UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF

Neurosciences, Biomedicine and Movement Sciences

GRADUATE SCHOOL OF

Life and Health Sciences

DOCTORAL PROGRAM IN

Biomolecular Medicine

XXXII cycle / 2016-2019

Influence of ONCONASE in the therapeutic potential of PARP and BRAF inhibitors in human A375 melanoma cells.

S.S.D. BIO/10

Coordinator: Prof. Lucia De Franceschi

Tutor: Prof. Marta Menegazzi

Doctoral Student: Alice Raineri

Influence of ONCONASE in the therapeutic potential of PARP and BRAF inhibitors in human A375 melanoma cells.

Alice Raineri
Tesi di Dottorato
Verona, Dicembre 2019

Sommario

Il melanoma è una delle forme più aggressive di cancro della pelle, caratterizzata da un alto tasso di mortalità a causa dell'elevato potenziale metastatico. Negli ultimi anni sono state approvate diverse terapie innovative come l'uso di farmaci che agiscono contro specifici bersagli molecolari. Ad esempio, gli inibitori di BRAF (BRAFi) sono molto utilizzati contro il melanoma che presenta il gene BRAF mutato. Sfortunatamente, fino ad ora tutte le strategie terapeutiche non hanno dato risultati soddisfacenti a causa della resistenza acquisita verso i farmaci antitumorali. Gli enzimi poli (ADP-ribosio) polimerasi (PARP) hanno un ruolo cruciale nella risposta ai danni al DNA e l'inibizione di PARP provoca l'accumulo di DNA danneggiato causando morte cellulare. I PARP inibitori (PARPi) sono comunemente usati in chemioterapia sia singolarmente che in associazione con altri farmaci. Il PARPi più usato nella terapia antitumorale è l'olaparib, anche se esso viene rapidamente espulso dalle cellule ad opera della P-glicoproteina, canale di membrana che favorisce l'efflusso dei farmaci dalla cellula. Per questo motivo è stata progettata una nuova generazione di PARPi di cui l'AZD2461 è un esempio selezionato per una più bassa affinità di legame alla P-glicoproteina.

Studi *in vitro* ed *in vivo* hanno dimostrato che la proteina Onconasi (ONC), grazie alla sua attività ribonucleasica, agisce da antitumorale in diversi tipi di cancro. Pertanto, in questo lavoro sono stati valutati gli effetti di ONC sulla linea cellulare A375 di melanoma umano, la quale presenta una mutazione nel gene codificante per l'enzima BRAF chinasi. I risultati dimostrano che ONC è capace di ridurre la vitalità di cellule A375, mentre nessuna citotossicità è stata riscontrata in melanociti umani normali. Inoltre, il trattamento ha prodotto una riduzione dell'incorporazione di BrdU e dell'espressione della proteina Ki67, indice di un effetto citostatico di ONC, come anche una riduzione della sintesi proteica. ONC ha anche azione citotossica in quanto il trattamento di cellule A375 con questa ribonucleasi ha prodotto un aumento della fluorescenza di Annexin V-FITC e dell'espressione della forma tagliata di PARP1, entrambi indici di apoptosi.

Per studiare i bersagli intracellulari di ONC, sono stati esaminati i cambiamenti di espressione delle proteine intracellulari, indotti da ONC, mediante analisi

proteomica in spettrometria di massa. È stata osservata una diminuzione dell'espressione delle proteine coinvolte nell'allungamento dei telomeri, nello sviluppo delle cellule mesenchimali e nella biogenesi delle subunità ribosomiali. Quando ONC è stata somministrata in combinazione con il PARPi AZD2461 non sono stati ottenuti forti benefici dal trattamento congiunto se confrontati con gli effetti dei farmaci somministrati singolarmente. Sia ONC che AZD2461 si sono dimostrati capaci di inibire la trascrizione genica di TNF-α e la capacità di NF-κB di legarsi al DNA, mentre non è stato osservato alcun effetto additivo quando i due farmaci sono stati usati in combinazione. Al fine di ottenere cellule resistenti all'AZD2461, le cellule A375 sono state trattate per due mesi con questo farmaco senza ottenere cloni resistenti. Ciò nonostante, le cellule trattate a lungo con AZD2461, rispetto alle parentali, sono risultate più sensibili all'azione pro-apoptotica di ONC. Successivamente, è stata creata una sottopopolazione di cellule A375 resistenti al dabrafenib (A375DR), selezionate dopo un lungo trattamento con il farmaco. Le cellule A375DR, rispetto alle parentali (A375P), hanno mostrato attivazione dei marcatori di cellule staminali tumorali CD133 e NANOG, ed una più alta espressione delle proteine legate alla transizione epitelio-mesenchimale: N-caderina e β-catenina nucleare. Inoltre, le cellule A375DR hanno mostrato un aumento del livello di fosforilazione di ERK1/2, suggerendo una riattivazione di queste chinasi solitamente bloccate dall'azione del dabrafenib. Confrontando A375P e A375DR, è stato dimostrato che ONC ha diminuito la vitalità totale e l'indice di proliferazione e ha indotto apoptosi in entrambe le sottopopolazioni cellulari. Tra i suoi effetti pleiotropici, ONC ha ridotto i livelli di p65 NF-κB nel nucleo e delle forme fosforilate di IκB kinasi, così come l'attività di metalloproteinasi-2 in entrambe le sottopopolazioni cellulari. Infine, ONC ha diminuito la formazione delle colonie, la migrazione e la capacità di invasione in modo più cospicuo nelle cellule A375DR rispetto alle A375P.

In conclusione, ONC ha dimostrato capacità di inibire il fenotipo maligno delle cellule A375 specialmente nella sottopopolazione di cellule resistenti al BRAFi. Quindi, ONC potrebbe diventare uno strumento terapeutico efficace per contrastare le recidive di melanoma.

ABSTRACT

Melanoma is one of the most aggressive form of skin cancer, characterized by high mortality rate due to the metastatic potential of its cells. Several therapies have been approved during last few years. Recently, the melanoma molecular characterization led to development of drugs acting against specific targets. For instance, BRAF inhibitors (BRAFi) have been tested against BRAF-mutated melanoma. Unfortunately, all chemotherapy strategies failed for the resistance acquired by tumor cells.

Poly (ADP-ribose) polymerase (PARP) enzymes are crucial in the DNA damage response and the PARP inhibition causes the accumulation of unrepaired DNA, inducing cell death. PARP inhibitors (PARPi) are commonly used in chemotherapy. The most used PARPi in cancer therapy is olaparib, although it can be quickly throwing outside the cells by the P-glycoprotein drug efflux transporter. For this reason, a new PARPi generation has been designed, such as AZD2461 that demonstrated a lower binding affinity to the P-glycoprotein than olaparib.

In vitro and *in vivo* studies showed that the protein onconase (ONC) exerted an antitumor effect in different cancer types, due to its ribonuclease activity. Therefore, in this work, the ONC effects on A375 human melanoma cell line that harbors a mutation in the BRAF kinase gene, has been evaluated. A reduction in A375 cell viability has been observed with ONC treatment, while no cytotoxicity was registered in normal human melanocytes. In A375 cells a reduction in BrdU incorporation and a decrease in Ki67 protein expression occurred, suggesting that ONC elicits a cytostatic effect. In addition, an increase of both Annexin V-FITC fluorescence and cleaved PARP1 expression level were observed, suggesting that ONC induces apoptotic cell death. In order to investigate the ONC intracellular targets, changes in the proteome profile have been investigated using mass spectrometry. A decreased expression level of proteins involved in telomere elongation, in the mesenchymal cell development and in the ribosomal subunit biogenesis has been obtained. A decrease in protein synthesis in ONC-treated cells was also demonstrated. Besides ONC, AZD2461 PARPi can reduce A375 cell

viability, conversely, no strong benefits were obtained when ONC was administered in combination with AZD2461, in comparison with each single drug treatment. ONC and AZD2461 have displayed an inhibitory effect on both TNF-α gene transcription and NF-κB DNA binding activity, but no additive effect was observed when they were used in combination. In order to obtain A375 cells resistant to AZD2461, cells were treated for two months with AZD2461, but this long treatment did not induce resistance. Nevertheless, the AZD2461 long-time treated cells resulted more responsive to the ONC pro-apoptotic action, if compared to the parental ones. Subsequently, resistance against the BRAFi dabrafenib has been induced in A375 since long time treatment with increasing drug concentrations of dabrafenib induced a sorting of an A375 resistant cell (A375DR) subpopulation. A375DR cells displayed activation of the cancer stem cells markers CD133 and NANOG and increase expression of epithelial-mesenchymal transition-related proteins N-cadherin and nuclear β-catenin, in comparison with A375 parental (A375P) cells. In addition, A375DR showed an increase in the ERK1/2 phosphorylation level, suggesting a reactivation of the MAPK pathway. By comparing A375P and A375DR, ONC treatment can inhibit the total viability and the proliferation rate in both cell subpopulations and induce apoptotic cell death. Moreover, among its pleiotropic effects, ONC reduced nuclear p65 NF-κB amount and IκB kinase phosphorylation level, as well as metalloproteinase-2 activity in both cell subpopulations. Finally, ONC decreased cell colony formation, migration and invasion capability more extensively in A375DR than in A375P cells.

In conclusion, ONC successfully counteracts malignant phenotype of A375 cells especially in BRAFi resistant cells and could become a helpful tool for therapy of melanoma recurrence.

TABLE OF CONTENTS

1. **INTRODUCTION**

1.1 MELANOMA

Melanoma is a malignant tumor that arises from neoplastic transformation of melanocytes, cells localized principally in the skin and eyes where they produce melanin, the pigment that defines skin and hair colour[1]. Malignant melanoma is classified into different subgroups, based on tumor localization: mucosal, uveal and cutaneous forms. The cutaneous is the most common and represents 90% of all cases, is one of the most aggressive form of skin cancer and is related with high mortality rate, due to its metastatic potential[2,3]. Melanoma progression is described by Clark into different clinically and histologically defined stages (Fig.1[4]): starting by the common melanocytic nevus, that accumulates mutations and gains hyperplasia and dysplasia, followed by a non-tumorigenic radial growth phase that could be in situ or invasive, carrying on with the vertical phase, characterized by invasion and tumorigenicity and finally leads in the metastatic phase[5].

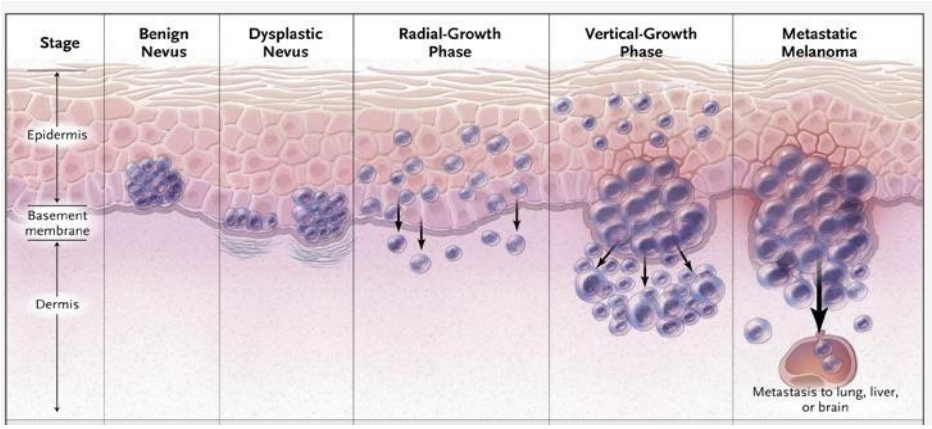


Fig.1. Biologic events and molecular changes in melanoma progression.

The incidence of malignant melanoma has been increasing worldwide, resulting in an important socio-economic problem. Starting from 1960s, the incidence of this cancer has increased in Caucasian population and, thus, melanoma has become one of the most frequent cancers in fair-skinned populations[6]. Melanoma is now the fifth most common cancer in men and the sixth in women in the United States, where the incidence is increased by 270% from 1973 to 2002. Currently, 1.6%

people in US population will develop melanoma during their lifetime. Melanoma, different to other cancers, affects a young and middle-aged population. The people average age at the diagnosis has estimated around 57 years and it was observed that the incidence increases linearly after 25 until 50 years and then reduces, especially in females. The incidence sex-related, according with Markovic *et al.*, is grater in women than men until 40 years, however, by 75 years, is 3-times higher in men than in women[7,8]. The pathogenesis of melanoma is a complex phenomenon in which environmental, genetic, and host factors play different roles. Several risk factors have been identified by epidemiological studies. Pigmentation has a significant influence on skin susceptibility to malignant changes. Indeed, subjects with red/blond hair, blue eyes, fair skin, and developing sunburns have higher melanoma risk than subjects with brown hair/eyes and skin[9]. Regarding genetic alterations, 10% of melanoma are hereditary due to a mutation in cyclin-dependent kinase inhibitor 2A/p16 (CDKN2A/p16), a tumor suppressor that plays an important role in cell cycle regulation. In the dysplastic nevus it has been found a large number of cells that display driver mutations, well known as gain of function mutations that activate the mitogen-activated protein kinase (MAPK) pathway. More than 50% of melanoma have mutations in the kinase domain of BRAF oncogenic protein that render the BRAF kinase constitutively active, independently of a previous activation by extracellular stimuli and causing over activation in the downstream MAPK/ERK pathway involved in cell proliferation and survival[10,11] (Fig.2[12]).

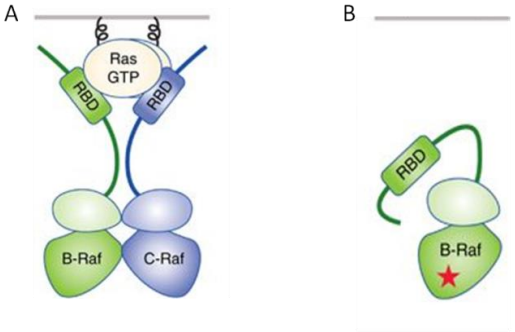


Fig.2. (A). Normal activation of Ras by extracellular factors. (B). Mutations induce constitutive activation of Raf, independent to the extracellular signals.

In 90% of epidemiological cases the mutation is a substitution of glutamic acid for valine at amino acid 600 (BRAF V600E)[13]. The most already known environmental factor, implicated in the development of cutaneous melanoma, is UV radiation. Epidemiologic studies, according to the world health organization (WHO), reveal that excessive sunlight exposure, also associated with sunburn, especially in childhood years, could trigger the transformation of benign melanocytes into a malignant phenotype and consequent melanoma development[14].

Early detection of malignant melanoma and its treatment remains the key factor in lowering mortality. The prognosis in melanoma is directly proportionate to the neoplastic stage. The cutaneous location is an advantage in melanoma cure, compared to other cancers, because permits its early detection through non-invasive approaches[15]. The European Society for Medical Oncology clinical practice guidelines for cutaneous melanoma highlight the importance of a detailed diagnosis for the establishment of the tumor stage and, in some cases, a mutation test is also required[16].

Several therapies have been approved by the US Food and Drug Administration (FDA). Depending on the features of the tumor (location, stage, and genetic profile), the therapeutic options may be surgical resection, chemotherapy, radiotherapy, photodynamic therapy, immunotherapy, or targeted therapy [17].

Two types of limitations are relevant in melanoma therapy: the adverse events (AEs), which can lead to skin and gastrointestinal toxicity usually related to immune reactions or lack of specificity of drugs for tumor cells, and a reduced treatment efficiency, which can be due to immune, chemo/targeted therapies resistance [18,19,20].

1.2 MELANOMA THERAPIES

Dacarbazine (DTIC), an alkylating agent, that causes brakes in DNA strands, has been approved in 1974 by FDA. DTIC is the standard chemotherapy medication for metastatic melanoma. In clinical it is administered intravenously, and it requires a metabolic activation. The first problem related to this drug is that it has no effect against brain metastasis, that are found in two-thirds of patients, because DTIC is not able to cross the blood-brain barrier[21]. Studies reported that a complete response

was achieved in <5% and 5-year survival in 2%–6% of patients, although DTIC is still used also in combination with other drugs[22]. Recently a novel alkylating agent, temozolomide (TMZ), a DTIC analog, has been tested against different solid tumors including metastatic melanoma. TMZ displays some advantages compared to DTIC: is administrated orally, it passes through the blood-brain barrier and is converted in the active form spontaneously in physiological condition[23,24,25]. With both drugs, the low chemotherapeutic response is due to intrinsic or acquired resistance, associated with increased expression of the DNA repair protein O6-alkylguanine DNA alkyltransferase[26,27]. The recent characterization of the molecular alterations in melanoma leads to the development of target therapies. These treatments are based on the specific molecular mutations and on the related intracellular signaling pathways[2]. Indeed, the discovery that the kinase BRAF is activated by mutations in a high percentage (50-60%) of melanoma species has opened the research on specific BRAF inhibitors (BRAFi). The first agent developed to target oncogenic BRAF was sorafenib, a multikinase inhibitor that inhibits BRAF (wild type or V600E) activity but even VEGFR, PDGFR, cKIT, and FLT3. This drug proved to be inefficient in the treatment of malignant melanoma[28]. In 2011 FDA has approved vemurafenib, a small-molecule kinase inhibitor directed on BRAF V600E mutation. It binds the nucleotide-binding pocket of BRAF mutated with higher affinity than ATP and renders the kinase unable to phosphorylate downstream targets. The reduction in BRAF phosphorylation activity, consequently, displays a reduction in ERK phosphorylation by causing a cell-cycle arrest[29]. However, nearly all tumors demonstrated reactivation of the MAP kinase pathway with promotion of ERK phosphorylation at the time of resistance due to different mechanisms involved in acquired resistance[30]. Vemurafenib has also been shown to be a substrate of ATP-binding cassette sub-family G member 2 (ABCG2) transporter. The overexpression of this efflux pump has described as a resistant mechanism observed in preclinical melanoma models[31]. Another drug, dabrafenib, more selective than vemurafenib for BRAF mutations, was approved in 2013 by FDA for unresectable or metastatic melanoma. Both vemurafenib and dabrafenib can be useful in controlling brain metastasis, because they display a good brain-barrier penetration[32]. This last BRAFi is usually used in

combination treatment with mitogen-activated protein kinase inhibitors (MEKi), this combined treatment extends the drug duration response during the therapy[33]. Unfortunately, cellular responses to the BRAFi are short live because patients in few months develop different mechanisms of acquired drug resistance that involve activation of alternative signaling pathways[34]. Also the expression of another RAF isoform, such as CRAF, can reduce the sensitivity to BRAFi and drive resistance via direct MEK activation or via paradoxical trans activation of RAF dimers and subsequent ERK signalling[35,36]. In some cell lines, a 61KDa variant form of BRAF (V600E), lacking the exons 4-8, has been discovered. This variant dimerizes in higher amount compare to full-length BRAF (V600E) and restores the proliferation signal through the activation of ERK[37].

It is known that tumors are linked with immune system functionality, indeed, cancer often develops in sites characterized by chronic inflammation[38]. During antitumoral responses, T-cells should recognize specific antigens on tumor cells, inducing T-cells immune activation and acquiring ability to destroy cells that exposed tumor specific antigens. Unfortunately, cancer cells can escape from T-cells control[39]. It has been shown that tumor infiltrating lymphocytes mediate the host immune response against cancer cells, because they are associated with positive outcome in patients affected by malignant melanoma[40]. Based on these observations, immunotherapy could be a promising option in the advanced melanoma treatment, and, in some cases, it has been demonstrated a durable complete response in patients better than with other previous treatments. Nevertheless, immunotherapy resistance can occur, due to a lack in antigen recognition by T-cells, that involves different components such as regulatory T-cells and M2 macrophages[41]. In addition, the immunogenic mediators in tumor microenvironment can influence the success of immunotherapy[42]. The first immunotherapy approved by FDA was INFα-2b, used as an adjuvant therapy for the treatment of resected stage 3 melanoma, although only few patients were responsive and the primary tumor ulceration was the predictive factor for IFNs sensitivity[43,44]. In 2011 FDA approved ipilimumab, an anti-CTLA-4 antibody that acts inducing the production of pro-inflammatory cytokines, favoring T-cells expansion and infiltration[45,46]. Melanoma patients must show immune-related

response criteria to undergo anti-CTLA-4 treatment because adverse effects, including autoimmune alterations, such as dermatitis, colitis and hepatitis, can occur[47]. Programmed cell death receptor protein 1 (PD-1) binding its ligands can suppress T-cells activation. In 2014 FDA approved nivolumab, a high affinity anti-PD-1 monoclonal antibody, that can inhibit the binding between PD-1 receptor and its ligands. This immune-drug was currently used in the treatment of metastatic melanoma inducing immune system responses and reducing tumor progression[48,49,50]. Unfortunately, similar to the previous immune-treatments, it shows some adverse effects[17]. For these reasons, new therapeutic strategies need to be developed against advanced melanoma.

1.3 CANCER CELLS RESISTANT TO THERAPY

Drugs resistance is the major problem in cancer therapy. The establishment of drug resistance can develop when a new tumor cell subpopulation becomes able to escape chemotherapy[51]. This process involved a lot of causes based on drug efflux, target alteration, DNA damage repair, epithelial-to-mesenchymal transition, alteration in cells receptor and so on. It has been shown that melanoma cells, that acquire resistance upon treatment with vemurafenib, display a cancer stem cells (CSC) phenotype producing melanosphere[52]. CSC are also able to undergo cell cycle arrest (quiescent state) that supports their ability to become resistant to chemo- and radiotherapy[53]. Indeed, common chemotherapeutic drugs target the proliferating cells. Although successful cancer therapy eradicates the bulk of proliferating tumor cells, a subpopulation of those, composed of CSC, can survive and promote cancer relapse[54]. CSC, also known as tumor-initiating cells, have been intensively studied in the past decade, focusing on the possible source, origin, cellular markers, mechanism, and development of therapeutic strategies targeting their survival pathways[55]. Although their origin is unclear and controversial, some hypotheses suggest that CSC might be derived from either adult stem cells, progenitor cells that have undergone mutation, or from differentiated cancer cells that obtained stem-like properties through dedifferentiation processes[53,56,57,58]. This CSC subpopulation can be recognized by some markers that belong either to undifferentiated or to embryonic state. CSC subpopulation is enriched by several

cell surface markers, for example CD271 and CD133, and by high expression of additional intracellular proteins, such as Sox2, Oct4 and NANOG, that are important determinants of cancer malignancy[59]. CSC are particularly resistant to therapy, this characteristic promotes tumor relapse followed by cells migration and new niches colonization, finally developing metastasis (Fig.3[60]). This behavior has been studied also in a mice model under vemurafenib treatment, in which scientists have seen tumor onset in mice implanted with a heterogeneous population of sensitive and vemurafenib-resistant A375 melanoma cells, compared to mice implanted exclusively with A375 sensitive cells, where they did not observed tumor growth[61].

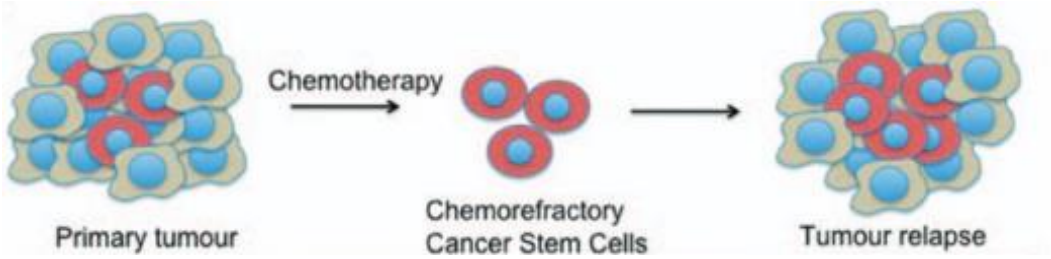


Fig.3. CSC are resistant to chemotherapy and radiotherapy; the tumor can relapse after treatment from CSC.

The BRAFi-resistant cells undergo to the epithelial-to-mesenchymal transition (EMT) process, downregulating the expression of cell-cell adhesion molecules, such as E-cadherin or integrins, typical markers of epithelial cells. At the same time, resistant cells acquire migratory and invasive capacity that permit the penetration through the extracellular matrix (ECM) in order to colonize new body sites[62,63]. Interesting, it is reported that melanoma cells become able to migrate one-by-one through channels crossing on the nearby stroma[64]. These cells prone to invasion often display loss in E-cadherin, induction of N-cadherin expression and translocation of the β-catenin in the nucleus[65]. Sinnberg et al. demonstrated that β-catenin is involved in the vemurafenib resistance and they confirmed this data, *in vitro*, with a vemurafenib resistant melanoma cells. They also reported that 50% of melanoma biopsies were positive to β-catenin localization in the nucleus [66]. Finally,

in order to cross the ECM, a mechanic barrier against cells mobility, cells need to overexpress matrix metalloproteinases (MMPs).

1.4 METALLOPROTEINASES (MMPs)

MMPs belong to zinc-finger endopeptidases family involved in degradation of ECM component, with both physiologic and pathologic activities. In cancer pathology those protein activity cover an essential step in cell invasion, migration and metastasis formation[67]. Most of them are secreted by the cells in an inactive form (proMMPs) requiring proteolytic multistep activation processes[68]. Their expression and activity are highly controlled by transcriptional and post-transcriptional mechanisms. Indeed, the MMPs activity is regulated by tissue inhibitors of metalloproteinase (TIMPs) through the binding of their catalytic domain[69]. The expression of MMPs and TIMPs have been widely studied in melanoma cell lines. Overexpression of TIMPs in B16-F10 murine melanoma cells has been shown to reduce tumor growth and metastasis formation when cells were injected in mice[70]. MMP-2, known as gelatinase, has been found in high amount in the melanoma cells conditioned medium. Rotte et al., studying melanoma biopsies from different stages, observed that MMP-2 expression level negatively correlates with patient survival[71,72]. In particular, the increased expression of MMP-2 and the presence of its active form has been associated with melanoma progression. Active MMP-2 was only observed in highly invasive cell lines, but was absent in poorly invasive ones[73]. MMP-2 activity directly modulates melanoma cell adhesion, suggesting that MMP-2 may also facilitate migration and invasion[74]. Indeed, cellular localization of the active form of MMPs plays a crucial role in cell invasion, in fact, integrin $\alpha_v\beta_3$ can bind and place the active MMP-2 on the cell surface of melanoma cells[75].

In conclusion, it is important to find different therapeutic approaches that, on the one hand cause cytotoxicity and, on the other, block cell migration, invasion and MMPs release.

1.5 Ki67

Abnormal cell proliferation is a characteristic feature observed in tumor onset and progression. The Ki67 protein is expressed in all cell cycle phases, except in G_0 and, for this reason, is consider a sensitive marker for cell proliferation[76]. Indeed, it is widely used in clinical laboratories as a diagnostic tool because is highly expressed in malignant compared to normal tissues[77,78]. Moreover, Ki67 protein expression tends to increase proportionally with a decreasing in tumor differentiation and is correlated with the presence of occult metastasis[79,80]. In the lesions of primary melanoma less than 1,5 mm thickness the Ki67 expression has been considered an indicator of poor prognosis since it is a predictor of metastatic progression[81]. Moreover, it has been demonstrated that antibodies against Ki67 microinjected in mice are able to interfere with the progression of the cell cycle, proving that the presence of Ki67 protein is essential for cell proliferation and tumor growth[82]. Accordingly, inhibition of cell proliferation has previously been shown by transfecting human IM-9 cells with an anti-sense oligonucleotide complementary to the translational start site of the Ki-67 transcript[83]. In the recent years, other authors, using RNA interfering technique, confirmed the antiproliferative effects obtained by Ki67 inactivation and suggested that Ki-67 protein can be an important target in anticancer therapy[80,84].

1.6 CELL DEATH

In the last decades, many authors have underlined the differences between an accidental cell death (ACD) opposed to a regulated cell death (RCD). ACD occurs after physical stresses, such as osmotic forces. RCD, instead, is programmed by defined machinery and can be modulated by drug therapy[85]. Different mechanisms of cell death share overlapping pathways which can be related to the emerging of drug-resistance. Apoptosis is the earliest characterized programmed cell death and it works as a homeostatic control of tissue growth. This RCD is mediated by initiators and effectors such as proenzymes and caspases, which propagate through a proteolytic cascade ended in cell death[86]. The apoptotic process at the early stage is also characterized by the flipping of phosphatidylserines (PS) from the inner to the outer surface of plasma membrane, this event works as a signal for the other

cells designed to engulf the apoptotic bodies[87]. Then, apoptotic cells change morphology, showing nuclear condensation and membrane blebbing. Apoptosis is activated by three different pathways: the extrinsic, known also as death receptor pathway, the intrinsic, described as mitochondrial way and the granzyme where T-cell mediated cytotoxicity and killing of cells. The extrinsic involves death receptors, that belong to the tumor necrosis factor (TNF) receptor superfamily[88]. Upon ligand binds to receptor, in the cytoplasm an adaptor is recruited and constitutes with caspase-8 a death-inducing complex, that auto-activate caspase-8[89,90]. Otherwise, the intrinsic one involves stimuli that cause changes in the inner mitochondrial membrane, followed by opening of the mitochondrial permeability transition pores, loss of the mitochondrial transmembrane potential and release into the cytosol of pro-apoptotic factors, such as cytochrome c and SMAC/DIABLO proteins. In this way, the intrinsic pathway can activate the caspase-dependent mitochondrial pathway, starting from the cytochrome-c/Apaf1/procaspase-9 interaction, constituting the apoptosoma[91,92]. Other proteins, such as apoptosis-inducing factor are release from the mitochondria as a late event after the induction to cell death[93]. To date, there are a lot of evidences that underline an influence between these mechanisms[94]. Finally, all the previous pathways converge on the activation of the effector caspase 3, that causes DNA damage and protein breakdown of several targets including the enzyme poly (ADP) ribose polymerase 1 (PARP1). The protein breakdown is triggered when active caspase 3 recognizes a specific conserved amino acid sequence present on its protein targets[95].

The evasion from the apoptotic cell death is essential for cancer cells leading to uncontrolled proliferation and tumor progression. Instead, the majority of drugs used in melanoma therapy are pro-apoptotic drugs, such as cisplatin that is an activator of the apoptotic mitochondrial pathway[96]. Indeed, cell apoptosis induction helps to reduce the tumor metastatic potential[97].

1.7 DNA DAMAGES

An important hallmark in cancer development is its genomic instability, which is associated with a propensity to accumulate mutations[98]. Afterwards, in the cancer progression, the high proliferative index is connected to high DNA damage and, if

this is not repair, cells are going to die. Thus, in tumor cells we observed an increased expression in elements that drive the DNA damage response (DDR), which include signaling events and enzyme activities involved in the detection and repairment of DNA damages. Chemotherapeutic drugs and radiotherapy produce single- or double-stranded DNA breaks, that induce an activation of DNA-damage sensors[99]. One of the most known sensors is PARP1, that belongs to the PARP family. PARP enzymes include 17 members, involved in transferring of the ADP-ribose negative group from NAD^+ to some amino acid residues present in the protein targets recognized as PARPs substrates[100]. This process, that takes place near the DNA damage site, is known as poly (ADP-ribose) ylation (PARylation), a post-translational protein modification that consists in the binding up to 200 ADP-ribose chains on the protein target. The high number of negative charges added to the protein targets leads to the recruitment of DNA repairing proteins in the damage site and to activate several biological processes, such as cell cycle arrest (Fig.4 [101])[102,103]. PARP1 itself, which is responsible for about 90% of polyADP ribosylation activities in the cells, is polyADP-ribosylated together with histones, nuclear repair enzymes and transcriptional factors[104]. PARylation is a transient and reversible protein modification because PARs turnover is also depending on poly (ADP-ribose) glycohydrolase (PARG) that degrades PARs[105].

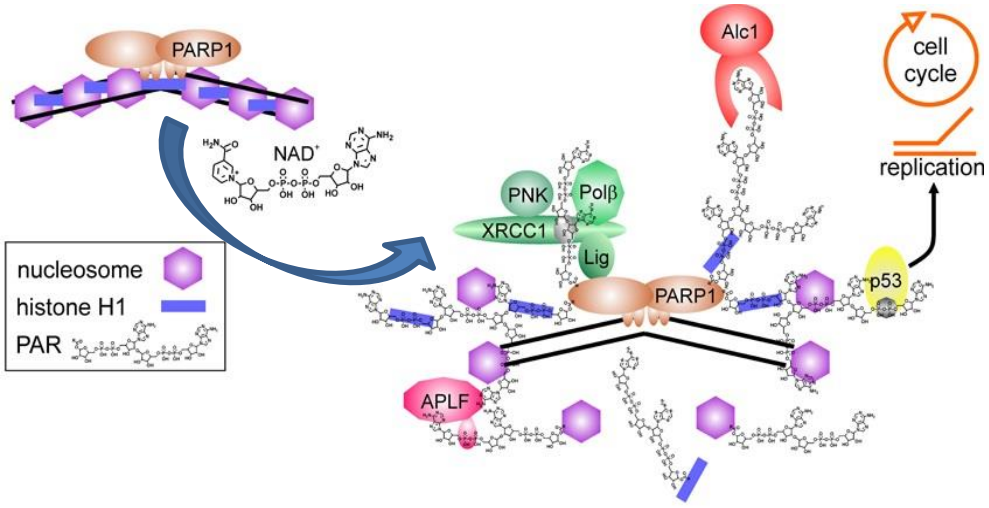


Fig.4. PARylation mechanism. Active PARP1 dimers recognize and bind DNA breaks, PARP1 synthesizes poly (ADP-ribose) (PAR) from NAD^+ by modifying itself and other proteins involved in DNA repair.

In addition, PARylation is followed by the phosphorylation of H2AX (γH2AX) histone variant, that is essential for the recruitment and retainment of several factors nearby DNA strand brakes[106]. Downstream to DNA sensors, transcription factors like p53 and NF-κB mediate the responses to damage. On the one hand, p53 induces cells cycle arrest and cell death, on the other, NF-κB favors cell survival[107,108]. Notably, the ability to escape apoptotic cell death could be an useful way for cancer cells to become resistant to chemotherapeutic drugs that induce DNA damage[109,110].

1.8 NF-κB

Nuclear Factor-κB (NF-κB) is a transcriptional factor composed by five subunits, NF-κB1/p105, NF-κB2/p100, RelA/p65, RelB, and c-Rel, which can interact in homo-hetero dimeric manner and bind to DNA at the promoter regions of several responsive genes. The upstream regulators of NF-κB are NF-κB inhibitors (IκBs) and IκB kinases α/β/γ activator (IKKs). In unstimulated conditions NF-κB is present in the cytosol in the inactive state because it is binding to one of the three inhibitor factors, IκBα, IκBβ, and IκBε, that blocks the NF-κB nuclear translocation[111,112]. NF-κB is activated by different extracellular or intracellular stimuli, such as Tumor Necrosis Factor α (TNFα), lipopolysaccharide (LPS), viral double-stranded RNA, ionizing radiation and intracellular reactive oxygen species (ROS). The NF-κB activation is trigged by two different pathways: canonical and non-canonical[113,114,115]. Both pathways have in common the activation of IKK complex (KK1/IKKα, IKK2/IKKβ, and NEMO/IKKγ), that phosphorylating the IκBs proteins, produce their ubiquitination and degradation in the proteasome. Hence, NF-κB is released from its inhibitor and translocates into the nucleus, where activates the transcription of several genes involved in inflammation, cell differentiation, proliferation, defense against infections and cellular stress. NFκB is also involved in the inhibition of apoptosis process since it induces the transcription of several anti-apoptotic proteins, such as Bcl-XL and the protein inhibitors of apoptosis (Fig.5[116])[112,117,118].

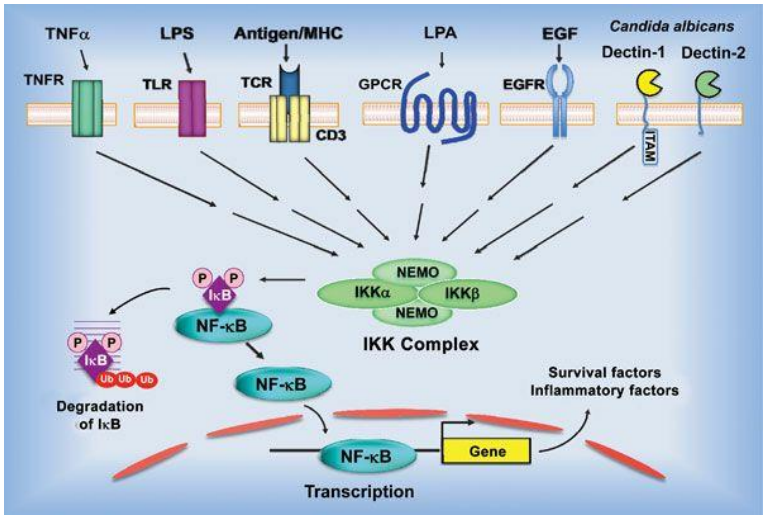


Fig.5. NF-κB signaling pathway.

Several members of NF-κB subunits have been found mutated in different types of cancer, especially in malignancy of hematopoietic origin, indeed, NF-κB2/p100 is frequently activated through chromosomal translocations in lymphoma and leukemia[119]. Instead, mutations that directly cause the NF-κB activation are very rare in solid tumors in which its constitutive activation is due to microenvironment influence[120]. In melanoma, recent studies show that some NF-κB components are overexpressed in dysplastic nevi and melanoma cells, compared to healthy melanocytes[121]. In some cases, it has been described an increase in IKKs activity, causing fast degradation of IκBs followed by NF-κB nuclear translocation. In Hs294T melanoma cells an imbalance between IκBs synthesis/degradation has been demonstrated[122]. The widely diffuse BRAF mutation in melanoma has been also associated with an increase in IKKs activity[123]. All these previous described processes increase melanoma cell survival.

Because NF-κB can activate the transcription of several genes eliciting cell growth, anti-apoptotic pathway and inflammation, NF-κB can be used as a target in the melanoma therapy[124]. Indeed, different compounds has been tested against this pleiotropic transcriptional factor, such as bortezomib, a proteasome inhibitor that inhibits IκBs degradation; curcumin, a polyphenol with anti-cancer properties that induces programmed cell death in B16-R melanoma cell resistant to doxorubicin; and BMS-345541, a selective inhibitor of IKKs[125,126]. *In vitro,* BMS-345541 induces inhibition of cells proliferation in different melanoma cell lines, including

A375 cell line, and it also induces apoptosis, as shown by the presence of PARP1 cleaved form, marker for the apoptotic cells death[127]. Unfortunately, all of the previously cited treatments have shown very important side effects in clinical trials, such as high hematological toxicity caused by bortezomib, as well as off-target effects because they inhibit not only cancer cells proliferation but also the immune response during the anti-cancer therapy[128,129,130]. The presence of PARP1 is important for NF-κB activity because PARP1 functions as a co-activator of NF-κB at the promoter sites[131]. A study reported that PARP1 silencing in malignant melanoma B16 cells reduces tumor onset, tumor progression and drug-resistance when this cell line is implanted in mice. Indeed, PARP1 silencing decreases the expression of the NF-κB dependent pro-inflammatory cytokines, for example TNF-α[132]. Many reports underline the importance of PARP inhibitors (PARPi) on the NF-κB activity and on its downstream signaling. Hence, NF-κB inhibition by PARPi could be more safety in cancer treatment compare to the use of specific NF-κB inhibitors[133,134].

1.9 PARP INHIBITORS

Clinical trials validated the antitumor effect of PARPi in patients with mutations in BRCA1/2 genes in breast and ovarian cancer because this mutation results in a deficient homologous recombinant (HR) DNA repair process[135]. In clinic, different PARPi have been used until now: olaparib, rucaparib, niraparib, talazoparib and veliparib[136]. After inhibitor treatment, PARP is trapped onto single strand break (SSB), blocking the replication fork progression followed by its collapse and generation of genotoxic double strand breaks (DSBs)[137]. Olaparib (Lynparza®) is the first one that has demonstrated efficacy in maintenance therapy in patients with ovarian cancer, approved by both FDA and European Medicines Agency (EMA)[138]. Olaparib demonstrates *in vitro* a nanomolar inhibitory activity against PARP1 and PARP2 enzymes[139]. Olaparib is well tolerated as a single agent. Recent reports demonstrate that some type of cancer cells acquire resistance against this drug through different mechanisms, including loss of p53-binding protein 1 (53BP1) or overexpression of ATP-binding cassette (ABC) drug transporter, a P-glycoprotein that prevents the accumulation of anticancer drug in cells[140,141]. 53BP1 is a crucial

player in maintaining the balance between HR and non-homologous end joining, loss of 53BP1 activity partially restores HR in BRCA1-deficient tumors, thereby reducing their hypersensitivity to PARP inhibition[142]. In order to overcome the resistance mechanism connecting to drug efflux outside the cells, olaparib has been administrated in combination therapy with tariquidar, the P-glycoprotein inhibitor, this approach has restored the PARPi sensitivity in breast tumor[143].

AZD2461 is a new generation PARPi, structurally analog to olaparib, but less prone to bind the P-glycoprotein compare to olaparib[144]. It has been shown that after the onset of olaparib resistance, when tumor was treated with AZD2461, it was responsive to treatment also in absence of tariquidar[142]. It is important to underline that for this new drug a Phase I clinical trial has been completed[145]. Anyway, in general it is known that PARPi work better in combination therapy with other drugs[146].

1.10 ONCONASE

To counteract extracellular RNA, onconase (ONC) is a 104 residues basic secretory protein extracted from the *Rana Pipiens* frog oocytes, which belongs to the "pancreatic-type" ribonuclease super-family[147] (Fig.6). ONC has a similar fold compared to the proto-type RNase A although they share only 30% of sequence homology[148].

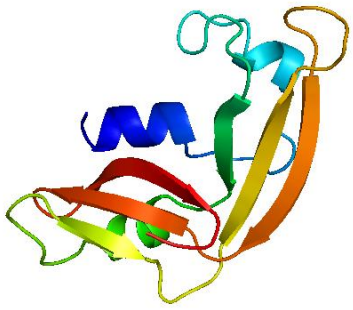


Fig.6. Onconase structure.

This low homology is explained by a deletion in some amino acid residues, that occurs predominantly in loops that are important sites of binding for the ribonuclease inhibitor (RI) present in the cytosol of all mammalian cells[149]. For this

reason, ONC, if internalized in the cells, is not sequestered by RI and can exert its ribonuclease activity. Conversely, other monomeric RNases of the same family are not cytotoxic because their ribonucleolytic action is hindered by RI binding[150]. Moreover, compared to normal cells, ONC is better internalized by cancer cells through energy-dependent endocytosis mediated by AP-2/clathrin[151]. Some studies describe that the electrostatic forces play a key role in ONC binding to the cell membrane. Indeed, its basic charge (PI>9.5) allows ONC internalization in cancer cells, because they have more negatively charged membranes than normal ones. In particular, it is known that cancer cells with high metastatic potential have stronger electronegative membrane[152]. This RNase gets access inside the cytosol bypassing the Golgi and Endoplasmic Reticulum. The current model of ONC-mediated cytotoxicity predicts that ONC, once inside the cells, first degrades tRNAs through its ribonuclease activity[151]. Although ONC effects are different from those exerted by other protein synthesis inhibitors, the tRNAs breakdown leads to the ubiquitous inhibition of protein synthesis, finally inducing cell death[153]. The ONC cytostatic and cytotoxic effects have been studied in human leukemic HL-60, submaxillary carcinoma A-253 and colon adenocarcinoma Coo 320-CM cells. The cytostatic effect is caused by a decrease in frequency of DNA replicating cells (S-phase blockage), and the cytotoxicity by the apoptosis induction[154]. It is already known that ONC is active against many human cancer cell types, such as glioma and lymphoma B cells[155,156]. Moreover, other results showed that it promotes cell death in human pancreatic adenocarcinoma cell lines through a ROS/autophagy dependent pathway[157]. Other evidences, in malignant pleural mesothelioma cell lines, show that ONC affects the expression of miRNAs, noncoding RNA which induce gene silencing through interaction with 3'-untraslated regions (3'-UTR) of the messenger RNAs. miRNAs control cell proliferation, invasion, migration, and cell death[158]. Among them, miR-622 is known to induce resistance to PARPi[159]. In malignant mesothelioma ONC is also able to inhibit the nuclear translocation of NF-κB induced by TNF-α, and to reduce the activity of MMP9 decreasing tumor invasion[160]. Another study in the same tumor type has underlined that ONC, through its RNase activity on miRNAs, downregulates the expression of NF-κB, decreasing colony formation, motility, invasion and cells proliferation[158]. The first

in vivo preclinical study has analyzed the survival of mice with M109 Madison carcinoma: mice treated with ONC had a survival rate 12-fold longer compared to untreated controls[161]. ONC has already been used in Phase III clinical trials against malignant pleural mesothelioma, an aggressive tumor characterized by a poor prognosis[162,163]. In this clinical trial, the ONC-treatment was combined with doxorubicin (DOX), and the life-extension was compared to DOX alone. A significant advantage for survival was found only for patients who experimented the ONC treatment after having previously received a DOX therapy. Phase II human clinical trials with ONC in metastatic kidney cancer was also completed[164]. It should be noted, however, that numerous clinical studies have found ONC to possess an acceptable safety profile, being generally well-tolerated by the majority patients[165]. Clinical trials evaluating ONC effects on non-resectable malignant mesothelioma, reported a reduction of tumor growth even though adverse side-effects in some patients were mentioned[162]. There are evidences that ONC produced synergic effects in many cancer types when administered with other drugs, including tamoxifen and cisplatin[166]. Despite its cytotoxic activity on cancer cells of different types of tumors *in vitro*, ONC displayed some problems during *in vivo* experimentation. Indeed, the fast clearance from circulation, caused by renal filtration due to its low molecular mass, produces ONC kidney retention and nefrotoxicity[167]. Homo-oligomerization or hetero-oligomerization with other proteins, as already experimented in preclinical studies by fusion protein with immunotoxin, could solve these problems[150].

2. AIM OF THE STUDY

Because malignant melanoma therapy is far from being successful and quickly drug-resistance is established either in *in vitro* studies using melanoma cell lines or in clinic during the treatment of patients, the principal aim of this thesis is to experiment alternative and more efficacious therapeutic strategies to counteract melanoma progression and relapse. In this context, the antitumor effect of the cytotoxic ribonuclease ONC, by itself or in association with other chemotherapeutic drugs, was tested on A375 cells, an extremely aggressive melanoma cell line.

Aims of the study are:

- To measure the ONC effects on cell viability in A375 cells and normal human melanocytes. Notably, no data are available, so far, concerning the antitumor effects of ONC in any cutaneous malignant melanoma cell line.
- To characterize the effects of ONC on A375 cell proliferation rate and programmed cell death.
- To characterize the effects of ONC in countering the metastatic potential of A375 cells. In particular, the ONC capability to decrease cell migration, invasion and colony formation.
- To measure the potential synergistic effect of ONC treatment in association with AZD2461.
- To investigate the cytotoxic effects of ONC on two A375 cell subpopulations: one treated for a long time with AZD2461 PARPi and the other that has become resistant to dabrafenib BRAFi.
- To characterize the capability of ONC to interfere with NF-κB activation, a signaling pathway that is important in tumor progression, tumor cell dissemination and metalloproteinase activity.

  To investigate, by proteomic analysis, all the protein expression modifications elicited by ONC in A375 cells in order to study its molecular mechanisms of action.

3. MATERIALS AND METHODS

All chemicals, solvents, and buffers were from Sigma-Aldrich (St.Louis, MO, USA) unless noted otherwise.

3.1 Protein production

Recombinant wide type-ONC was produced from *E. Coli* and purified using a Superdex 75 HR 10/300 column equilibrated with 0.1M Tris-acetate, 0.3M NaCl, pH 8.4 by Prof. Gotte, Neuroscience, Biomedicine and Movement Sciences Department, University of Verona.

3.2 Cell cultures

Human melanoma cell line (A375) and Normal human epidermal melanocytes (NHEM) (ATCC, Manassas, VA, USA), were cultured in high glucose Dulbecco's Eagle's Medium (DMEM) supplemented with 10% FBS, 1% Antibiotic Antimycotic Solution (Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA), at 37° C in a humidified atmosphere of 5% CO_2. A375 cells were treated for a long period with increasing AZD2461 concentration (AZD long treated), starting form 5 µM up to 80 µM, to investigate if cells could develop resistance mechanisms. The human melanoma A375 cell line was induced to generate dabrafenib resistance (DR) with the aim of selecting a A375DR subpopulation. In order to do this, A375 cells were cultured at 37 °C with different concentrations of dabrafenib (Selleckchem, Houston, TX, USA) that gradually increased from 1 nM up to 5 µM, until a subpopulation grew in the constant presence of this concentration. Subsequently, A375DR cells were maintained in culture with 5 µM dabrafenib for six months.

3.3 Cells Viability assay

NHEM and both dabrafenib-sensitive parental, A375P, and A375DR A375 cells were seeded in a 96-well plate (2.9 x 103 cells/well). After 24 h culture, A375 were incubated with different concentrations of dabrafenib (Selleckchem, Houston, TX, USA), AZD2461, olaparib (Calbiochem, CA, USA) or ONC and cells were harvested at 72 h after treatment. Drug combination studies were performed by combining ONC and AZD2461, seeding cells in 24-well plates ($17*10^3$ cells/well)

In a parallel experiment, NHEM cells were treated with ONC to compare their cell viability with the ones of both A375 subpopulations. In the experiments performed with A375DR cells, dabrafenib was removed from the culture medium one day before starting the 72 h incubation with ONC or with dabrafenib. To measure cell viability, each cell sample was washed at the end of treatment with 1X PBS solution (Gibco BRL Invitrogen Corp., Carlsbad, CA, USA) and stained with crystal violet (0.75% crystal violet powder, 8% formaldehyde, 50% ethanol) for 5 min. Crystal violet was solubilized in PBS/1% SDS solution, and OD_{595} was measured using Tecan NanoQuant INFinite M200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). Six replicates were performed for each condition/time point.

3.4 Br-deoxy-Uridine (BrdU) cell proliferation Elisa kit (colorimetric)

Cells proliferation was evaluated with a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis. A375P and A375DR cells were seeded in 96-well plates ($2.9*10^3$ cells/well). After 24 h, cells were incubated with ONC (0.5 and 1μM) for 24, 48 and 72 h, and, subsequently, for other 4 h with BrdU (Abcam, Cambridge, UK). Medium was aspirated and cells were fixed for 30 min. Plates were washed three times and anti-BrdU detector antibody was added for 1h at room temperature (RT). Plates were then washed three times, incubated for 30min at RT with peroxidase goat anti-mouse IgG conjugate and then washed three times again. The tetramethybenzidine chromogenic peroxidase substrate (TMB) was added and plates were incubated for 30 min in dark. Finally, stop solution was added and absorbance was measured at 450nm using a plate reader, Tecan NanoQuant INFinite M200 Pro.

3.5 Protein synthesis assay kit

A375P were seeded in 96-well clear bottom, black plates ($2.9*10^3$ cells/well). After 24 h cells were treated with 1μM ONC and then protein synthesis assay was done. This assay is based on the incorporation in live cells of O-Propargyl-puromycin (OPP), that is a puromycin analog containing an alkyne moiety. It is cell-permeable and, once inside cells, it is incorporate into the C-terminus of translating polypeptide chains, thereby stopping translation. The truncated C-terminal alkyne-

labeled proteins can subsequently be detected via copper-catalyzed click chemistry (FAM-azide). Cycloheximide was used for negative control. OPP working solution was added for 30 min, cells were fixed and washed. Cells were incubated with FAM-azide in dark for 30 min. Plates were washed and FITC fluorescence (ex/em 485/535) was detected by a plate reader (GENios Pro, Tecan, Männedorf, Switzerland).

3.6 Annexin V-FITC assay

Cells were seeded in 96-well clear bottom, black plates (2.9×10^3 cells/well) and, the day after, treated with 1 μM ONC for 48 h. Cells were washed and fixed with 2% paraformaldehyde in PBS at room temperature for 30 min. Cells were washed twice with PBS and incubated with Annexin V/FITC (eBioscience, San Diego, CA, USA) in binding buffer solution (10 mM HEPES/HCl pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 10 min in the dark. Cells were washed with binding buffer and fluorescence was measured with a plate reader (ex/em 485/535) (GENios Pro, Tecan, Männedorf, Switzerland). Fluorescence values were normalized on cell content by crystal violet assay.

3.7 Wound closure cell migration assay

A375P cells were seeded in 6-well plates (2×10^5 cells/well) and, upon reaching confluence, the monolayer was scratched with a sterile 200 μL pipette tip. A375DR cells were seeded in 6-well plates (4×10^5 cells/well) and, when they reached confluence, the monolayer was scratched with a sterile 10 μL pipette tip. Wells were then washed with complete medium to remove detached cells and, afterwards, refilled with fresh medium containing 10% FBS. 1 μM ONC was added and samples were compared with ONC-free one. A375P cells were incubated for 17 h (1020min), while A375DR cells for 54h (3240min) and both cell subpopulations were monitored with EVOS FL Auto Cell Imaging System. Cells were kept at 37°C with 5% CO2 in humified atmosphere in an EVOS Onstage Incubator (Thermo Fisher Scientific, Waltham, MA, USA). A375P cells movement frames were captured every 30 min for 17 h, with a 5X magnification, while the A375DR cells frames every 2 h during the mentioned 54 h, with a 10X magnification. A time-

lapse video was created with the acquired frames, and the relative images were quantitatively analyzed by using the ImageJ computing software, MRI Wound Healing Tool. In the graphic, the mean value of each point calculated from four independent experiments ± S.D. are reported.

3.8 Total and nuclear protein purification

In order to purify the total proteins content, cell pellets were resuspended in ~50-100 μL of radioimmunoprecipitation assay buffer (RIPA) (150mM NaCl, 5mM EDTA pH 8.0, 50mM Tris pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease and phosphatase inhibitors (Proteoloc protease inhibitor cocktails, and Phosphatase Inhibitor Cocktail 2, respectively; Tema Ricerca, Bologna, Italy). Samples were incubated on ice for 20 min and centrifuged for 25 min at 25000 g at 4 °C. The supernatant, containing soluble proteins, was collected. Nuclear extracts were prepared using a detergent solution (HEPES 10mM pH 7.9, Nonidet-P40 0.1%, KCl 10mM, $MgCl_2$ 1.5mM) in the presence of protease and phosphatase inhibitors, incubated for 10 min in ice, centrifugated for 10 min a 2000g. The supernatant was discarded, and the proteins were extracted from nuclei with a hypertonic solution (HEPES 20mM pH 7.9, NaCl 420mM, $MgCl_2$ 1.5mM, glycerol 25%, EDTA 0.2mM), in the presence of protease and phosphatase inhibitors and incubated on ice for 15 min, centrifuged for 25 min at 25000 g at 4 °C.

Proteins concentration was measured using Bradford assay (Thermo Scientific, Waltham, MA, USA) in a Jasco V-650 spectrophotometer. In order to prepare the samples for western blot analysis, a reducing sample buffer was added (Sample buffer: Tris-HCl 625mM pH 6.8, 2% SDS, 8% Glycerol, 0.25% Bromophenol blue, 5% β-mercaptoethanol). Proteins sample containing 40 μg of total protein extract or 15 μg of nuclear protein extract were boiled for 3 min and placed in ice to aid protein denaturation.

3.9 Western blot assay

Protein extracts were electrophoresed by a 5-10 % polyacrylamide SDS-PAGE, then were transferred on polyvinylidene difluoride membrane (PVDF, Merck-

Millipore, Burlington, MA, USA). Membranes were blocked with TBST (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% bovine albumin serum (BSA, Serva, Heidelerg, Germany) for 1 h at RT, then were incubated overnight on a rocker at 4 °C, with a 5% BSA solution containing primary antibodies for: cleaved PARP1, p65 NF-κB, pIKKα/β (Ser176/180), pERK1/2 (Cell Signaling, Danvers, CO, USA), N-cadherin, NANOG, CD133, γH2AX, β-catenin (Genetex, San Antonio, Texas, USA), Ki-67 (Dako, Santa Clara, CA, USA). After that, membranes were washed three times with TBST buffer for 30, then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (anti-rabbit 1:4000, Cell Signaling, Danvers, CO, USA), and washed with TBST three times again. All protein extracts were normalized with β-actin (1:1000) or Lamin B (1:1000) for nuclear extract (Cell Signaling, Danvers, CO, USA). Immunodetection was carried out with ECL kit (GE-Healthcare, Chicago, IL, USA) and the chemiluminescence signals were detected with ChemiDoc (Bio-Rad, Hercules, CA, USA).

3.10 Proteomic analysis

Whole protein extracts from A375P control and treated cells were used in proteomic analysis. Analysis was done by prof. Cecconi, Department of Biotechnology, University of Verona, using Mascot and Protein Pilot FDR 1% for protein identification. Proteins were quantified by Skyline software and normalized by MS Stat.

3.11 Drug combination studies

Drug combination analysis were performed using ONC in combination with AZD2461. Cells were seeded in 12-well plates (43×10^3 cells/well) and the day after incubated with AZD2461, ONC and with AZD/ONC combination. After 72 h cells were stained with a crystal violet solution and the dye was solubilized in PBS containing 1% SDS. OD was measured at 595 nm using a plate reader. All values are means (±SD) of three independent biological replicates, each performed using six technical replicates.

3.12 Transwell invasion assay

Both A375P and A375DR cells were incubated with 1μM ONC for 72 h. Afterwards, cells were detached with trypsin, counted and seeded in transwell composed of polycarbonate membrane inserts of 8 μm pore size, and coated with a uniform layer of dried basement membrane matrix solution. Cell invasion was measured by using CytoSelectTM cell invasion assay kit (CELL BIOLABS, San Diego, CA, USA) according to the manufacturer instructions. 0.5 mL of complete media was added to the lower chamber wells as chemoattractant, while 0.3 mL of cell suspension consisting of 1 x 10^6cells/mL in DMEM serum-free were seeded on the upper chamber. Invasion capability was analyzed after 48 h. Non-invading cells were removed by cotton swab scrubbing, and the invading cells were stained with staining solution. Finally, the OD_{450} was measured using the Tecan NanoQuant infinite M200 Pro plate reader.

3.13 Gelatin zymography

Both A375P and A375DR cells subpopulations were cultured in presence or absence of ONC for 48 h, then, they were serum-starved for 30 h. The conditioned medium was collected and concentrated 10X by using an Amicon Ultra-2 Centrifugal Filter Unit (Millipore, Burlington, MA, USA). Proteins concentration was determined with Coomassie (Thermo Scientific, Waltham, USA) and samples were prepared with non-reducing sample buffer. In order to separate proteins, a 7.5% SDS-PAGE, contained 4 mg/mL gelatin, was performed. Gels were washed with 50mM Tris-HCl pH 7.5, 5mM CaCl2, 1μM ZnCl2 supplemented with 2.5% Triton X-100 (Serva Electrophoresis, Heidelberg, Germany) buffer, and kept 24 h in the incubation buffer (1% Triton X-100, 50mM Tris-HCl pH 7.5, 5mM CaCl2, 1μM ZnCl2) to allow the digestion by the MMP-2 gelatinase. This buffer contained the cofactors necessary to keep MMP-2 active so that it could degrade the gelatin present in the gel. The gel was stained with 0.5% Coomassie Brilliant Blue R-250 in 20% Ethanol-10% Acetic acid and the gelatinase activity was detectable by evaluating presence and intensity of clear bands on the blue background.

3.14 Real time PCR analysis

RNA was extracted with the Pure link RNA kit (Ambion, Invitrogen Corp., Carlsbad, CA, USA), quantified with the Nanodrop UV-vis spectrophotometer and its quality checked by 1% agarose gel. Reverse transcription into cDNA was performed (500 ng RNA in 10 μl) by using the SuperScript VILO cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA), according to manufacturer's instructions. The expression levels of specific genes were determined with real time (RT)-PCR by using the SensiFAST Sybr No-ROX kit (BIOLINE, London, UK). TATA-box binding protein (TBP) was used to normalize the gene expression level in each sample. PCR reactions were performed in triplicate in a Rotorgene Q (Qiagen, Hilden, Germany), with the following amplification primers:

Gene	For (5'-3')	Rev (5'-3')
CD133	GCATTGGCATCTTCTATGGTT	CGCCTTGTCCTTGGTAGTGT
NANOG	AGTCCCAAAGGCAAACAACCCAGTTC	TGCTGGAGGCTGAGGTATTTCTGTCTC
TNF-α	GAGCACTGAAAGCATGATCC	CGAGAAGATGATCTGACTGCC
TBP	TGTATCCACAGTGAATCTTGG	ATGATTACCGCAGCAAACC

A comparative quantification of gene expression was performed using a Pfaffl's efficiency corrected calculation[168].

3.15 Soft agar colony formation assay

Cells were seeded in flasks and treated with 1μM ONC for 72 h. Before seeding, 6 wells plates were prepared with soft agar as follows: 1% agarose low gelling temperature and DMEM 2X supplemented with 10% FBS and 1% Antibiotic Antimycotic Solution (Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA) was placed at the bottom layer, while with 0.6% agarose and DMEM 2X supplemented with 10% FBS and 1% Antibiotic Antimycotic Solution was placed on its top. Treated and control cells were detached, counted and seeded in 6 wells plates (5000 cells/well), together with the upper soft agar solution. 100 μL of fresh media/well was added twice a week. After 21 days, crystal violet was used to stain cell colonies. The semi-solid cultures were performed in triplicate and images were captured with the EVOS FL Auto Cell Imaging System (Thermo scientific, Waltman, USA).

3.16 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared in the presence of protease inhibitors. After measuring protein concentration, nuclear extracts (6 μg) were incubated at RT for 20 min with ^{32}P-labelled double-stranded oligonucleotide (5′-GATCATGTGGGATTTTCCCATG-3′), in a 15 μl reaction mixture containing pH 7.9 20mM HEPES, 50mM KCl, 10% glycerol, 0.5mM DTT, 0.1mM EDTA and 2 μg poly(dIdC). Products were separated on a non-denaturing 5% polyacrylamide gel in TBE (Tris-Borate-EDTA buffer, 0.5X). The oligonucleotide, whose sequence derives from the IL6 promoter, reproduces the DNA-binding site of NF-κB and, besides this, also the CBF1 DNA-binding site. Thus, in the same gel can be visible either the two faint and low mobility shift bands belonging to NF-κB dimers or the band relative to the DNA-binding of the constitutively expressed CBF1 transcription factor. This latter large band with high mobility has been used as a loading control. The competition experiment was performed using 200 X non-labelled free probe, and the intensity of the retarded bands measured with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

3.17 Statistics

All results are reported as a mean value ± SD. Unless differently noted otherwise, p values were determined using unpaired, two-tailed Student's t test. For each type of experiment, a minimum of three independent biological replicates were performed.

4. RESULTS

4.1 ONC treatment decreases A375 cells viability in a concentration-dependent manner.

To determine the effect of ONC on cells viability, A375 and NHEM cells were treated with different ONC concentrations (A375: 0.1, 0.2, 0.35, 0.5, 1 µM; NHEM: 0.5, 1, 2, 4 µM) for 72 h. Crystal violet assay results highlighted a significant reduction in cell viability in ONC-treated A375 cell, in a concentration dependent manner, with an IC_{50} of 0.48 µM (Fig.7 A). Instead, NHEM cell viability was not significantly affected also by the highest ONC concentration (Fig.7 B). This behavior could be explained by the melanoma cell phenotype, that makes tumor cells more sensitive to ONC than normal melanocytes, due to the high negative tumor membrane charges[152].

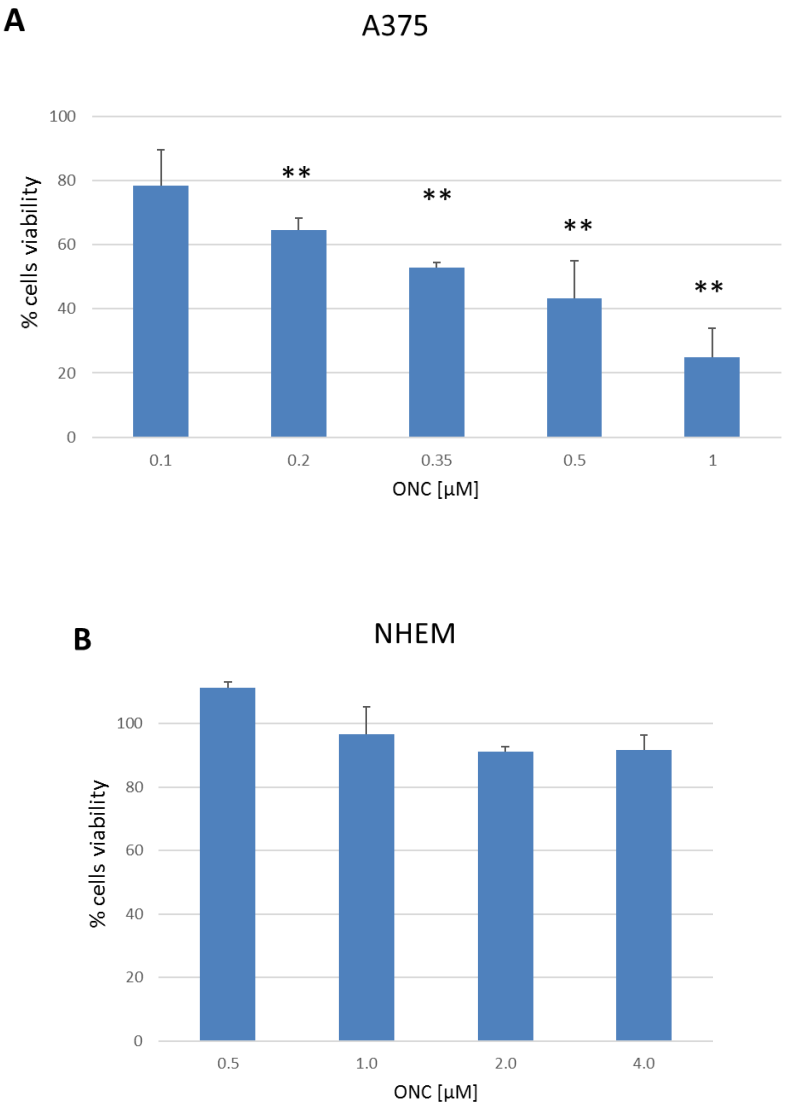


Fig.7. ONC treatment affects cells viability only in A375 cells line.

(A). A375 cell viability (% of ONC-free control) evaluated by crystal violet assay after 72 h treatment with increasing concentration of ONC. The IC_{50} is 0.48 µM. (**) Differences between treatments and control are statistically significant ($p < 0.01$).

(B). NHEM viability (% of ONC-free control) evaluated by crustal violet assay after 72 h treatment with increasing concentration of ONC. Differences between treatments and control are not statistically significant.

Each histogram column reported represents the average of three independent experiments performed in sextuplicate ± S.D.

4.2 ONC induced cytostatic and cytotoxic effects in tumor cells.

Changes in cell viability could be a consequence of cytostatic outcome, that affects the proliferation rate, and/or cytotoxic effect, which causes cell death and the related cell mass decrease. In order to study the cytostatic effect, the cells proliferation index has been evaluated by BrdU assay, based on the incorporation of this thymidine analog into DNA of dividing cells. After 24 and 48 h a 4 h pulse of BrdU on live cells showed that the proliferation index is lower in A375 ONC-treated cells than in ONC-free A375 control cells (Fig.8 A). This result is also confirmed by a significant decrease in Ki67 protein expression level at 48 h, measured by WB analysis. As previously reported, Ki67 expression is correlated with cell proliferation and widely used in clinic to evaluate tumor growth[76] (Fig.8 B).

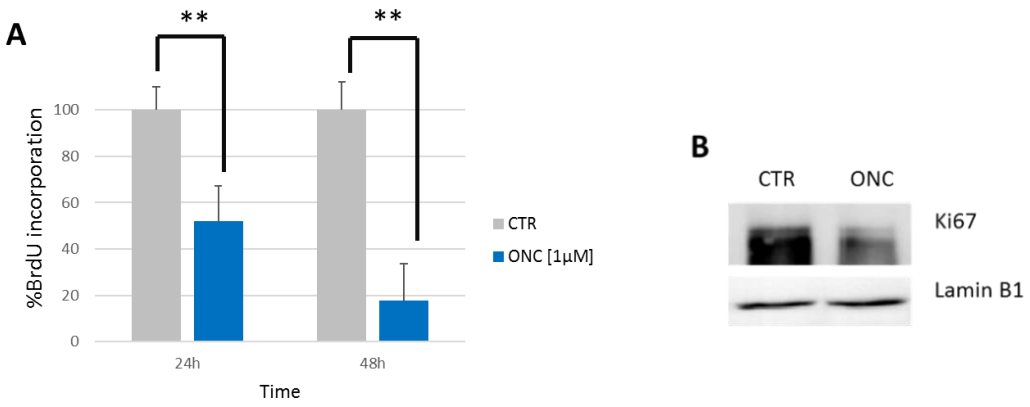


Fig.8. Cytostatic effect of ONC treatment.

(A). Cells proliferation measured by BrdU incorporation. Reduction in the proliferation index in A375 cells after 24 or 48 h from ONC (1 µM) administration. (**) Difference between ONC-treated A375 cells and each control is statistically significant ($p < 0.01$). Each histogram column reported represents the average of three independent experiments ± S.D.

(B). A375 cells treated with 1µM ONC for 48 h show a strong reduction in the Ki67 protein expression level measured by WB analysis. Lamin B1 is used as a loading control. This blot is a representative result of three independent experiments.

In order to analyze if a cytotoxic effect was also present, the early stages of apoptosis have been investigated. Annexin V-FITC assay was used to evaluate the exposure of the phosphatidylserines on the outer surface of cell membrane. This modification is one of the first phenotypic changes occurring in a programmed cell death. 48 h after ONC treatment, Annexin V-FITC fluorescence, that is proportional to the phosphatidylserine exposure on the surface of cells membrane, increased in treated cells compared to the control sample (Fig.9 A). To confirm this data, the expression level of cleaved PARP1 in the nuclei has been analyzed. PARP1 is cleaved by activated effectors caspases 3 or 7 in the apoptotic cell death pathway[169]. After 48 h of treatment, an increase in cleaved PARP1 expression level was measured in ONC-treated cells compared to the control cells (Fig.9 B).

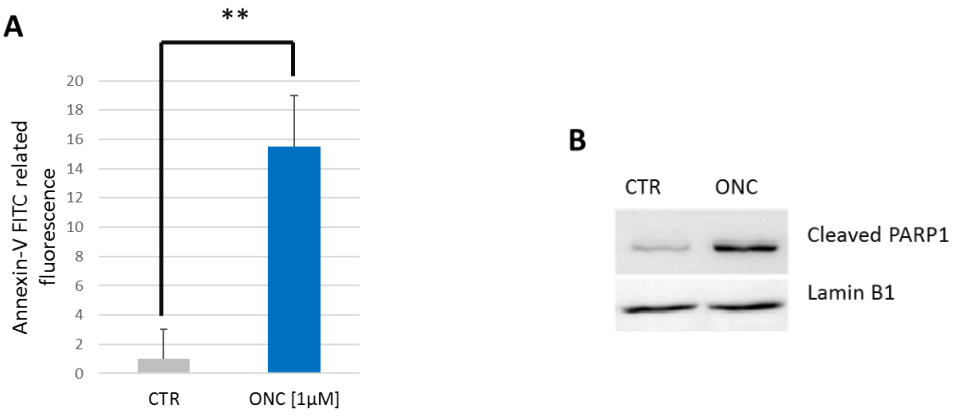


Fig.9. Cytotoxic ONC-treatment effect.

(A). ONC-treated A375 cells show an increase of Annexin V-FITC fluorescence related to the exposure of phosphatidylserine on the outer site of cell membrane. Each histogram column reported represents the average of three independent experiments ± S.D. (**) The difference between ONC-treated and control cells is statistically significant ($p < 0.01$).

(B). Increase of cleaved PARP1 form measured by WB analysis in A375 cells after ONC (1µM) administration compared to not treated cells (CTR). Lamin B1 is used as a loading control. This blot is a representative result of three independent experiments.

4.3 Proteomic analysis of A375 cells; evaluation of ONC influence on protein expression compared to control cells.

In collaboration with prof. Cecconi (Biotechnology Department, University of Verona), after 48 h of ONC administration a proteomic analysis has been performed. It is know that the ONC intracellular ribonuclease activity is multitargets, in particular it affects the stability of tRNAs and miRNAs[153,158]. Thus, the proteome's changes have been evaluated in order to understand the effect of ONC activity on the protein expression profile of A375 cell line. Results show that 48 protein were downregulated and 52 upregulated (Fig.10 A). The biggest modulation is related to a decrease in the expression of proteins involved in telomeres elongation. It is known that this effect can produce a lack of telomerase activity and a consequent loss in tumor cell immortalization[170]. Other modulations involve a down-expression of small ribosomal subunits and a decrease in the expression of proteins clustered in the mesenchymal cells' development groups (Fig.10 B). This data could explain the reduction in cell proliferation connected to a decrease in telomerase activity, as well as the failure in protein production, as described in the next chapter. A switching to a less aggressive cells phenotype connecting to a reduction in the expression of mesenchymal markers was suggested. In summary, the ONC-elicited modifications in protein expression could justify the loss of the typical behavior of tumor cells.

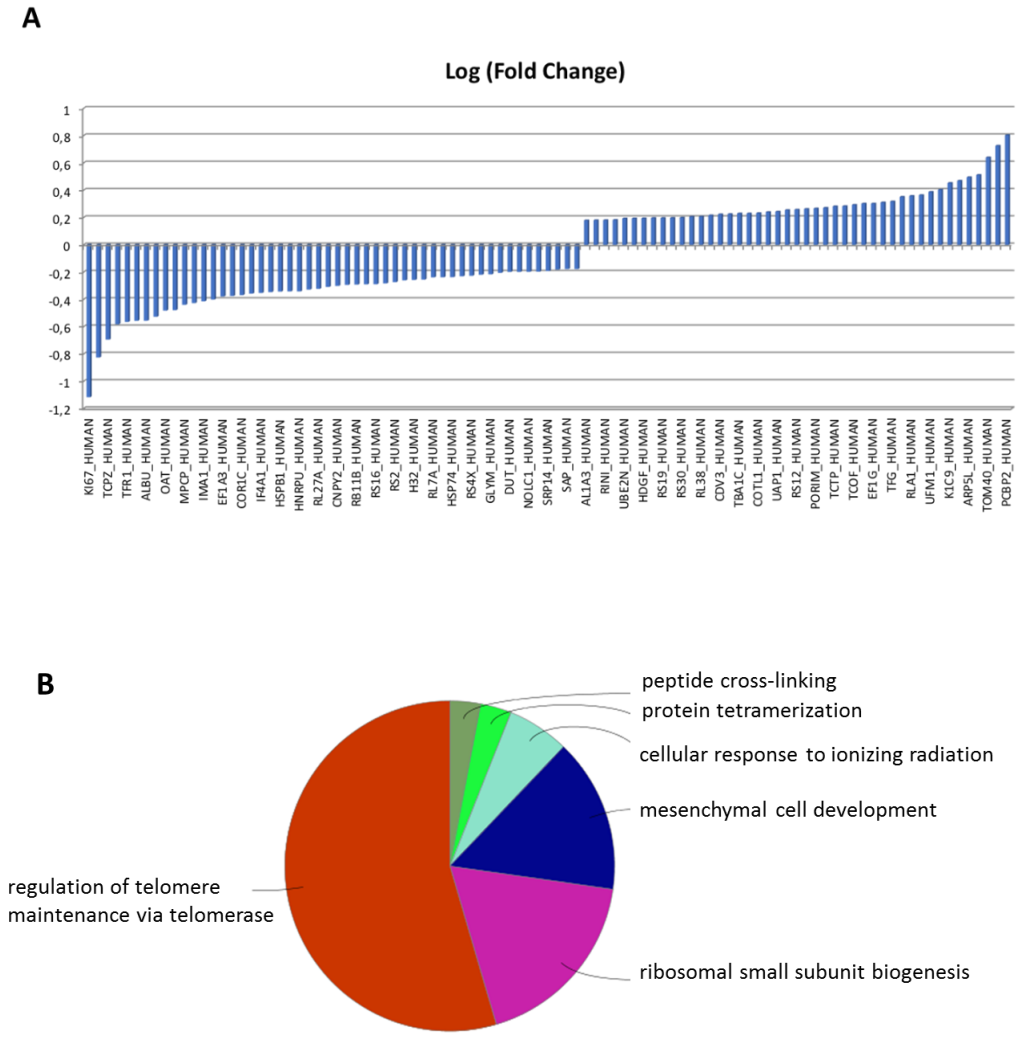


Fig.10. Proteomic analysis of ONC-treated versus control cells at 48 h.

(A). Figure shows the proteomic analysis at 48 h after ONC administration in comparison with ONC-free A375 cells. 48 proteins were downregulated and 52 upregulated.

(B). The biggest clusters include proteins involved in telomeres regulation, in ribosomal biogenesis and in mesenchymal cell development.

Since the proteomic analysis underlined a reduction in the expression level of ribosomal proteins and chaperons, has been proposed that ONC treatment could affect protein synthesis. Thus, the protein synthesis activity after 24 and 48 h of treatment was quantified by measuring the incorporation of a puromycin analog. In ONC-treated cells, a significant reduction in the fluorescence signal, that is directly related with a reduction in the protein synthesis, was detected (Fig.11).

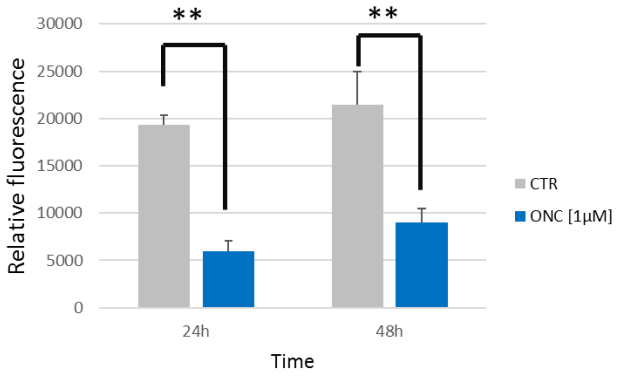


Fig.11. ONC treatment reduced protein synthesis in A375 cells.

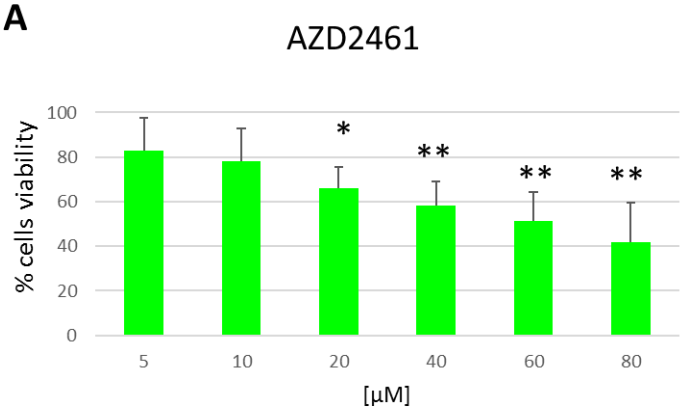
Histogram shows the quantification of de novo protein synthesis measured in A375 cells after 24 and 48 h from ONC (1 µM) administration, compared to control cells (CTR). Each histogram column reported represents the average of three independent experiments ± S.D. (**) At each time point, the differences between ONC-treated and control cells are statistically significant ($p < 0.01$).

4.4 Effect on A375 cell viability after AZD2461 or olaparib single treatments and combined ONC/AZD2461 treatment.

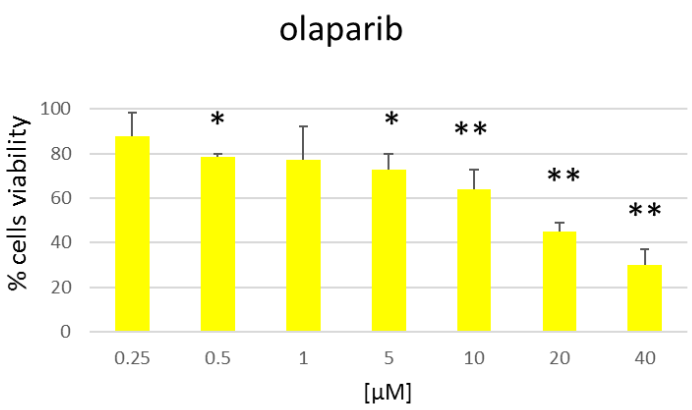
The antitumor effect of the PARP inhibitors olaparib and AZD2461, in comparison with ONC, was evaluated (Fig.12). After 72 h, AZD2461 alone induced a decrease in A375 cell viability, concentration-dependently, with an IC_{50} of 60 μM (Fig.12 A). Thus, the effect of the new AZD2461 PARPi was compared to the well-known PARPi olaparib. Olaparib decreased A375 cell viability with an IC_{50} of 22 μM (Fig.12 B). Notably, ONC was even more effective than both PARPi since the administration for 72 h of only 0.5 μM of this RNase induced a 50% reduction of A375 cell viability (Fig.12 C).

To test the effect of combined treatments, A375 cells were treated for 6 h with low ONC concentrations (0.1, 0.2, 0.35 μM). Then, AZD2461 was added to the culture medium at the increasing concentrations of 5, 10, 20, 40 μM. The concentrations used in the combined treatments were chosen below the IC_{50} value measured in each single treatment. After 72 h, crystal violet assays were performed. Only the treatment with 0.35/20 μM (respectively ONC/AZD2461) was statistically more effective to reduce A375 cell viability in comparison with each drug administered alone at the same concentration (Fig.12 D). Instead, no strong benefit was obtained upon performing a combined therapy with the other drug concentrations used.

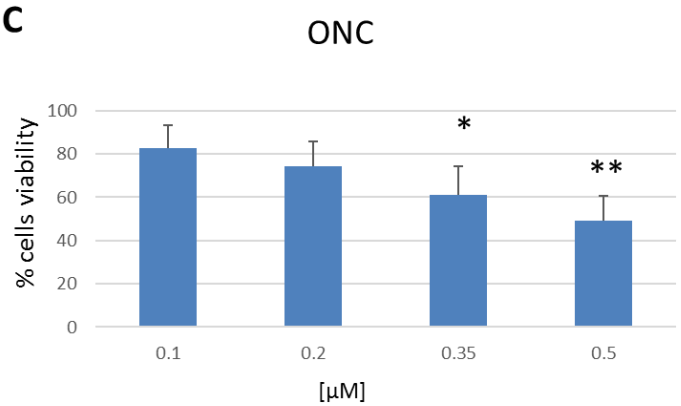
A


AZD2461
% cells viability
100
80
60
40
20
0
5
10
20
40
60
80
[µM]
*
**
**
**

B


olaparib
% cells viability
100
80
60
40
20
0
0.25
0.5
1
5
10
20
40
[µM]
*
*
**
**
**

C


ONC
% cells viability
100
80
60
40
20
0
0.1
0.2
0.35
0.5
[µM]
*
**

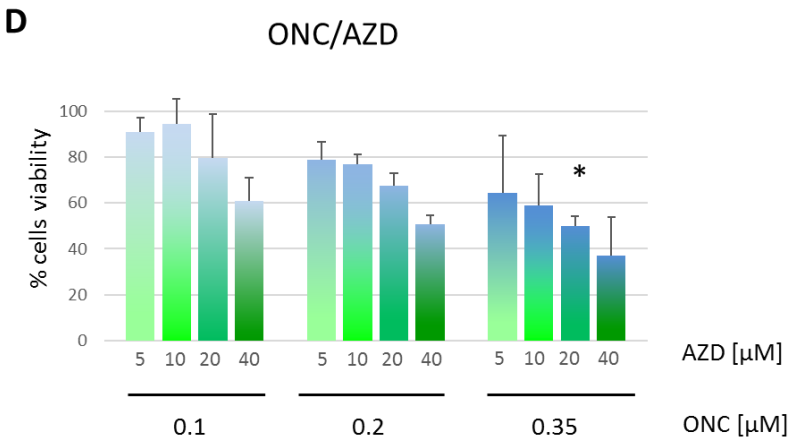


Fig.12. A375 cell viability after incubation with AZD2461, olaparib, ONC single treatment, and ONC/AZD2461 combined.

Cell viability of A375 upon 72 h incubation with increasing concentrations of (A) AZD2461, (B) olaparib, (C) ONC, or (D) ONC/AZD2461. Each histogram column reported represents the average of four to ten independent experiments, each performed in sextuplicate ± S.D.

(A, B, C). (*) ($p < 0.05$) (**) ($p < 0.01$) Differences between treatments with their control are statistically significant.

(D). (*) Differences between the ONC/AZD2461 combined treatment in comparison to both (ONC or AZD2461) single treatments at the same concentration are statistically significant ($p < 0.05$).

4.5 Effects of single or combined treatment on A375 DNA damage and apoptosis.

It is known that the H2AX histone variant is phosphorylated (γH2AX) in response to DNA damage[106]. Thus, the expression level of γH2AX was measured in A375 melanoma cells to evaluate the damage effects of each drug on DNA. A375 cells in basal condition, after 72 h culture, are characterized by low level of phosphorylated H2AX, as shown by western blot analysis (Fig.13 A). The average of three independent blots revealed a slight and not significant increase in the intensity of γH2AX band in 0.35 μM ONC-treated A375 cells (Fig.13 B), instead, the level of γH2AX was higher in the cell sample treated with 40 μM AZD2461. In Fig.13 was shown that the co-treatment, performed with 0.35/40 μM (ONC/AZD2461), induced the same amount of γH2AX than those obtained with AZD2461 single treatment.

The pro-apoptotic activity of AZD2461 and ONC alone, or in combination, were analyzed by measuring the expression level of cleaved PARP1. In the cells, the activation of effector caspase 3 or 7 triggers PARP1 protein cleavage, resulting in two fragments of 85 and 24 KDa. The intensity of the 85 KDa band is directly related to the number of cells undergoing apoptosis. A375 cells treated for 72 h with 0.35 μM ONC showed a slight increase of cleaved PARP1 amount. A remarkable increase was also visible upon the treatment with 40 μM AZD2461 in comparison with control cells (Fig.13). The incubation performed with the combination of two drugs induced the same effect detected with each single treatment.

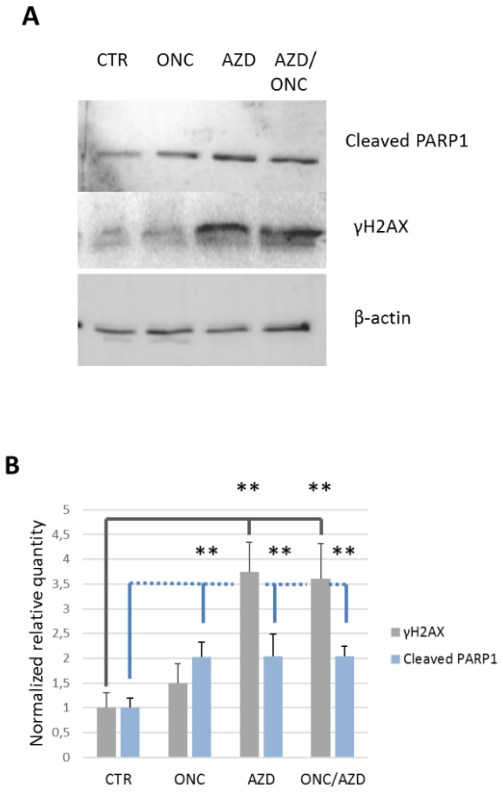


Fig.13. WB analysis of the cleaved PARP1 and γH2AX histone variant.

(A). A representative WB experiment relative to the expression level of the cleaved PARP1 and the phosphorylated histone variant H2AX (γH2AX) in A375 cells after incubation with 40 μM AZD2461 and 0.35 μM ONC, administered alone or in combination. The β-actin expression level was shown as loading control.

(B). The histogram reports the average ± S.D. of three independent experiments. (**) Differences are statistically significant in comparison to each control sample ($p < 0.01$).

4.6 AZD2461 and ONC effects on NF-κB DNA-binding activity and TNF-α gene expression.

In order to study the capability of NF-κB transcription factor to bind the specific sequence on the promoter of its responsive genes, EMSA experiments were performed, by using nuclear protein extracts from A375 cell cultures at 24, 48 and 72 h (Fig.14 A). The oligonucleotide sequence used for the experiment has been selected from the human IL-6 promoter that contain both the NF-κB and CBF1 binding sites very close each other. The DNA-binding of NF-κB dimers resulted in two low mobility weak bands in the gel, whereas the DNA-binding of the constitutively expressed CBF1 transcription factor, that could be used as a loading control, appeared as a large band with higher mobility. The NF-κB DNA-binding bands in the control cells were very weak after 24 h culture, but their intensity increased during cell culture (48 and 72 h). This effect may be due to some activator factors secreted by the cells and accumulated in the medium after two- or three-days culture. Notably, the ONC-incubation almost completely blocks NF-κB DNA binding, while the band representing CBF1 binding shows the same intensity of the control one (Fig.14 A). Differently to the ONC incubation, the treatment with AZD2461 was not able to reduce NF-κB DNA-binding, whereas the reduction of NF-κB bands intensity caused by ONC alone was maintained in the samples treated with a combination of the two drugs (Fig.14 B).

In order to investigate the transcriptional activity of NF-κB, a real-time PCR was performed to measure the mRNA level of TNF-α. TBP gene expression was used as internal standard. Notably, the TNF-α promoter holds a NF-κB binding site, hence TNF-α is a NF-κB-responsive gene. Here, a significant reduction of TNF-α gene expression was measured in A375 cells treated for 72 h with 0.1 and 0.35 μM ONC, while treatments with 20 and 40 μM AZD2461 induced a lower reduction of TNF-α mRNA level with an uncertain concentration dependence. The combination of the two drugs had no additive effect (Fig.15).

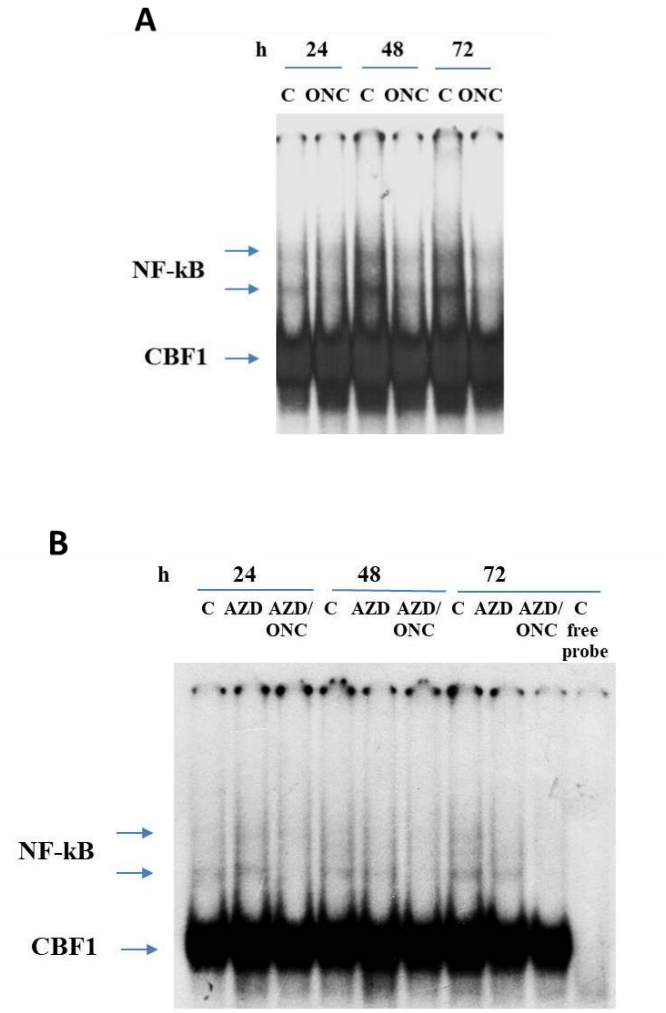


Fig.14. EMSA shows NF-κB DNA binding activity.

NF-κB DNA-binding activity upon A375 incubation with (A) 0.35 μM ONC and (B) 40 μM AZD2461 alone or in combination with 0.35 μM ONC. DNA-binding of CBF1 transcription factor has been used as a loading control. In the last line of the panel B was reported EMSA result obtained with control sample after 72 h of culture in the presence of 200X of unlabeled probe for competition.

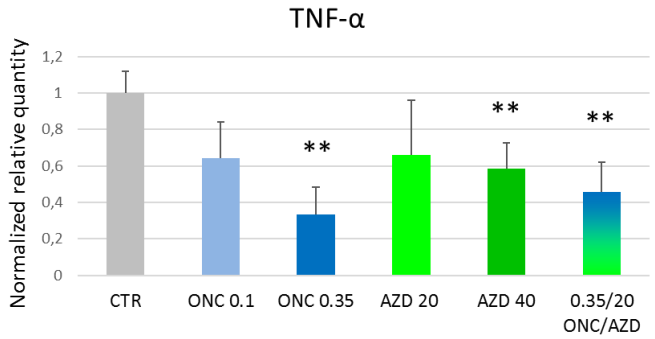


Fig.15. TNF-α mRNA expression level.

Histogram representing the normalized mRNA expression level of TNF-α obtained by RT-PCR experiments. The expression level of TATA box binding protein (TBP) housekeeping gene has been used as internal standard. Each histogram column reported represents the average of three independent experiments ± S.D. (**) Differences between ONC-treated and ONC-free samples are statistically significant ($p < 0.01$).

4.7 Cell viability and protein expression in A375 melanoma cells treated with AZD2461 for two months.

Because A375 melanoma cells are sensitive to AZD2461, it has been investigated if these cells progressively decrease their drug sensitivity. A375 cells were treated for two months with increasing AZD2461 concentrations, starting from 5 up to 80 μM. Data demonstrate that no significant differences in drug sensitivity emerged: in fact, cells remained sensitive to AZD2461 (Fig.16 A). The cells response to ONC treatment has been analyzed also in these cells treated for long-time with AZD2461. Therefore, the AZD2461 administration was stopped one day before the ONC incubation, so AZD2461 was not present in the cell culture medium during the ONC incubation. Unexpectedly, despite no drug-resistance was found in the cells pre-treated with AZD2461 for two months, a subsequent treatment of these cells with low ONC concentrations was more efficacious than that observed in parental ones (Fig.16 B). For all ONC concentrations, except 0.1 μM ONC, differences in cell

viability between A375P and A375 long treated with AZD2461 are statistically significant ($p<0.05$).

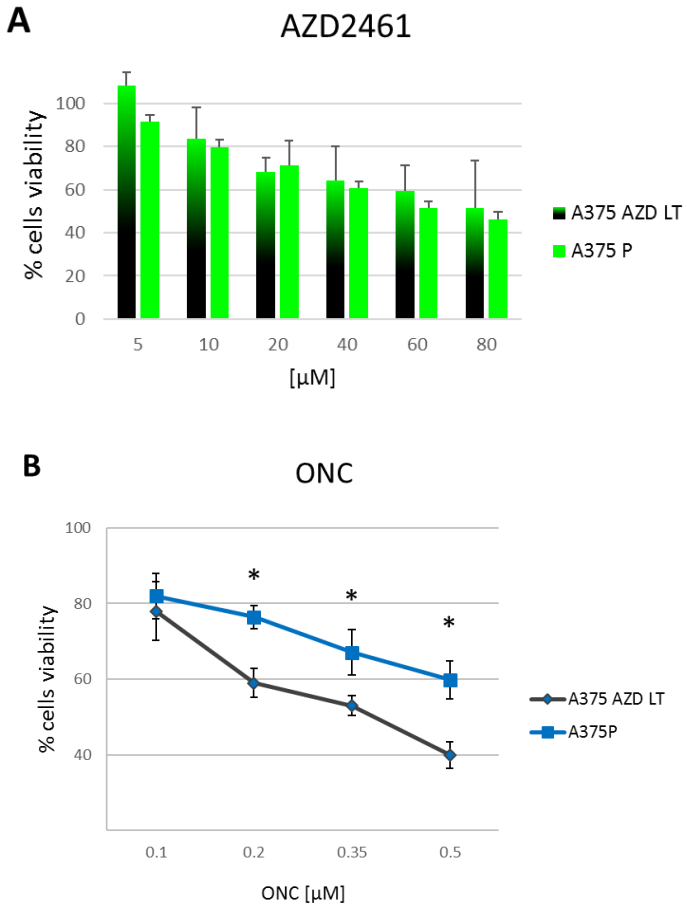


Fig.16. Viability in parental and AZD2461 long time treated A375 cells.

(A). Cell viability of A375 parental (A375P) cells treated for 72 h with AZD2461 in comparison with A375 cells previously treated for two months with AZD2461 (A375 AZD LT) and treated for 72 h with the same drug. Differences in viability between the two populations (A375P and A375 AZD LT) are not statistically significant.
(B). Cell viability of A375P cells compared to A375 AZD LT after 72 h treatment with ONC. (*) Differences between the two subpopulations are statistically significant ($p < 0.05$).
For (A) and (B) Each histogram column reported represents the average of four independent experiments performed in sextuplicate ± S.D.

According to the viability results, cytotoxicity, studied by western blot analysis, showed higher cleaved PARP1 expression level in the cells pre-treated for two months with AZD2461 and then incubated with 0.35 μM ONC for 72 h compared to the ONC-treated parental ones (Fig.17 A and B). This result is promising, because the pro-apoptotic effect of ONC in A375 melanoma cells has been potentiated by a previously two-month treatment with AZD2461.

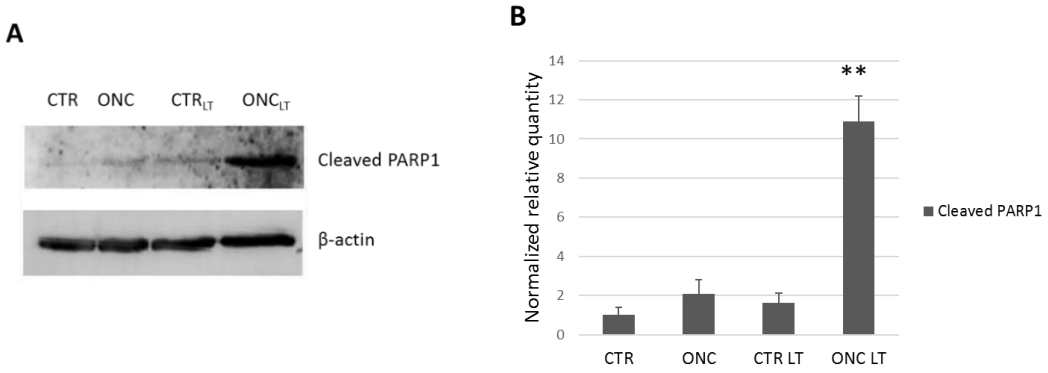


Fig.17. Cleaved PARP1 amount in A375P and A375 long treated with AZD2461.

(A). A representative western blot shows the cleaved PARP1 expression level detected after 72 h culture with or without 0.35 μM ONC in A375P and A375AZD LT cells. The β-actin bands were used as loading control.

(B). Quantification of WB analysis for cleaved PARP1. Each histogram column reported represents the average of three independent experiments ± S.D. (**) Differences between ONC-treated A375AZD LT cells and all other samples are statistically significant ($p < 0.01$).

4.8 ONC strongly affects cell viability of both parental and dabrafenib resistant A375 cells.

Because loss of sensitivity to the treatment is one of the major problems in malignant melanoma therapy with BRAFi[27], A375 cell line, harboring a mutation in the BRAF gene, was cultured with increasing concentrations of the BRAFi dabrafenib to induce drug-resistance. Indeed, one month after, A375 cells started to acquire resistance to dabrafenib (A375DR). Afterward, cells were maintained under drug selective pressure. To confirm this data, the drug sensitivity was measured at different dabrafenib concentrations, on both A375P and A375DR cells with crystal violet assay. As shown in Fig.18 A, A375P cells were highly sensitive to dabrafenib, with an IC_{50} of 2.7 nM. Instead, the A375DR cells viability was not significantly affected until 50 nM dabrafenib. Consequently, the relative IC_{50} value was not measurable or definitively >>5 µM since A375DR cells showed growth also in the presence of this drug concentration. All differences in the viability observed between A375P and A375DR cells are statistically significant, except 1 nM (Fig. 18 A). Subsequently, the effect of ONC on A375P versus A375DR subpopulation was evaluated. Fig.18 B shows that both cell subpopulations have the same viability reduction after 72 h ONC treatment with the calculated IC_{50} value of 0.40 and 0.32 µM for A375P and A375DR cells, respectively.

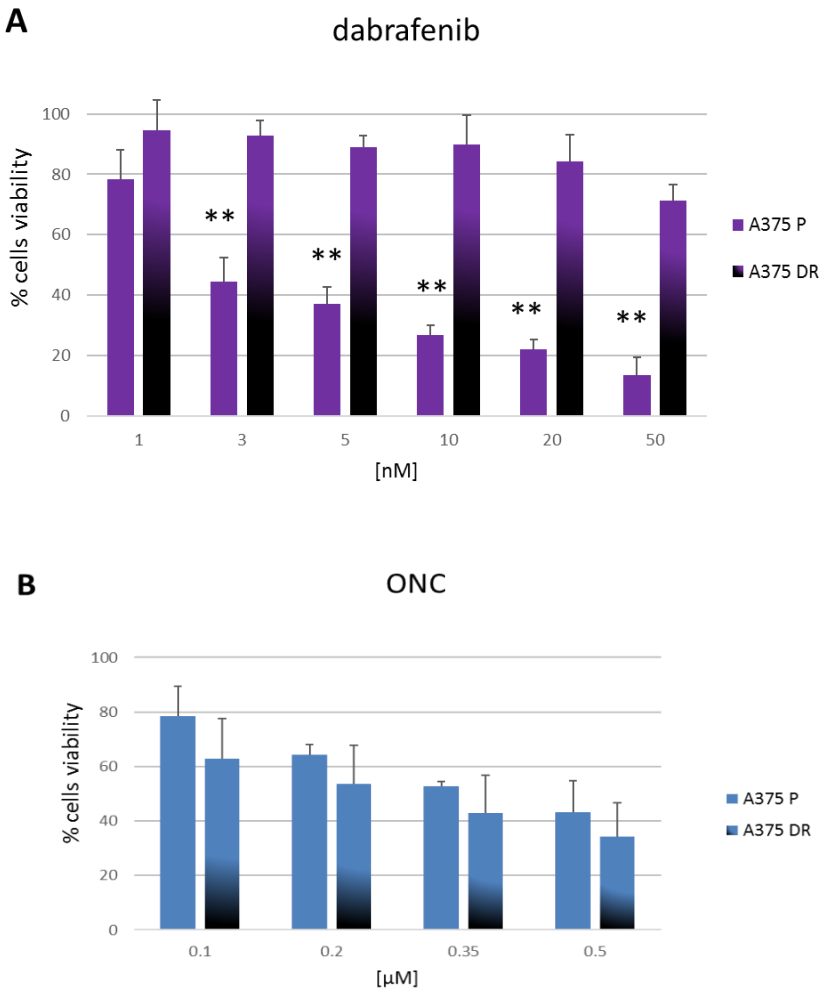


Fig.18. Effects of dabrafenib or ONC in melanoma A375P and A375DR cell subpopulations.

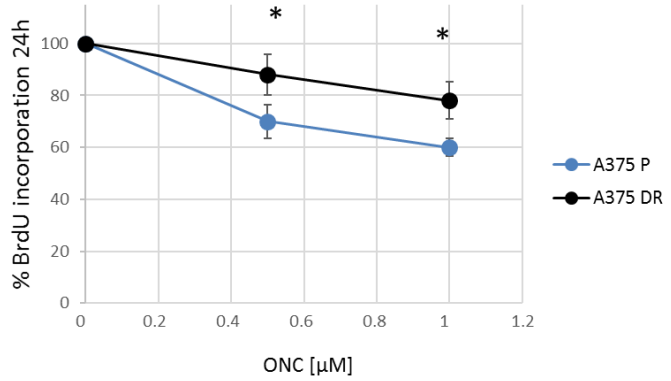
Parental (A375P) and dabrafenib resistant (A375DR) cell viability evaluated after 72 h incubation with increasing concentrations of dabrafenib (A), and ONC (B). (**) For each dabrafenib concentration tested, almost all comparisons between A375P and A375DR are statistically significant ($p < 0.01$), while differences found upon ONC incubations are not statistically significant. Each histogram column reported represents the average of four to five independent experiments performed in sextuplicate, ± S.D.

4.9 ONC decreases the proliferation rate of both A375P and A375DR cell subpopulations.

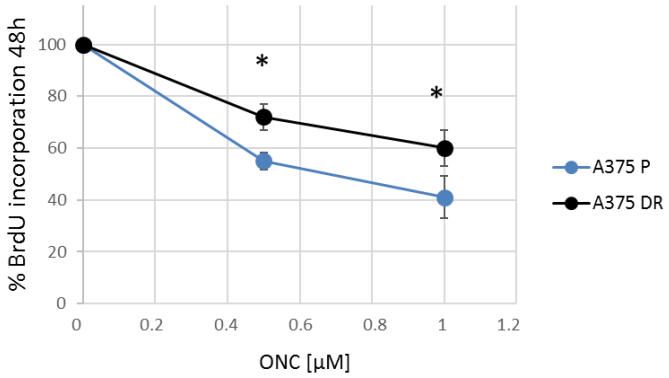
To identify the causes related to the ONC-elicited viability reduction the A375P and A375DR cell proliferation rates were compared, by performing BrdU assay. After 24, 48, 72 h culture, A375P and A375DR cells have been incubated for 4h with BrdU. Both A375 cell subpopulations displayed a significative reduction of the proliferation rate in the ONC-treated samples in comparison with each ONC-free control. Nevertheless, ONC-treated cells have shown a smaller reduction of the BrdU incorporation level in A375DR than in A375P (Fig.19 A, B and C). At all the time-point the differences, emerging by comparing the two ONC-treated cell subpopulations, are statistically significant, except for 1 µM ONC at 72 h. It should be highlighted that A375DR control cells incorporated a lower amount of BrdU than A375P control ones (Fig.19 D), suggesting that, with or without ONC, A375DR cell subpopulation replicate slower than its parental counterpart.

In conclusion, these data indicate that ONC decrease the proliferation rate of both A375P and A375DR cells, although, in comparison with A375P one, A375DR cell subpopulation is less responsive to the antiproliferative effect of ONC.

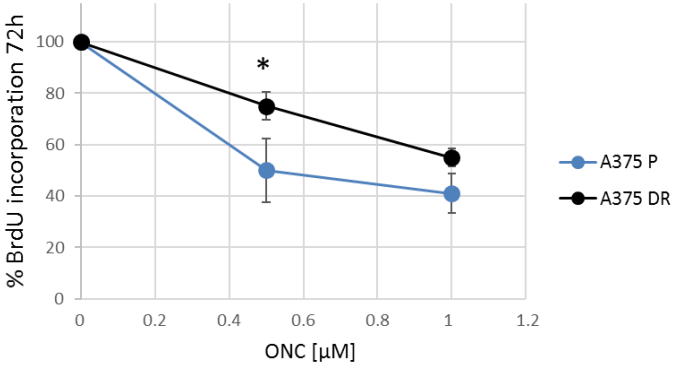
A


% BrdU incorporation 24h
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
1.2
ONC [µM]
*
*
A375 P
A375 DR

B


% BrdU incorporation 48h
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
1.2
ONC [µM]
*
*
A375 P
A375 DR

C


% BrdU incorporation 72h
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
1.2
ONC [µM]
*
A375 P
A375 DR

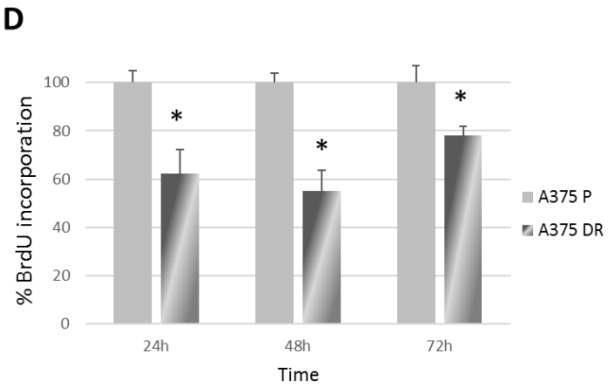


Fig.19. Proliferation rate in two A375 cell subpopulations.

The graphs show % of BrdU incorporation measured at 24 (A), 48 (B) and 72 (C) h in A375P or A375DR cells, after their incubation with 0.5 or 1 µM ONC, in comparison with each ONC-free control. In figure D is represented the different proliferation rate between A375P and A375DR ONC-free control cells. (*) Differences are statistically significant ($p < 0.05$).
Each histogram column reported represents the average of four independent experiments performed in triplicate, ± S.D.

4.10 ONC induces cell death by triggering apoptosis principally in A375 DR cells.

To verify if ONC treatment induce apoptosis in both cell subpopulations, the expression levels of cleaved PARP1 were compared. A representative WB was shown in Figure 20 A, while the average of cleaved PARP1 expression level, relative to four independent experiments and quantified after normalization with β-actin expression, was reported in Figure 20 B. The amount of cleaved PARP1 increased significantly in all ONC-treated cells compared to each control sample, thus confirming that 1 µM ONC concentration induced the A375 apoptotic cell death. Remarkably, ONC-treated A375DR cells show a higher increase of cleaved PARP1 amount in comparison with ONC-treated A375P ones. In conclusion, although ONC-treated A375DR cells showed less reduction in cell proliferation rate than ONC-treated A375P ones, they are more prone to undergo apoptotic cell death.

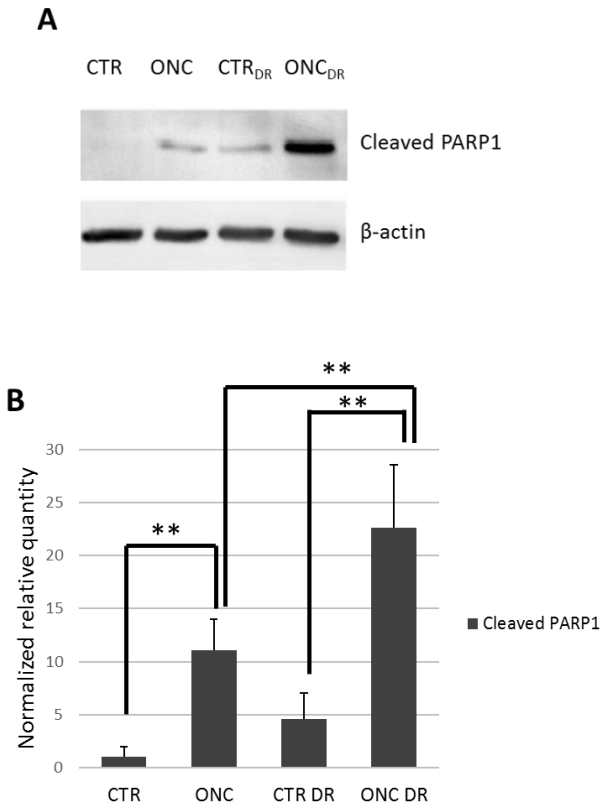


Fig.20. Changes in the amount of cleaved PARP1, in both A375P and A375DR cell subpopulations with or without ONC (1 µM).

(A). Representative WB for cleaved PARP1. (CTR=ONC-free A375P cells, ONC=ONC-treated A375P cells, CTR_{DR}=ONC-free A375DR cells, ONC_{DR}=ONC-treated A375DR cells).

(B). Quantification of cleaved PARP1 expression level after normalization with β-actin expression that was used as internal control. Data reported are the average of four independent WB experiments ± S.D. (**) All differences showed in figure are statistically significant ($p < 0.01$).

4.11 ONC affects p65 NF-κB nuclear localization.

It is known that NF-κB is highly activated in cancer cells, its activation induces survival pathways by promoting cell growth and allowing the tumor to escape from apoptotic cells death. It has been also demonstrated that NF-κB can be a specific target in melanoma therapy[124]. By western blot analysis we documented the presence of p65 NF-κB subunit in the cell nuclei of both A375P and A375DR control cell subpopulations. Instead, ONC-treated A375DR cells showed a significant decrease in the amount of nuclear p65 (Fig.21 A). For this reason, the phosphorylation level of IκB kinases-α/β (pIKK), that trigger NF-κB activation, was measured. As shown in WB analysis (Fig.21 B), the pIKK level was slightly higher in non-treated A375DR cells compared to A375P ones, while ONC decreased the pIKK level in both A375P and A375DR cell subpopulations. Therefore, our data suggest that ONC reduces NF-κB nuclear localization by inhibiting IKK phosphorylation (WB quantification are reported in Fig.21 C).

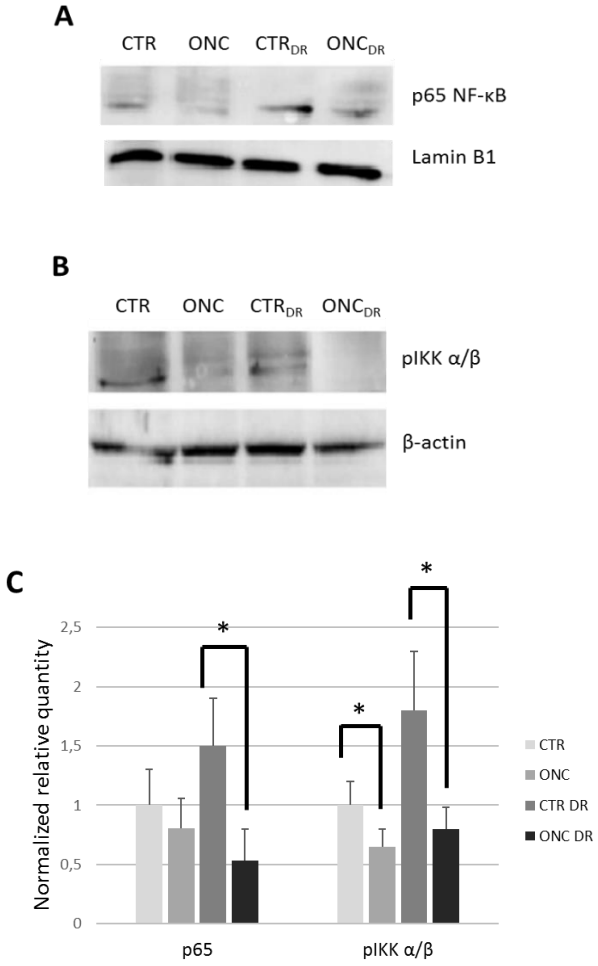


Fig.21. Nuclear localization of p65 NF-κB and phosphorylation level of IKK in A375P and A375DR with or without ONC.

A representative WB shows (A) the amount of p65 NF-κB subunit localized in the nucleus and (B) pIKK expression level in A375P and A375DR cells. β-actin and Lamin B1 expression level are used as internal controls.
(C). Quantification of WB reported as average of four independent experiments ± SD. (*) Differences are statistically significant ($p < 0.05$).

4.12 The A375DR cell subpopulation displays CSC biochemical markers.

A375DR cells displayed smaller size, rounder morphology, and a lower proliferation rate than A375P ones. Notably, all these properties characterize CSC[59]. In ONC-free A375P and A375DR cells the mRNA expression levels of either CD133, a transmembrane glycoprotein expressed on the surface of normal and cancer stem cells, and NANOG, a transcription regulator involved in the maintenance of an embryonic stem cell state were measured by real-time PCR. CD133 and NANOG mRNA expression levels showed 2.1-fold and 2.4-fold increases, respectively, in comparison with the relative levels detected in A375P ones (Fig.22 A). The amount of TBP mRNA was used here as an internal standard. These results demonstrate that A375DR cells display high mRNA amount of both surface (CD133) and intracellular (NANOG) markers of undifferentiating and embryonic stem cells.

Thereafter, the protein expression levels of CSC and of EMT markers were also investigated in both cell subpopulations. A representative WB, showing the differences existing between the two A375 cell subpopulations in the protein amount of CD133, NANOG, N-cadherin, nuclear β-catenin and pERK1/2, was displayed in Figure 22 B. The expression level of β-actin was used to normalize the total protein amount loaded into the gel.

In conclusion, results show that A375DR cell subpopulation expresses high levels of CSC markers, such as CD133 and NANOG and it undergoes EMT by increasing nuclear β-catenin and N-cadherin protein levels. Finally, has been confirmed that A375DR cells can escape to the BRAF inhibition by restoring high levels of the phosphorylated and active form of ERK1/2 kinases.

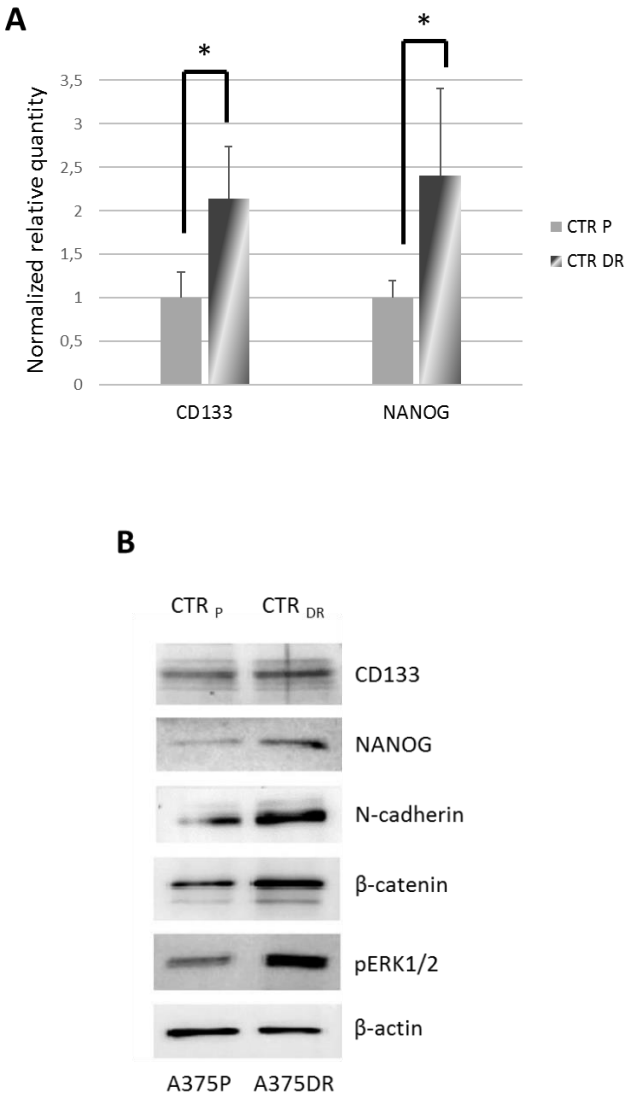


Fig.22. Changes in the expression level of malignancy markers in ONC-free A375DR vs A375P cells.

(*A). mRNA expression level of CD133 glycoprotein and NANOG transcription regulator measured by RT-PCR in A375P and A375DR ONC-free cells. Data reported are the average of three independent experiments, each performed in triplicate; ± S.D.; (*) differences are statistically significant ($p < 0.05$).*

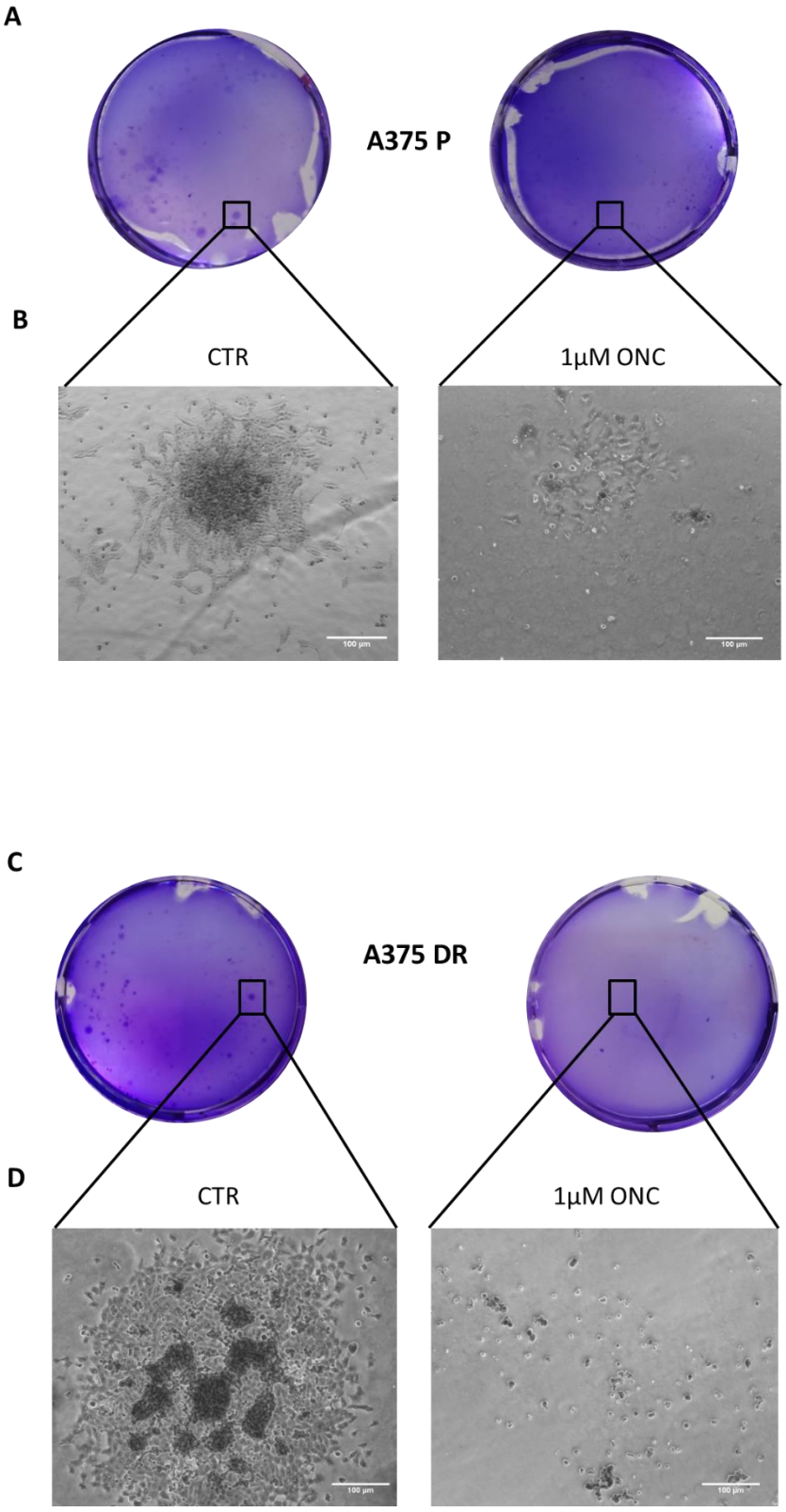
(B). A representative WB showing the expression levels of CD133, NANOG, N-cadherin, nuclear β-catenin, pERK1/2 proteins, relative to the two ONC-free A375 cell subpopulations. β-actin was used as loading control.

4.13 ONC reduces the colonies formation in soft agar.

Anchorage-independent growth is a carcinogenesis hallmark presents in transformed cells that permit their growth regardless of a solid surface.

The soft agar colony formation assay is a deep-rooted method for characterizing this capability *in vitro* and is considered one of the most stringent test for malignant transformation in cells[59,27]. For this reason, soft agar assay has been performed for three weeks. A higher number of colonies has grown in A375P control cells than in ONC-treated ones (Fig.23 A-B). Besides, the number of colonies was higher in A375DR cells than in A375P ones, and it got down around zero upon incubation of A375DR cells with 1 µM ONC (Fig.23 C-D).

Hence, the remarkable malignancy of A375DR cells is further demonstrated here by their ability to form a high number of colonies in soft agar assay (Fig.23 E). Significantly, this ability was suppressed by ONC at a higher extent in A375DR than in A375P.


A
A375 P
B
CTR
1µM ONC
C
A375 DR
D
CTR
1µM ONC

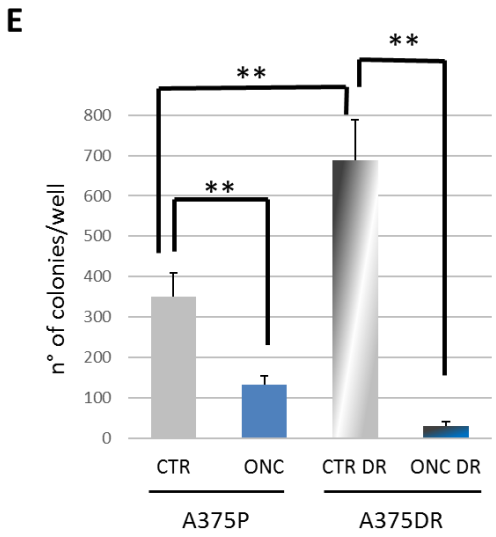


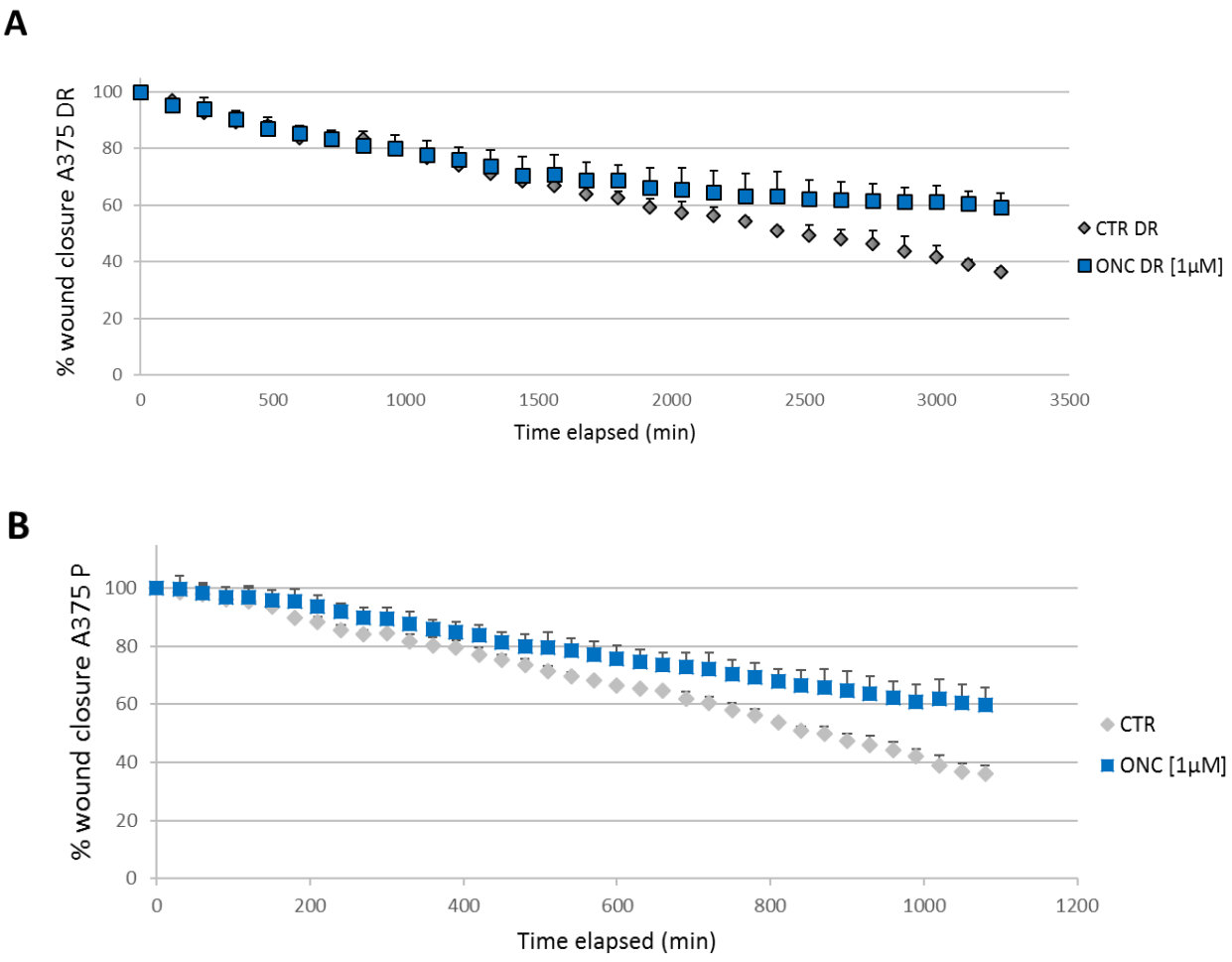
Fig.23. ONC effect on colonies formation in the two A375 cell subpopulations.

Representative images of the stained agar in well (A and C), of colonies formed by A375P (A and B) and A375DR cells (C and D) treated or not with ONC. Images were captured with 5X magnification, Zeiss Axio Vert. A1 AxioCAM/cm^{-1} 60N-C1" 1,0X camera.

(E). Number of colonies grown in soft agar by A375P and A375DR cells after being incubated with or without 1 µM ONC for 72 h. Data reported are relative to three independent experiments ± S.D. (**) All differences indicated were statistically significant ($p < 0.01$).

4.14 ONC reduces cell migration rate in both A375P and A375DR cell subpopulations.

A wound closure cell assay was performed to measure the cell migration ability of both A375P and A375DR subpopulations, in the presence or absence of ONC (Fig.24). Notably, the time necessary to close the wound was different for the two subpopulations: in detail, A375DR cells migrated and proliferated slower than A375P ones. For this reason, the experiment with parental cells was carry out for 17 h, while 54 h were needed for A375DR to close the wound. 1 µM ONC significantly reduced the migration rate of either A375DR or A375P cells, as it is visible in Fig.24 A and B, respectively. Figure 24 C shows images taken at T_0 and at T_{54h} for the ONC-treated and untreated A375DR cells, while figure 24 D represents images capture at T_0 and $T_{17\ h}$ for ONC-treated and untreated A375P cells.



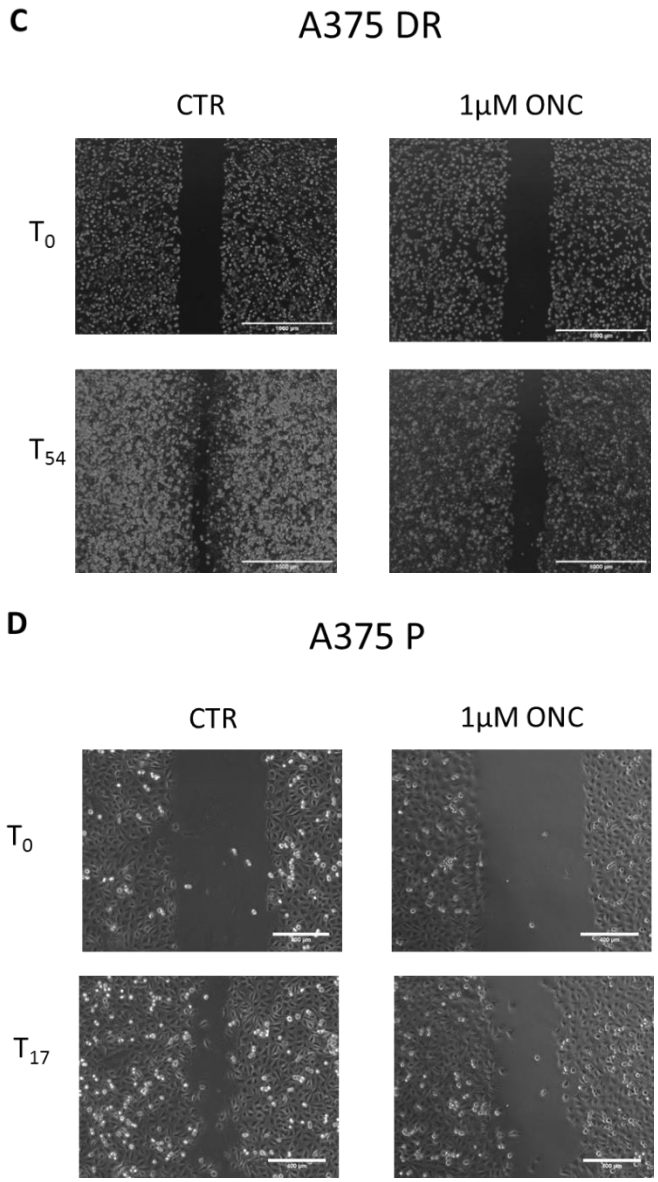


Fig.24. Wound closure cells assay time elapsed in two A375 cell subpopulations.

Wound closure measure (%) in (A) A375DR and (B) A375P starting from time zero until the end of the experiments, initial (t_0) and final (t_{54h} for A375DR and t_{17h} for A375P). Representative images of A375DR cells (4X) (C), and of A375P (10X) (D) treated or not with 1 µM ONC. Images were captured with the EVOS FL Auto Cell Imaging System.

4.15 ONC reduces cells invasion and MMP-2 activity in both A375P and A375DR cells.

Both A375 cell subpopulations were compared in order to verify if they acquired an invasive phenotype, and the potential of ONC to affect cells ability to degrade the basal membrane and pass through the transwell barrier, has been measured.
Cells were treated for 72 h with or without 1µM ONC, then were detached. Each cell sample was seeded in a transwell upper chamber, and complete DMEM was added to the lower chamber as a chemo-attractant. After 48 h, the invading cells were stained and quantified. Both subpopulations invaded the Matrigel, although A375DR cells showed a slight, but not significant, increase in their invasion potential compared to A375P ones. Instead, treatment with 1µM ONC caused a significant 33% reduction of the A375DR cells invasion capability in comparison with not treated A375DR ones. Not statistically significant decrease was found in ONC-treated A375P cells in comparison with their not treated counterpart (Figure 25).

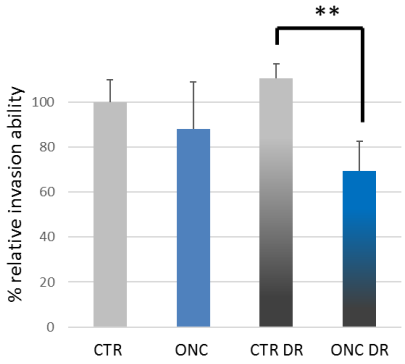


Fig.25. ONC effect on the invasion potential.

Invasion potential of A375 cells in the presence or absence of 1 µM ONC. A375P (CTR) and A375DR (CTR DR) and the respective ONC-treated cells were cultured for 72 h, detached, counted and seeded in the transwell. After 48 h, cells were stained and quantified at 460nm with Tecan NanoQuant Infinite M200 Pro Plate reader, as reported in the Materials & Methods Section. Each histogram column reported represents the average of three independent experiments ± S.D. (**) Differences between ONC_{DR} vs CTR_{DR} cells are statistically significant ($p < 0.01$).

In order to measure MMP-2 activity, both A375 cell subpopulations were cultured with or without 1 µM ONC for 48 h. Thereafter, cells were serum-starved for 30 h and the conditioned media collected to measure the MMP-2 activity by gelatin zymography. The results shown in Figure 26 A & B demonstrate that ONC significantly decreases MMP-2 activity at a similar extent either in A375P or in A375DR cells. Data relative to MMP-9 are not quantified because of the low activity level found in both cell subpopulations.

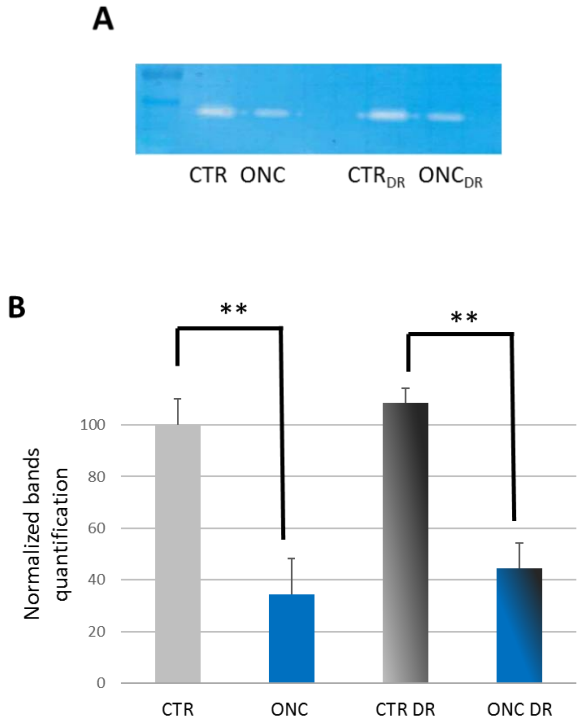


Fig.26. ONC effect on MMP-2 activity.

(A). Representative picture of a gelatin zymography to measure the amount of gelatinase (MMP-2) secreted in the culture medium.

(B). Quantifications of the intensities of the digested bands measured with the ImageJ software. Each histogram column reported represents the average of four independent experiments ± S.D. (**) All differences found within controls and ONC-treated cells of each subpopulation are statistically significant ($p < 0.01$).

5. DISCUSSION

In the last decays melanoma incidence is increased worldwide, becoming the most frequent skin malignancy in the Caucasian population[6]. Several therapies have been approved based on surgical resection for non-invasive stages and pass through chemotherapy for invasive phenotypes[17]. The first drug approved for melanoma therapy has been DTIC, an alkylating agent that causes breaks in the DNA strand, but it showed a low response in patients due to acquired chemoresistance[27]. The characterization of a specific mutation on BRAF kinase gene, that is present in the majority of melanoma patients and causes a constitutive activation of the MAPK pathway, led new therapeutic tools based on the development of drugs headed to a specific target[28]. Different BRAF inhibitors, such as dabrafenib and vemurafenib, have been approved in the last few years. Unfortunately, cellular responses to BRAF inhibitors are short-lived because patients in few months develop different mechanisms of resistance that involve the activation of alternative signaling pathways[34]. For these reasons, new therapeutic strategies for the treatment of patients with advanced melanoma need to be developed.

ONC is a small secretory ribonuclease, discovered in *Rana Pipiens* oocytes[147]. For its structure and biochemical features, ONC is better internalized in cancer cells compared to non-malignant cells and, contrarily to the majority of other secretory RNases, can escape from the binding to the intracellular ribonuclease inhibitor[149,152]. Previous studies in several different cell lines showed that ONC cytostatic effect was related to a decrease in frequency of DNA replicating cells (block before S-phase), while its cytotoxic effect was linked to the apoptosis induction[154]. The fist *in vivo* study revealed that mice with Ml09 Madison carcinoma treated with ONC displayed 12-fold longer life compared to untreated control group[161]. Moreover, ONC has already been tested in phase III clinical trial against mesothelioma, an aggressive tumor characterized by poor prognosis[162]. It has been previously reported that ONC can affect the viability of two conjunctival melanoma cell lines, although no data are available on its action's mechanism[171]. For these reasons, ONC activity has been tested on A375 human cell line from BRAF-mutated cutaneous melanoma and a dose-dependent reduction in A375 cell

viability was observed. In order to validate the ONC specific activity on malignant cells, ONC effect was also tested on NHEM, as a control. Indeed, a slight but not significative reduction of NHEM viability has been registered only at ONC concentrations higher than these used in A375 cells. This result could be explained by the high basic ONC charge that promote its better internalization in cancer cells which display higher negative charges on the outer surface membrane compared to non-transformed cells[152]. Thereafter, we investigated if the ONC-elicited decrease in A375 cell viability could be caused by cytostatic and/or cytotoxic activities. It has been reported that, the proliferative rate of malignant melanoma cells, studied by autoradiography after incorporation of tritiated thymidine, is directly correlated with a high risk for the early metastases onset[172]. A reduction in the A375 BrdU incorporation, both at 24 and 48 h after ONC administration, has been observed suggesting that a lower number of cells can enter the S-phase in ONC-treated A375 cells in comparison with non-treated ones. To confirm this result, the Ki67 protein expression level was investigated, because this protein is considered a sensitive marker for proliferating cells and is also used in clinic as a diagnostic tool[76,77]. Indeed, the high expression of Ki-67 protein and the correlated increase of tumor cell proliferation has been described as a poor prognostic factor in patient with vertical growth phase melanoma[173]. A significative reduction of Ki67 protein level in A375 cells after 48 h ONC administration has been observed by WB and further confirmed by proteomic analysis. Thereafter, it has been investigated if a cytotoxic effect also occurred. In this regard, it is known that the majority of drugs used in melanoma therapy, such as cisplatin and DTIC, can promote cell death by inducing DNA damage, proliferation arrest and activation of the mitochondrial apoptotic pathway[174,175]. Indeed, A375 melanoma cell line displayed an increase in two apoptotic markers, such as active caspase 3 and cleaved PARP1, after DTIC treatment[176]. In order to evaluate the cytotoxic effect of ONC, Annexin-V FITC assay has been used for detecting the exposure of phosphatidylserine on the outer surface of cell membrane as an early signal event in the programmed cell death[87]. After 48 h ONC treatment, an increase in fluorescence signal, due to Annexin V-FITC binding to the exposed phosphatidylserine, was detected. To support this result, a WB analysis was performed to measure the amount of the cleaved form of

PARP1, which came from the splitting of PARP1 protein in two parts carried out by the active caspases 3 and 7 operating in the late phase of apoptosis[169]. Data showed that ONC-treated cells displayed a higher amount of PARP1 cleaved form than ONC-free control cells. This result agrees with data obtained by Mihail et al., who demonstrated that ONC triggers apoptosis in Hela cell line[153].

Moreover, in collaboration with Prof. Cecconi, from the Biotechnology Department (University of Verona), a proteomic analysis was performed in order to study how ONC affects A375 proteome. 48 h after ONC administration, the proteins clusters highly modulated are involved in the regulation of telomere maintenance, in the mesenchymal cell development and in the ribonuclear subunit biogenesis. Recent studies demonstrate that telomerase not only regulates the synthesis of telomers but is also involved in different processes, because it directly regulates NF-κB-dependent transcription[177] and stimulates EMT process by inducing stemness of cancer cells[178]. All these telomerase activities contribute significantly to the oncogenic process[170]. Given that telomerase expression is upregulated in the majority of carcinomas, several therapeutic strategies against telomerase have been developed to counteract tumorigenesi[179,180]. These promising first data will be studied more deeply in the future.

Wu et al. demonstrated that ONC inhibited 9L cells protein synthesis, measured by the [^{14}C] leucine incorporation in the proteins[181]. Since in our proteomic analysis a reduction in chaperons' expression was also detected, we decided to evaluate if protein synthesis could be affected in A375 cells by ONC treatment. A significant reduction in protein synthesis in ONC-treated A375 cells, at both 24 and 48 h, was observed.

An hallmark of cancer is its genomic instability and the propensity to accumulate DNA damages[98]. In this context, the role of DDR machinery is fundamental to sustain a high level of tumor cell proliferation. Indeed, any error occurring during this process must be repaired before cells' division by activating DDR that detects and corrects DNA lesions. DDR inhibition in cancer cells leads to the accumulation of excessive DNA damage resulting in cell death[182]. In this context, DNA repair enzymes are important targets for anticancer therapy, and PARP family enzymes play a central role in the responses to DNA damage[183]. For long time, the potential

of PARPi was driven by the enhancement of the effects observed with commonly used cytotoxic chemotherapies, such as temozolomide (TMZ)[146,184]. Recently, it has been shown that ABT-888 PARPi, caused pro-apoptotic effects on different melanoma cell lines[185]. Moreover, depletion of PARP1 protein inhibits melanoma growth and counteracts drug resistance[132]. Recently, olaparib, a PARPi largely used in clinic, was employed with successful results in a ligase 4-deficient melanoma cells[186]. Nevertheless, some reports describe increase in cancer cells resistance to olaparib through different mechanisms, such as loss of p53-binding protein 1 expression or overexpression of ATP-binding cassette drug transporter, a P-glycoprotein that prevents the accumulation of the anticancer drugs in malignant cells[141,142]. AZD2461, is a new generation PARPi structurally analog to olaparib that is less prone to be expelled from the cell by the P-glycoprotein in comparison with olaparib[144]. Remarkably, this new PARPi has been successfully tested in a phase I clinical trial[145]. In order to discover if AZD2461 could display a synergic effect in combination with ONC, their antitumor activities were measured on A375 cells viability. Data showed that AZD2461 alone, after 72 h administration, affects A375 cells viability in a dose-dependent manner with an IC_{50} of 60 μM, in comparison olaparib is active a lower concentration since it displays an IC_{50} of 22 μM. Thereafter, a combination of ONC/AZD2461 was evaluated. Thus, ONC was added in the culture medium and, 6 h later, AZD2461 was administrated. The concentrations of both drugs were chosen at lower values of each respective IC_{50}. Only the 0.35/20 μM (ONC/AZD2461) combined treatment resulted more effective compared to each single drug at the same concentration. Hence, data suggest that the two drugs display no crucial synergic effects.

In order to investigate if single or combined drugs cause DNA damage, the phosphorylation of the H2AX histone variant was measured. It is known that the phosphorylation level of H2AX (γH2AX) increases proportionally with the occurrence of DNA strand breaks[106]. After 72 h culture, an increase in the amount of γH2AX has been registered with either AZD2461 alone or ONC/AZD2461 combined treatment. Conversely, ONC single treatment did not induced increased γH2AX expression level and, therefore, DNA damage. This suggest that different

mechanisms, such as the protein synthesis inhibition consequence of tRNA and rRNA degradation, underlie the antitumor effects of ONC[187,149].

Thereafter, the onset of apoptosis has been evaluated in A375 cells, measuring the expression level of the cleaved form of PARP1 by WB analysis. Both single (ONC or AZD2461) and combined (ONC/AZD2461) treatment after 72 h incubation showed analogous increases, compared to control samples, in the protein amount of cleaved PARP1. In summary, the low synergism found in cell viability was also confirmed by the expression of cleaved PARP1 and γH2AX, in which the combination of the treatments did not show significant differences compared to the effects seen by each single drug.

Recent studies showed that NF-κB is overexpressed in dysplastic nevi and in full-blown melanoma compared to non-transformed melanocytes. Notably, this transcription factor displays pleiotropic effects, acting on cell growth, apoptosis inhibition and inflammation, thus becoming an helpful target in melanoma therapy[121,124]. Different drugs have been discovered acting specifically against NF-κB activation but all of them have been characterized by important side effects, such as toxicity in normal cells and off-target activity[128,130]. Notably, PARP1 has been described as a co-activator of NF-κB transcriptional activity, indeed, PARP1 silencing is connect with a decrease in NF-κB dependent pro-inflammatory cytokines induction, in particular in the expression of TNF-α[131,132]. Therefore, the use of PARP inhibitors can indirectly reduce the NF-κB pro-cancer effects[133,134]. Using EMSA technique, the capability of this transcription factor to bind the promoter sequence of IL-6 gene has been studied. Data demonstrated a significant DNA-binding level of NF-κB in control cells, that increased during the cell's cultivation time. This agrees with Yang et al. data that reported a constitutive level of NF-κB activity in unstimulated A375 melanoma cells[129]. Remarkable, the incubation with 0.35 μM ONC completely inhibited the NF-κB activation under basal conditions. The same reduction in NF-κB DNA binding activity was detectable in sample treated with the combination of ONC/AZD2461 drugs. Conversely, AZD2461 single treatment was not able to inhibit NF-κB DNA binding. Even though this last result was negative, a recent study in a lung inflammation system reported that PARP inhibition suppressed p65

phosphorylation without altering NF-κB activation[188]. Hence, it cannot be rule out that AZD2461 could affect NF-κB transcriptional activity with a similar mechanism. As concern the ONC effect, its capability to inhibit NF-κB activity agrees with data, previously reported by other authors, showing a reduction of NF-κB nuclear translocation in pleural mesothelioma and in Jurkat T-lymphocytes[189,158].

Anyway, considering that NF-κB inhibition is crucial in melanoma also to overcome drug resistance[190], the drug effects on NF-κB downstream genes should be investigated. Hence, the influence of the treatments on TNFα, a NF-κB target gene, was evaluated measuring its mRNA expression level by Real-time PCR. Although the drug combination has no additive effect, results show a decrease of TNF-α expression in either ONC or AZD2461 single treatment. This result is remarkable, because high TNF-α expression level is recognized to be a poor prognostic index because it is up-regulated in advance melanoma in comparison with the expression level found in melanoma at the early stage[191]. Notably, TNF-α could be either an activator or a target of NF-κB activation. So, ONC inhibitory activity could be primary addressed to NF-κB activation followed by blocking of the TNF-α transcription, or TNF-α expression could be firstly inhibited by ONC not allowing the subsequent NF-κB activation[192]. In any case, these data are relevant because a suppression of both NF-κB activity and TNF-α expression is helpful because they control cell proliferation, apoptosis and invasiveness in melanoma[193].

AZD2461 is a new generation PARPi developed to overcome the P-glycoprotein efflux transporter action. In order to investigate the possibility to the induction of drug resistance, the A375 cells culture medium was supplemented for two months with increased AZD2461 concentrations. Data showed that A375 cells did not acquire resistance to this drug, so far. If this will be confirmed for longer treatment, AZD2461 could be a promising option in melanoma treatment instead of olaparib which induces chemoresistance both in cells and in patients. Thereafter, the response to ONC in the cell subpopulation treated for long time with AZD2461 was analyzed. Surprisingly, in these cells ONC displayed more cytotoxic effects in comparison with parental A375 cells. Indeed, the amount of cleaved PARP1 protein

in ONC-treated cells was higher in AZD2461 long-time treated A375 subpopulation than that observed in parental one (A375P). This promising result demonstrates that long incubation with AZD2461 can induce a sustained cytotoxic response whether cells were subsequently treated with ONC.

The major problem in melanoma therapy is the development of tumor resistance against almost all drugs used in clinic[27]. A375 cells harbors a mutation in the BRAF gene that is frequently present in melanoma patients[15,13]. The BRAF mutation causes a constitutive activation of this kinase also in the absence of extracellular stimuli, so A375 cells can be target of BRAFi. Thus, cell medium was supplemented with increasing concentrations of the BRAFi dabrafenib, to induce drug resistance. Cells were maintained in selective condition with increasing doses of dabrafenib for few months. After that period, whether A375P remained highly sensitive to dabrafenib, with an IC_{50} of 2.7 nM, the selected resistant cells (A375DR) were not significantly affected by the drug until 50 nM. Nevertheless, ONC significantly affect cell viability in a similar extent in both cell subpopulations since, after 72 h from ONC administration, the IC_{50} value was 0.40 and 0.32 μM for A375P and A375DR cells, respectively. These results demonstrated that the ONC sensitivity is maintained in A375 cells that acquired resistance to dabrafenib. To explore more deeply the causes of reduction in cell viability elicited by ONC, the A375P and A375DR cell proliferation rate has been analysed. ONC-treated cells of two subpopulations displayed a lower BrdU incorporation compared to each respective control and this is confirmed at all time point. However, ONC-treated A375DR cells displayed a smaller reduction in the BrdU incorporation than ONC-treated A375P ones. Because a similar reduction of BrdU incorporation was registered in not-treated A375DR cell subpopulation in comparison with not-treated A375P one, this suggests that, in all condition, A375DR cells displayed a lowest replication rate. In this regard, it has been reported that the BRAFi vemurafenib can induce senescence in melanoma cells[194]. Thus, it could be possible that part of the A375DR cell subpopulation, becoming senescent, was no longer susceptible to the anti-proliferative effect of ONC.

To verify if A375DR cell subpopulation can preserve their responsiveness to ONC cytotoxicity, apoptosis was measured by quantification of the cleaved PARP1

expression level. Firstly, differences in cleaved PARP1 amount between ONC-free A375P and ONC-free A375DR cells were remarkable suggesting a higher propensity of A375DR cells to die for apoptosis. As expected, both ONC-treated subpopulations displayed a significant increase in the amount of cleaved PARP1 in comparison to each ONC-free sample. Notably, the high expression level of cleaved PARP1 in ONC-treated A375DR cell was very impressive so indicating that ONC elicited an extensive cell death in this cell subpopulation. The balance between higher cell death and, conversely, less reduction in cell proliferation rate of ONC-treated A375DR in comparison with ONC-treated A375P cells could explain a similar decrease on cells viability for the two cell subpopulations.

Many evidences correlate NF-κB activation with cancer cell growth and the ability for tumor cells to escape from death[124]. Hence, as already discussed previously, some papers indicate that ONC inhibits NF-κB in different tumor cell types[160,189]. Thus, the activation of NF-κB in the two cell subpopulations measuring the nuclear localization of the p65 NF-κB subunit, has been compared. As expected, p65 was present in the nuclear extract of ONC-free cells of the two cell subpopulations and ONC treatment reduced p65 nuclear presence. Moreover, the activation of IKKαβ, the kinases that trigger NF-κB activation, was evaluated. Similar to p65 nuclear expression level, ONC was able to decrease the protein phosphorylation and activation of IKKαβ, especially in A375DR cells. In summary, data suggest that ONC alters the p65 NF-κB subunit nuclear localization by inhibiting the phosphorylation of IKK upstream kinases.

Differences in cell morphology, replication rate and size, recognized as all features related to CSC phenotype[59,195], has been observed in A375DR cell subpopulation. By using RT-PCR, the expression of CD133, a glycoprotein exposed on membrane cell surface of CSC, and NANOG, a transcriptional factor involved in embryonic stem cell state, have been quantified in the two cell subpopulations. A significant increase in the mRNA level of CD133 and NANOG, in not-treated A375DR compared to not-treated A375P cells, was registered. Differences in the protein expression of CSC and EMT markers were also evaluated. Protein expression levels of N-cadherin and nuclear β-catenin, as EMT markers, were increased in A375DR compared to A375P cells, as well as high expression levels of CD133 and NANOG

proteins were found confirming data obtained with the mRNA quantification. Finally, accordingly with literature data[196,197,198], an over-activation of ERK1/2 in A375DR control cells revealed that A375DR cells can escape from BRAF inhibition reactivating the MAPK pathway. Furthermore, an upregulation in CD133 has previously been associated with the capability of forming sphere in culture, a characteristic that belongs to CSC[59,199]. Indeed, the anchorage-independent growth is another hallmark connected to high malignant phenotype[200], and the most used method to evaluate this capability in the cell cultures is the agar colony formation assay. In the present work, in agreement with Cordaro et al[196], the ability of A375DR subpopulation in comparison with A375P to form a higher colonies number, has been demonstrated. Contrarily, this ability disappeared in ONC-treated cells, especially in ONC-treated A375DR cell subpopulation. Thereafter, a migration assay has been performed to measure the cells' migration capability. Although A375P cell subpopulation migrated faster than A375DR one, after 1 µM ONC treatment, a significant inhibition of cells migration was observed equally in both A375P and A375DR subpopulations in comparison with each control sample. To support this result, the cells invasion potential, that permits cell colonization far from the original site by the degradation of the extracellular matrix[63], has been studied. For this analysis, transwell plate-insert with 8µm pore-size, coated with a basement membrane, were used. This support allows the evaluation of the cells capability to degrade and pass through the transwell barrier. Data showed that both A375P and A375DR control subpopulations can are able to degrade the matrix, instead, ONC treated cells displayed a reduction in the invasion potential even if this difference was significative only for A375DR cell subpopulation. The invasion capability is dependent on the proteolytic degradation of ECM component[67]. The high level of MMPs regularly secreted by melanoma cells can be crucial in this mechanism, in particular the amount of MMP-2 and MMP-9 involving in the degradation of type IV collagen, the major ECM component[201]. Certainly, in two hepatocarcinoma cell lines[202], Liu et al. demonstrated that MMP-2 knockdown significantly decreased cell proliferation and cell invasion capability. MMP-2 is highly secreted in invasive melanoma cell lines, and it has also been associated with melanoma progression since its expression has been inversely correlated with the

patient survival[73,72.] By using the gelatin zymography, the presence of MMP-2 and its activity has been evaluated. In our model, a significant decrease in MMP-2 activity in both ONC-treated A375P and A375DR cell subpopulations was documented. Since the NF-κB activation has been implicated in enhancing MMP-2 expression[203], the ONC capability to hindering NF-κB activation could almost partially explain the inhibition of MMP-2 activity as well as the invasion capability reduction in our cell system.

6. CONCLUSIONS

This study underlined, for the first time, that ONC selectively affects the viability of a very aggressive human cutaneous melanoma cell line. Indeed, the A375 human melanoma cells, differently from normal human melanocytes, display high responsivity to the cytostatic and cytotoxic effects of ONC. In these cells, the ONC antitumor effects exhibit a time and concentration dependency. Moreover, in A375 cells, an ONC-elicited increase of apoptotic cell death was shown, as well as inhibition of cells migration, invasion and colony formation. Among the multitarget actions of ONC, at the molecular level the inhibitory effects of this RNase on NF-κB DNA binding, p65 nuclear translocation and MMP-2 gelatinase activity has been showed. Notably, all these effects are determinant in melanoma progression and metastasis formation.

Because the major problem in melanoma therapy is the onset of drug resistance, different combination treatments were used to obtain the best strategy in order to block melanoma progression and recurrence. No advantageous effects were found by combining the ONC treatment with AZD2461. Instead, both A375 cells treated with AZD2461 PARPi for a long time and A375 dabrafenib BRAFi resistant cell subpopulations increased their apoptotic cell death after being incubated with ONC. In summary, the apoptotic cell death driven by ONC occurred either if a previous treatment did not induce resistance[204] or if the cells had already lost their responsiveness to the cytotoxic effect of a BRAFi[205]. Furthermore, ONC treatment in A375DR cell subpopulation inhibits colony formation and invasion capability at a higher extent than in A375 parental cells. Recalling Reck et al., reported data concerning a phase III clinical trial on ONC and Doxorubicin (DOX) therapy in unresectable malignant mesothelioma[206]. In this trial the authors documented that patients had a significant advantage for survival only when they were previously treated with DOX before they were given ONC. Therefore, the highest sensitivity to ONC of both dabrafenib resistant and long-time AZD2461 treated A375 cell subpopulations is in line with this important clinical result[206].

REFERENCES

1. Gray-Schopfer, V.; Wellbrock, Cl.; Marais, R. Melanoma biology and new targeted therapy. *Nature* **445**, 851–857 (2007).
2. Bertolotto, C. Melanoma : From Melanocyte to Genetic Alterations and Clinical Options. *Scientifica (Cairo).* (2013).
3. Leonardi, GC.; Falzone, L.; Salemi, R.; Zanghì, A.; Spandidos, DA.; Mccubrey, JA.; Candido, S.; Libra, M. Cutaneous melanoma: From pathogenesis to therapy (Review). *Int. J. Oncol.* **52**, 1071–1080 (2018).
4. Miller, AJ., Mihm, M. Mechanisms of disease Melanoma. *N. Engl. J. Med.* **335**, 51–65 (2006).
5. Clark, WH.; Elder, DE.; Guerry, D.; Epstein, MN.; Greene, MH.; Van Horn, M. A study of tumor progression: The precursor lesions of superficial spreading and nodular melanoma. *Hum. Pathol.* **15**, 1147–1165 (1984).
6. Caini, S.; Gandini, S.; Sera, F.; Raimondi, S.; Fargnoli, M.; Boniol, M. . Meta-analysis of risk factors for cutaneous melanoma according to anatomical site and clinico-pathological variant. *Eur. J. Cancer* **45**, 3054–3063 (2009).
7. Markovic, SN.; Erickson, LA.; Rao, R. . et al. Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. *Mayo Clin Proc* **3**, 364–380 (2007).
8. Rigel, D. Epidemiology of melanoma. *Semin Cutan Med Surg* **4**, 204–209 (2010).
9. D'Orazio, J.; Jarrett, S.; Amaro-Ortiz, A.; Scott, T. UV Radiation and the Skin. *Int J Mol Sci.* **14**, 12222–12248 (2013).
10. Goldstein, AM.; Chan, M.; Harland, M.; Hayward, K.; Demenais, F.; Bishop, TD.; Azizi, E.; Bergman, Wi.; Bianchi-scarra, G.; Bruno, Wi.; Calista, D.; Albright, A.; Chaudru, V.; Chompret, A.; Cuellar, F.; Elder, DE.; Gillanders, EM.; Gruis, NA.; Hansson, J., L. Features associated with germline CDKN2A mutations: A GenoMEL study of melanoma-prone families from three continents. *J Med Genet* **44**, 99–106 (2007).
11. Elder, D. Melanoma progression. *Pathology* **48**, 147–154 (2016).
12. Durrant, DE.; Morrison, D. Targeting the Raf kinases in human cancer: The Raf dimer dilemma. *Br. J. Cancer* **118**, 3–8 (2018).

13. Tentori, L.; Lacal, PM.; Graziani, G. Challenging resistance mechanisms to therapies for metastatic melanoma. *Trends Pharmacol. Sci.* **34**, 656–666 (2013).

14. Pennello, G.; Devesa, S.; Gail, M. Association of Surface Ultraviolet B Radiation Levels with Melanoma and Nonmelanoma Skin Cancer in United States Blacks. **9**, 291–297 (2000).

15. Ali, Z.; Yousaf, N.; Larkin, J. Melanoma epidemiology , biology and prognosis. *EJC Suppl.* **11**, 81–91 (2013).

16. Eichenauer, D. A. *et al.* Hodgkin lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **29**, 19–29 (2018).

17. Batus, M.; 0 Waheed, S.; Ruby, C.; Petersen, L.; Bines, SD.; Kaufman, H. Optimal management of metastatic melanoma: current strategies and future directions. *Am J Clin Dermatol.* **14**, 179–94

18. Widakowich, C.; de Castro, G.; de Azambuja, E.; Dinh, P.; Awada, A. Review: Side Effects of Approved Molecular Targeted Therapies in Solid Cancers. *Oncologist* **12**, 1443–1455 (2007).

19. Li, J. *et al.* Recent advances in targeted nanoparticles drug delivery to melanoma. *Nanomedicine Nanotechnology, Biol. Med.* **11**, 769–794 (2015).

20. Austin, E.; Mamalis, A.; Ho, D.; Jagdeo, J. Laser and light-based therapy for cutaneous and soft-tissue metastases of malignant melanoma: a systematic review. *Arch. Dermatol. Res.* **309**, 229–242 (2017).

21. Fonkem, E.; Uhlmann, EJ.; Floyd, SR.; Mahadevan, A.; Kasper, E.; Eton, O.; Wong, E. Melanoma brain metastasis: overview of current management and emerging targeted therapies. *Expert Rev Neurother* **12**, 1207–15 (2012).

22. Kim, C. *et al.* Long-Term Survival in Patients with Metastatic Melanoma Treated with DTIC or Temozolomide. *Oncologist* **15**, 765–771 (2010).

23. Agarwala, SS.; Kirkwood, J. Temozolomide, a Novel Alkylating Agent with Activity in the Central Nervous System, May Improve the Treatment of Advanced Metastatic Melanoma. *Oncologist* **5**, 144–151 (2000).

24. Patel, M.; McCully, C.; Godwin, K.; Balis, F. Plasma and cerebrospinal fluid pharmacokinetics of intravenous temozolomide in non-human primates. *J Neurooncol* **61**, 203–7 (2003).

25. Bleehen, NM.; Newlands, ES.; Lee, SM.; Thatcher, N.; Selby, P.; Calvert, AH.;

Rustin, GJ.; Brampton, M.; Stevens, M. Cancer Research Campaign phase II trial of temozolomide in metastatic melanoma. *J Clin Oncol* **13**, 910–3 (1995).

26. Zheng, M.; Bocangel, D.; Ramesh, R.; Ekmekcioglu, S.; Poindexter, N.; Grimm, EA.; Chada, S. Interleukin-24 overcomes temozolomide resistance and enhances cell death by down-regulation of O6-methylguanine-DNA methyltransferase in human melanoma cells. *Mol Cancer Ther* **7**, 3842–51 (2008).

27. Uhara, H. Recent advances in therapeutic strategies for unresectable or metastatic melanoma and real‐world data in Japan. *Int. J. Clin. Oncol.* **24**, 1508–1514 (2019).

28. Wilhelm, S. *et al.* Discovery and development of sorafenib: A multikinase inhibitor for treating cancer. *Nat. Rev. Drug Discov.* **5**, 835–844 (2006).

29. Trunzer, K.; Pavlick, AC.; Schuchter, L.; Gonzalez, R.; McArthur, GA.; Hutson, TE.; Moschos, ST.; Flaherty, KT.; Kim, KB.; Weber, JS.; Hersey, P.; Long, GV.; Lawrence, D.; Ott, PA.; Amaravad, RK.; Ribas, A. Pharmacodynamic Effects and Mechanisms of Resistance to Vemurafenib in Patients With Metastatic Melanoma. *J. Clin. Oncol.* **31**, 1767–1774 (2013).

30. Sullivan, RJ.; Flaherty, K. Resistance to BRAF-targeted therapy in melanoma. *Eur. J. Cancer* **49**, 1297–1304 (2013).

31. Wu, CP.; Sim, HM.; Huang, YH.; Liu, YC.; Hsiao, SH.; Cheng, HW.; Li, YQ.; Ambudkar, SV.; Hsu, S. Overexpression of ATP-binding cassette transporter ABCG2 as a potential mechanism of acquired resistance to vemurafenib in BRAF(V600E) mutant cancer cells. *Biochem Pharmacol* **85**, 325–34 (2013).

32. MittapallI, RK.; Vaidhyanathan, S.; Dudek, AZ.; Elmquist, W. Mechanisms limiting distribution of the threonine-protein kinase B-RaF(V600E) inhibitor dabrafenib to the brain: implications for the treatment of melanoma brain metastases. *J Pharmacol Exp Ther* **344**, 655–64 (2013).

33. Flaherty, K.;Infante, J.;Daud, A.;Gonzalez, R.;Kefford, R.; Sosman, J.; Hamid, O.;Schuchter, L.; Cebon, J.; Ibrahim, N.; Kudchadkar, R:, Burris, H:, et al. Combined BRAF and MEK Inhibition in Melanoma with BRAF V600 Mutations. *N. Engl. J. Med.* **367**, 1694–1703 (2012).

34. Aplin, AE.; Kaplan, FM.; Shao, Y. Mechanisms of resistance to RAF inhibitors: literature review. *Oncologie* **14**, 2–4 (2012).

35. Doudican, N.A.; Orlow, S. J. Inhibition of the CRAF/prohibitin interaction reverses

CRAF-dependent resistance to vemurafenib. *Oncogene* **36**, 423–428 (2017).

36. Heidorn, SJ.; Milagre, C.; Whittaker, S.; Nourry, A.; Niculescu-Duvas, I.; Dhomen, N.; Hussain, J.; Reis-Filho, JS.; Springer, CJ.; Pritchard, C.; Marais, R. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* **140**, 209–21 (2010).

37. Poulikakos, PI.; Persaud, Y.; Janakiraman, M.; Kong, X.; Ng, C.; Moriceau, .; Shi, H.; Atefi, M.; Titz, B.; Gabay, MT.; Salton, M.; Dahlman, KB.; Tadi, M.; Wargo, JA.; Flaherty, KT.; Kelley, MC.; Misteli , T.; Chapman, PB.; Sosman, JA.; Graeber, TG.; Riba, D. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature* **480**, 387–90 (2011).

38. Balkwill, F., Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* **357**, 539–545 (2001).

39. Sharma, P.; Allison, J. P. The future of immune checkpoint therapy. *Science (80-.).* **348**, 56–61 (2015).

40. Gata, V.A.; Lisencu, C.I.; Vlad, C.I.; Piciu, D.; Irimie, A. . A.-C. P. Tumor infiltrating lymphocytes as a prognostic factor in malignant melanoma. Review of the literature. *J Buon* **22**, 592–598 (2017).

41. Sharma, P.; Hu-Lieskovan, S.; Wargo, J.; Ribas, A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* **168**, 707–723 (2017).

42. Gasser, S1.; Lim, LHK.; Cheung, F. The role of the tumour microenvironment in immune therapy. *Endocr Relat Cancer* **24**, 283–295 (2017).

43. Kirkwood, JM.; Ibrahim, JG.; Sosman, JA.; Sondak, VK.; Agarwala, SS.; Ernstoff, MS.; Rao, U. High-dose interferon alfa-2b significantly prolongs relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIB-III melanoma: results of intergroup trial E1694/S9512/C509801. *J Clin Oncol* **19**, 2370–80 (2001).

44. Eggermont, AM.; Suciu, S.; Rutkowski, P.; Kruit, WH.; Punt, CJ5.; Dummer, R.; Salès, F.; Keilholz, U.; de Schaetzen, G.; Testori, A. . E. M. G. Long term follow up of the EORTC 18952 trial of adjuvant therapy in resected stage IIB-III cutaneous melanoma patients comparing intermediate doses of interferon-alpha-2b (IFN) with observation: Ulceration of primary is key determinant for IFN-sensitivity. *Euro J Cancer* 111–21 (2016).

45. Ribas, A. Anti-CTLA4 Antibody Clinical Trials in Melanoma. *Cancer Ther* **2**, 133–

9 (2007).

46. Ribas, A1.; Comin-Anduix, B.; Economou, JS.; Donahue, TR.; de la Rocha, P.; Morris, LF.; Jalil, J.; Dissette, VB.; Shintaku, IP.; Glaspy, JA.; Gomez-Navarro, J.; Cochran, A. Intratumoral immune cell infiltrates, FoxP3, and indoleamine 2,3-dioxygenase in patients with melanoma undergoing CTLA4 blockade. *Clin. Cancer Res.* **15**, 190–9 (2009).

47. Wolchok, JD.; Neyns, B.; Linette, G.; Negrier, S.; Lutzky, J.; Thomas, L.; Waterfield, W.; Schadendorf, D.; Smylie, M.; Guthrie, T Jr.; Grob, JJ.; Chesney, J.; Chin, K.; Chen, K.; Hoos, A.; O'Day, SJ.; Lebbé, C. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol* **11**, 155–64 (2010).

48. Melero, I.; Grimaldi, AM.; Perez-Gracia, JL.; Ascierto, P. Clinical development of immunostimulatory monoclonal antibodies and opportunities for combination. *Clin Cancer Res* **19**, 997–1008 (2013).

49. Raedler, L. Opdivo (Nivolumab): Second PD-1 Inhibitor Receives FDA Approval for Unresectable or Metastatic Melanoma. *Am Heal. Drug Benefits* **8**, 180–3 (2015).

50. Specenier, P. Nivolumab in melanoma. *Expert Rev Anticancer Ther* **16**, 1247–1261 (2016).

51. Housman, G.; Byler, S., Heerboth, S.;Lapinska, K.; Longacre, M.; Snyder, N.; Sarkar, S. Drug Resistance in Cancer: An Overview. *Cancers (Basel).* **6**, 1769–1792 (2014).

52. Marzagalli, M.; Moretti, RM.; Messi, E.; Montagnani Marelli, M:; Fontana, F.; Anastasia, A.; Bani, MR.; Beretta, G.; Limonta, P. Targeting melanoma stem cells with the Vitamin E derivative δ-tocotrienol. *Nature* **587**, (2018).

53. Singh, A.; Settleman, J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29**, 4741–51 (2010).

54. Zeng-JieYang, R. . J. W.-R. Hit 'Em Where They Live: Targeting the Cancer Stem Cell Niche. *Cancer Cell* **11**, 3–5 (2007).

55. Valent, P.; Bonnet, D.; De Maria, R.; Lapidot, T.; Copland, M.; Melo, JV.; Chomienne, C.; Ishikawa, F.; Schuringa, JJ.; Stassi, G.; Huntly, B.; Herrmann, H.; Soulier, J.; Roesch, A.; Schuurhuis, GJ.; Wöhrer, S., Arock, M.; Zuber, J.; Cerny-Reiterer S, Joh, C. Cancer stem cell definitions and terminology: the devil is in the details. *Nat Rev Cancer* **12**, 767–75 (2012).

56. Bonnet, JB.; Dick, J. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3**, 730–737 (1997).

57. López-Lázaro, M. The stem cell division theory of cancer. *Crit Rev Oncol Hematol* **123**, 95–113 (2018).

58. Nouri, M.; Caradec, J.; Lubik, AA.; Li, N.; Hollier, BG., Takhar, M.; Altimirano-Dimas, M.; Chen, M.; Roshan-Moniri, M.; Butler, M.; Lehman, M.; Bishop, J.; Truong, S.; Huang, SC.; Cochrane, D.; Cox, M.; Collins, C.; Gleave, M.; Erho, N.; Alshalafa, M.; D, R. Therapy-induced developmental reprogramming of prostate cancer cells and acquired therapy resistance. *Oncotarget* **8**, 18949–18967 (2017).

59. Marzagalli, M.; Raimondi, M.; Fontana, F.; Montagnani Marelli, M.; Moretti, RM.; Limonta, P. Cellular and molecular biology of cancer stem cells in melanoma: Possible therapeutic implications. *Semin Cancer Biol* **S1044-579X**, 30002–1 (2019).

60. O'Flaherty, JD.; Barr, M.; Fennell, D.; Richard, D.; Reynolds, J.; O'Leary, J.; O'Byrne, K. The cancer stem-cell hypothesis: Its emerging role in lung cancer biology and its relevance for future therapy. *J. Thorac. Oncol.* **7**, 1880–1890 (2012).

61. Obenauf, A. *et al.* Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature* **520**, 368–372 (2015).

62. Heerboth, S.; Housman, G.; Leary, M.; Longacre, M.; Byler, S.; Lapinska, K.; Willbanks, A. . S. EMTand tumor metastasis. *Clin. Transl. Med* **4**, (2015).

63. Yilmaz, M.; Christofori, G.; Lehembre, F. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol. Med.* **13**, 535–541 (2007).

64. Friedl, P. Prespecification and plasticity: Shifting mechanisms of cell migration. *Curr. Opin. Cell Biol.* **16**, 14–23 (2004).

65. Brabletz, T.; Hlubek, F.; Spaderna, S.; Schmalhofer, O.; Hiendlmeyer, E.; Jung, A.; Kirchner, T. Invasion and metastasis in colorectal cancer: Epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and β-catenin. *Cells Tissues Organs* **179**, 56–65 (2005).

66. Sinnberg, T.; Makino, E.; Krueger, MA.; Velic, A.; Macek, B.; Rothbauer, U.; Groll, N.; Pötz, O.; Czemmel, S.; Niessner, H.; Meier, F.; Ikenberg, K.; Garbe, C.; Schittek, B. A Nexus Consisting of Beta-Catenin and Stat3 Attenuates BRAF Inhibitor Efficacy and Mediates Acquired Resistance to Vemurafenib. *EBioMedicine* **8**, 132–149 (2016).

67. Birkedal-Hansen, H.; Moore, WG.; Bodden, MK.; Windsor, LJ.; Birkedal-Hansen, B.; DeCarlo, A.; Engler, J. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* **4**, 197–250 (1993).

68. Nagase, H. Activation mechanisms of matrix metalloproteinases. *Biol Chem* **378**, 151–160 (1997).

69. Gomez, D.E.; Alonso, D.F.; Yoshiji, H.; Thorgeirsson, U. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* **74**, 111–122 (1997).

70. Khokha, R. Suppression of the tumorigenic and metastatic abilities of murine B16–F10 melanoma cells in vivo by the overexpression of the tissue inhibitor of the metalloproteinases-1. *J Natl Cancer Inst.* **86**, 299–304 (1994).

71. Malaponte, G.; Zacchia, A.; Bevelacqua, Y.; Marconi, A.; Perrotta, R.; Mazzarino, MC.; Cardile, V.;Stivala, F. Co-regulated expression of matrix metalloproteinase-2 and transforming growth factor-beta in melanoma development and progression. *Oncol Rep* **24**, 81–7 (2010).

72. Nikkola, J. *et al.* High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. *Clin. Cancer Res.* **11**, 5158–5166 (2005).

73. Kurschat, P.; Zigrino, P.; Nischt, R. . et al. Tissue inhibitor of matrix metalloproteinase-2 regulates matrix metalloproteinase-2 activation by modulation of membrane-type 1 matrix metalloproteinase activity in high and low invasive melanoma cell lines. *J Biol Chem* **274**, 21056–21062 (1999).

74. Ray, G. ;. tetler-S. W. Gelatinase A activity directly modulates melanoma cell adhesion and spreading. *Embo J* **14**, 908–917 (1995).

75. Brooks, PC.; Strömblad, S.; Sanders, L. et al. Localization of matrix metalloproteinase, MMP, -2 to the surface of invasive cells by interaction with integrin alpha v beta, 3. *Cell* **85**, 683–693 (1996).

76. Brown, D. C.; Gatter, K. C. Ki67 protein: The immaculate deception? *Histopathology* **40**, 2–11 (2002).

77. Viale, G. Pathological work up of the primary tumor: getting the proper information out of it. *Breast* **20**, (2011).

78. Hu, HY.; Liu, H.; Zhang, JW.; Hu, K.; Lin, Y. Clinical significance of Smac and

Ki-67 expression in pancreatic cancer. *Hepatogastroenterology* **3**, 2640–3 (2012).

79. McCormick, D.; Chong, H.;Hobbs, C., Datta, C.; Hall, P. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. *Histopathology* **22**, 355–60 (1993).

80. Fernandez, EB.; Sesterhenn, IA.; McCarthy, WF.; Mostofi, FK.; Moul, J. Proliferating cell nuclear antigen expression to predict occult disease in clinical stage I nonseminomatous testicular germ cell tumors. *J Urol* **152**, 1133–8 (1994).

81. Moretti, S.; Spallanzani, A.; Chiarugi, A.; Fabiani, M.; Pinzi, C. Correlation of Ki-67 expression in cutaneous primary melanoma with prognosis in a prospective study: Different correlation according to thickness. *J. Am. Acad. Dermatol.* **44**, 188–92 (2001).

82. Starborg, M.; Gell, K.; Brundell, E.; Höög, C. The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. *J Cell sci* **109**, 143–53 (1996).

83. Schlüter, C.; Duchrow, M.; Wohlenberg, C.; Becker, MH.; Key, G.; Flad, HD.; Gerdes, J. The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. *J Cell Biol* **123**, 513–22 (1993).

84. Kausch, I., Lingnau, A.; Endl, E.; Sellmann, K.; Deinert, I.; Ratliff, TL.; Jocham, D.; Sczakiel, G.; Gerdes, J.; Böhle, A. Antisense treatment against Ki-67 mRNA inhibits proliferation and tumor growth in vitro and in vivo. *Int J Cancer* **105**, 710–16 (2003).

85. Galluzzi, L.; Vitale, I.; Aaronson, SA.; Abrams, JM.; Adam, D.; Agostinis, P. . et al. Molecular mechanisms of cell death: recommendations of the nomenclature committee on Cell death. *Cell Death Differ* **25**, 486–541 (2018).

86. Adewale, FO.; Basiru, AO.; Ayorinde, OO.; Israel, OI.; Oluwafemi, O. Regulation of apoptotic and necroptotic cell death in skin cancer. *J Cancer Biol Res* **17**, 1108–1117 (2017).

87. Suzuki, J.; Denning, DP.; Imanishi, E.; Horvitz, HR.; Nagata, S. Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science (80-.).* **341**, 403–6 (2013).

88. Locksley, RM.; Killeen, N.; Lenardo, M. The TNF and TNF receptor superfamilies:

integrating mammalian biology. *Cell* **104**, 487–501 (2001).

89. Hsu, H.; Xiong, J.; Goeddel, D. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* **81**, 495–504 (1995).

90. Kischkel, FC.; Hellbardt, S.; Behrmann, I.; Germer, M.; Pawlita, M.; Krammer, PH.; Peter, M. Cytotoxicity-dependent APO-1 (Fas/CD95)- associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14**, 5579–88 (1995).

91. Saelens, X.; Festjens, N.; Vande Walle, L.; van Gurp, M.; van Loo, G.; Vandenabeele, P. Toxic proteins released from mitochondria in cell death. *Oncogene* **23**, 2861–74 (2004).

92. Chinnaiyan, A. The apoptosome: heart and soul of the cell death machine. *Neoplasia* **1**, 5–15 (1999).

93. Joza, N.; Susin, SA.; Daugas, E.; Stanford, WL.; Cho, SK.; Li, CY.; Sasaki, T.; Elia, AJ.; Cheng, HY.; Ravagnan, L.; Ferri, KF.; Zamzami, N.; Wakeham, A.; Hakem, R.; Yoshida, H.; Kong, YY.; Mak, TW.; Zuniga- Pflucker, JC.; Kroemer, G.; Penninger, J. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* **410**, 549–54 (2001).

94. Igney, FH.; Krammer, P. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* **2**, 277–88 (2002).

95. Berger, N. Poly(ADP-Ribose) in the Cellular Response to DNA Damage. *Radiat. Res.* **101**, (1985).

96. Jordan, P.; Carmo-Fonseca, M. Molecular mechanisms involved in cisplatin cytotoxicity. *Cell Mol Life Sci* **57**, 1229–35 (2000).

97. Hashimoto, S.; Koji, T.; Kohara, N.; Kanematsu, T.; Nakane, P. Frequency of apoptosis relates inversely to invasiveness and metastatic activity in human colorectal cancer. *Virchows Arch.* **431**, 241–8 (1997).

98. O'Connor, M. Targeting the DNA Damage Response in Cancer. *Mol Cell* **60**, 547–60 (2015).

99. Kauffmann, A.; Rosselli, F.; Lazar, V.; Winnepenninckx, V.; Mansuet-Lupo, A.; PDessen.; van den Oord, j.; Spatz, A.; Sarasin, A. High expression of DNA repair pathways is associated with metastasis in melanoma patients. *Oncogene* **27**, 565–573 (2008).

100. Jungmichel, S. *et al.* Proteome-wide identification of poly(ADP-Ribosyl)ation targets in different genotoxic stress responses. *Mol. Cell* **52**, 272–285 (2013).

101. Beneke, S. Regulation of chromatin structure by poly(ADP-ribosyl)ation. *Front. Genet.* **3**, 1–16 (2012).

102. Jacobson, MK.; Alvarez-Gonzale, R. Characterization of Polymers of Adenosine Diphosphate Ribose Generated in Vitro and in Vivo. *Biochemistry* **26**, 3218–3224 (1987).

103. Schreiber, V.; Dantzer, F.; Amé, JC.; De Murcia, G. Poly(ADP-ribose): Novel functions for an old molecule. *Nat. Rev. Mol. Cell Biol.* **7**, 517–528 (2006).

104. Zaffini, R.; Gotte, G.; Menegazzi, M. Asthma and poly(ADP-ribose) polymerase inhibition: A new therapeutic approach. *Drug Des. Devel. Ther.* **12**, 281–293 (2018).

105. Gibson, G. . B. A. . W. L. New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nat. Rev. Mol. Cell Biol.* **13**, 411–424 (2012).

106. Celeste, A. *et al.* Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat. Cell Biol.* **5**, 675–679 (2003).

107. Vousden, KH.; Lu, X. Live or let die: The cell's response to p53. *Nat. Rev. Cancer* **2**, 594–604 (2002).

108. Janssens, S.; Tschopp, J. Signals from within: The DNA-damage-induced NF-κB response. *Cell Death Differ.* **13**, 773–784 (2006).

109. Stilmann, M.; Hinz, M.; Arslan, SÇ.; Zimmer, A.; Schreiber, V.; Scheidereit, C. A Nuclear Poly(ADP-Ribose)-Dependent Signalosome Confers DNA Damage-Induced IκB Kinase Activation. *Mol. Cell* **36**, 365–378 (2009).

110. Bailey, S. T. *et al.* NF-κB activation-induced anti-apoptosis renders HER2-positive cells drug resistant and accelerates tumor growth. *Mol. Cancer Res.* **12**, 408–420 (2014).

111. Espinosa, L.; Bigas, A.; Mulero, M. C. Novel functions of chromatin-bound IκBα in oncogenic transformation. *Br. J. Cancer* **111**, 1688–1692 (2014).

112. Xia, L.;Lin, Ji.;Wang, H.;Oyang, Li.;Liu, L.;Su, Mi.;Wang, H.; Cao, D. Role of the NF κ B-signaling pathway in cancer. *Onco Targets Ther* 2063–2073 (2018).

113. Kinpara, S. *et al.* Involvement of double-stranded RNA-dependent protein kinase and antisense viral RNA in the constitutive NFκB activation in adult T-cell

leukemia/lymphoma cells. *Leukemia* **29**, 1425–1429 (2015).

114. He, H.;Chang, R.;Zhang, T.; Yang, C.; Kong, Z. ATM mediates DAB2IP-deficient bladder cancer cell resistance to ionizing radiation through the p38MAPK and NF-κB signaling pathway. *Mol. Med. Rep.* **16**, 1216–1222 (2017).

115. Pak, C.; Miyamoto, S. A new alpha in line between KRAS and NF-κB activation? *Cancer Discov* **3**, 613–615 (2013).

116. Blonska, M.;Lin, X. NF-κB signaling pathways regulated by CARMA family of scaffold proteins. *Cell Res.* **21**, 55–70 (2011).

117. Kwon, H. Choi, GE.; Ryu, S.; Kwon, SJ.; Kim, SC.; Booth, C.; Nichols, K.; Kim, H. Stepwise phosphorylation of p65 promotes NF-k B activation and NK cell responses during target cell recognition. *Nat. Commun.* **7**, (2016).

118. Vlantis, k.; Wullaert, A.; Polykratis, A., Kondylis, V.; Dannappel, M.; Schwarzer, R.; Welz, P.; Corona, T.; Walczak, H.; Weih, F.; Klein, U.; Kelliher, M.; Pasparakis, M. NEMO Prevents RIP Kinase 1-Mediated Epithelial Cell Death and Chronic Intestinal Inflammation by NF-κB-Dependent and -Independent Functions. *Immunity* **44**, 553–567 (2016).

119. Neri, A.; Chang, C.; Lombardi, L.; Salina, M.; Corradini, P., Maiolo, AT.; Chaganti, RSK.; Dalla-Favera, R. B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. *Cell* **67**, 1075–1087 (1991).

120. Xia, Yi.;Shen, S.;Verma, I. NF-κB, an active player in human cancers. *Cancer Immunol. Res.* **2**, 823–830 (2014).

121. McNulty, SE.; del Rosario, R.; Cen, D.; Meyskens, FL Jr.; Yang, S. Comparative expression of NFkappaB proteins in melanocytes of normal skin vs. benign intradermal naevus and human metastatic melanoma biopsies. *Pigment Cell Res* **17**, 173–80 (2004).

122. Richmond, RL.; Shattuck-Brandt, A. Enhanced Degradationof I-kBalpha Contributes to Endogenous Activation of NF-icB in Hs294T Melanoma Cells. *Cancer Res.* **57**, (1997).

123. Liu, J.; Suresh Kumar, KG.; Yu, D.; Molton, SA.; McMahon, M.; Herlyn, M.; Thomas-Tikhonenko, A.; Fuchs, S. Oncogenic BRAF regulates beta-Trcp expression and NF-kappaB activity in human melanoma cells. *Oncogene* **26**, 1954–8 (2007).

124. Madonna, G.; Ullman, CD.; Gentilcore, G.; Palmieri, G.; Ascierto, P. NF-κB as potential target in the treatment of melanoma. *J. Transl. Med.* **10**, 53 (2012).

125. Adams, J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* **4**, 346–60 (2004).

126. Odot, J.; Albert, P.; Carlier, A.; Tarpin, M.; Devy, J.; Madoulet, C. In vitro and in vivo anti-tumoral effect of curcumin against melanoma cells. *Int J Cancer* **111**, 381–7 (2004).

127. Ianaro, A.; Tersigni, M.; Belardo, G.; Di Martino, S.; Napolitano, M.; Palmieri, G.; Sini, M.; De Maio, A.; Ombra, M.; Gentilcore, G.; Capone, M.; Ascierto, M.; Satriano, RA.; Farina, B.; Faraone-Mennella, M.; Ascierto, PA.; Ialenti, A. NEMO-binding domain peptide inhibits proliferation of human melanoma cells. *Cancer Lett* **274**, 331–6 (2009).

128. Croghan, GA.; Suman, VJ.; Maples, WJ.; Albertini, M.; Linette, G.; Flaherty, L.; Eckardt, J.; Ma, C.; Markovic, SN.; Erlichman, C. A study of paclitaxel, carboplatin and bortezomibin the treatment of metastatic malignant melanoma: a phase II consortium study. *Cancer* **116**, (2010).

129. Yang, J.; Amiri, KI.; Burke, JR.; Schmid, JA.; Richmond, A. BMS-345541 targets inhibitor of κB kinase and induces apoptosis in melanoma: Involvement of nuclear factor κB and mitochondria pathways. *Clin. Cancer Res.* **12**, 950–960 (2006).

130. Erstad, DJ.; CusackJr, J. Targeting the NF-κB Pathway in Cancer Therapy. *Surg. Oncol. Clin. N. Am.* **22**, 705–746 (2013).

131. Hassa, PO.; Hottiger, M. PARP-1 as Novel Coactivator of NF-κB in Inflammatory Disorders. in *Molecular Biology Intelligence Unit* 79–50 (2006).

132. Tentori, L.; Muzi, A.; Dorio, A.; Bultrini, S.; Mazzon, E.; Lacal, P.; Shah, G.; Zhang, Ji.; Navarra, P.; Nocentini, G.; Cuzzocrea, S.; Graziani, G. Stable depletion of poly (ADP-ribose) polymerase-1 reduces in vivo melanoma growth and increases chemosensitivity. *Eur. J. Cancer* **44**, 1302–1314 (2008).

133. Qingying, Z.; Yang, L.; Yanhao, L.; Zunzhen, Z. The role of NF-κB in PARP-inhibitor-mediated sensitization and detoxification of arsenic trioxide in hepatocellular carcinoma cells. *J. Toxicol. Sci.* **40**, 349–363 (2015).

134. Kwon, Mi.; Jang, H.; Kim, E.; Hye, R.; Jong, L. Efficacy of poly (ADP-ribose) polymerase inhibitor olaparib against head and neck cancer cells: Predictions of drug sensitivity based on PAR–p53–NF-κB interactions. *Cell Cycle* **15**, 3105–3114

(2016).

135. Bitler, Benjamin G.; Watson, Zachary L.; Wheeler, L. J. . PARP inhibitors: Clinical utility and possibilities of overcoming resistance. *Gynecol. Oncol.* **147**, 695–704 (2017).

136. Brown, JS.;Kaye, SB.;Yap, T. PARP inhibitors: The race is on. *Br. J. Cancer* **114**, 713–715 (2016).

137. Murai, J.; Huang, S.; Brata Das, B.; Renaud, A.; Zhang, Y.; Doroshow, J.; Ji, J.; Takeda, S.; Pommier, Y. Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. *Cancer Res.* **72**, 5588–99 (2012).

138. Frampton, J. Olaparib: A Review of Its Use as Maintenance Therapy in Patients with Ovarian Cancer. *BioDrugs* **29**, 143–150 (2015).

139. Menear, KA.; Adcock, C.; Boulter, R.; Cockcroft, X. . et al. 4-[3-(4-cyclopropanecarbonylpiperazine-1-carbonyl)-4-fluorobenzyl]-2H-phthalazin-1-one: a novel bioavailable inhibitor of poly(ADP-ribose) polymerase-1. *J Med Chem* **51**, 6581–91 (2008).

140. Hurley, R. *et al.* 53BP1 as a potential predictor of response in PARP inhibitor-treated homologous recombination-deficient ovarian cancer. *Gynecol. Oncol.* **153**, 127–134 (2019).

141. Jiang, Xuan; Li, Xiaoying; Li, Weihua; Bai, Huimin; Zhang, Z. PARP inhibitors in ovarian cancer: Sensitivity prediction and resistance mechanisms. *J. Cell. Mol. Med.* **23**, 2303–2313 (2019).

142. Jaspers, JE.; Kersbergen, A.; Boon, U.; Sol, W.; Van Deemter, L.; Zander, SA.; Drost, R. Wientjens, El.; Ji, J.; Aly, A.; Doroshow, JH.; Cranston, A.; Martin, N.; Lau, A.; O'Connor, MJ.; Ganesan, S.; Borst, Pi.; Jonkers, J.; Rottenberg, S. Loss of 53BP1 causes PARP inhibitor resistance in BRCA1-mutated mouse mammary tumors. *Cancer Discov.* **3**, 68–81 (2013).

143. Gogola, E.; Rottenberg, S.; Jonkers, J. Resistance to PARP Inhibitors: Lessons from Preclinical Models of BRCA-Associated Cancer. *Annu. Rev. Cancer Biol.* **3**, 235–254 (2019).

144. O'Connor, L.; Rulten, S.; Cranston, AN.; Odedra, R.; Brown, H.; Jaspers, JE.; Jones, L.; Knights, C.; Evers, B.; Ting, A. . *et al.* The PARP inhibitor AZD2461 provides insights into the role of PARP3 inhibition for both synthetic lethality and tolerability with chemotherapy in preclinical models. *Cancer Res.* **76**, 6084–6094

(2016).

145. Infante, J. ClinicalTrials.gov Identifier: NCT01247168.

146. Ashworth, J.; Christopher, A. PARP inhibitor combination therapy. *Crit. Rev. Oncol. Hematol.* **108**, 73–85 (2016).

147. Ardelt, W.; Mikulski, S. M.; Shogen, K. Amino acid sequence of an anti-tumor protein from Rana pipiens oocytes and early embryos. Homology to pancreatic ribonucleases. *J. Biol. Chem.* **266**, 245–251 (1991).

148. Mosimann, S.C.; Ardelt, W.; James, M. N. G. Refined 1·7 Å X-ray crystallographic structure of P-30 protein, an amphibian ribonuclease with anti-tumor activity. *J. Mol. Biol.* **236**, 1141–1153 (1994).

149. Rutkoski, TJ.; Raines, R. Evasion of Ribonuclease Inhibitor as a Determinant of Ribonuclease Cytotoxicity. *Curr Pharm Biotechnol* **9**, 185 (2008).

150. Gotte, G.; Menegazzi, M. Biological activities of secretory RNases: focus on their oligomerization to design antitumor drugs. *Front Immunol* (2019).

151. Ribó, M.; Benito, A.; Vilanova, M.; Beaumelle, B.; Ribó, M.; Benito, A.; Vilanova, M.; Beaumelle, B. Intracellular pathway of onconase that enables its delivery to the cytosol. *J. Cell Sci.* **120**, 1405–1411 (2007).

152. Leland, P A.; Raines, R. Cancer chemotherapy - Ribonucleases to the rescue. *Chem. Biol.* **8**, 405–413 (2001).

153. Iordanov, MS.; Ryabinina, OP.; Wong, J.; Dinh, YH.; Newton, DL.; Rybak, SM.; Magun, B. Molecular Determinants of Apoptosis Induced by the Cytotoxic RibonucleaseOnconase: Evidence for Cytotoxic Mechanisms Different from Inhibitionof Protein Synthesis. *Cancer Res.* **60**, 1983–1994 (2000).

154. Darzynkiewicz, Z.; Carter, S. P.; Mikulski, S. M.; Ardelt, W. J.; Shogen, K. Cytostatic and Cytotoxic Effects of Pannon (P-30 Protein), A Novel Anticancer Agent. *cell Prolif.* **21**, 169–182 (1988).

155. Wang, Xi.;Guo, Z. Recombinant expression, different downstream processing of the disulfide-rich anti-tumor peptide Ranpirnase and its effect on the growth of human glioma cell line SHG-44. *Biomed. reports* **1**, 747–750 (2013).

156. Smolewski, P. *et al.* Cytotoxic activity of the amphibian ribonucleases onconase and r-amphinase on tumor cells from B cell lymphoproliferative disorders. *Int. J. Oncol.* **45**, 419–425 (2014).

157. Fiorini, Cl.;Cordani, M.;Gotte, G.;Picone, D.;Donadelli, M. Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 549–560 (2015).

158. Goparaju, CM.; Blasberg, JD.; Volinia, S.; Palatini, J.; Ivanov, S.; Donington, JS.; Croce, C.; Carbone, M.; Yang, H.; Pass, H. Onconase mediated NFKβ downregulation in malignant pleural mesothelioma. *Oncogene* **30**, 2767–2777 (2011).

159. Choi, Y. *et al.* Platinum and PARP Inhibitor Resistance Due to Overexpression of MicroRNA-622 in BRCA1-Mutant Ovarian Cancer. *Cell Rep.* **14**, 429–439 (2016).

160. Nasu, M.; Carbone, M.; Gaudino, G.; Ly, BH.; Bertino, Pi.; Shimizu, D.; Morris, P.; Pass, HI.; Yang, H. Ranpirnase interferes with NF-κb pathway and MMP9 activity, inhibiting malignant mesothelioma cell invasiveness and xenograft growth. *Genes and Cancer* **2**, 576–584 (2011).

161. Mikulski, SM.; Ardelt, W.; Shogen, K.; Bernstein, EH.; Menduke, H. Striking increase of survival of mice bearing M109 Madison carcinoma treated with a novel protein from amphibian embryos. *J Natl Cancer Inst.* **82**, 151–153 (1990).

162. Mikulski, S.M.; Costanzi, J.J.; Vogelzang, N.J.; McCachren, S.; Taub, R.N.; Chun, H.; Mittelman, A.; Panella, T.; Puccio, C.; Fine, R.; Shogen, K. Phase II trial of a single weekly intravenous dose of ranpirnase in patients with unresectable malignant mesothelioma. *J Clin Oncol* **20**, 274–81 (2002).

163. ClinicalTrial.gov. ONCONASE Plus Doxorubicin Versus Doxorubicin Alone For Patients With Malignant Pleural or Peritoneal Mesothelioma Who Have Had No More Than One Prior Chemotherapy Regimen. *ClinicalTrials.gov Identifier: NCT00003034*

164. Vogelzang, NJ.; Aklilu, M.; Stadler, WM.; Dumas, MC.; Mikulski, S. A phase II trial of weekly intravenous ranpirnase (Onconase), a novel ribonuclease in patients with metastatic kidney cancer. *Invest New Drugs* **19**, 255–60 (2001).

165. Pavlakis, N.; Vogelzang, N. . Ranpirnase--an antitumour ribonuclease: its potential role in malignant mesothelioma. *Expert Opin Biol Ther* **6**, 391–9 (2006).

166. Mikulski, S. M.; Viera, A.; Darzynkiewicz, Z.; Shogen, K. Synergism between a novel amphibian oocyte ribonucléase and lovastatin in inducing cytostatic and cytotoxic effects in human lung and pancreatic carcinoma cell lines. *Br. J. Cancer*

66, 304–310 (1992).

167. Maack, T.; Johnson, V.; Kau, ST.; Figueiredo, J.; Sigulem, D. Renal filtration, transport, and metabolism of low-molecular-weight proteins: a review. *Kidney Int* **16**, 251–70 (1979).

168. Pfaffl, MW.; Horgan, GW.; Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**, 36 (2002).

169. Kaufmann, SH.; Desnoyers, S.; Ottaviano, Y.; Davidson, NE.; Poirier, G. Specific Proteolytic Cleavage of Poly (ADP-ribose) Polymerase : An Early Marker of Chemotherapy-induced Apoptosis Specific Proteolytic Cleavage of Poly (ADP-ribose) Polymerase : An Early Marker of. *Cancer Res* **53**, 3976–3985 (1993).

170. Jafri, MA.; Ansari, SA.; Alqahtani, MH.; Shay, J. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome Med* **8**, (2016).

171. Westekemper, H.; Freistuehler, M.; Bornfeld, N.; Steuhl, KP.; Scheulen, M.; Hilger, R. Chemosensitivity of conjunctival melanoma cell lines to target-specific chemotherapeutic agents. *Graefes Arch Clin Exp Ophthalmol* **251**, 279–84 (2013).

172. Pierard, GE.; Pierard-Franchimont, C. The proliferative activity of cells of malignant melanomas. *Am J Dermatopathol* **6**, 317–23 (1984).

173. Straume, O.; Sviland, L., Akslen, A. Loss of Nuclear p16 Protein Expression Correlates with Increased Tumor Cell Proliferation (Ki-67) and Poor Prognosis in Patients with Vertical Growth Phase Melanoma. *Mol. Oncol. Markers, Clin. Correl.* **6**, 1845–1855 (2000).

174. Mattia, G.; Puglisi, R.; Ascione, B.; Malorni, W.; Carè, A.; Matarrese, A. Cell death-based treatments of melanoma:conventional treatments and new therapeutic strategies. *Cell death Dis* **9**, 112 (2018).

175. Maverakis, E.; Cornelius, LA.; Bowen, GM.; Phan, T.; Patel, FB.; Fitzmaurice, S.; He, Y.; Burrall, B.; Duong, C.; Kloxin, AM.; Sultani, H.; Wilken, R.; Martinez, SR.; Patel, F. Metastatic melanoma - a review of current and future treatment options. *Acta Derm Venereol.* **95**, 516–24 (2015).

176. Al-Qatati, A.; Aliwaini, S. Combined pitavastatin and dacarbazine treatment activates apoptosis and autophagy resulting in synergistic cytotoxicity in melanoma cells. *Oncol Lett* **14**, 7993–7999 (2017).

177. Ghosh, A.; Saginc, G.; Leow, SC.; Khattar, E.; Shin, EM.; Yan, TD.; Wong, M.; Zhang, Z.; Li, G.; Sung, WK.; Zhou, J.; Chng, WJ.; Li, S.; Liu, E.; Tergaonkar, V. Telomerase directly regulates NF-κB-dependent transcription. *Nat Cell Biol* **14**, 1270–81 (2012).

178. Liu, Z.; Li, Q.; Li, K.; Chen, L.; Li, W.; Hou, M.; Liu, T.; Yang, J.; Lindvall, C., Björkholm, M.; Jia, J.; Xu, D. Telomerase reverse transcriptase promotes epithelial-mesenchymal transition and stem cell-like traits in cancer cells. *Oncogene2* **32**, 4203–13 (13AD).

179. Bernal, A.; Tusell, L. Telomeres: Implications for Cancer Development. *Int J Mol Sci* **19**, 294 (2018).

180. Forsyth, NR.; Wright, WE.; Shay, J. Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation* **69**, 188–97 (2002).

181. Wu, Y.; Mikulski, SM.; Ardelt, W.; Rybak, SM.; Youle, R. A cytotoxic ribonuclease. Study of the mechanism of onconase cytotoxicity. *J Biol Chem* **268**, (1993).

182. Lecona, E.; Capetillo, O. Targeting ATR in cancer. *Nat. Rev. Cancer* **18**, 586–595 (2018).

183. Jain, PG.; Patel, B. Medicinal chemistry approaches of poly ADP-Ribose polymerase 1 (PARP1) inhibitors as anticancer agents - A recent update. *Eur. J. Med. Chem.* **165**, 198–215 (2019).

184. Tentori, L.; Muzi, A.; Dorio, AS.; Scarsella, M.; Leonetti, C.; Shah, GM.; Xu, W.; Camaioni, E.; Gold, B.; Pellicciari, R.; Dantzer, F.; Zhang, J.; Graziani, G. Pharmacological inhibition of poly(ADP-ribose) polymerase (PARP) activity in PARP-1 silenced tumour cells increases chemosensitivity to temozolomide and to a N3-adenine selective methylating agent. *Curr Cancer Drug Targets* **10**, 368–83 (2010).

185. Fratangelo, F.; Camerlingo, R.; Carriero, MV.; Pirozzi, G.; Palmieri, G.; Gentilcore, G.; Ragone, C.; Minopoli, M.; Ascierto, PA.; Motti, M. Effect of ABT-888 on the apoptosis, motility and invasiveness of BRAFi-resistant melanoma cells. *Int J Oncol* **53**, 1149–1159 (2018).

186. Czyż, M.; Toma, M.; Gajos-Michniewicz, A.; Majchrzak, K.; Hoser, G.; Szemra, j J.; Nieborowska-Skorska, M.; Cheng, P.; Gritsyuk, D.; Levesque, M.; Dummer, R.,

Sliwinski, T.; Skorski, T. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. *Oncotarget2* **7**, 75551–75560 (2016).

187. Ardelt, W.; Ardelt, B.; Darzynkiewicz, Z. Ribonucleases as potential modalities in anticancer therapy. *Eur J Pharmacol* **625**, 181–9 (2009).

188. Dharwal, V.; Naura, A. PARP-1 inhibition ameliorates elastase induced lung inflammation and emphysema in mice. *Biochem Pharmacol* **150**, 24–34 (2018).

189. Tsai, SY.; Ardelt, B.; Hsieh, TC.; Darzynkiewicz, Z.; Shogen, K.; Wu, J. Treatment of Jurkat acute T-lymphocytic leukemia cells by onconase (Ranpirnase) is accompanied by an altered nucleocytoplasmic distribution and reduced expression of transcription factor NF-kappaB. *Int J Oncol2* **25**, 1745–52 (2004).

190. Levati, L.; Ruffini, F.; Muzi, A.; Umezawa, K.; Graziani, G.; D'Atri, S.; Lacal, P. Placenta growth factor induces melanoma resistance to temozolomide through a mechanism that involves the activation of the transcription factor NF-κB. *Int J Oncol* **38**, 341–7 (2011).

191. Moretti, S.; Pinzi, C.; Spallanzani, A.; Berti, E.; Chiarugi, A.; Mazzoli, S.; Fabiani, M.; Vallecchi, C.; Herlyn, M. Immunohistochemical evidence of cytokine networks during progression of human melanocytic lesions. *Int J Cancer* **84**, 160–8 (1999).

192. Pahl, H. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene1* **18**, 6853–66 (1999).

193. Luo, Q.; Li, Y.; Lai, Y.; Zhang, Z. The role of NF-κB in PARP-inhibitor-mediated sensitization and detoxification of arsenic trioxide in hepatocellular carcinoma cells. *J Toxicol Sci* **40**, 349–63 (2015).

194. Haferkamp, S.; Borst, A.; Adam, C.;Becker, TM.; Motschenbacher, S.; Windhövel, S.; Hufnagel, AL.; Houben, R.;Meierjohann, S. Vemurafenib induces senescence features in melanoma cells. *J. Invest. Dermatol.* **113**, 1601–1609 (2013).

195. Grichnik, JM.; Burch, JA.; Schulteis, RD.; Shan, S.; Liu, J.; Darrow, Tl.; Vervaert, CE.; Seigler, H. Melanoma, a tumor based on a mutant stem cell? *J Invest Dematol* **126**, 142–53 (2006).

196. Cordaro, FG.; De Presbiteris, AL.; Camerlingo, R.; Mozzillo, N.; Pirozzi, G.; Cavalcanti, E.; Manca, A.; Palmieri, G.; Cossu, A.; Ciliberto, G.; Ascierto, PA.; Travali, S.; Patriarca, EJ.; Caputo, E. Phenotype characterization of human melanoma cells resistant to dabrafenib. *Oncol Rep* **38**, 2741–2751 (2017).

197. Wagle, N.; Emery, C.; Berger, MF.; Davis, MJ.; Sawyer, A.; Pochanard, P.; Kehoe, SM.; Johannessen, CM.; Macconaill, LE.; Hahn, WC.; Meyerson, M.; Garraway, L. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol2* **29**, 3085–96 (2011).

198. Cvetanova, B.; Shen, YC.; Shyur, L. Cumingianoside A, a Phyto-Triterpenoid Saponin Inhibits Acquired BRAF Inhibitor Resistant Melanoma Growth via Programmed Cell Death. *Front Pharmacol* 10–30 (2019).

199. Calvani, M.; Bianchini, F.; Taddei, ML.; Becatti, M.; Giannoni, E.; Chiarugi, P.; Calorini, L. Etoposide-Bevacizumab a new strategy against human melanoma cells expressing stem-like traits. *Oncotarget* **7**, 51138–51149 (2016).

200. De Larco, J. E.; Todaro, G. J. Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 4001–4005 (1978).

201. Moro, N.; Mauch, C.; Zigrino, P. Metalloproteinases in melanoma. *Eur J Cell Biol2* **93**, 23–9 (2014).

202. Liu, J.; Li, X.; Huang, J.; Liu, Y. Matrix Metalloproteinase 2 Knockdown Suppresses the Proliferation of HepG2 and Huh7 Cells and Enhances the Cisplatin Effect. *Open Med* **14**, 384–391 (2019).

203. Philip, S.; Bulbule, A.; Kundu, G. Matrix metalloproteinase-2: Mechanism and regulation of NF-κB-mediated activation and its role in cell motility and ECM-invasion. *Glycoconj. J.* **21**, 429–441 (2004).

204. Raineri, A.; Prodomini, S.; Fasoli, S.; Gotte, G.; Menegazzi, M. Influence of onconase in the therapeutic potential of PARP inhibitors in A375 malignant melanoma cells. *Biochem. Pharmacol.* **167**, 173–181 (2019).

205. Raineri, A.; Fasoli, S.; Campagnari, R.; Gotte, G.; Menegazzi, M. Onconase restores cytotoxicity in dabrafenib-resistant A375 melanoma cells and affects cell migration, invasion and colony formation capability. *Int. J. Mol. Sci.* **20**, (2019).

206. Reck, M.; Krzakoski, M.; Jassem, J.; Eschbach, C.; Kozielski, J.; Costanzi, J.J.; Gatzemeier, U.; Shogen, K.; von Pawel, J. Randomized, multicenter phase III study of ranpirnase plus doxorubicin (DOX) versus DOX in patients with unresectable malignant mesothelioma (MM). *J. Clin. Oncol.* **27**, (2009).