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Original Paper

Protein-Bound Polysaccharides from *Coriolus Versicolor* Induce RIPK1/RIPK3/MLKL-Mediated Necroptosis in ER-Positive Breast Cancer and Amelanotic Melanoma Cells

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Key Words
Coriolus versicolor • Protein-bound polysaccharides • Melanoma • Breast cancer • Necroptosis

Abstract
Background/Aims: The induction of necroptosis, a form of caspase-independent cell death, represents one of the most promising anticancer therapeutic modalities, as necroptosis serves as an alternative way to eliminate apoptosis-resistant tumor cells. Here, we investigated whether protein-bound polysaccharides (PBPs) derived from the fungus *Coriolus versicolor* (CV) induce the necroptotic death pathway in breast cancer and melanoma cells. ***Methods:*** MCF-7 and SKMel-188 cells were exposed to PBPs either alone or in combination with necrostatin-1 (Nec-1), GSK'872 or necrosulfonamide (NSA), pharmacological inhibitors of the kinases receptor-interacting protein 1 kinase (RIPK1), receptor interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL), respectively, which are involved in necroptotic processes. The effects of cellular treatment with these inhibitors were quantified by measuring cell viability and reactive oxygen species (ROS) generation *via* 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2',7'-dichlorofluorescein diacetate (DCF-DA) assays, respectively. The morphological changes induced in these cells were detected using holotomographic (HT) microscopy. Activation of the TNF-α/TNFR1 pathway in the PBP-stimulated cells was evaluated using TNF-α-neutralizing antibody, qRT-PCR and immunofluorescence-based assays. ***Results:*** PBPs showed effective antitumor activity against MCF-7 and SKMel-188 cells. Cotreatment of the cells with Nec-1, GSK'872 or NSA abrogated PBP-induced cell death, and the cells were protected against membrane rupture. Moreover,

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breast cancer cell death caused by PBPs was mediated by induced activation of the TNF-α/TNFR1 pathway. Interestingly, the melanoma cells did not express TNF-α or TNFR1 after PBP stimulation; instead, PBPs triggered intracellular ROS generation, which was partially diminished by the inhibitors Nec-1, GSK'872 and NSA. ***Conclusion:*** These results suggest that PBPs from the fungus CV induce RIPK1/RIPK3/MLKL-mediated necroptosis in breast cancer and melanoma cells, providing novel insights into the molecular effects of PBPs on cancer cells.

Introduction

Cell death can be classified into several major forms, including apoptosis, necrosis, autophagic death and delayed cell death associated with mitotic catastrophe, according to the morphological appearance and biochemical characteristics of the lethal process [1, 2]. The two most distinctive and best-studied cell death modalities are apoptosis and necrosis. Apoptosis, a highly regulated ATP-dependent biochemical process, was originally thought to be the only form of programmed cell death. Necrosis was discovered long ago and considered to be an unregulated, uncontrollable and energy-independent mode of cell death [3, 4]. However, this assumption was changed by studies performed by the group of Dr. Peter Vandenabeele, who provided the first evidence that necrosis can be regulated [5]. In 2005, Dr. Junying Yuan and colleagues applied the term 'necroptosis' to the programmed necrosis they found when TNF-α-induced necrosis was inhibited by the receptor-interacting protein 1 kinase (RIPK1)-specific inhibitor necrostatin-1 (Nec-1) [4, 6]. Necroptosis, considered to be a regulated cell death process, is morphologically characterized by an increase in cell volume, cytoplasmic swelling and vacuolization, plasma membrane rupture and permeabilization, cellular collapse and finally release of the cellular contents [7]. These characteristics clearly distinguish necroptosis from apoptosis, which is characterized by nuclear condensation and an intact plasma membrane [8]. Biochemically, necroptosis has no active protein-related processes comparable to the hallmarks of apoptosis, such as caspase activation or the caspase-mediated cleavage of cellular proteins [9]. The molecular mechanism of the pathway that executes necroptotic cell death involves the activation and integration of a trio of downstream signaling pathways formed by RIPK1, receptor interacting protein kinase 3 (RIPK3), and mixed lineage kinase domain-like protein (MLKL) [7]. A plethora of different stimuli, including signaling through tumor necrosis factor receptor-1 (TNFR1), TNF-α-related apoptosis-inducing ligand (TRAIL) receptors, Toll-like receptors 3 and 4, and Fas, and treatments, including multiple anticancer drugs, photodynamic therapy and ionizing radiation, can initiate the necroptotic cell death pathway [10, 11]. The induction of necroptosis is one of the most promising modalities of anticancer therapy and mainly serves as an alternative way to eliminate apoptosis-resistant tumor cells. Previously, we showed that protein-bound polysaccharides (PBPs), natural compounds isolated from the Chinese fungus *Coriolus versicolor* (CV), induce the death of ER-positive breast cancer cells [12] and amelanotic melanoma cells by Bcl-2- and caspase-independent pathways [13]. In the current study, we evaluated whether PBP extract triggers the necroptotic pathway in MCF-7 breast cancer cells and SKMel-188 amelanotic melanoma cells. Our results revealed that PBPs from CV induced RIPK1/RIPK3/MLKL-dependent necroptosis in both cancer cell lines; however, the induction of this process by PBPs differed in the breast cancer and melanoma cells.

Materials and Methods

Chemicals and reagents

CV capsules were purchased from MycoMedica Company (Czech Republic), and PBPs were extracted following previously described protocols [12-14]. The final PBP concentration in the stock solution was determined based on the manufacturer's certificate of analysis (MycoMedica Company) and had been tested with the CP2010-UV method. The carbohydrate and protein contents in the fractions were measured us-

ing phenol sulfuric acid and bicinchoninic acid assays, respectively. Nec-1,3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma-Aldrich (Germany). GSK'872 was purchased from R&D Systems (USA), and necrosulfonamide (NSA) was purchased from Tocris Bioscience (UK). Anti-human TNF-α monoclonal mouse antibody (cat. nr MAB210) and a mouse IgG1 isotype control (cat. nr MAB002) were purchased from R&D Systems. An EXTRACTME Total RNA Plus kit was obtained from Blirt company (Poland), and iScript Reverse Transcription Supermix for qRT-PCR, SSoAdvanced Universal SYBR® Green Supermix, the PrimePCR™ SYBR® Green Assay, human TNF (qHsaCED0037461), human TNFRSF1A (qHsaCED0037739) and human ACTB (qHsaCED0036269) were purchased from Bio-Rad (USA). Primary rabbit polyclonal antibody against TNFR1 (cat. nr orb100329) was from Biorbyt Ltd. (UK), and goat anti-rabbit IgG (H+L) secondary antibody conjugated to Alexa Fluor 488 (cat. nr A-11034) was purchased from Thermo Fisher Scientific (USA). Mounting medium with propidium iodide (PI) (cat. nr ab104129) was purchased from Abcam (UK). All the reagents used for cell culture were provided by Sigma-Aldrich.

Cell lines

The MCF-7 breast cancer cell line was obtained from the European Collection of Cell Cultures (lot 13K023; UK). The MCF-7 cells were cultured in RPMI 1640 medium (with L-glutamine) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and nonessential amino acids (1×) at 37°C in a humidified atmosphere with 5% CO_2. The human SKMel-188 melanoma cell line was a gift from Dr. Chakraborty at Yale University. SKMel-188 cells were cultured in Ham's F10 medium (with L-tyrosine at a low concentration (10 μM) to obtain an amelanotic phenotype) supplemented with 5% FBS and 1% antibiotics (penicillin/streptomycin/amphotericin) at 37°C in a humidified atmosphere with 5% CO_2 as described previously [13]. Both cell lines were provided with fresh medium every 2 days.

Viability assay

The effects of the PBP extract, inhibitors of necroptosis-related kinases and TNF-α neutralization on MCF-7 and SKMel-188 cell viability were determined by MTT assay. Briefly, cells (1×10^4/well) were stimulated with PBP extract (100 or 200 μg/ml) alone or with Nec-1 (30 μM), GSK'872 (10 μM), NSA (1.5 μM) or anti-human mouse TNF-α monoclonal antibody for the indicated time periods. Then, the cells were incubated with a 0.5 mg/ml MTT solution for 4 h at 37°C in an incubator, and the resulting purple formazan crystals were dissolved in DMSO. The optical density at 570 nm (with a reference wavelength of 630 nm) was measured using a Synergy HT Multi-Mode microplate reader (BioTek; Winooski, VT, USA). The cytotoxicity was determined as follows:

cytotoxicity (%) = (absorbance of treated cells/absorbance of control cells)×100%.

Cell morphology analysis

MCF-7 and SKMel-188 cells (3×10^5) were plated on TomoDishes designed for live-cell imaging using a Tomocube HT-1S microscope (Daejeon, Inc., Korea). After overnight preincubation, the cells were treated with PBP extract (200 μg/ml) alone or in combination with Nec-1, GSK'872 or NSA. Based on the three-dimensional refractive index (RI) distributions of the cells, cellular images at 72 h after treatment were rendered in two dimensions and three dimensions with Tomostudio™ software (Korea). Necroptotic cells were identified based on cell morphology.

Detection of intracellular reactive oxygen species (ROS)

Intracellular ROS formation was analyzed with the fluorescent probe 2′,7′-dichlorofluorescein diacetate. For the experiments, 25×10^3 cells/well were plated in 96-well Transwell plates, and the same protocol used for the MTT assay was followed. Briefly, before treatment, the cells were loaded with 20 μM DCF-DA in HBSS and incubated at 37°C for 45 min in the dark. Following incubation, the DCF-DA solution was removed, and the cells were washed with PBS. After cell stimulation for the indicated time period, the fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a fluorescence microplate reader (BioTek Synergy HT). The results are expressed in relative fluorescence units (RFU).

Quantitative real-time PCR

Total RNA was extracted from MCF-7 and SKMel-188 cells treated for 24 h with 0, 100 or 200 μg/ml PBP extract using an EXTRACTME Total RNA Plus kit, and first-strand cDNA was synthesized with the use of iScript Reverse Transcription Supermix for RT-qPCR according to the manufacturer's protocol. qPCR was performed using SSoAdvanced Universal SYBR® Green Supermix. Target mRNA expression was normalized to that of the reference gene, β-actin (ACTB). The primers used to detect ACTB, TNF and TNFRSF1A by qPCR were purchased and validated by Bio-Rad. RT-qPCR and qPCR were performed with a CFX Connect Real-Time PCR detection system (Bio-Rad, USA), and the data analysis was performed using CFX Maestro™ software for CFX Real-Time PCR instruments. All assays were performed according to the MIQE guidelines [15]. The relative expression of TNF-α and TNFR1 was calculated using the comparative threshold cycle (CT), 2^(−ΔΔCT), method.

Immunofluorescence staining for TNFR1

TNFR1 expression in the MCF-7 and SKMel-188 cells was examined by immunofluorescent staining. The treated cells were fixed with 4% formalin in PBS for 20 min, washed and permeabilized with 0.5% Triton X-100 in PBS. Then, the cells were blocked with 2% BSA for 1 h at RT and incubated overnight with anti-TNFR1 antibody diluted in 0.1% Triton X-100 in PBS at 4°C. After extensive rinsing in PBS, Alexa 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody was applied for 1 h at RT, after which the cells were mounted in medium with PI (red). The samples were examined at 20× magnification with a BX-50 epifluorescence microscope (Olympus, Tokyo, Japan) and photodocumented using a ColorView III digital camera and AnalySIS software (Olympus, Tokyo, Japan).

Statistical analysis

All values are reported as the means ± standard errors of the means (SEMs) and were determined by analysis of variance followed by Bonferroni multiple comparison test, with the level of significance set at $p < 0.05$. Statistical analyses were performed with GraphPad Prism 7.0 (La Jolla, CA, USA). The significance of differences in qPCR data between groups was determined with unpaired Student's t-test. Differences for which the p value < 0.05 were considered significant. All significant differences were based on comparisons with control cells or PBP-stimulated cells and are indicated in the figures with asterisks (*p<0.05, **p<0.01, and ***p<0.001).

Results

RIPK1, RIPK3 and MLKL inhibition abrogates PBP-induced cell death in melanoma and breast cancer cells

The necrosome complex (consisting of RIPK1 and RIPK3), which is essential for necroptosis activation, is commonly assembled upon caspase-8 inhibition [16-18]. Previously, we showed that the expression of caspase-8 as well as downstream caspases 3/7 was unchanged upon the treatment of melanoma cells with PBPs [13]. To elucidate the involvement of RIPK1, RIPK3 and MLKL (downstream effector molecules of necroptosis) in the observed PBP-induced death of breast cancer and melanoma cells (Fig. 1a and 2a), we manipulated the functions of the abovementioned kinases by using inhibitors. Nec-1 is a first-in-class inhibitor of RIP1 kinase activity [19]. The other specific inhibitors, GSK'872 and NSA, are widely used in various experimental models to target the kinase activities of RIPK3 and MLKL, respectively, to elucidate the roles of necroptosis components [2, 20]. Our cytotoxicity assays revealed that pretreatment of breast cancer cells with Nec-1, GSK'872 or NSA abolished PBP-induced cell death (Fig. 1b and c). A similar analysis performed on SKMel-188 amelanotic melanoma cells confirmed the inhibitory potency of PBP extract in reducing cancer cell viability (Fig. 2a). The use of inhibitors of kinases involved in the necroptosis process remarkably attenuated cell death induced by PBPs at a concentration of 200 μg/ml (Fig. 2c).

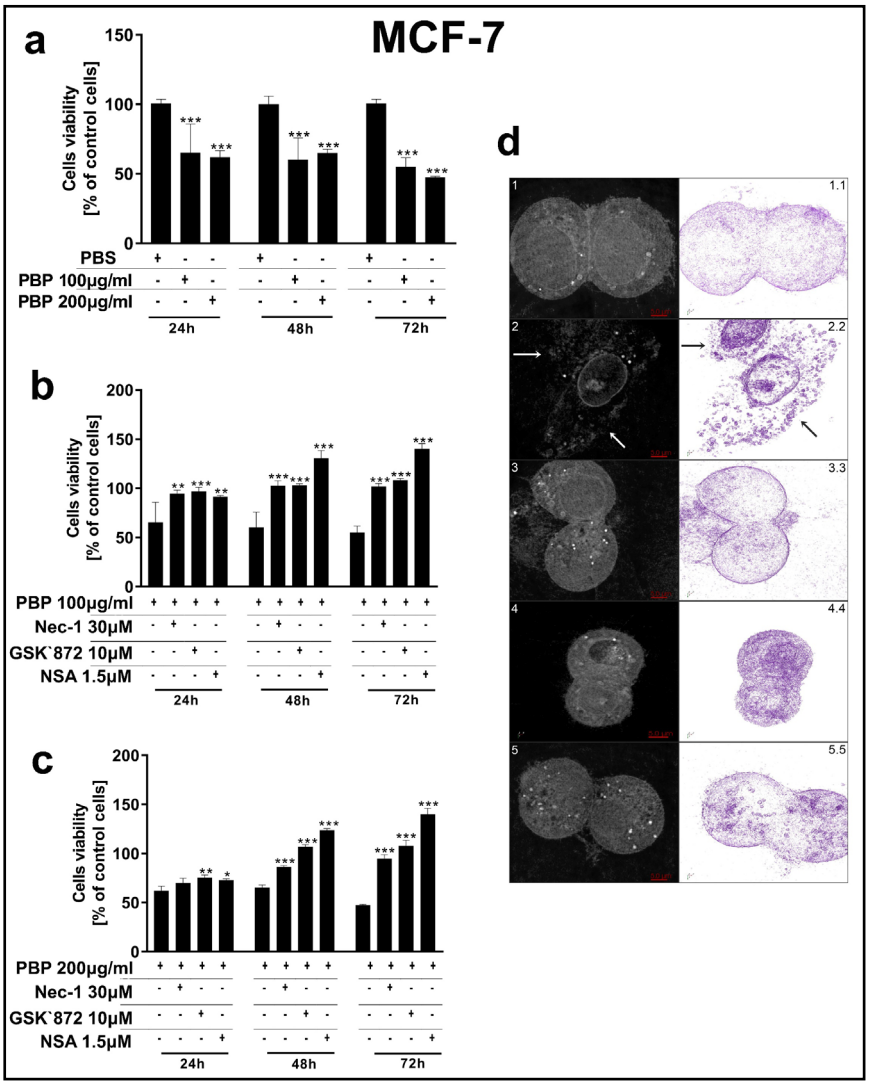

Fig. 1. The inhibition of RIPK1, RIPK3 and MLKL restores the PBP-mediated reduction in breast cancer cell viability and protects MCF-7 cells against PBP-induced morphological changes. Viability of cells treated with PBPs at a concentration of 100 or 200 µg/ml (a) or cotreated with PBPs at a concentration of 100 µg/ml (b) or 200 µg/ml (c) and Nec-1 (30 µM), GSK'872 (10 µM) or NSA (1.5 µM) for 24, 48 and 72 h. Data are reported as the means ± SEMs of three independent experiments, with six wells in each experiment. (d) Two-dimensional tomographs of breast cancer cells: control cells (1), cells treated with PBPs at a concentration of 200 µg/ml (2) and cells cotreated with PBPs at a concentration of 200 µg/ml and Nec-1 (3), GSK'872 (4) or NSA (5). A three-dimensional model of the membrane of control cells (1.1), cells stimulated with 200 µg/ml PBPs (2.2) and cells coincubated with 200 µg/ml PBPs and Nec-1 (3.3), GSK'872 (4.4) or NSA (5.5). Visualizations were rendered with Tomostudio™ software (Korea).

Inhibitors of RIPK1, RIPK3 and MLKL protect cancer cells against PBP-induced morphological changes

Based on the abovementioned results, we investigated whether blocking the activity of kinases engaged in the necroptosis process with their specific inhibitors, Nec-1, GSK'872 and NSA, would modulate the morphological changes in breast cancer and melanoma cells induced by PBPs (Fig. 1d and Fig. 2d, respectively). Holotomographic (HT) microscopy revealed that PBP-stimulated MCF-7 and SKMel-188 cells exhibited plasma membrane rupture (Fig. 1d-2, 2.2 and 2d-2, 2.2; white and black arrows). However, the treatment of cells with PBPs and inhibitors of RIPK1, RIPK3 or MLKL maintained plasma membrane integrity (Fig. 1d-3, 3.3; 4, 4.4; 5, 5.5 and Fig. 2d-3, 3.3; 4, 4.4; 5, 5.5).

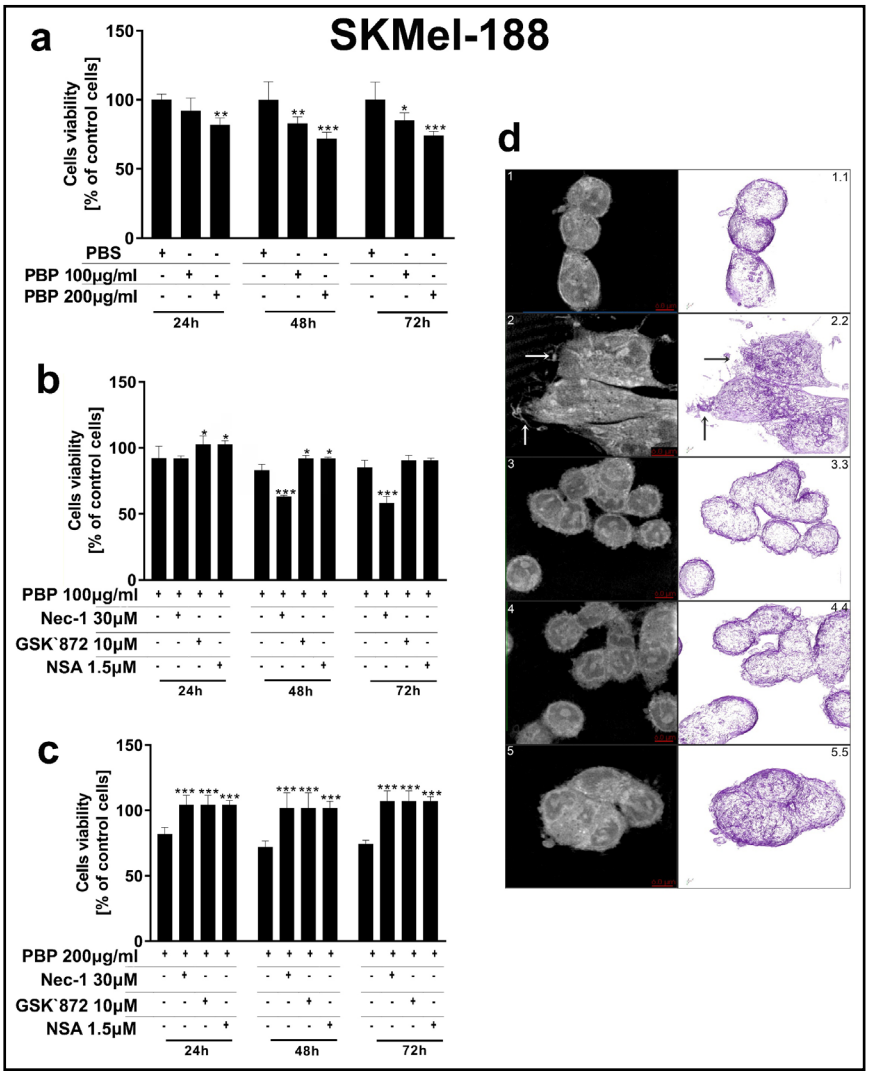

Fig. 2. The inhibition of RIPK1, RIPK3 and MLKL restores the PBP-mediated reduction in amelanotic melanoma cell viability and protects SKMel-188 cells against PBP-induced morphological changes. Viability of cells treated with PBPs at a concentration of 100 or 200 µg/ml (a) or cotreated with PBPs at a concentration of 100 µg/ml (b) or 200 µg/ml (c) and Nec-1 (30 µM), GSK'872 (10 µM) or NSA (1.5 µM) for 24, 48 and 72 h. Data are reported as the means ± SEMs of three independent experiments, with six wells in each experiment. (d) Two-dimensional tomographs of amelanotic melanoma cells: control cells (1), cells treated with PBPs at a concentration of 200 µg/ml (2) or cells cotreated with PBPs (200 µg/ml) and Nec-1 (3), GSK'872 (4) or NSA (5). Three-dimensional model of the cell membrane of control cells (1.1), cells stimulated with 200 µg/ml PBPs (2.2) and cells co-incubated with 200 µg/ml PBPs and Nec-1 (3.3), GSK'872 (4.4) or NSA (5.5). Visualizations were rendered with Tomostudio™ software (Korea).

Intracellular ROS generation in PBP-stimulated cells is partially inhibited by Nec-1, GSK'872 and MLKL

We previously showed that the cytotoxic effects of PBPs towards human melanoma cells are associated with deregulation of the cellular redox status [13]. Herein, we tested whether interference with the levels of intracellular ROS induced by PBPs is universal to all cancer cell types or rather cell-type specific and whether the intracellular generation of ROS in cancer cells is modulated by treatment with inhibitors of RIPK1, RIPK3 or MLKL. Although the treatment of MCF-7 cells with PBPs at both concentrations tested (100 and 200 µg/ml) significantly reduced cell viability (Fig. 1a), PBPs had little effect on ROS formation. PBPs at a concentration of 100 µg/ml induced a 1.25±0.07-, 1.25±0.08-, and 1.17±0.05-fold increase in ROS generation after 24, 48 and 72 h of stimulation, respectively, compared to ROS generation in the control cells, whereas in cells stimulated with PBPs at a concentration of 200 µg/ml, the following values were obtained at the corresponding time periods: 1.62±0.02, 1.31±0.05 and 0.88±0.11 (Fig. 3a and b). The addition of Nec-1, GSK'872 or NSA

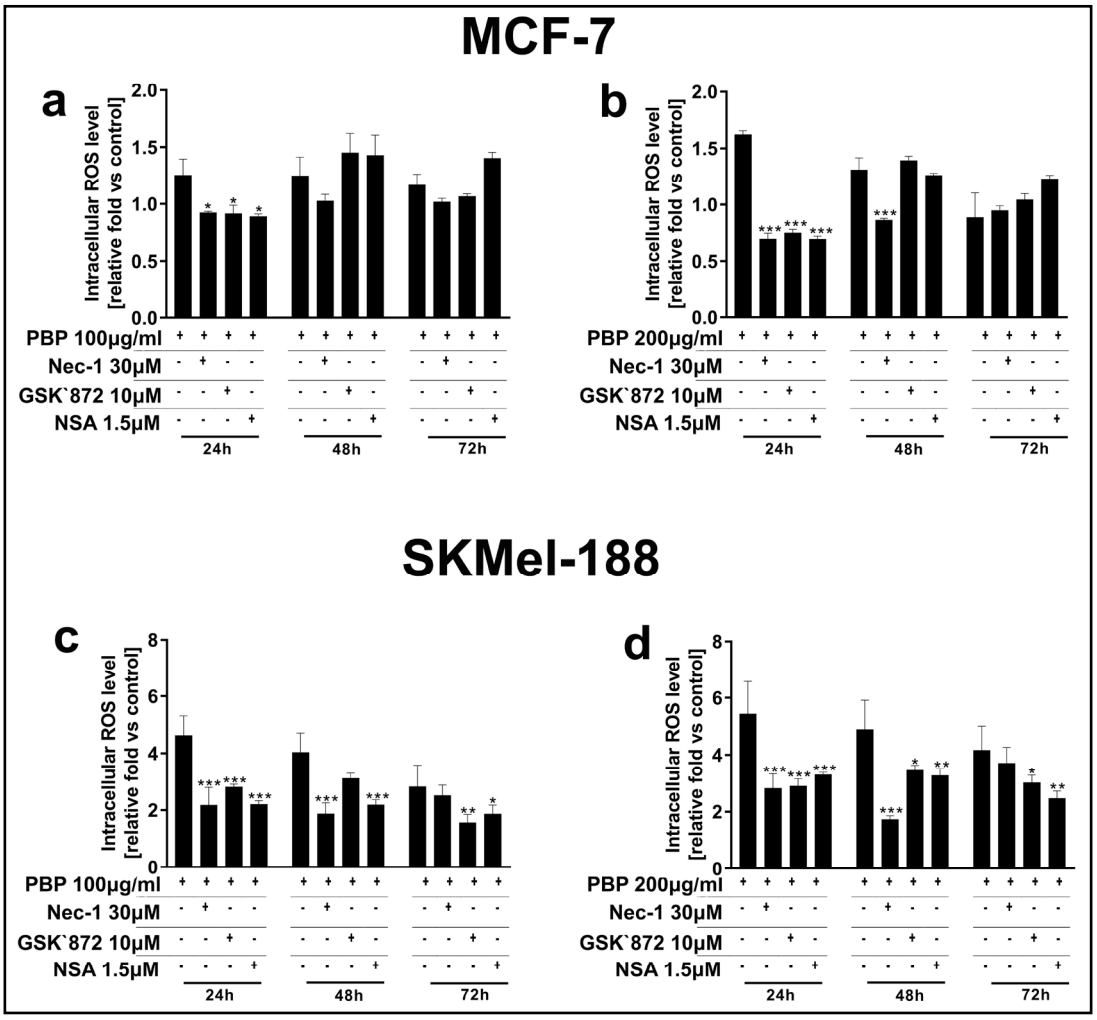

Fig. 3. PBP-mediated ROS generation is modified by the inhibition of kinases involved in necroptosis. MCF-7 and SKMel-188 cells were cotreated with PBPs at a concentration of 100 µg/ml (a, c) or 200 µg/ml (b, d) and Nec-1 (30 µM), GSK'872 (10 µM) or NSA (1.5 µM). ROS production at 24, 48 and 72 h after stimulation was measured using DCF dye. Data are the means ± SEMs based on values obtained from three independent experiments and expressed in relative fluorescence units.

to the cell culture reduced the level of ROS to that of the control cells (cells incubated with PBS), but this was only observed after 24 h of culture. After treatment of the cells for longer time periods (48 and 72 h), GSK'872 and NSA did not influence the cellular redox status (Fig. 3a and b). RIPK1 inhibition also reduced ROS formation after 48 h of culture compared to that in cells treated with only PBPs (200 µg/ml); however, after 72 h of incubation, this effect was no longer observed (Fig. 3b). A similar analysis of PBP-stimulated SKMel-188 melanoma cells confirmed our previous findings [13] and showed significantly upregulated ROS formation compared to that in the control cells. PBPs at a concentration of 100 µg/ml induced a 4.63±0.35-, 4.03±0.34-, and 2.81±0.38-fold increase in ROS generation after 24, 48 and 72 h of stimulation, respectively, compared to ROS generation in the control cells, while PBPs at a higher concentration (200 µg/ml) induced a 5.43±0.57-, 4.88±0.51- and 4.16±0.42-fold increase in ROS generation at the corresponding time periods (Fig. 3c and d). PBP-stimulated cells treated with Nec-1, GSK'872 or NSA for 24 and 48 h showed significant decreased ROS production compared to that in cells treated with only PBPs (Fig. 3c and d). After 72 h, inhibition of ROS generation was still observed in cells cotreated with PBPs and the inhibitors GSK'872 or NSA (Fig. 3c and d).

PBPs alter the expression of TNF-α/TNFR1 in breast cancer cells but not in melanoma cells

To explore the underlying molecular signaling pathways by which PBPs induce necroptosis in breast cancer and melanoma cells, we examined the expression of TNF-α/TNFR1 by qPCR. As shown in Fig. 4c, the mRNA levels of TNF-α and TNFR1 were increased in a dose-dependent manner in MCF-7 cells treated with PBPs compared to the control cells. Compared to the control cells, breast cancer cells stimulated with PBPs at a concentration of 200 μg/ml showed a 3.9±0.19-fold increase in TNF-α mRNA expression and a 5.27±0.55-fold increase in TNFR1 mRNA expression (Fig. 4c). Subsequent immunohistochemical detection

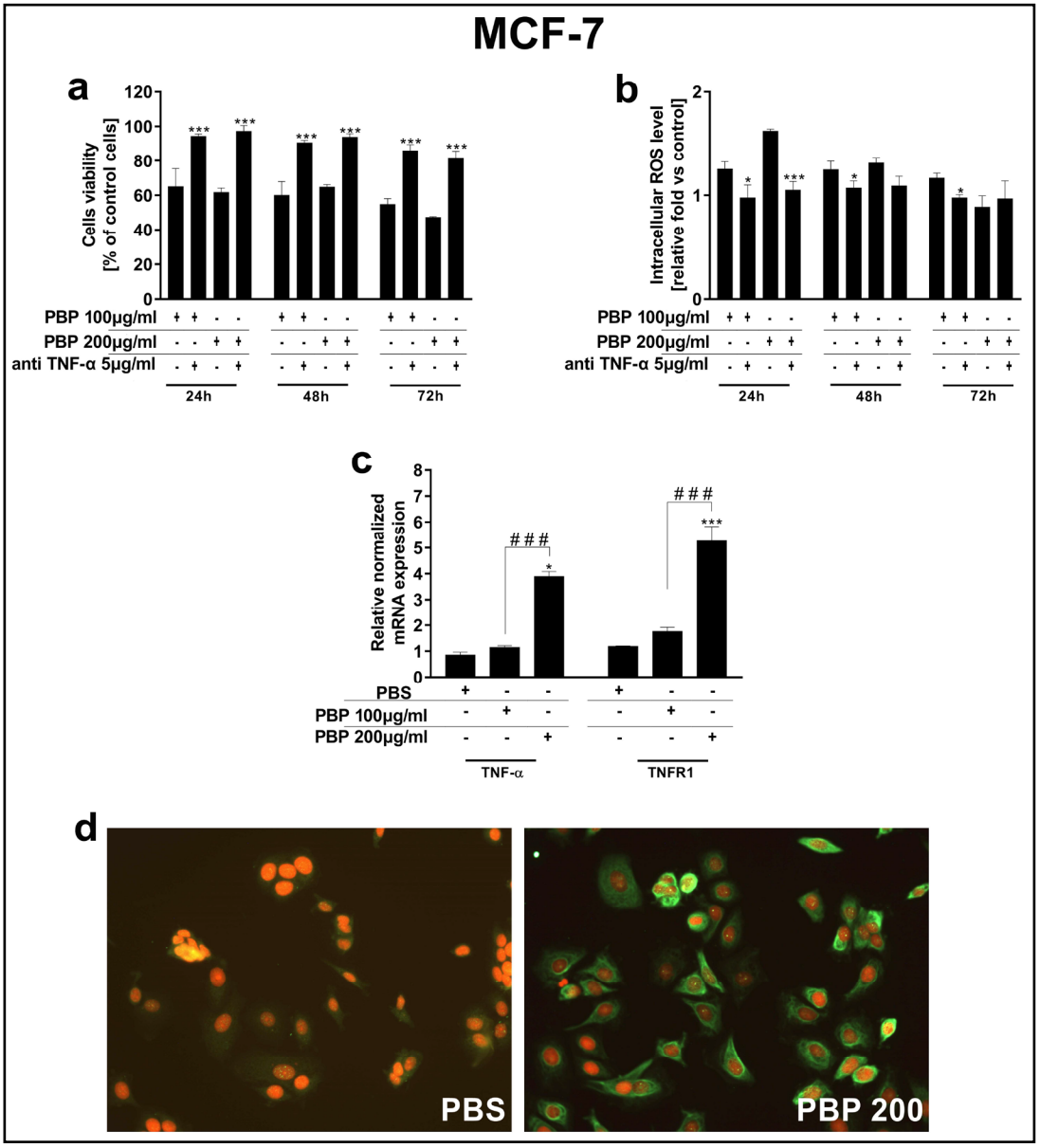

Fig. 4. PBPs enhance autocrine production of TNF-α, which is required for the necroptosis of breast cancer cells. (a) Viability and (b) ROS generation in MCF-7 cells pretreated (1 h) with neutralizing antibody (5 μg/ml) against TNF-α prior to treatment with PBPs (100 μg/ml or 200 μg/ml). (c) mRNA expression levels of TNF-α and TNFR1 in MCF-7 cells treated with PBPs for 24 h, as determined by quantitative real-time PCR. (d) Detection of TNFR1 expression (green) using immunohistochemical assays of MCF-7 cells incubated with PBS (control) or stimulated with PBPs (200 μg/ml) for 48 h. Cell nuclei were counterstained with PI (red).

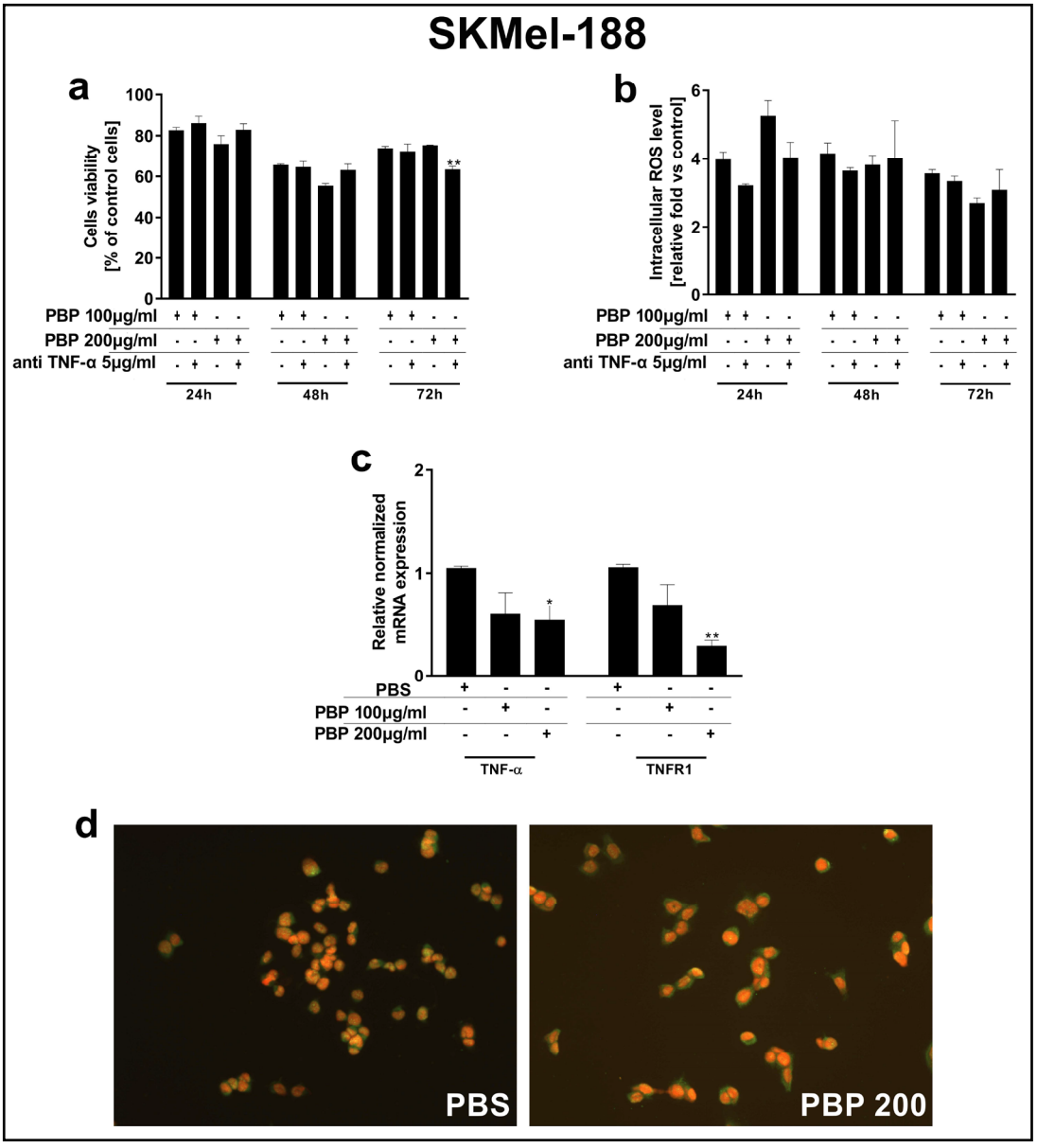

Fig. 5. PBPs do not enhance the autocrine production of TNF-α in amelanotic melanoma cells. (a) Viability and (b) ROS generation in SKMel-188 cells pretreated (1 h) with neutralizing antibody (5 µg/ml) against TNF-α prior to treatment with PBPs (100 µg/ml or 200 µg/ml). (c) The mRNA expression levels of TNF-α and TNFR1 in SKMel-188 cells treated with PBPs for 24 h, as determined by quantitative real-time PCR. (d) Detection of TNFR1 expression (green) using immunohistochemical assays with SKMel-188 cells incubated with PBS (control) or stimulated with PBPs (200 µg/ml) for 48 h. Cell nuclei were counterstained with PI (red).

of TNFR1 in PBP-stimulated MCF-7 cells confirmed the upregulation of TNFR1 (Fig. 4d). Interestingly, qPCR analysis of PBP-stimulated SKMel-188 cells did not reveal the upregulation of either TNF-α or TNFR1 mRNA expression. The mRNA expression of these molecules was even downregulated in SKMel-188 cells stimulated with PBPs at a concentration of 200 µg/ml compared to the control cells (TNF-α, 0.54±0.14; TNFR1, 0.29±0.06) (Fig. 5c). Immunohistochemical analysis to detect TNFR1 also showed that its expression was not upregulated in PBP-stimulated SKMel-188 cells compared to untreated cells (Fig. 5d).

PBP-induced necroptosis in breast cancer cells is mediated by TNF-α

To determine whether TNF-α is critical for the necroptotic cell death of PBP-stimulated breast cancer cells, we inhibited TNF-α for 1 h by preincubating cells with a neutralizing antibody prior to the addition of PBPs, after which we measured cell viability and intracellular ROS formation. First, we confirmed that the neutralization of TNF-α restored breast cancer cell viability after treatment with PBPs (Fig. 4a). Furthermore, TNF-α blockade resulted in the downregulation of ROS compared to that in PBP-stimulated MCF-7 cells. This effect was observed after 24 h of stimulation (Fig. 4b). To confirm the abovementioned findings indicating the negligible role of TNF-α in necroptosis induction in amelanotic melanoma cells, we performed additional analysis with TNF-α-neutralizing antibody. Indeed, the preincubation of SKMel-188 melanoma cells with this monoclonal antibody had no effect on their viability (Fig. 5a) or intracellular ROS generation (Fig. 5b).

Discussion

In recent decades, the search for alternative anticancer agents in traditional medicine has rapidly increased, and these agents are considered a potential strategy for complementary and alternative medical therapy [21, 22]. CV is a well-known traditional Chinese fungus that has been used for medical purposes for over 2000 years [23]. PBPs are major bioactive components in mushroom extracts. Numerous *in vitro* and *in vivo* studies and some clinical trials have reported the immunostimulatory and anticancer effects of CV extract, which led to its adoption as an adjunct therapy for cancer in many Eastern countries [24]. However, the use of PBPs as an immunotherapy throughout the world requires complete knowledge about the exact molecular mechanisms induced in different cancer cell types and the cells engaged in host immunological defense. Previously, we showed that PBPs, acting as a biological response modifier (BRM), have strong immune-enhancing effects towards blood lymphocytes [12]. Interestingly, at the same concentration at which they are effective BRMs, PBPs induced cytotoxicity in MCF-7 breast cancer cells. We have also demonstrated that PBPs cause Bcl-2- and caspase-independent cell death in amelanotic melanoma cells, while pigmented melanoma cells were found to be resistant to the anticancer activity of PBPs due to their melanin content [13]. In this study, we explored a signaling pathway leading to breast cancer and amelanotic melanoma cell death.

Our results showed that PBPs induced RIPK1/RIPK3/MLKL-mediated necroptosis in breast cancer and amelanotic melanoma cell cultures. Since no specific markers for necroptosis are available, the identification of programmed cell death requires the use of combined detection methods. Herein, we used the pharmacological inhibitors of the kinases RIPK1 (Nec-1), RIPK3 (GSK'872) and MLKL (NSA), which antagonize the necroptotic pathway and therefore rescue cells from necroptosis [6, 20, 25, 26]. To identify the morphology of the necroptotic cells, we used HT microscopy, which enables measurement of the three-dimensional RI tomography of cells and imaging of the cellular membrane and subcellular organelles without the use of exogenous labeling agents. Our results revealed that PBP-mediated breast cancer and melanoma cell death is dose-dependent and might be fully suppressed by inhibitors of RIPK3 and MLKL. The blockade of RIPK1 by its specific inhibitor restored the viability of PBP-stimulated breast cancer cells. In amelanotic melanoma cells, this effect was observed in only cultures coincubated with Nec-1 and PBPs at a concentration of 200 μg/ml, which was more potent in inducing melanoma cell death than PBPs at the lower concentration tested. Therefore, HT visualization of PBP-induced cancer cells death and the effect of necroptosis kinases inhibition was observed in both, the MCF-7 and SKMel-188, cell lines stimulated with PBPs at the higher concentration tested, 200 μg/ml.

We showed that PBP-stimulated MCF-7 and SKMel-188 cells exhibited rupture of the cell membrane, a feature of programmed cell death specific to necroptosis. The molecular pathway responsible for this process has been extensively characterized. After the activation of RIPK1 and recruitment of RIPK3, necrosome formation is observed. MLKL is then oligo-

merized and translocates to the plasma membrane, leading to the execution of necroptosis, which causes necrotic plasma membrane permeabilization and ultimately cell death [26]. This whole cascade seems to be essential for PBP-induced necroptosis, since the addition of the inhibitor Nec-1, GSK'872 or NSA completely abolished the morphological changes triggered by PBPs in breast cancer and melanoma cells.

Previously, we showed that PBP-induced amelanotic melanoma cell death is dependent on ROS [13]. Knowing that ROS play a role in regulating necroptosis in many cell types [27], we sought to determine whether blockade of the activity of kinases involved in necroptosis would affect the ROS generation induced by PBPs in breast cancer and melanoma cells. As revealed by the DCF-DA assay, Nec-1, GSK'872 and NSA diminished the ROS generation triggered by PBPs in amelanotic melanoma cells, whereas the pretreatment of breast cancer cells with an inhibitor of RIPK1, RIPK3 or MLKL decreased ROS formation after only 24 h of stimulation, with no effect observed after 48 or 72 h of culture. Interestingly, intracellular ROS levels in melanoma and breast cancer cells treated with only PBPs differed significantly compared to those in the control cells. A significant increase in ROS was observed in SKMel-188 cells, but in MCF-7 cells, this effect was diminished. The observed reduction in ROS production in melanoma cells cotreated with PBPs and Nec-1, GSK'872 or NSA in this study suggests that ROS overproduction is a component of the necroptotic pathway following PBP treatment. However, in the case of breast cancer cells, the induction of necroptosis by PBPs is independent of ROS.

To further characterize the molecular mechanism of PBP-induced necroptosis, we focused on upstream molecules that may be critical for the initiation of programmed cell death. TNF-α-induced necroptosis is the best characterized necroptotic pathway [28]. In this study, to determine whether TNF-α is required for PBP-induced cell death, we inhibited the activity of this molecule with neutralizing antibody prior to the addition of PBPs and then measured cell viability and ROS formation. Importantly, the inhibition of TNF-α had no effect on PBP-induced cytotoxicity in amelanotic melanoma cells or on intracellular ROS generation. However, the preincubation of breast cancer cells with TNF-α-neutralizing antibody abolished PBP-induced cell death, suggesting the crucial role of TNF-α in necroptosis initiation, independent of ROS formation. Furthermore, the results of qPCR analysis showed a significant increase in the mRNA expression of TNF-α and its receptor, TNFR1, in breast cancer cells stimulated with PBPs at a concentration of 200 μg/ml compared to control cells. Immunohistochemical analysis of TNFR1 expression confirmed that this receptor was upregulated in breast cancer cells stimulated with PBPs. Based on the abovementioned results, we concluded that PBPs indeed induce necroptotic breast cancer cell death, which is mediated by the TNF-α/TNFR1 signaling pathway. The downregulation of TNF-α and TNFR1 expression in SKMel-188 cells after PBP treatment suggests TNF-α-independent activation of necroptosis in melanoma cancer cells.

Multiple chemotherapeutic agents and a growing number of natural compounds have been reported to act on cancer cells by triggering the necroptosis pathway [26]. Shikonin, a naturally occurring naphthoquinone, was found to induce necroptosis *via* ROS production and the formation of RIPK1/RIPK3 necrosomes in osteosarcoma, pancreatic cancer and glioma cells [29-31]. Staurosporine, an alkaloid originally extracted from the bacterium *Streptomyces staurosporeus*, has been reported to induce the RIPK1/MLKL-dependent necroptosis pathway in leukemia cells [32]. Neoalbaconol, isolated from the fungus *Albatrellus confluens*, was reported to induce necroptosis by stimulating the autocrine secretion of TNF-α and remodeling cellular metabolism in nasopharyngeal carcinoma cells [33, 34]. Finally, resibufogenin, a bioactive compound extracted from toad venom, was shown to inhibit the growth and metastasis of colorectal cancer by inducing RIPK3-mediated necroptosis [35]. Our study demonstrated that PBPs from the CV fungus induce RIPK1/RIPK3/MLKL-mediated necroptosis in breast cancer and melanoma cells, as indicated by the restoration of cell viability and protection of cells from membrane rupture due to inhibition of kinases involved in the necroptosis process. We showed that PBP-mediated induction of necroptosis was mediated by activation of the TNF-α/TNFR1 pathway in only breast cancer cells. The amelanotic mela-

noma cells did not express TNF-α after PBP stimulation; instead, PBPs triggered intracellular ROS generation, which was partially diminished by the inhibitors Nec-1, GSK'872 and NSA.

For decades, agents have been designed to trigger apoptosis in cancer cells and applied in clinical oncology to eliminate malignant cells [36, 37]. However, the activation of anti-apoptotic systems allows tumor cells to escape this type of programmed cell death, leading to resistance to cancer therapeutics [38]. Thus, inducing necroptosis as an anticancer therapy has promising potential for cancer treatment. Our findings on this approach provide novel insights into the molecular effects of PBPs on cancer cells *in vitro*. While PBPs are clinically important, further studies are needed to validate them *in vivo*.

Conclusion

We conclude that PBPs from the fungus *Coriolus versicolor* triggered RIPK1/RIPK3/MLKL-dependent necroptosis in both breast cancer and melanoma cell lines. However, stimulation of the TNF-α/TNFR1 pathway by PBPs seemed to activate this form of programmed cell death in only breast cancer cells, whereas in melanoma cells, PBPs triggered intracellular ROS generation.

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Author contributions

M.P.: conceived and designed the study, acquired data, analyzed and interpreted the data, and drafted the article; T.J.: analyzed and interpreted the data for the article; A.A.B.: supplied the immunohistochemistry data; S.W.: critically revised the work for important intellectual content.

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Disclosure Statement

The authors declare no financial or commercial conflicts of interest.

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