in melanoma cells. In addition, it synergised with vemurafenib, dacarbazine and cisplatin, and re-sensitized vemurafenib-resistant melanoma cells. SLMP53-2 also exhibited antitumor activity in human melanoma xenograft mouse models by repressing cell proliferation and EMT, while stimulating cell death.

Conclusions and implications: This work discloses the p53-activating agent SLMP53-2 with promising therapeutic potential in advanced melanoma, either as a single agent or in combination

therapy. By targeting p53, SLMP53-2 may counteract major features of melanoma aggressiveness, particularly metastasis and therapeutic resistance.

Keywords: Melanoma; Metastasis; Drug resistance; Targeted therapy; p53; Tryptophanol-derived oxazoloisoindolinone

Bullet point summary:

What is already known:

- Advanced melanoma is a highly metastatic and therapeutic resistant cancer with low survival rates
- Inactivation of the p53 tumour suppressor protein is a frequent event in melanomagenesis

What this study adds:

- The p53-activating agent SLMP53-2 displays promising *in vitro* and *in vivo* anti-tumour activity in melanoma
- SLMP53-2 (re)sensitizes melanoma cells to clinically used therapeutic agents

Clinical significance:

- SLMP53-2 represents a new therapeutic opportunity for melanoma, particularly in combination with MAPK pathway-targeting drugs

Abbreviations:

BRAF - B-Raf proto-oncogene, serine/threonine kinase

CDKN2A - Cyclin-dependent kinase inhibitor 2A

CHX - Cycloheximide

C.I. - Combination index

Co-IP - Co-immunoprecipitation

D.R.I. - Dose reduction index

MAPK - Mitogen-activated protein kinase

MDM2 - Mouse double minute 2

MDMX - Mouse double minute X

MDR – Multidrug resistance

MEK - Mitogen-activated protein kinase kinase

MutBRAF - Mutant BRAF

Mutp53 - Mutant p53

SRB - Sulforhodamine B

Vem - Vemurafenib

Wtp53 - Wild-type p53

1. Introduction

Skin cancer is among the most commonly occurring cancer type worldwide, particularly in the Caucasian population. Although only representing 5% of all skin cancers, melanoma is the most lethal subtype. This has been mainly attributed to its high metastatic potential and therapeutic resistance (Domingues et al., 2018; Ferlay J, 2018).

Melanoma is a highly heterogeneous tumour, comprising various genetic and molecular alterations (Paluncic et al., 2016). Over the last years, the main driver mutations/alterations associated with melanoma have been described. In particular, the $BRAF^{V600}$ mutation occurs in over 50% of melanoma cases (V600E substitution is the most frequent), leading to the constitutive activation of the mitogen-activated protein kinase (MAPK) pathway (Shtivelman et al., 2014). Accordingly, targeted therapies for melanoma, aiming $BRAF^{V600E/K}$-expressing tumours, have involved the use of the BRAF inhibitor, vemurafenib, which was approved by Food and Drug Administration (FDA) for unresectable or stage IV melanomas (Kim et al., 2014). The efficacy of vemurafenib as a single agent was demonstrated with improved overall and progression-free survival in 50% of treated patients (Sosman et al., 2012). Nonetheless, acquired resistance to the clinically approved MAPK pathway-targeting drugs for melanoma, BRAF and mitogen-activated protein kinase kinase (MEK) inhibitors, have been frequently reported. As such, there are many expectations about forthcoming clinical trials investigating new drugs targeting different pathways to overcome chemoresistance in melanoma (Box et al., 2014).

The p53 tumour suppressor protein is a major hub in a molecular network controlling cell proliferation and death. As a central player in carcinogenesis (Kastenhuber and Lowe, 2017), p53 is found mutated in the majority of human cancers (Schulz-Heddergott and Moll, 2018).

However, melanoma commonly harbours wild-type (wt)p53 (over 80-95% of melanoma cases; (Chin et al., 2006; Hodis et al., 2012)). The low frequency of mutant (mut)p53 in melanoma has been mostly attributed to the inactivation of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus, encoding $p16^{INK4A}$ and $p14^{ARF}$, which potentially renders the p53 mutation unwarranted. In fact, $p14^{ARF}$ directly inhibits mouse double minute 2 (MDM2), which is the major ubiquitin ligase involved in p53 degradation and inactivation. As such, under $p14^{ARF}$ deletion, p53 remains highly inhibited by MDM2 and unable to counteract tumour progression (Box et al., 2014; Kwon et al., 2017). Notably, many chemotherapeutic agents have often proved ineffective due to an impairment of the p53 pathway. The combination of p53-activating agents, particularly inhibitors of the p53 interaction with MDM2 (e.g. nutlin-3a), with BRAF and MEK inhibitors, might therefore represent an appealing therapeutic strategy, potentially overcoming therapeutic resistance and improving disease-free survival of melanoma patients (Ji et al., 2013; Box et al., 2014).

Recently, we disclosed the tryptophanol-derived oxazoloisoindolinone SLMP53-2 as a new p53-activating agent with *in vitro* and *in vivo* anti-tumour activity against hepatocellular carcinoma (Gomes et al., 2019). The p53-dependent anti-tumour activity of SLMP53-2 was demonstrated through reestablishment of the wt-like function to mutp53. This work also highlighted the low toxicity of SLMP53-2 against normal cells and the absence of *in vivo* undesirable side effects.

Herein, we aimed to explore the anti-tumour potential of SLMP53-2, either as a single agent or in combination therapy, in advanced melanoma. By targeting the p53 pathway, we intended to counteract major features of melanoma aggressiveness, particularly tumour dissemination and therapeutic resistance.

2. Methods

2.1. Human cell lines and growth conditions

Human melanoma A375 (CLS Cat# 300110/p852_A-375, RRID:CVCL_0132) and SK-MEL-5 (CLS Cat# 300157/p634_SK-MEL-5, RRID:CVCL_0527) cells were purchased from CLS Cell lines service (Eppelheim, Germany). G361 and MEWO human melanoma cells were kindly provided by Dr Paula Soares (i3S, Porto, Portugal). Tumour cells A375, SK-MEL-5 and MEWO were cultured in RPMI-1640 medium with UltraGlutamine (Lonza, VWR, Carnaxide, Portugal) and G361 cells were culture in McCoy's 5A Medium (Lonza). The culture mediums were supplemented with 10% FBS (Gibco, Alfagene, Lisboa, Portugal). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Routine testing for Mycoplasma was performed using the MycoAlert™ PLUS detection kit (Lonza).

2.2. Sulforhodamine B (SRB) assay

4.5 x 10^3 A375 or A375 resistant to vemurafenib (Vem-res) cells/well and 5.0 x 10^3 SK-MEL-5 cells/well were seeded in 96-well plates and allowed to adhere for 24 h. Cells were then treated with serial dilutions of the compound, for an additional 48 h incubation period. Effects on cell proliferation were measured by SRB assay, as described (Soares et al., 2015). IC_{50} values were determined for the tested cell lines using the GraphPad Prism software version 7.0 (RRID:SCR_002798, La Jolla, CA, USA).

2.3. Colony formation assay

5.0 x 10^2 A375 or MEWO cells/well, 2.0 x 10^3 SK-MEL-5 cells/well and 7.0 x 10^2 G361 cells/well were seeded in six-well plates and treated at the seeding time with a range of concentrations of SLMP53-2 for 11 days. Formed colonies were fixed with 10% methanol and 10% acetic acid for 10 min and then stained with 0.5% crystal violet (Sigma-Aldrich, Sintra, Portugal) in 1:1 methanol/H_2O for 15 min. Colonies containing more than 20 cells were counted.

2.4. Cell cycle and apoptosis analyses

The analyses were performed essentially as described (Soares et al., 2015). Particularly, 1.2 x 10^5 A375 cells/well were seeded in 6-well plates and allowed to adhere overnight, followed by treatment with 12 μM SLMP53-2 for 24 h (cell cycle) or 72 h (apoptosis). For cell cycle analysis, cells were stained with propidium iodide (Sigma-Aldrich) and were analysed by flow cytometry; cell cycle phases were identified and quantified using the FlowJo X 10.0.7 Software (RRID:SCR_008520, Treestar, Ashland, OR, USA). For apoptosis, cells were stained using the Annexin V-FITC Apoptosis Detection Kit I from BD Biosciences (Enzifarma, Porto, Portugal), according to the manufacturer's instructions. The Accuri™ C6 flow cytometer and the BD Accuri C6 software (RRID:SCR_014422, BD Biosciences) were used.

2.5. Western blot analysis

1.2 × 10^5 A375 or A375 Vem-res cells/well and 1.5 × 10^5 SK-MEL-5 cells/well were seeded in six-well plates for 24 h, followed by treatment with 6 and 12 μM (for A375 cells), 10 and 20 μM (for SK-MEL-5 cells) and 2 μM SLMP53-2 (for A375 Vem-res cells) for the indicated treatment periods. In particular, protein extracts were quantified using the Bradford reagent (Sigma-Aldrich). Proteins were run in SDS-PAGE and then transferred to a Whatman

nitrocellulose membrane from Protan (VWR). Membranes were blocked in 5% milk and labelled with specific primary antibodies followed by HRP-conjugated secondary antibodies (described in Supporting Information, Table S1). GAPDH was used as loading control. The signal was detected with the ECL Amersham kit from GE Healthcare (VWR). For signal detection the ChemiDoc™ XRS Imaging System from Bio-Rad Laboratories (Amadora, Portugal) was used. Band intensities were quantified using the Image Lab software version 5.2.1 (RRID:SCR_014210, Bio-Rad Laboratories). Signal intensity is relative to the respective loading and normalized to control (DMSO), set as 1.

2.6. Transfection of p53 siRNA

A375 cells were seeded in six-well plates and allowed to growth until 50% confluence. Thereafter, cells were transfected with 100 nM siRNAs against p53 (SMARTpool p53) and nonspecific siRNAs (Non-targeting Pool), both from Thermo Scientific (Bioportugal, Porto, Portugal), using Lipofectamine 2000 (Invitrogen, Alfagene, Lisboa, Portugal), according to manufacturer's instructions. After 24 h of transfection, 1.2 x 10^5 cells/well of control and transfected cells were seeded in six-well plates and immediately treated with a range of concentrations of SLMP53-2. For control of the transfection efficiency, cells were harvested for western blot analysis of p53 expression levels, as described in 2.5.

2.7. RNA extraction and RT-qPCR

1.2 × 10^5 A375 cells/well were seeded in six-well plates for 24 h, followed by treatment with 6 and 12 μM SLMP53-2 for 24 h. Total RNA was extracted from the cells using the Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare, Enzymatic, Loures, Portugal). For

cDNA synthesis, 1 μg of RNA was used with the NZY M-MuLV Reverse Transcriptase from Nzytech (RRID:SCR_016772, Lisboa, Portugal) in 20 μL final volume, following the manufacturer's instructions. RT-qPCR assays were performed in a 96-well plate on a Real-Time PCR Detection System (Bio-Rad, version 3.1), starting with 16.5 ng of cDNA. The NZY qPCR Green Master Mix (Nzytech) and specific forward and reverse primers for *MDM2* (F-GGCCTGCTTTACATGTGCAA, R-GCACAATCATTTGAATTGGTTGTC), *CDKN1A* (p21; F-CTGGAGACTCTCAGGGTCGAA, R-GATTAGGGCTTCCTCTTGGAG), *TNFRSF10B* (KILLER; F-TGACTCATCTCAGAAATGTCAATTCTTA, R-GGACACAAGAAGAAAACCTTAATGC), *BAX* (F-CCTGGAGGGTCCTGTACAATCT, R-GCACCTAATTGGGCTCCATCT) from Stabvida (Caparica, Portugal) and *TP53* (p53; F-CTCTGACTGTACCACCATCCACTA, R-GAGTTCCAAGGCCTCATTCAGCTC) from Eurofins (MWG, Milan, Italy) were used; *GAPDH* was used as a reference gene.

2.8. Cycloheximide (CHX) assay

A375 cells were seeded in 6-well plates at 1.2×10^5 cells/well for 24 h, followed by 24 h treatment with 12 μM SLMP53-2 or solvent. After that, cells were treated with 150 μg/mL CHX (Sigma-Aldrich) for 0, 0.5, 1, 1.5 and 2 h. p53 protein expression was detected by western blot, as described in section 2.5.; GAPDH was used as loading control.

2.9. Co-immunoprecipitation (Co-IP) assay

For the Co-IP assay, the Pierce Classic Magnetic IP and Co-IP Kit from Thermo Scientific (Dagma, Carcavelos, Portugal) were used. 5.0×10^5/flask A375 cells were treated with 12 and 18 μM SLMP53-2 for 4 h; after cell lysis and protein lysate separation, 300 μg of total

protein was incubated with 10 µL of mouse monoclonal anti-p53 (DO-1) or mouse immunoglobulin G (IgG, negative control) from Santa Cruz Biotechnology (Frilabo, Porto, Portugal), overnight at 4 °C. The immunoprecipitation of the immunocomplexes was performed using magnetic beads. The western blot analysis was performed, as in Section 2.5., for detection of p53 and MDM2 in whole cell lysate (input) and in immunoprecipitated proteins. GAPDH was used as loading control.

2.10. MicroRNAs (miRNA) analysis

Total RNA was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA concentration and purity were measured in NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed by gel electrophoresis. miRNA levels were evaluated using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA). cDNA was synthesized (MyCycler Thermal Cycler, Bio-Rad) using RNA, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and gene specific stem-loop Reverse Transcription primers (Applied Biosystems). qPCR reactions were performed in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using cDNA, has-miR-145, hsa-miR-23a, or small nuclear RNA U6 (snRNA U6) TaqMan probes (Applied Biosystems) and SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA). snRNA U6 was used as reference gene. Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines (Bustin et al., 2009).

2.11. Generation of melanoma spheroids

A375 cells were resuspended in RPMI-1640 culture medium containing 10% FBS. 6 x 10^2 A375 cells/well were plated in 96-well plates coated with 1% agarose (Sigma-Aldrich). At the seeding time, cells were treated with a concentration range of SLMP53-2 and allowed to growth for 10 days. In another experimental condition, after seeding, spheroids were allowed to grow for 3 days and then treated with SLMP53-2 for 8 days. Fresh medium with drugs was added to the wells each two days. To evaluate the synergistic effect of SLMP53-2 with vemurafenib in spheroids development, 3-days old melanoma spheroids were treated with 2 μM SLMP53-2 and/or 0.027 μM vemurafenib, for additional 8 days. Fresh medium with the drugs was added to the wells each two days.

Spheroids were photographed using an inverted Nikon TE 2000-U microscope from Nikon Instruments Inc. (Izasa, Carnaxide, Portugal), at ×100 magnification, with a DXM1200F digital camera and NIS-Elements microscope imaging software (RRID:SCR_014329, Nikon Instruments Inc.). Determination of spheroids diameter was performed using Image J software (v1.8.0, RRID:SCR_003070, Madison, WI, USA) (Schneider et al., 2012).

2.12. Combination therapy assay

For the assessment of synergistic effects of SLMP53-2 with known chemotherapeutic agents, A375 cells were treated with 2 μM SLMP53-2 and/or increasing concentrations of vemurafenib (0.03–0.5 μM), dacarbazin (0.25–4 μM) and cisplatin (0.3–5 μM) for 48 h. The SRB assay was used to assess the effect of the combined treatments on cell proliferation. For each combination, the combination index (C.I.) and the dose reduction index (D.R.I.) values were calculated using the CompuSyn Software version 1.0 (ComboSyn, Inc., Paramus, NJ, USA), according to the following equation: $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$, where the numerators

$(D)_1$ and $(D)_2$ are the concentrations of each drug in the combination $[(D)_1 + (D)_2]$ that inhibit x %, and the denominators $(D_x)_1$ and $(D_x)_2$ are the concentrations of drug one and two alone that inhibit x%; D.R.I. measures how much the dose of a drug may be reduced in synergistic combination compared to the dose of each drug alone; C.I. values < 1, 1 < C.I. < 1.1 and > 1.1 indicate synergistic, additive and antagonistic effects respectively (Chou and Talalay, 1984).

For the 3D spheroid model, the area of melanoma spheroids was assessed using the following equation: $A = \pi ab$, where (a) corresponds to the major axis and (b) to the minor. The synergistic effect was determined using the Additive model: a positive drug combination effect occurs when the observed combination effect (E_{AB}) is greater than the expected additive effect given by the sum of the individual effects ($E_A + E_B$). The C.I. was calculated as described (Jonsson et al., 1998; Foucquier and Guedj, 2015): $C.I. = \frac{EA + EB}{EAB}$.

2.13. Establishment of vemurafenib-resistant cells

To generate A375 cells resistant to vemurafenib (Res-vem), cells were exposed to several rounds of selection with increasing concentrations of this drug, as previously reported (Ogawara et al., 2009; Yan et al., 2017), starting at the IC_{50} value, and using concentrations 1.5-fold higher in each round until a maximum of 5.0 μM. Vemurafenib was added to culture medium for 24 h, followed by a recovery period of two days in fresh medium without treatment. For the maintenance of the resistance, the Res-vem cells were kept in the presence of vemurafenib at the maximum concentration used to induce resistance and grown in medium without drug for 3 to 4 days before experiments. The same passage number of both parental and resistant cells was used

in the experiments. The IC_{50} values of SLMP53-2 and vemurafenib, in parental and Res-vem A375 cells, were determined by SRB assay.

2.14. Acquired resistance studies

A375 cells were exposed to six rounds of treatments with increasing concentrations of SLMP53-2 (6, 9, 12, 18, 24 and 30 μM). Compound was added to culture medium for 24 h, followed by a recovery time of two days, with fresh medium without treatment. Cells were harvested, seeded and treated twice for each concentration (one round). At the end of each round, IC_{50} values were determined by SRB assay, after 48 h treatment. The passage number for both control and surviving resistant cells was the same for each round.

2.15. *In vitro* migration and invasion assays

For tumour cells migration analysis, both the wound-healing assay and the QCM 24-Well Fluorimetric Chemotaxis Cell Migration Kit (8 μm) from Merck Millipore (Taper, Sintra, Portugal), were performed as described (Soares et al., 2016). Briefly, for the wound-healing assay, 5×10^5 A375 cells/well and 7×10^5 SK-MEL-5 cells/well were grown to confluence in six-well plates, and a fixed-width wound was created in the cell monolayer using a sterile 10 uL micropipette tip. Cells were treated with SLMP53-2 at 2 μM (for A375 cells) and 4 μM (for SK-MEL-5 cells); images of the wound were captured at different time points, using an inverted Nikon TE 2000-U microscope from Nikon Instruments Inc. (Izasa) at 100× magnification with a DXM1200F digital camera (Nikon Instruments Inc.) and a NIS-Elements microscope imaging software (version 4; Nikon Instruments Inc.). For calculation of the wound closure, the subtraction of the 'wound' area (measured using ImageJ Software) at the indicated time point of

treatment to the 'wound' area at the starting point was made. For Chemotaxis Cell Migration assay and the Fluorimetric Cell Invasion Assay, 1.2×10^5 A375 and SK-MEL-5 cells (cultured in serum-free medium for 24h) were prepared in serum-free medium for each tested condition. Melanoma cells were treated with SLMP53-2 at 2 μM (for A375 cells) and 4 μM (for SK-MEL-5 cells). The prepared cell suspensions were distributed in 24-well plates (300 μL per insert), followed by an addition of 500 μL medium containing 10% FBS to the lower chamber. After 24 h, cells that migrated or invaded through the ECMatrix layer (with 8 μm pore membranes) were eluted, lysed and stained with a green-fluorescence dye that binds to cellular nucleic acids. The number of migrating/invading cells was proportional to the fluorescence signal measured using the Bio-Tek Synergy HT plate reader (Izasa), at 480/520 nm (ex/em).

2.16. Measurement of extracellular lactate

Using the Lactate-Glo™ assay kit (Promega, VWR), the lactate levels exported by A375 and SK-MEL-5 cells to the culture medium were determined, according to the manufacturer's instructions. Briefly, 1.2×10^5 A375 cells/well and 1.5×10^5 SK-MEL-5 cells/well were seeded in six-well plates, followed by treatment with a concentration range of SLMP53-2. After 8 h treatment, the culture medium was collected and diluted in phosphate saline buffer (1:200). Then, 50 μL was transferred to a 96-well assay plate and 50 μL of Lactate Detection Reagent Mix was added. The plate was kept 60 min at room temperature followed by luminescence assessment using the Bio-Tek Synergy HT plate reader (Izasa).

2.17. *In vivo* anti-tumour assay

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology. All animals were housed in polycarbonate cages (two to six per cage) and kept on a 12 h light/dark cycle. Food and water were given *ad libitum*. Studies were reviewed by the Animal Ethics Committee and Animal Welfare Body of the i3S (reference 2016/22), authorized by the national authority *Direção Geral de Alimentação e Veterinária* (DGAV reference 0421/000/000/2017). Animal studies were performed using the C57BL/6-Rag2$^{-/-}$IL2rg$^{-/-}$ mice model (negative for B and T cells to allow tumour growth), generously provided by Prof. James Di Santo (Institute Pasteur, Paris, France). 9.0 x 10^6 A375 cells (in PBS/Matrigel 1:1; Corning, Enzifarma, Porto, Portugal) were subcutaneously inoculated in the right flank of male and female mice with 7 to 11 weeks (in each experimental group three male and four female mice were used). Tumours volume were routinely measured using a calliper and the formula (a x b^2)/2 (where a and b represent the longest and shortest tumour axis, respectively). Twice-weekly intraperitoneal injections of 50 mg·kg^{-1} SLMP53-2 or vehicle (seven animals per group) were started for tumours with approximately 100 mm^3 (7 days after implantation). Six administrations were performed with continuous monitoring of tumour volume (endpoint set at 2000 mm^3), animal weight (endpoint set at 10% of weight loss), and signs of morbidity. At the end of treatment, animals were sacrificed by cervical dislocation.

2.18. Immunohistochemical (IHC) analysis

Tumour tissues from xenografts of human A375 melanoma cells were analysed by IHC. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm, and stained with haematoxylin and eosin (H&E) or antibodies, as described (Soares et al., 2016). Briefly, antigen

retrieval was performed by boiling the sections for 20 min in citrate buffer (pH 6.0). Antibodies used are listed in (Supporting information, Table S1). Immunostaining was performed using the UltraVision Quanto Detection System HRP DAB Kit, from Lab Vision Thermo Scientific (Taper), according to the manufacturer's instructions. Evaluation of DAB (3,3′-diaminobenzidine) intensity and quantification of stained cells were performed using ImageJ software version 1.8.0. (Madison, WI, USA). Images were obtained using an Eclipse E400 fluorescence microscope (Nikon) with ×200 magnification, with a Digital Sight camera system (Nikon DS-5Mc) and software Nikon ACT-2U (Izasa).

2.19. Randomization and blinding

For *in vivo* experiments, the animals were randomized to each treatment group, seven animals by group. Additionally, the tumours removed from the mice were collected for histological analyses. The slides obtained from paraffin-embedded tumours were labelled with numbers and the analysis was performed under blinded conditions.

For *in vitro* studies, blinded analysis was not performed due to the nature of the assays *per se*. However, to minimize the possible operator bias, raw data were acquired directly from the experimental techniques and analysed through standardized procedures.

2.20. Data and statistical analysis

Data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are presented as mean ± SEM of 'n' samples, where 'n' refers to independent experiments, not replicates. Values of 'n' and number of technical replicates, if performed, are given in figure legends. Where

replicates were used, their values were averaged to provide a single value to the data set. Data analyses were carried out using GraphPad Prism Software version 7.0 (La Jolla). All assays with five or more independent experiments were subjected to statistical analysis. In some data sets, log transformation to generate Gaussian-distributed data set was carried out. Normalization was made for controlling unwanted sources of variation, and data analysis was performed setting controls (DMSO or non-threated cells) as 100% or as one for comparison purposes. For comparison of two groups, unpaired Student's *t*-test was used. For comparison of multiple groups, statistical analysis relative to controls was performed using one-way or two-way ANOVA followed by post hoc Tukey's, Sidak's or Dunnet's multiple comparison tests. Statistical significance was set as $*p < 0.05$. Post hoc tests were run only if F achieved $p < 0.05$ and when no significant variance inhomogeneity was observed.

2.21. Materials

Tryptophanol-derived oxazoloisoindolinone SLMP53-2 was prepared using the method previously described (Gomes et al., 2019; Barcherini et al., 2020).

Cisplatin was purchased from Enzo Life Science (Taper), vemurafenib was from Santa Cruz Biotecnology and Dacarbazine were purchased from Sigma-Aldrich. All tested compounds were dissolved in DMSO (Sigma-Aldrich), except cisplatin which was dissolved in saline. In all experiments, the correspondent solvent was included as control in a concentration range that did not affect cell proliferation (maximum concentration used 0.5%).

2.22. Nomenclature of targets and ligands

Key proteins and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (RRID:SCR_013077) (Harding et al., 2018), and are permanently archived in the Concise guide to Pharmacology (Alexander et al., 2019).

3. Results

3.1. SLMP53-2 inhibits the growth of human melanoma cells through induction of cell cycle arrest and apoptosis

In our previous work, the small-molecule SLMP53-2 (Figure 1A) was unveiled as a new activator of wt and mutp53 with promising anti-tumour activity, particularly in hepatocellular carcinoma (Gomes et al., 2019). Herein, it was investigated the effectiveness of SLMP53-2 against cutaneous melanoma, which is a hard-to-treat tumour with a compromised p53 pathway. To that end, the effect of SLMP53-2 on the proliferation and survival of melanoma cells expressing wtp53 (A375, SK-MEL-5, G361) and mutp53 (MEWO) was assessed by colony formation assay. Using this cell survival assay, SLMP53-2 led to a 50% reduction of cell growth at 3.3 to 8.5 μM, having its lowest growth inhibitory effect on mutp53-expressing MEWO cells (Figure 1B, C).

For an in-depth analysis of the molecular mechanism underlying the anti-tumour activity of SLMP53-2 in melanoma cells, we focused on A375 cells. The pronounced antiproliferative effect of SLMP53-2 on these cells was further evidenced by SRB assay (IC_{50} of 6.0 ± 1.0 μM, n=6; Supporting information, Figure 1S). This growth inhibition caused by SLMP53-2, in A375 cells, was associated with changes in cell morphology (Figure 1D), induction of apoptosis (Figure 1E) and G2/M-phase cell cycle arrest (Figure 1F), at 12 μM. The growth inhibitory effect

of SLMP53-2 was also evaluated in a 3D spheroid model of A375 cells. A marked reduction in spheroid area (Figure 1G, H) and formation (Figure 1I, J) was achieved after 8 and 10 days of treatment, respectively, in particular at 6 and 12 μM of SLMP53-2.

3.2. SLMP53-2 has a p53-dependent growth inhibitory effect through enhancement of p53 transcriptional activity, in melanoma cells

To assess the dependence of SLMP53-2 growth inhibitory activity on p53, the colony formation assay was performed in p53 siRNA silenced A375 cells (Figure 2A). In these cells, the growth inhibitory effect of SLMP53-2 was significantly reduced at 3.5, 4 and 5 μM, when compared to control siRNA cells (CTRL; Figure 2B, C).

In A375 cells, SLMP53-2 also enhanced the p53 transcriptional activity by regulating the protein and mRNA levels of several p53 transcriptional targets. In fact, 6 and 12 μM SLMP53-2 up-regulated the protein levels of p53, MDM2, PTEN, as well as of proteins involved in cell cycle arrest (p21 and GADD45) and apoptosis (PUMA, BAX and KILLER). In addition, it downregulated the levels of the anti-apoptotic proteins BCL-2 and BCL-xL and of the regulator of cell cycle progression Cyclin D1 (Figure 2D, E). By RT-qPCR, we further confirmed that SLMP53-2 up-regulated the mRNA levels of the p53 target genes *TP53*, *CDKN1A* (p21), *BAX*, *TNFRSF10B* (KILLER) and *MDM2*, mainly at 12 μM (Figure 2F).

It was further verified that SLMP53-2 induced wtp53 stabilization. In fact, an enhancement of p53 half-life by SLMP53-2 was observed upon inhibition of protein synthesis with cycloheximide (Figure 2G, H). To further understand the mechanism underlying p53 stabilization, we started by checking the ability of SLMP53-2 to promote the p53 interaction with heat shock proteins (Hsp) involved in wtp53 stabilization, particularly Hsp70 and Hsp90

(Boysen et al., 2019). In fact, in mutp53-expressing hepatocellular carcinoma cells, SLMP53-2-restored wt-like conformation and transcriptional activity of mutp53 by promoting its interaction with Hsp70 (Gomes et al., 2019). However, by Co-IP analysis, we did not observe an enhancement of Hsp70 or Hsp90 binding to wtp53, in melanoma (Supporting Information, Figure 2S). We next investigated whether SLMP53-2 could disrupt the p53 interaction with MDM2, a major p53 interactor involved in its inactivation and degradation by the ubiquitin-proteasome pathway (Kwon et al., 2017). For that, the Co-IP analysis was performed, in A375 cells treated with 12 and 18 μM SLMP53-2 (Figure 2I, J). Notably, SLMP53-2 reduced the amount of MDM2 bound to p53, particularly at 18 μM, which indicated an inhibition of the p53-MDM2 interaction by the compound.

The relevance of the miRNA network on melanoma pathogenesis led us to also check the interference of SLMP53-2 on the levels of the tumour suppressors miR-145 and miR23a, which are direct targets of p53 regulation and crucial players in different melanomagenesis phases. The results showed that SLMP53-2 increased miR-145 and miR23a expression levels, particularly at 12 μM, in A375 cells (Figure 2K). Based on the pronounced enhancement observed for miR-145, the protein expression levels of its targets were also evaluated. Accordingly, 6 and 12 μM SLMP53-2 downregulated the protein levels of TLR4, FSCN1 and NRAS (Figure 2L, M).

3.3. SLMP53-2 reduces melanoma cell migration and invasion

Considering metastization the major cause of melanoma-related deaths, we investigated the potential of SLMP53-2 to prevent the migration and invasion of A375 and SK-MEL-5 melanoma cells. The SK-MEL-5 cells were also included in the study once it was obtained from a metastatic site of an axillary node. For further analysis with SK-MEL-5 cells, the IC_{50} of

SLMP53-2 in this cell line was determined by SRB assay (9.5 ± 1.1 μM, n = 6; Supporting Information, Figure 1S).

The anti-migratory activity of SLMP53-2 was firstly evaluated by wound healing assay. For the evaluated timepoints, 2 μM (in A375 cells) and 4 μM (in SK-MEL-5 cells) of SLMP53-2 (concentrations with no significant effect on cell proliferation) significantly reduced the wound closure (Figure 3A, B). Consistently, at the same concentrations, SLMP53-2 also inhibited the migration of A375 and SK-MEL-5 cells through a microporous membrane, in the chemotaxis cell migration assay (Figure 3C), as well as the ability of these cells to invade through an ECMatrix layer (Figure 3D).

Considering that lactate secretion by tumour cells, with subsequent acidification of tumour microenvironment, is a well-known stimulator factor of their evasion (Smallbone et al., 2007; Liberti and Locasale, 2016), the levels of extracellular lactate were measured in melanoma cells treated with SLMP53-2. The results evidenced a marked reduction of extracellular lactate secreted by A375 (at 6 and 12 μM SLMP53-2) and SK-MEL-5 (at 10 and 20 μM SLMP53-2) cells (Figure 3E).

Accordingly, we also verified that SLMP53-2 inhibited epithelial-to-mesenchymal transition (EMT) markers, in A375 and SK-MEL-5 cells. In fact, in A375 cells, 6 - 12 μM SLMP53-2 increased E-cadherin, while decreasing N-cadherin, Vimentin, Slug, MMP-2, β-catenin and Twist protein levels (Figure 3F, G). Of note that a decrease of the angiogenic factor VEGF by 6 - 12 μM SLMP53-2 was also detected (Figure 3F, G). In SK-MEL-5 cells, 10 - 20 μM SLMP53-2 reduced the protein levels of MMP-2, β-catenin, Twist, Vimentin and Slug (Figure 3H, I).

3.4. SLMP53-2 sensitizes melanoma cells to currently available chemotherapeutic agents

The potential synergistic combination of SLMP53-2 with chemotherapeutic drugs currently used in melanoma therapy was assessed by SRB in A375 cells. For that, a concentration of SLMP53-2 with no significant effect on melanoma cell growth (2 μM) was tested with a range of concentrations of vemurafenib, dacarbazine and cisplatin (Figure 4A-C). The results showed that SLMP53-2 significantly increased the antiproliferative activity of these anticancer drugs, when compared to their effects as single agents. Using the CompuSyn software, a multiple drug-effect analysis was performed for each combination and a C.I. and a D.R.I. value was calculated. Based on C.I. values, a synergistic effect was obtained for most of the tested concentrations (C.I. < 1.0). The only exception occurred with cisplatin, which only synergised with SLMP53-2 at its highest concentration tested. These results were further corroborated in a 3D spheroid model of A375 cells, in which we evaluated the combination of SLMP53-2 with vemurafenib (a $BRAF^{mutV600E/K}$ inhibitor commonly used in melanoma targeted therapy). In particular, 3-day-old spheroids were treated with 2 μM SLMP53-2 alone and in combination with 0.027 μM vemurafenib. As single agents, none of the compounds significantly interfered with melanoma spheroid growth, at the tested concentrations (Figure 4D, E). However, in a combination regimen, a synergistic effect was achieved (C.I. = 0.60) with a marked reduction in the spheroid area (Figure 4D, E).

3.5. SLMP53-2 does not induce resistance in melanoma cells and re-sensitizes vemurafenib-resistant cells

The acquisition of resistance, particularly to BRAF inhibitors as vemurafenib, remains one of the most reported drawbacks in melanoma targeted therapy (Luebker and Koepsell, 2019).

To address this issue, we started by evaluating whether SLMP53-2 was able to induce resistance in A375 cells. After six rounds of treatment with increasing concentrations of SLMP53-2, melanoma cells did not develop resistance, as evidenced by the constant IC_{50} values of the compound in successive generations, compared to parental cells (Figure 4A).

Since multiple drug resistance (MDR) is a common event in cancer cells, we next analysed whether vemurafenib-resistant (Vem-res) A375 cells could develop cross-resistance to SLMP53-2. For that, we started by establishing Vem-res A375 cells, as evidenced by the lower anti-proliferative effect of vemurafenib (IC_{50} of 4.3 ± 1.1 μM, n = 6), when compared to non-treated cells (parental; IC_{50} of 0.17 ± 0.02 μM, n = 6) (Figure 5B, C). Interestingly, the established Vem-res A375 cells presented a partial loss of PTEN expression (inhibitor of the PI3K/AKT pathway that is found exacerbated in resistant melanomas (Kozar et al., 2019)) and increased protein levels of phosphorylated forms of ERK (p-ERK) and AKT (p-AKT) (common mechanisms of acquired resistance after BRAF inhibitor treatment; (Luebker and Koepsell, 2019)), as well as of MDR-1 (Figure 5D, E). We next analysed the effect of SLMP53-2 on the growth of parental and Vem-res A375 cells. The results showed a similar sensitivity of both cells to SLMP53-2, which demonstrated that cross-resistance was not acquired by Vem-res A375 cells (Figure 5F). Importantly, we also verified that SLMP53-2 led to a re-sensitization of Vem-res A375 cells to vemurafenib. In fact, the combination of 2 μM SLMP53-2 with a concentration range of vemurafenib resulted in a greater growth inhibitory effect than vemurafenib alone (Figure 5G, H). Indeed, the synergistic effect between SLMP53-2 and vemurafenib could be evidenced by a C.I. < 1.0 for all tested concentrations. Consistently, the D.R.I. values also revealed a notable reduction of the effective dose of vemurafenib by its combination with SLMP53-2, in Vem-res melanoma cells (Figure 5G).

To further understand the mechanism of re-sensitization of Vem-res melanoma cells by SLMP53-2, we checked the protein levels of relevant players in melanoma therapeutic response. Particularly, 2 μM SLMP53-2 increased PTEN, while reducing p-AKT and MDR-1 protein levels (Figure 5I, J). Consistently, considering the common resistance of cancer cells to cell death induced by chemotherapeutic drugs, a downregulation of BCL-2 protein levels was also observed in Vem-res A375 cells treated with SLMP53-2 (Figure 5I, J).

3.6. SLMP53-2 displays *in vivo* anti-tumour activity against melanoma

The *in vivo* anti-tumour potential of SLMP53-2 was evaluated in human tumour xenograft mouse models of A375 cells. Six intraperitoneal administrations of 50 $mg \cdot kg^{-1}$ SLMP53-2 inhibited the growth of melanoma tumours, when compared to vehicle (Figure 6A). Consistently, the weight of the collected tumours was significantly reduced in SLMP53-2 treated tumours (Figure 6B). Moreover, no significant body weight loss or morbidity signs were observed in SLMP53-2−treated mice compared to vehicle throughout the experiment (Figure 6C). Additionally, no significant differences were observed between the weight of heart, spleen, kidney and livers of SLMP53-2-treated mice and vehicle (Figure 6D).

The IHC staining of the tumour sections showed that, when compared to vehicle, SLMP53-2 decreased Ki-67, Vimentin and β-catenin, while increasing BAX staining (Figure 6E, F). These results support a potent *in vivo* anti-tumour activity of SLMP53-2 through inhibition of cell proliferation and EMT and stimulation of cell death.

4. Discussion and conclusions

Although not representing the most incident form of skin cancer, melanoma is undoubtedly the most aggressive and deadly. One of the main features attributed to this type of tumour is the frequency of intrinsic or acquired resistance mechanisms (Kozar et al., 2019). In fact, despite the notable increase of overall survival of treated patients, a significant percentage of melanoma patients do not effectively respond to the available therapies. In most cases, the monotherapy has led to resistance scenarios that have not been settled by combining drugs directed to the MAPK pathway. Given the major role of the p53 tumour suppressor protein and its reported implication in MAPK-driven melanomas (Bardeesy et al., 2001; Goel et al., 2009), the combination targeting of MAPK and p53 signalling pathways has been highlighted as a promising therapeutic strategy for melanoma patients (Gembarska et al., 2012; Lu et al., 2013; Box et al., 2014; Shattuck-Brandt et al., 2020).

In our recent work, the small-molecule SLMP53-2 was disclosed as a new p53-activating agent able to restore wt-like function to mutp53 (Gomes et al., 2019). In that work, SLMP53-2 also displayed potent growth inhibitory activity in hepatocellular carcinoma cells expressing either wt or mutp53. That work also reinforced a potent *in vivo* antitumor activity of SLMP53-2 with favourable toxicological profile.

Herein, the anti-tumour potential of SLMP53-2 against melanoma cells was investigated, either as a single agent or in combination therapy. The tumour growth inhibitory effect of SLMP53-2 was confirmed in a panel of melanoma cell lines expressing wt or mutp53. The ability of SLMP53-2 to reduce proliferation of melanoma cells expressing wtp53 was also substantiated in 3D spheroid models of melanoma cells. Further reinforcing the p53-dependent anti-tumour activity of SLMP53-2, unveiled in previous work (Gomes et al., 2019), silencing of wtp53 in melanoma cells significantly decreased the tumour growth inhibitory activity of

SLMP53-2. Consistently, in wtp53-expressing melanoma cells, SLMP53-2 induced cell cycle arrest and apoptosis, and markedly increased p53 transcriptional activity, as evidenced by the regulation of mRNA and protein expression levels of several p53 transcriptional targets. In particular, SLMP53-2 increased the levels of MDM2 and PTEN. Notably, PTEN is a tumour suppressor protein with a major role in cell proliferation, death, migration and adhesion, which expression is found lost in approximately 20% of melanomas (Tsao et al., 2004). Moreover, in accordance with a G2/M-phase cell cycle arrest, SLMP53-2 increased the expression of p21 and GADD45, while decreasing cyclin D1 expression, which is consistent with its negative regulation by p21. SLMP53-2 also regulated the expression levels of several p53 targets involved in apoptosis, upregulating PUMA, BAX and KILLER and downregulating the anti-apoptotic proteins BCL-2 and BCL-xL. It was further demonstrated that SLMP53-2 induced wtp53 stabilization through disruption of the p53 interaction with MDM2. In fact, as previously mentioned, since *TP53* mutations are rare in melanoma, activation of wtp53, by releasing it from the prominent MDM2 inhibitory effect, is considered a promising strategy in melanoma therapy (Box et al., 2014; Kwon et al., 2017; Wu et al., 2018).

Compelling evidence have shown that miRNAs are important regulators in melanoma progression and dissemination. Since miRNAs are frequently dysregulated in several types of cancer, they have been considered effective therapeutic targets. From the numerous miRNAs dysregulated in melanoma, some are directly regulated by p53, including miR-145 and miR-23a, which are tumour suppressors with crucial roles in distinct phases of melanomagenesis (Loureiro et al., 2020). In fact, these miRNAs are frequently downregulated in melanoma, which has been correlated with poor prognosis in melanoma patients. Accordingly, their expressions have been associated with improved long-term survival in metastatic melanoma patients and a significant

reduction of cell proliferation, migration, and drug resistance (Loureiro et al., 2020). In the present work, we showed that SLMP53-2 significantly increased the expression levels of miR-145 and miR-23a. In particular, the pronounced enhancement of miR-145 levels by SLMP53-2 was correlated with downregulation of its targets TLR4, FSCN1 and NRAS. Particularly, it has been suggested that TLR4 may contribute to melanoma progression and migration (Takazawa et al., 2014) and that FSCN1 promotes EMT events (Lin et al., 2020). Notably, in other works, this negative regulation of NRAS expression by miR-145 has been associated with inhibition of proliferation, invasion, and migration of melanoma cells (Liu et al., 2017).

Consistently, SLMP53-2 displayed potent anti-invasive and anti-migratory properties in melanoma cells (including metastatic site-derived cells). Accordingly, the expression levels of relevant markers of EMT inhibition were evaluated in melanoma cells treated with SLMP53-2. EMT has been described as a crucial event in tumours dissemination, orchestrating alterations in the integrity of cell–cell junctions and cell-extracellular matrix, loss of polarity and epithelial markers (e.g. E-cadherin), which subsequently result in loss of contact between adjacent cells. As such, the acquisition of a more mesenchymal-like phenotype prompts cells to become more prone to migrate and invade the nearby tissues (Li et al., 2015; Pearson, 2019). In melanoma, throughout the radial growth phase (RGP), the interactions of melanoma cells with keratinocytes decrease, mainly due to the loss of E-cadherin expression. The successive vertical growth phase (VGP) is then characterized by a transmigration of melanoma cells from the epidermis, across the basal lamina, to the dermis (Bonaventure et al., 2013). In accordance with the crucial role of p53 in suppressing major players of classic metastasis pathways, including cell adhesion, motility, invasion and EMT (Powell et al., 2014), SLMP53-2 increased E-cadherin and decreased N-cadherin, MMP-2 (relevant in degrading extracellular matrix components), Vimentin, β-

catenin, Slug and Twist expression levels. It should be noted that despite controversial results regarding the role of β-catenin in melanoma dissemination, our results are in line with several reports sustaining a pro-migratory and pro-invasive role of β-catenin in melanoma cells (Sinnberg et al., 2011; Grossmann et al., 2013). SLMP53-2 also decreased the levels of the angiogenesis-inducing factor VEGF and markedly reduced the lactate levels secreted to the cell medium. Actually, the lactate secretion from tumour cells (due to metabolic changes) favours a low pH in the microenvironment associated with an enhancement of angiogenesis through upregulation of VEGF production by tumour cells (Shi et al., 2001). It is interesting to note the correlation between the reduction of β-catenin levels and the observed decrease of FSCN1, which is transcriptionally regulated by β-catenin in tumour cells (Lin et al., 2020)

One of the main causes of therapeutic inefficiency in cancer patients is drug resistance, which in most cases has features of MDR, with an insensitivity of tumour cells to multiple drugs (Ullah, 2008). To infer the ability of our compound to enhance the antitumor effect of currently adopted chemotherapeutic drugs in melanoma treatment (Dummer et al., 2015), we assessed its combination potential with vemurafenib, dacarbazine and cisplatin. SLMP53-2 displayed synergistic effects with all tested drugs. In line with the relevance of a functional p53 pathway for the effectiveness of many chemotherapeutic agents, the calculated D.R.I. indicated that SLMP53-2 markedly reduced the effective doses of each chemotherapeutic agent. In fact, further supporting the predictive clinical impact of multitargeting p53 and MAPK pathways, a promising C.I. was obtained combining SLMP53-2 with vemurafenib, in a 3D spheroid model of melanoma cells. This strategy reveals to be particularly relevant in a context of acquired resistance to BRAF-targeting therapy due to reactivation of the MAPK pathway or an exacerbation of the PI3K-AKT pathway to compensate the inhibition of MAPK pathway (Kozar

et al., 2019; Luebker and Koepsell, 2019). Also corroborating the compensatory phenomenon underlying developed resistance by MAPK-targeting therapy, the loss of expression of PTEN (a p53 transcriptional target and inhibitor of the PI3K-AKT pathway) can be also found in some melanomas (Aguissa-Touré and Li, 2012; Kozar et al., 2019). It is also noteworthy that parental and Vem-res melanoma cells did not develop (cross)resistance to SLMP53-2. Importantly, SLMP53-2 enhanced the sensitivity of Vem-Res cells to vemurafenib, potentially due to an inhibition of the PI3K-AKT pathway through an enhancement of PTEN expression and downregulation of p-AKT levels. In accordance with this, SLMP53-2 downregulated BCL-2 and MDR-1 protein levels in Vem-res cells treated with SLMP53-2.

SLMP53-2 also displayed *in vivo* antitumor activity, in a xenograft mouse model, suppressing the growth of human melanoma tumours, without interfering with body and organs weight of animals. The *in vivo* tumour suppressive activity of SLMP53-2 was associated with decreased cell proliferation and increased cell death. The inhibition of EMT by SLMP53-2 was also confirmed *in vivo* by reduction of Vimentin and β-catenin expression in melanoma tumours treated with SLMP53-2.

In conclusion, melanoma is a highly metastatic disease with a frequent resistance profile to a broad panel of drugs. The continuous increase in melanoma incidence in western countries and its high clinical aggressiveness have made necessary the development of more effective therapeutic options against melanoma. This work discloses the p53-activating agent SLMP53-2 with encouraging therapeutic potential in melanoma, either as a single agent or in combined regimes. Notably, besides its promising effect on melanoma proliferation, SLMP53-2 also revealed great potential against metastatic melanoma, and counteracted melanoma resistance to clinically used therapeutic agents. It is still noteworthy that besides the activation of wtp53, our

previous studies have demonstrated the ability of SLMP53-2 to also reactivate wt-like function to mutp53. Despite its lower frequency in melanomas, the appearance of mutp53 can also occur as a genetic, environmental (namely induced by ultraviolet radiation), or even as a consequence of several cycles of treatment (Stretch et al., 1991). Hence, SLMP53-2 may represent a great contribution to the advance of personalized melanoma therapy, namely in comparison to other p53-MDM2 interaction inhibitors, such as nutlin-3a, only effective against wtp53-expressing tumours. It may also be the starting point for the development of improved pharmacological agents against advanced melanoma that still lack effective therapeutic options.

Figure legends

Figure 1. SLMP53-2 inhibits melanoma cell growth through induction of cell cycle arrest and apoptosis. (A) Chemical structure of SLMP53-2. **(B)** IC_{50} values of SLMP53-2 in A375, G361, MEWO and SK-MEL-5 melanoma cells obtained by colony formation assay; data were normalized to DMSO and correspond to mean ± SEM, n = 5 (two replicates each). **(C)** Colony formation assay for A375, G361, MEWO and SK-MEL-5 melanoma cells treated with SLMP53-2 for the indicated concentrations. Images are representative of five independent experiments. (**D**) Effect of SLMP53-2 on growth and morphology of A375 cells for the indicated time-points; images are representative of five independent experiments (scale bar = 100 μm, magnification = ×100). (**E**) Apoptosis (Annexin V-positive cells) was evaluated in A375 cells after 72 h treatment with 12 μM SLMP53-2. (**F**) Cell cycle analysis in A375 cells was determined after 24 h treatment with 12 μM SLMP53-2. In **E** and **F**, data are mean ± SEM, n = 5; values significantly different from DMSO: *p<0.05, unpaired Student's t-test. (**G, H**) Effect of SLMP53-2 on three−day−old A375 spheroids, for up to 8 days treatment. In **H**, data are mean ± SEM, n = 5; values

significantly different from DMSO: *p<0.05, one-way ANOVA followed by Tukey's test. (**I, J**) Evaluation of spheroids formation after 10 days treatment with SLMP53-2; treatment was performed at the seeding time of A375 cells. In **J**, data are mean ± SEM, n = 5; values significantly different from DMSO: *p<0.05, one-way ANOVA followed by Tukey's test. In **G** and I images are representative of five independent experiments; scale bar = 100 μm; magnification = 100×.

Figure 2. SLMP53-2 has p53-dependent growth inhibitory effect in melanoma cells with enhancement of p53 stabilization and transcriptional activity. (A-C) Colony formation assay for silenced p53 (sip53) and control (CTRL) A375 cells treated with SLMP53-2. In **A**, silencing efficacy of p53 by siRNA; immunoblots are representative of five independent experiments, GAPDH was used as loading control; data plotted were normalized to CTRL and correspond to mean ± SEM, n = 5; values significantly different from CTRL: *p<0.05, unpaired Student's t-test. In **B**, images are representative of five independent experiments. In **C**, data were normalized to DMSO and correspond to mean ± SEM, n = 5; values of sip53 cells significantly different from CTRL cells: *p<0.05, two-way ANOVA followed by Sidak's test. **(D, E)** Proteins levels of p53 transcriptional targets, in A375 cells treated with SLMP53-2 for 24 h (p53, MDM2, PTEN, Cyclin D1, p21 and KILLER) or 48 h (GADD45, PUMA, BCL-2, BCL-xL and BAX). In **D**, immunoblots are representative of three independent experiments; GAPDH was used as loading control. In **E**, quantification of protein expression levels; values with DMSO were set as 1; data are means ± SEM, n = 3. (**F**) mRNA levels of p53 target genes, in A375 cells after 24 h treatment with SLMP53-2, were determined by RT-qPCR; fold of change is relative to DMSO; data are mean ± SEM, n = 5; values significantly different from DMSO: *p<0.05, two-way ANOVA with

Dunnett's multiple comparison test. (**G**) p53 protein levels in A375 melanoma cells treated for 24h with 12 μM SLMP53-2 or solvent followed with cycloheximide treatment from 0 h to 2 h (CHX; 150 μg/mL). (**H**) Quantification of p53 protein expression levels; immunoblots are representative of three independent experiments; GAPDH was used as loading control. Values for cells non-treated with cycloheximide (0 h) were set as 1; data are mean ± SEM, $n = 3$. (**I**) Co-IP was performed in A375 cells treated with SLMP53-2 for 4 h. In **I**, representative immunoblots are shown; whole-cell lysate (Input). p53 from IP was used as loading control. In **J**, quantification of protein expression levels relative to DMSO (set as 1). Data shown are means ± SEM, $n = 3$. **(K)** Expression levels of miR-145 and miR-23a, in A375 cells after 24 h treatment with SLMP53-2, was determined by RT-qPCR; fold of change is relative to DMSO; data are mean ± SEM, $n = 5$; values significantly different from DMSO: $*p<0.05$, one-way ANOVA followed by Tukey's test. **(L, M)** Protein levels of miR-145 target genes, in A375 cells treated with SLMP53-2 for 24h. In J, immunoblots are representative of three independent experiments; GAPDH was used as loading control. In **M**, quantification of protein expression levels; values with DMSO were set as 1; data are mean ± SEM, $n = 3$.

Figure 3. SLMP53-2 inhibits melanoma cell migration and invasion. (A) A375 and SK-MEL-5 confluent cells were treated with 2 or 4 μM SLMP53-2, respectively; cells were observed at different timepoints in the wound-healing assay. Images are representative of five independent experiments; scale bar = 100 μM; magnification = 100×. (**B)** Quantification of wound closure using randomly selected microscopic fields (six fields per sample). Data are mean ± SEM, $n = 5$; values significantly different from DMSO: $*p<0.05$, two-way ANOVA followed by Sidak's test. (**C)** Effect of 2 μM SLMP53-2 on migration of A375 and SK-MEL-5 cells after 24 h treatment;

the relative number of migratory cells were determined by analysis of fluorescence signal intensity; values with DMSO were set as 1. Data are mean ± SEM, n = 5 (two replicates each); values significantly different from DMSO: *p<0.05, Student's t-test. **(D)** Effect of 2 μM SLMP53-2 on the invasion of A375 and SK-MEL-5 cells for 24 h; cells able to invade through an ECMatrix layer were quantified by fluorescence signal; values with DMSO were set as 1. Data are mean ± SEM, n = 5 (two replicates each); values significantly different from DMSO: *p<0.05, Student's t-test. **(E)** Effect of SLMP53-2 on lactate secretion by A375 and SK-MEL-5 cells, after 8 h treatment. Cell density for each sample was used to normalize relative luminescence units (RLU) signal. Data are mean ± SEM, n = 5 (two replicates each); values significantly different from DMSO: *p<0.05; unpaired Student's t-test. **(F-I)** Protein expression levels of crucial regulators of EMT and angiogenesis, in A375 **(F, G)** and SK-MEL-5 **(H, I)** melanoma cells after 48 h treatment with SLMP53-2 (in A375 cells, β-catenin was detected for 8 h and E-cadherin and TWIST for 24 h treatment). Immunoblots are representative of three independent experiments; GAPDH was used as a loading control. In **G** and **I**, quantification of protein expression levels; values with DMSO were set as 1; data are means ± SEM, n = 3.

Figure 4. SLMP53-2 sensitizes melanoma cells to chemotherapeutic agents. (A-C) Cells were treated with a concentration range of vemurafenib (**A**), dacarbazine (**B**) and cisplatin (**C**), alone and in combination with 2 μM SLMP53-2, for 48 h, and the growth analysed by SBR assay. Growth with DMSO was set as 100%. For each combination, the C.I. and D.R.I. values were obtained. Data are means ± SEM, n = 5 (two replicates each); values significantly different from chemotherapeutic drug alone: *p<0.05; two-way ANOVA followed by Sidak's test. (**D, E**) Effect of 2 μM SLMP53-2 on combination with 0.027 μM Vemurafenib (Vem) on three-day-old

A375 spheroids, for up to 8 days treatment. For the combination, the C.I. value was obtained. Images are representative of five independent experiments; scale bar = 100 μm; magnification = 100×. In **E**, data are mean ± SEM, n = 5; values significantly different from DMSO: *p<0.05, one-way ANOVA followed by Tukey's test.

Figure 5. Melanoma cancer cells does not develop resistance to SLMP53-2: vemurafenib-resistant melanoma cells show no cross-resistance to SLMP53-2 and are re-sensitized to vemurafenib by SLMP53-2. (A) A375 cells were exposed to six rounds of treatment with 6, 9, 12, 18, 24 and 30 μM of SLMP53-2. IC_{50} values were determined at the end of each round by SRB assay after 48 h treatment. Data were normalized to DMSO and correspond to mean ± SEM, n = 5 (two replicates each); values not significantly different from parental cells: p>0.05, two−way ANOVA followed by Sidak's test. (**B**) Representative images of parental, vemurafenib-resistant (Vem-res) A375 cells; scale bar = 100 μm; magnification = 100×. **(C)** Concentration-response curves for vemurafenib in parental and Vem-res A375 cells after 48 h treatment. Data were normalized to DMSO and correspond to mean ± SEM, n = 6 (two replicates each); values of Vem-res cells significantly different from parental cells: *p<0.05; two−way ANOVA followed by Sidak's test. **(D, E**) Protein levels of p-AKT/AKT, p-ERK/ERK, PTEN and MDR-1 in parental and Vem-res A375 cells untreated. In **D**, immunoblots are representative of three independent experiments; GAPDH was used as loading control. In **E**, quantification of protein expression levels; values with DMSO were set as 1; data are mean ± SEM, n = 3. **(F)** Concentration-response curves for SLMP53-2 in parental and Vem-res A375 cells, after 48 h treatment. Data were normalized to DMSO and correspond to mean ± SEM, n = 6 (two replicates each); values of Vem-res cells are not significantly different from parental cells: two−way

ANOVA followed by Sidak's test. **(G)** Vem-res A375 cells were treated with a concentration range of vemurafenib alone and in combination with 2 μM of SLMP53-2. Cell growth was evaluated for 48 h treatment; growth obtained with DMSO was set as 100%. For each combination, the C.I. and D.R.I. values were obtained. Data are mean ± SEM, n = 6 (two replicates each); values significantly different from vemurafenib alone: *p<0.05, two−way ANOVA followed by Sidak's test. **(H)** Representative images of Vem-res A375 cells treated with DMSO, 2 μM SLMP53-2, 1.3 μM vemurafenib (Vem) and the combination (SLMP53-2+Vem); images are representative of five treatments; scale bar = 100 μm; magnification = 100×. **(I, J)** Proteins levels of PTEN, BCL-2, MDR-1 and p-AKT/AKT in Vem-Res after 48 h treatment with 2 μM SLMP53-2. In **I**, immunoblots are representative of three independent experiments; GAPDH was used as loading control. In **J**, quantification of protein expression levels; values with DMSO were set as 1; data are means ± SEM, n = 3.

Figure 6. *In vivo* melanoma anti-tumour activity of SLMP53-2. C57BL/6-Rag2$^{-/-}$IL2rg$^{-/-}$ mice carrying A375 xenografts were treated with 50 mg·kg^{-1} SLMP53-2 or vehicle, by intraperitoneal injection twice a week, for a total of six administrations. **(A)** Tumour volume curves of mice carrying A375 xenografts treated with SLMP53-2 or vehicle; fold of change is relative to the start of treatments; data are mean ± SEM, n = 7; values significantly different from vehicle: *p<0.05, two−way ANOVA followed by Sidak's test. Representative images of the tumours treated with SLMP53-2 or vehicle at the end of the experiment. **(B)** Tumour weights measured at the end of the *in vivo* experiment; data are mean ± SEM, n = 7; values significantly different from vehicle: *p<0.05, unpaired Student's t-test. **(C)** Body weight of the mice registered during the course of the experiment; data are mean ± SEM, n = 7; values are not

significantly different from vehicle: p>0.05, two−way ANOVA followed by Sidak's test. **(D)** Weight of heart, spleen, kidney and livers from animals treated with SLMP53-2 or vehicle; data are mean ± SEM, *n* = 7; values are not significantly different from vehicle: p>0.05, two−way ANOVA followed by Sidak's test. **(E)** Representative images of Ki-67, Vimentin, BAX and β-catenin detection in tumour tissues of A375 xenografts treated with SLMP53-2 or vehicle, collected at the end of treatment (scale bar = 5 μm; magnification = 200×); haematoxylin and eosin (H&E). **(F)** Quantification of immunohistochemistry of A375 xenograft tumour tissues treated with SLMP53-2 or vehicle; quantification of the number of Ki-67 positive and negative cells (*n* = 5; values significantly different from vehicle: **p*<0.05, two−way ANOVA followed by Sidak's test) and of the Vimentin, BAX and β-catenin staining, quantified by evaluation of 3,3′-diaminobenzidine (DAB) intensity (*n* = 5; values significantly different from vehicle: *p < 0.05, unpaired Student's t-test).

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