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”Counteracting resistance to chemo-radiotherapy and to targeted therapy in solid tumors”

Francesco Sabbatino

Università degli Studi di Napoli Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

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Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore", CNR, Naples, Italy
Istituto Superiore di Oncologia, Italy

Italian Faculty

Salvatore Maria Aloj
Francesco Saverio Ambesi Impiombato
Francesco Beguinot
Maria Teresa Berlingieri
Bernadette Biondi
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Giuseppe Viglietto
Mario Vitale

“Counteracting resistance to chemo-radiotherapy and to targeted therapy in solid tumors”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1) Multidisciplinary Approach to Patient with Malignant Melanoma. Scarpati GD, Fusciello C, Sabbatino F, Ferrone S, Caponigro F, Perri F, Carlomagno C, Pepe S. Anticancer Agents Med Chem. 2012 Dec 14. In press.

2) Can the "Right" EGFR-Specific mAb Dramatically Improve EGFR-Targeted Therapy? Sabbatino F, Ferrone S. Clin Cancer Res. 2013;19:958-60.

3) p53 modulates the enhanced cytotoxicity induced by Poly(ADP-Ribose) Polymerase-1 inhibition to Topoisomerase-I inhibitor and radiotherapy in human glioblastoma cell lines. Sabbatino F, Fusciello C, Leonardi A, Pacelli R, Poudel R, Pepin D, Ferrone S, Carlomagno C, Pepe S. To be submitted.

LIST OF ABBREVIATIONS

AE	Adverse events
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promote
Bcl	B-cell lymphoma
BMF	Bcl-2-modifying factor
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRAF-I	BRAF inhibitors
CDK	Cyclin-dependent protein kinases
EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
FDG-PET	Fluorodeoxyglucose- positron emission tomography
GIST	Gastrointestinal Stromal Tumors
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HSP90	Heat shock protein 90
IL	Interleukin
IGF-1R	Insuline growth factor-1 receptor
KAs	keratoacanthomas
MCL	Myeloid cell Leukemia
Mcl-1	Myeloid cell leukemia sequence 1
MAPK	Mitogen-activated protein kinase
NSCLC	Non Small Cell Lung Cancer
OS	Overall survival
PARP1	Poly (ADP-ribose) polymerase 1
PCNA	Proliferating cell nuclear antigen
PDGFR	Platelet-derived growth factor receptor
PDL1	Programmed cell death ligand 1
PTEN	Tumor suppressor phosphatase and tensin homolog
RAF	Rapidly growing Fibrosarcoma
Rb	Retinoblastoma
RTK	Receptor tyrosine kinases
SCC	Cutaneous squamous cell carcinomas
SH2	Sequence homology 2
SOS	Son of sevenless
TA	Tumor antigen
TGFβ	Transforming growth factor beta
T reg	T cell regulators
VEGFR	Vascular endothelial growth factor receptor

INTRODUCTION

The resistance to anticancer drugs (cytotoxic compounds and molecular targeted agents) and radiotherapy is the main obstacle for an effective treatment of human cancers. In this study are presented several results regarding:

a) The molecular basis of resistance to treatment with novel inhibitor of the tyrosine-kinase encoded by the mutated form of the gene BRAF in melanoma carrying the mutant BRAF V600E and potential therapeutic strategies to counteract this resistance.
b) The role of specific inhibition of PARP1 enzyme to potentiate the cytotoxicity of chemo-radiotherapy in human glioblastoma cells.

The frequency of BRAF mutations varies in human cancers. In over 50% of metastatic melanoma patients melanoma cells harbor the BRAF V600E point mutation (T1799A). Clinical trials have demonstrated that the BRAF inhibitor PLX4032, now known as vemurafenib, and other inhibitors in its class (GSK2118436 or dabrafenib) can induce tumor regression in > 50% of patients with metastatic melanoma harboring the BRAF V600E mutation and increase the overall survival of metastatic melanoma patients as compared to dacarbazine. However in spite of the drug's high efficacy, melanomas develop resistance to the treatment with BRAF-I and there is the need to identify the molecular mechanisms underlying this resistance.

Another line of research is devoted to the study of the inhibition of the activity of a specific DNA-repair pathway mediated by the poly (ADP-ribose) polymerase 1 (PARP1) as a therapeutic strategy capable of significantly increasing the activity of conventional cytotoxic and radiation treatment in solid tumors. PARP-1 also known as NAD^+ ADP-ribosyltransferase 1 or poly[ADP-ribose] synthase 1 has a role in repair of single-stranded DNA breaks. Knocking down intracellular PARP1 levels with siRNA or inhibiting PARP1 activity with small molecules reduces

repair of DNA breaks. As PARP1 enzyme activity is stopped, when these breaks are encountered during DNA replication, the replication fork stalls, and double-strand DNA breaks accumulate. These double strand DNA breaks are repaired via homologous recombination repair. Inhibition of PARP1 activity is found highly toxic for cells that are deficient in BRCA1 or BRCA2 since their gene products are involved in homologous recombination repair. To date, PARP1 inhibitors are studied as specific treatment for BRCA deficient cancers (i.e. BRCA 1 -/- breast and ovarian cancer). However several forms of cancer seem to be more dependent on PARP1 than regular cells. These findings makes PARP1 as an attractive target for cancer therapy to be used as single agents or in combination with conventional strategies such as chemotherapeutic agents or radiotherapy causing DNA damage.

ABSTRACT

Control of mutant BRAF (V600E) metastatic melanomas by the selective BRAF inhibitor (BRAF-I), vemurafenib, is limited by the the lack of complete response due to intrinsic resistance and the often rapid development of acquired resistance . By utilizing melanoma cell lines with acquired BRAF-I resistance, we demonstrate for the first time an association between BRAF-I resistance and PDGFRα upregulation *in vitro* and *in vivo*. PDGFRα inhibition by PDGFRα-specific shRNA restores melanoma cells' sensitivity to BRAF-I *in vitro*. These effects are mediated by inhibition of ERK and AKT activation, which is associated with BRAF-I resistance. Combining vemurafenib with a PDGFRα inhibitor (sunitinib or imatinib) *in vitro* and *in vivo* demonstrate a significantly greater anti-proliferative and pro-apoptotic effect than either agent individually. These effects reflect the inhibition of ERK and AKT activation, which inhibits the proliferation and induces apoptosis. We corroborated this finding by demonstrating PDGFRα upregulation in melanomas harvested from patients who demonstrated disease progression following treatment with BRAF-I. Furthermore, analysis of matched biopsies of BRAF-I treated melanoma patients before treatment, after 1-2 weeks of treatment and at the time of disease progression demonstrated that PDGFRα upregulation correlated with less tumor regression (based on RECIST criteria) and a shorter time to disease progression. Our results demonstrate that PDGFRα inhibitors (sunitinib or imatinib) in combination with vemurafenib can overcome BRAF-I resistance mediated by PDGFRα. Although we could not associate baseline PDGFRα with clinical outcome, our data suggest that monitoring patients for early up regulation of PDGFRα may identify those for whom combination therapy would be most appropriate.

1. BACKGROUND

1.1. Melanoma.

Melanoma is an aggressive form of skin cancer arising from malignantly transformed melanocytes[1]. The incidence and mortality from melanoma are currently rising, placing significant demands on healthcare provision and representing a major public health issue[2]. The reasons for the higher incidence of melanoma remain unclear but increased exposures to sun or ultraviolet radiation are some of the major risk factors. Family history of melanoma, genetic susceptibility, environmental factors, and age-related immunosuppressions are also some of the contributing factors that could influence the incidence rates[3, 4].

In many cases melanoma begins with the transformation of a benign nevus that develops into a dysplastic lesion before progressing into a radial and vertical-growth phase that can invade into the dermis, regional lymph nodes, and from there disseminates to distant organs, leading to metastatic melanoma[4]. However, not all melanomas arise from nevus and many arise through direct transformation of normal skin cells[3].

While surgical excision is still the standard of care for the treatment of primary melanomas, until 2011 only chemotherapy with dacarbazine and immunotherapy with high dose of IL-2 were approved by U.S. Food and Drug Administration (FDA) for metastatic melanoma treatment although both of these did not demonstrate to increase the overall survival (OS) of metastatic melanoma patients[5-7]. However in the last 10 years significant advances have been facilitated by an improved understanding of the driving genetic aberrations of melanoma. Molecular profiling and genome sequencing have shown that melanomas are a heterogeneous and complex group of malignancies whose progression is driven by distinct patterns of oncogenic mutation[8-12].

The discovery of activating mutations in the serine/threonine kinase BRAF (v-raf murine sarcoma viral oncogene homolog B1) in approximately 50% of all

melanomas[8] has led to the development of novel targeted therapy which, in less than 10 years, has led to the U.S. FDA approval of the BRAF inhibitors (BRAF-I)[13-15]. These agents have provided a strong improvement in objective responses and in overall survival of melanoma patients carrying mutant BRAF. However like other oncogene directed therapies, such as imatinib in Myeloid cell Leukemia (MCL) and Gastrointestinal Stromal Tumors (GIST)[16, 17] or erlotinib in Non Small Cell Lung Cancer (NSCLC)[17], treatment with BRAF-I is effective for a limiting time and complete responses are infrequently seen due to onset of resistance. Therefore identification and characterization of the mechanisms mediating BRAF-I resistance is now need and essential for the rational design of novel targeted strategies to improve melanoma patient responses and survival.

1.2. Biology of mutated BRAF and its role in melanoma development and progression.

BRAF is a member of the RAF (Rapidly growing Fibrosarcoma) family of serine/threonine kinases. The RAF family includes three members: ARAF, BRAF, and CRAF. All RAF proteins are part of the RAS/RAF/MEK/ERK mitogen-activated protein kinase (MAPK) signaling pathway (**Fig. 1**) which is constitute by all serine/threonine-specific protein kinases[18]. This signaling cascade promotes proliferation, survival, and invasion by linking cell surface growth factor receptors (such a receptor tyrosine kinases [RTKs]) to the transcription of genes involved in cell cycle progression and anti apoptotic activity[19]. Canonical activation of the MAPK pathways occurs when growth factors bind the corresponding growth factor receptors which lead to the activation of a RAS family member, any of the three isoform H/N/or KRAS. Subsequent to RAS activation, RAF isoforms are activated by the interaction of RAS with the RAS binding domain on RAF[20-22].

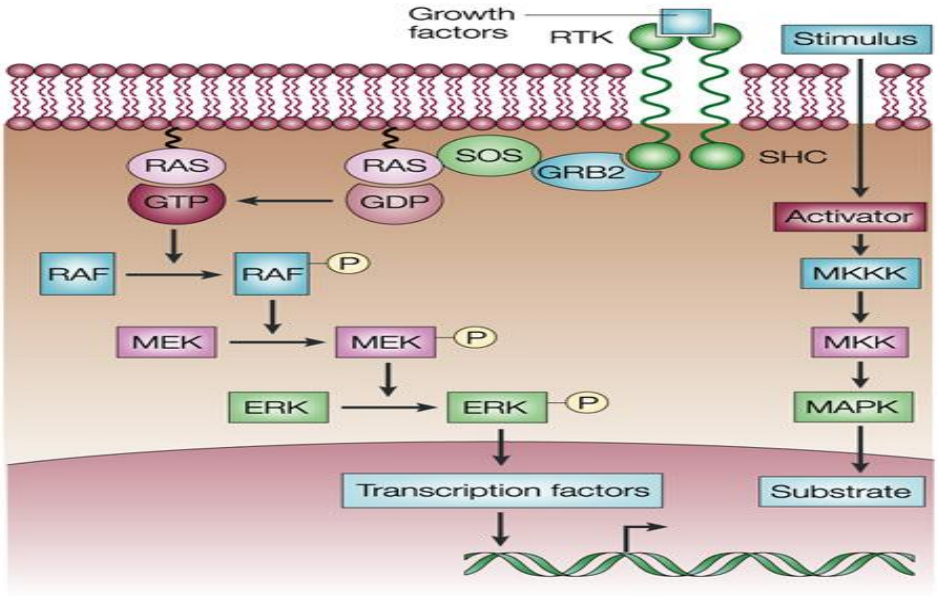


Figure 1. MAPK pathway. The MAPKs are part of the phospho-relay system that translate a plethora of extracellular signals into diverse cellular responses. A simplified MAPK signalling module is illustrated here with the RAS–RAF–MEK–ERK pathway. After binding of ligands, such as growth factors, to their respective RTK, receptor dimerization triggers the intrinsic tyrosine-kinase activity. This is followed by autophosphorylation of specific tyrosine residues on the intracellular portion of the receptor. These phosphorylated tyrosine residues then bind the sequence homology 2 (SH2) domains of adaptor proteins such as GRB2. Such a complex formation recruits SOS (son of sevenless), a cytosolic protein, into close proximity to RAS on the plasma membrane. Like other G proteins, RAS (HRAS, NRAS and KRAS) cycles between the GDP-bound inactive form and the GTP-bound active form. In the quiescent state, RAS exists in the GDP-bound form. The binding of SOS to RAS causes a change in the RAS conformation and leads to the dissociation of GDP and binding of GTP. GTP-bound RAS is the activator of this signaling module. It initiates the signal cascade by phosphorylating a RAF (CRAF, ARAF and BRAF). RAF, in turn, phosphorylates the MEK (MEK1 and MEK2), which then phosphorylates the ERK (extracellular signal regulated kinases, ERK1 and ERK2). Activated ERKs then translocate into the nucleus where they phosphorylate specific substrates that are involved in the regulation of cellular responses [23].

In cells without mutations in BRAF, RAF activation occurs only after the formation of homo- or hetero-dimers (between ARAF, BRAF or CRAF)- RAF activation leads to the phosphorylation of MEK which activates ERK through its phospshorylation[24, 25].

In melanoma activation of MAPK signaling by oncogenic mutations is found in up to 90% of cases. BRAF is the most common oncogene to be mutated in the MAPK signaling in melanoma with approximately 60% of all melanomas harbor activating mutations in BRAF. Although over 50 distinct mutations in BRAF gene have been identified, more than 80% of mutations in BRAF result in the substitution of valine to glutamic acid at amino acid 600 (BRAF V600E mutation)[8, 9, 26, 27]. Other point common mutations identified at same level include substitution of valine to an aspartic acid (V600D) or a lysine (V600K) or an arginine (V600R). The BRAF V600D, V600K and V600R mutations account for 16% and 3% of the BRAF mutations[28]. Mutations of BRAF cause the destabilization of the inactive conformation of the BRAF kinase which shifts the equilibrium to the active state[29]. Activated BRAF in turn activates the downstream components of the MAPK pathway such as MEK and ERK (**Fig. 2**).

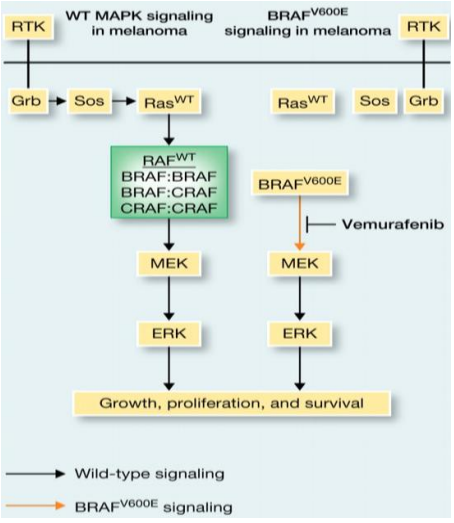


Figure 2. MAPK pathway in BRAF V600E mutant melanoma. A preponderance of melanomas are driven by MAPK signaling. Under wild-type conditions (left panel), RAF isoforms dimerize upon upstream stimulation of receptor tyrosine kinases and signaling through RAS to RAF. RAF dimers signal through MEK and ERK, eventually leading to downstream growth, proliferation, and survival. In BRAF V600E mutant melanoma (right panel), signaling between RAS and RAF is disconnected because of the constitutive, oncogenic signaling by mutant BRAF[30].

Several mechanisms mediated by MEK and ERK activation have been demonstrated to drive cellular proliferation and oncogenic activity in melanoma.

1) Signaling through the MAPK pathway determines up-regulation of Cyclin D1 expression and down-regulation of the cell cycle inhibitor p27[31]. These alterations cause deregulation of Cyclin/ cyclin-dependent protein kinases (CDK) complex which promotes tumor progression through the G_1-S phase of the cell cycle.

2) MAPK activation by BRAF increases melanoma cell survival by regulating the expression and function of a number of pro apoptotic and anti apoptotic proteins such as BIM, Bcl-2-modifying factor (BMF), Bcl-2-associated death promote (BAD), and myeloid cell leukemia sequence 1 (Mcl-1)[32-35]. Phosphorylation of BIM at Ser69 by ERK activity leads to proteasomal degradation of BIM and inhibition of its activity binding to pro survival proteins of the B-cell lymphoma 2 (Bcl-2) family such as Bcl-2, Bcl-w, Bcl-XL, and Mcl-1[36, 37]. MAPK activation also regulate anti apoptotic signals by controlling the stability and inhibiting the degradation of Mcl-1 expression by its phosphorilation on Thr163 residue[33].

3) Constitutive activity in the BRAF/MEK/ERK pathway in melanoma drives the invasive and motile behavior of melanoma cells through the reorganization of the cytoskeleton, activation of the cells' migratory machinery and up-regulation of matrix metalloproteinase expression[38, 39]. Mechanistically, mutated BRAF down-regulates the expression of the cyclic GMP phosphodiesterase PDE5A, which facilitates melanoma cell invasion by increasing intracellular levels of cGMP. These alterations lead to the release of cytosolic calcium and the phosphorylation of myosin light chain 2 which favorite the motility and invasiveness of melanoma cells[40].

4) BRAF oncogene and MAPK activation regulates the interaction of melanoma cells with the host microenvironment by alteration of human leukocyte antigen (HLA) Class I antigens, programmed cell death ligand 1 (PDL1) and melanoma tumor antigen (TA) expression. These alterations lead to the lack of recognition of

tumor cells by the immune cells and inhibition of cytotoxic T cell activation[41-44]. Furthermore BRAF oncogene by ERK activation increases the production of immunosuppressive cytokines such as Interleukin 10 (IL-10) and transforming growth factor beta (TGFβ) by melanoma cells. Increased secretion of IL-10 and TGFβ leads to increase number of T cell regulators (T reg) in the tumor microenviroment which in turn inhibits cytotoxic T cell activation[45].

These evidences demonstrate that mutant BRAF is oncogenic and drives tumor cell proliferation, anti apoptotic signals and tumor escape mechanisms. However it is noteworthy that presence of BRAF V600E is not sufficient to develop spontaneous melanoma. *In vivo* studies demonstrated that transgenic mice carrying BRAF V600E develop spontaneous melanomas only in presence of concomitant inactivation of the tumor suppressor phosphatase and tensin homolog (PTEN). The latter is a tumor suppressor gene which regulates the activation of AKT also known as Protein Kinase B, a component of the major prosurvival signaling pathway. Thus, although mutated BRAF may be an initiating factor in melanomagenesis, its alteration is not sufficient to lead to malignancy and other co-operating events are also required for melanoma development.

1.3. Inhibition of BRAF V600E.

Because of the relatively high frequency of mutations (approximately 50%) as well as its oncogenic potential, BRAF has represented a prime therapeutic target. Sorafenib is the first drug to have been investigated in inhibiting BRAF and the melanoma growth[46]. Besides RAF including BRAF wild type and BRAF mutated, sorafenib inhibits tyrosine kinases such as the vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR3, platelet-derived growth factor receptor (PDGFR), p38 MAPK, FLT, cKIT, FMA, and RET[47]. *In vitro* studies demonstrated that sorafenib abrogated the MAPK signaling and inhibited the growth of melanoma cells. However early clinical trials failed to show any activity

of sorafenib as monotherapy or in combination with standard chemotherapy in patients with metastatic melanoma even when patients were selected for their BRAF mutational status[47]. It is supposed that sorafenib failure reflects the lack in targeting selectively mutant BRAF. This effect caused intolerable off-target side effects through inhibition of wild type BRAF and other target proteins.

Although sorafenib has failed in the treatment of BRAF mutant melanoma, during the last few years novel agents have been proven to be effective in targeting BRAF V600E melanoma. The latest generation of BRAF-I has offered a significant improvement over sorafenib in potency against mutant BRAF and far fewer off-target effects. Compounds that have been evaluated pre-clinically include AZ628, XL281, GDC-0879, SB590885, GSK2118436, PLX4032 and its analog PLX4720 [48, 49]. So far, PLX4032 (vemurafenib) and GSK2118436 (dabrafenib) are the most extensively studied BRAF-I both preclinically and clinically.

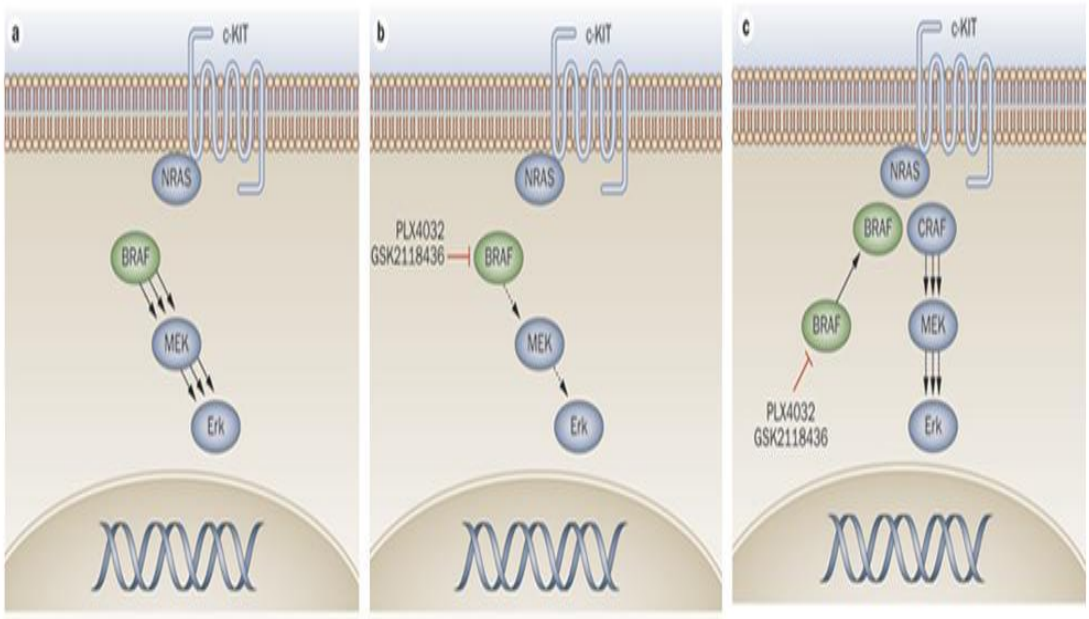
Vemurafenib is an orally available adenosine triphosphate (ATP)-competitive RAF inhibitor that potent inhibits BRAF V600E [drug concentration required for 50% inhibition of BRAF V600E kinase activity (IC50), 31 nM; CRAF IC50, 48 nM; wild type BRAF IC50, 100 nM]. Biochemical assays showed that vemurafenib exhibits selectivity against a broad range of kinases but the vast majority of them is inhibited by doses of vemurafenib much higher than the clinically achievable drug concentrations[49]. In *in vitro* and *in vivo* studies treatment with vemurafenib demonstrated to inhibit the growth of melanoma cell lines harboring not only BRAF V600E mutations[50] but also BRAF V600D, V600R and V600K mutations[51]. Analysis of signaling pathways showed that the growth inhibitory effects induced by vemurafenib are mediated by the inactivation of the RAF-MEK-ERK pathway[52], the induction of G1-phase cell cycle arrest and the increase of the fraction of apoptotic cells correlated with increased BIM expression[50, 53]. When tested in melanoma patients vemurafenib has demonstrated to be an effective treatment for metastatic melanoma patients carrying mutant

BRAF. In a phase I study, treatment with vemurafenib demonstrated substantial tumor regression in 81% of patients with metastatic melanoma who had a BRAF V600E mutation[54]. In all cases objective responses were associated with a strong inhibition of proliferation as demonstrated by the inhibition of intra-tumoral ERK activation, reduction of Ki-67 positivity, and inhibition of glucose uptake as measured by FDG-PET. In a phase III randomized control trial (BRIM3) BRAF V600E melanoma patients were randomized to receive vemurafenib or dacarbazine. Analysis of the clinical data demonstrated that treatment with vemurafenib improved response rate (48% versus 5%), progression free survival (5.3 versus 1.6 months), and increased the percent of patients alive at six months (84% versus 64%) with a reported 75% of reduction in risk of death[13].

Dabrafenib as well as vemurafenib is an orally available ATP-competitive inhibitor that selectively inhibits the BRAF V600E kinase[14]. The drug concentration required for 50% inhibition of BRAF V600E kinase activity (IC50) is five times lower than that for BRAF wild type or CRAF[49]. Preclinical data demonstrated that dabrafenib inhibits the MAPK pathway in BRAF V600E melanoma cells, leading to decreased proliferation and regression in xenograft mouse models. Although more clinical data are available for vemurafenib than dabrafenib, the latter demonstrates a comparable activity to vemurafenib in melanoma patients. In phase I study the response rates in patients treated with dabrafenib was comparable to that achieve using vemurafenib[55]. Initial results of phase III trial (BREAK3) demonstrated that treatment with dabrafenib compared to dacarbazine in melanoma patients carrying BRAF V600E improved response rates (50% versus 6%) and progression free survival (6.7 versus 2.9 months). However OS data of this trial are not yet mature[14].

Vemurafenib and dabrafenib are characterized not only for their high clinical efficacy in BRAF V600E melanoma patients but also for their uncommon toxicity. The most frequent adverse events (AEs) with selective BRAF-I include arthralgia,

rash, nausea, photosensitivity, fatigue, pruritus and palmar-plantar dysesthesia. However the most important toxicity related to BRAF-I is accelerated growth of cutaneous squamous cell carcinomas (SCC) and keratoacanthomas (KAs)[13, 47]. The development of these skin lesions has been attributed to a differential effect of BRAF-I on the MAPK pathway in cells carrying BRAF wild type. This phenomenon has been coined "paradoxical activation" of MAPK signaling. *In vitro* studies have shown that a paradoxical activation of MAPK signaling arose in cells carrying both BRAF wild-type and oncogenic RAS mutations or upstream constitutive RTK activity (such as in HER2) after treatment with BRAF-I[56-59]. This paradoxical activation of MAPK signaling seems to be caused by the differential ability of BRAF-I to inhibit BRAF and CRAF. The high inhibition of wild type BRAF compared to the low inhibition of CRAF by BRAF-I causes MERK and ERK activation via CRAF which can be activated by upstream components of MAPK signaling such as oncogenic RAS mutations or activated RTK (**Fig. 3**)[56].



BRAF inhibitors. a) Signal transduction downstream of oncogenic BRAF in the setting of activating mutations, **b)** inhibition of ERK/MAPK signaling with class BRAF inhibitors. **c)** Paradoxical CRAF activation by BRAF. Upstream signaling from oncogenic RTK or RAS results in preferential recruitment of CRAF to the plasma inner membrane, which transduces oncogenic RAS signaling through the MAPK pathway. In this case, wild type BRAF remains cytosolic in an inactive conformation. In the presence of BRAF-I, the inhibited wild-type BRAF is recruited to the plasma membrane by RAS, where it serves as a scaffold to enhance CRAF heterodimer activity and consequently increase MAPK pathway signaling output. This would not happen with RAF inhibitors with similar activity against CRAF and BRAF, where inhibition of both isoforms would inhibit MAPK signaling [60].

It is noteworthy that HRAS mutations are commonly found in SCC and in KAs. Analysis of a large cohort of KAs from melanoma patients treated with BRAF-I demonstrated their significant enrichment for RAS mutations[61]. The pre-existence of keratinocytes harboring HRAS mutations as upstream activator of CRAF provides the perfect context for a growth stimulatory effect to produce the SCC with KA features which are observed in patients treated with selective BRAF-I.

1.4. Limitations of BRAF-I.

BRAF-I such as vemurafenib and dabrafenib have been demonstrated to be effective in the treatment of BRAF mutant melanoma patients. However like many other oncogene directed therapies, such as imatinib in MCL and GIST or erlotinib in NSCLC, treatment with BRAF-I is limited in time (5.3 months) and complete responses are infrequently seen (only 5%) due to onset of resistance.

Resistance to targeted agent might be caused by clonal selection of pre existing cells (tumor heterogeneity) which carrying different genetic and biologic properties. These cells which do not respond to the treatment support tumor growth, invasion, and metastasis and determine lack of complete response to target therapy. On the other hand resistance to targeted agent might be caused by the presence of additional alterations in tumor cells which also can drive tumor growth but coexist with the targeted alterations. These additional alterations are present before starting the treatment and determine a decreased sensitivity to the targeted agent (intrinsic resistance). Lastly, new alterations in oncogene or oncosuppressor genes, which will coexist with targeted alteration, might occur when the treatment is already started. These new alterations drive new proliferative and antiapoptotic signals and determine the lack of sensitivity to the targeted agent (acquired resistance).

Conflicting data have been published about the tumor heterogeneity of BRAF V600E melanoma. Both intra- and inter-tumor heterogeneity in BRAF V600E expression in melanoma cells isolated from different regions of the primary lesions have been described[62]. BRAF V600E positive melanoma cells have been described to coexist in primary lesions with tumor cells harboring wild type BRAF. Furthermore, although clinical data demonstrated that BRAF V600E and NRAS mutations are mutually exclusive[63], BRAF V600E melanoma cells and NRAS mutated melanoma cells have been also described to coexist in primary and in metastatic melanoma [62, 64, 65].

On the other hand many alterations have been identified to drive intrinsic and acquired BRAF-I resistance. In both cases BRAF-I resistance is likely to be caused by MAPK-dependent and/or -independent pathway activation (**Fig. 4**).

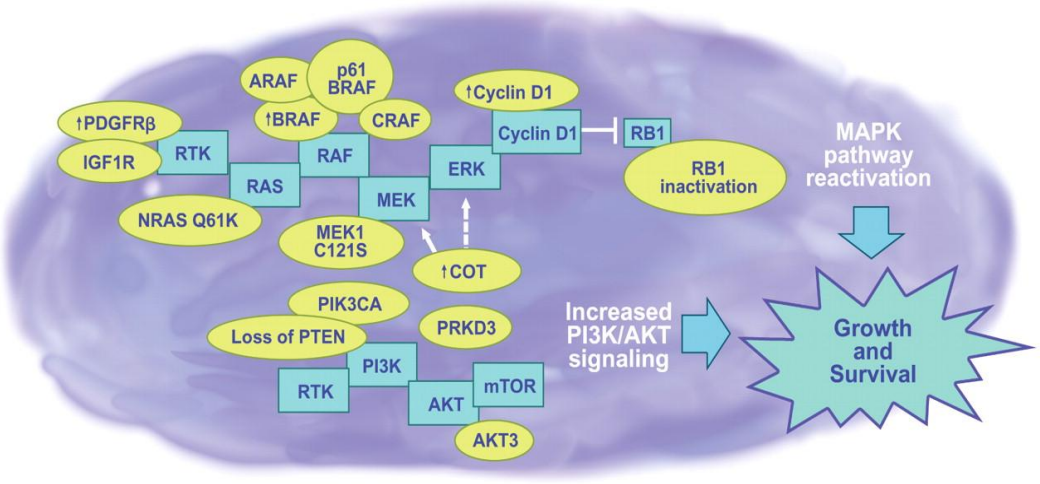


Figure 4. Mechanisms of BRAF-I resistance in BRAF-mutated melanomas. Several mechanisms (shown in yellow balloons) have been identified to drive BRAF-I resistance in BRAF-mutated melanomas. They include PDGFRβ and IGF1R receptor tyrosine kinase signaling, secondary NRAS mutations, BRAF V600E amplification, BRAF V600E p61 splice variant, RAF isoform signal switching, $MEK1^{C121S}$ mutation, COT amplification, increased AKT activity, loss of PTEN, PRKD3, amplified cyclin D1, and RB1 inactivation. The central theme of BRAF-I escape is the reactivation of MAP kinase and/or increased PI3K/AKT/mTOR signaling (skeleton pathways shown in blue boxes), which leads to melanoma growth and survival. As a further complication to the incredibly diverse resistance landscape, it has been demonstrated that multiple mutations, and possibly others that have yet to be identified, can occur within the same melanoma tumor or cell line, resulting in intratumor heterogeneity[66].

1.4.1. Intrinsic resistance to BRAF-I.

An intrinsic resistance to BRAF-I has been reported in both preclinical and clinical studies. *In vitro* experiment demonstrated that a significant proportion of BRAF V600E mutated melanoma cell lines had different sensitivity to the growth inhibitory effect of BRAF-I with a wide range of IC50 values for vemurafenib and

other BRAF-I [35, 67, 68]. It is known that melanomas carry a mutational profiles with multiple alterations in many genes including MITF, AKT3, COT, Cyclin D1, CDK2, CDK4, and retinoblastoma (Rb). However is still unclear how some of these alterations can modulate the biological behavior of melanoma cells and the response to BRAF-I.

Alteration of Cyclin D1 have been shown to decrease the cytotoxic sensitivity to BRAF-I[69]. Cyclin D1 is a protein that in humans is encoded by the CCND1 gene. The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. Specifically cyclins are positive regulators of CDK which represent the catalytic subunit of the Cyclin/CDK complex. Negative regulators of Cyclin/CDK complex are represented by serine/threonine kinases such as p21 and p16. In case of Cyclin D1, this protein forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity are required for cell cycle G1/S transition. Cyclin D1/CDK4 complex induces G1/S transition by phosphorylation of tumor suppressor Rb gene product which plays a major role in preventing the cell from replicating damaged DNA during G1 into S phase transition. Rb binds and inhibits transcription factors of the E2F family, which are composed of dimers of an E2F protein and a DP protein. The latter complex when is activated by Rb release, by transcripting the DNA, pushes a cell into S phase. Furthermore when E2F is free it activates factors like cyclins (e.g. Cyclin E and A), which push the cell through the S phase of cell cycle by activating CDK, and a molecule called proliferating cell nuclear antigen, or PCNA, which speeds DNA replication and repair by helping to attach polymerase to DNA and become active. As long as E2F-DP is inactivated, the cell remains stalled in the G1 phase. When Rb is bound to E2F, the complex acts as a growth suppressor and prevents progression through

the cell cycle. The Rb-E2F/DP complex also attracts a histone deacetylase (HDAC) protein to the chromatin, reducing transcription of S phase promoting factors, further suppressing DNA synthesis. Cyclin D1/CDK4 complex by phosphorylation of Rb allows E2F-DP to dissociate from Rb. In several tumors mutations, amplification and overexpression of Cyclin D1 have been observed. Cyclin D1 amplification has been described in clinical samples and in melanoma cell lines simultaneously with BRAF V600E mutation[69]. As previously described, in mutant BRAF V600E melanoma cells tumor growth is associated with uncontrolled cell cycle progression mediated by MAPK activation. MAPK activation leads to increasing Cyclin D1 expression. The inhibition of BRAF signaling by BRAF-I leads to the attenuation of the MAPK signaling, attenuation of Cyclin D1 expression, and arrest of cell cycle in the G1-phase. Therefore, an independent Cyclin D1 overexpression leads to intrinsic resistance to BRAF-I by facilitating cell cycle entry even when BRAF is inhibited. Similar alterations in the Cyclin/CDK complex such as aberrations in CDK4 or alterations in its negative regulators such as p16 or p53 have been also described[70] as well as alterations in Rb[71].

An intrinsic resistance to BRAF-I, have been also described to be mediated by alterations of PTEN gene[35, 71]. PTEN alterations have been reported in more than 30% and 50% of BRAF V600E melanomas and patients with PTEN alterations have been shown to display a decreased response rate to BRAF-I. PTEN is a tumor suppressor gene which acts through its phosphatase protein product in regulating cell cycle and survival. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. Specifically, PTEN negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating the PI3K/AKT signaling pathway. In absence of PTEN activity the PI3K/AKT pathway is iperactivated and leads to increased survival of tumor cells. This effect

is mediated by suppression of the pro-apoptotic protein BAD which is phosphorilated and subsequently inactivated by AKT at Ser99. Phosphorilation of BAD prevents its binding to Bax, a proapoptotic proteins, and relieves the antagonism of Bax on Bcl-2 and Bcl-XL which act as anti-apoptotic proteins[34]. In addition increased AKT signaling suppresses the expression of oncosuppressor gene product, BIM. Its phosphorylation by AKT determines the nuclear export of the transcription factor FOXO3a which regulates several oncosuppressor genes[35]. Therefore the decreased sensitivity to BRAF-I, when inactivating mutations of PTEN are present, is mediated by an increased AKT mediated survival of melanoma cells. In BRAF V600E melanoma similar findings have been also described for upregulation of the insuline growth factor-1 receptor (IGF-1R)[72], a RTK. Its activation leads to an increased AKT activation by activation of its upstream components PI3K which as well as PTEN alteration determine an intrinsic BRAF-I resistance.

Lastly, in BRAF V600E melanoma an intrinsic resistance to BRAF-I have been described to be mediated by alteration of tumor microenvironment[73]. In this case a decreased sensitivity to BRAF-I is not caused by tumor cell-intrinsic mechanisms but by alteration of the interaction between melanoma cells and tumor microenvironment. By definition, tumor microenvironment or "tumor stroma" includes all those components that are not cancer cells such as endothelial cells, fibroblasts, and infiltrating leukocytes, as well as extracellular matrix proteins in the cancer microenvironment. It has been demonstrated that BRAF-I resistance results to be mediated by fibroblast production of growth factors such as the hepatocyte growth factor (HGF)[73]. In presence of BRAF-I, HGF increased production by activating its natural ligand c-Met (HGF receptor with tyrosine-kinase activity) caused reactivation of MAPK pathway (MAPK dependent) or activation of AKT pathway (MAPK independent) which leads to BRAF-I intrinsic resistance.

1.4.2. Acquired resistance to BRAF-I.

Several mechanisms of acquire resistance have been described *in vitro* and have been validated in BRAF V600E melanoma specimens. Unlike the acquired resistance mechanisms to targeted therapy found in other malignancies, large-scale sequencing analysis of melanoma specimens demonstrated that acquired resistance to BRAF-I is not caused by the development of secondary mutations (so-called gatekeeper) in BRAF oncogene[65, 74]. Generation of acquired resistance, by continuous exposition of BRAF V600E melanoma cell lines to BRAF-I *in vitro*, has demonstrated that different mechanism are involved in the acquired BRAF-I resistance.

So far, the mechanisms described of acquired BRAF-I resistance include activation of constitutive signaling in receptor tyrosine kinases (IGF-1R and PDGFRβ)[65, 75], increased expression of the MAPK family member COT (MAP3K8, TPL-2)[76], BRAF V600E amplification[77], aberrantly spliced BRAF V600E[78], elevated CRAF activity[79] and acquisition of mutation in NRAS[65] and MEK1[80]. For all these mechanisms it has been shown that BRAF-I resistance is mediated by reactivation of ERK (MAPK dependent) or activation of alternative pathways such as PI3K/AKT (MAPK independent).

In the context of BRAF V600E inhibition, ERK reactivation can be caused by activation of up-stream components or down- stream components in MAPK pathway of BRAF as well as for the incapacity of BRAF-I in inhibiting BRAF V600E. Thus an ERK reactivation by up-stream components of BRAF V600E is mediated by activation of CRAF or ARAF which bypasses the BRAF inhibition (paradoxical MAPK activation, see before). CRAF or ARAF activation can be caused by upstream activation of RTK such as IGFR-1 or PDGFRβ, activating mutations in NRAS or by amplification of ARAF or CRAF. On the other hand, an ERK reactivation by the lack of ability of BRAF-I in inhibiting BRAF V600E is mediated by truncated BRAF V600E spliced isoforms. In this case although BRAF

maintains the mutation at the 600 position (i.e. V600E), it loses the binding site for BRAF-I and dimerises readily in the presence of a BRAF inhibitor, thereby hyper activating the MAPK pathway. Similar mechanisms have been also described for amplification of BRAF V600E. In this case BRAF monodimer inhibition by BRAF-I has been shown to be not sufficient to inhibit BRAF dimerization which in turn leads to ERK reactivation. An ERK reactivation by BRAF down-stream components can be mediated by a BRAF independent-MEK or ERK activation. Thus an increased expression of the MAPK family member COT or activating mutations of MEK leads to ERK reactivation by a direct phosphorilation of ERK.

MAPK play a major role in melanoma growth. However in the context of BRAF V600E and subsequently MAPK inhibition, activation of alternative pathways can restore tumor growth. Several signaling pathways have been shown to induce an increase proliferation, prosurvival signals, antiapoptotic signals as well as proangiogenetic signals. Thus an increased prosurvival signaling mediated by activation of PI3K/AKT has been demonstrated in BRAF V600E melanoma resistant to BRAF-I. It can be mediated by upregulation of RTK such as IGF-1R, PDGFRβ and epidermal growth factor receptor (EGFR), by activating mutations of PI3K or AKT, and by acquired mutations of PTEN as previously described for the intrinsic resistance of BRAF-I.

All together these findings demonstrate that several mechanisms can drive a BRAF-I intrinsic or acquired resistance. However it is still unknown whether therapeutic escape arises as the result of an evolutionary process within the melanoma or from the selection of pre-existing ‘‘resistant’’ clones that are already present prior to the initiation of therapy. In addition, to date, the resistance mechanisms described account for less than 50% of patients whose disease relapses while being treated with a BRAF-I and the remaining alterations need to be defined. Lastly many of the described mechanisms of BRAF-I resistant which have been demonstrated *in vitro* still required to be validated in clinical samples

and for most of them the molecular event induced by BRAF-I still need to be determined.

1.5. Strategies to overcome BRAF-I resistance

Development of BRAF-I resistance in melanoma patients as well as drug resistance to treatment with targeted agents in other malignancies demonstrated that tumor growth is driven by several alterations. Therefore to achieve a sustainable therapeutic response an effective strategy has to target multiple alterations. In melanoma the characterization of the molecular mechanisms underlies resistance in BRAF V600E melanoma has lead a rational designed of targeted combinatorial therapies. Most of the proposed combinatorial strategies to overcome BRAF-I resistance have been demonstrated *in vitro*, some of them are in clinical development but to date few of them have been already validated in clinical setting.

Although different mechanisms have been described for BRAF-I resistance most of them have demonstrated to lead the reactivation of the MAPK pathways or activation of the PI3K/AKT pathways. Several novel inhibitors are in clinical development to target almost all components of these two pathways. The simultaneous inhibition of the activation of different components of the MAPK and PI3K/AKT pathways has led to the develop of combinatorial strategies such as the combination of BRAF-I and MEK- inhibitor (MEK-I), BRAF-I/MEK-I and mTOR/PI3K/AKT inhibitor, BRAF-I and IGF-1R inhibitor, and BRAF and PDGFRβ inhibitor[75, 81-83].

In the context of the MAPK pathway reactivation, the combination of BRAF-I and MEK-I has demonstrated in several *in vitro* studies to be effective in delaying and overcoming the BRAF-I resistance[49]. Since MEK is a down-stream component of the MAPK pathway its inhibition has been shown *in vitro* to lead ERK inhibition in presence of BRAF-I resistance mediated by RTK, NRAS, BRAF, CRAF,

ARAF, COT and MEK alterations. The combination of BRAF-I and MEK-I has been also tested in clinical setting. A phase I trial in patients who disease progressed after BRAF-I treatment did not demonstrate a clinical responses to MEK-I in the treated patients[84]. Another phase I-II trail in patients who disease progressed after BRAF-I combined BRAF-I and MEK-I demonstrated short survival and low number of response rate. However a recent phase II trial evaluating the combination of BRAF-I and MEK-I in naïve melanoma patients carrying BRAF V600E or V600K demonstrated an increase in progression free survival in the combinatorial treatment compared to administration of BRAF-I alone (9.2 months in dabrafenib 150 mg/die and trametenib 1mg/die arm, 9.4 months in dabrafenib 150 mg/die and trametenib 2mg arm, 5.8 in dabrafenib 150mg/die arm as single agent, $p<0.001$)[85]. The rate of complete or partial responses with combination 150 mg /2 mg of BRAF-I/MEK-I therapy was 76%, as compared with 54% with BRAF-I monotherapy (P=0.03). Furthermore a decreased incidence of KA and SCC in BRAF-I/MEK-I combination was found compared to BRAF-I. These data validate the role of rebound BRAF inhibitor mediated MEK/ERK signaling (paradoxical MAPK activation) in the development of these types of tumors[85].

In case of resistance mediated by increased IGF-1R signaling it has been shown *in vitro* that dual MEK and PI3K inhibition or BRAF-I and IGFR-1 inhibition might be an effective strategy. Interesting *in vitro* resistance mediated through increased PDGFRβ signaling has been shown to be not overcome by PDGFR inhibitors sunitinib and imatinib but by the combination of the mTOR/PI3K/AKT inhibitor and MEK/BRAF inhibitor[86]. Clinical trials have been initiated to examine the PI3K/mTOR inhibitor BEZ235 in combination with the MEK-I MEK162 (NCT01337765). This trial is currently enrolling patients who have BRAF mutations and those who have NRAS mutations.

In case of resistance mediated by increased fibroblast secretion of HGF the combination of BRAF-I and c-Met inhibitor have been shown *in vitro* to sensitize resistant melanoma cells to BRAF-I[73].

Another approach used to delay and overcome BRAF-I resistance have been also proposed. Simultaneous administration of BRAF-I and an inhibitor of the heat shock protein 90 (HSP90) have been demonstrated *in vitro* to overcome an acquired resistance to BRAF-I mediated by NRAS mutation, PDGFRβ upregulation and COT overexpression[87]. HSP90 is a cellular chaperone required for the refolding of denatured proteins, cellular survival under stress conditions, and the maturation of a subset of proteins. Therefore it has been shown that inhibition of HSP90 induces instability and degradation of client proteins such as MEK, BRAF and AKT overcoming resistance to BRAF-I[87].

These evidences demonstrate that several strategies can be effective to overcome and delay the lack of sensitivity of BRAF V600E melanoma cells to BRAF-I. However several open questions remain to be determined. First, there is still the need to identify molecular biomarkers which allow the selection of melanoma patients and predict the most effective therapeutic strategy. Second, most of the proposed strategies need to be validated *in vivo* (i.e., mTOR-/PI3K-/AKT-I and MEK-/BRAF-I or HSP90 inhibitor and BRAF-I) and their reliability in terms of toxicity still need to be determined. It is noteworthy that targeting downstream components of signaling pathways as well as targeting a broad range of client proteins with HSP90 inhibitor is expected to be associated with an increased toxicity. Third, some of mechanisms and the strategies described to overcome the BRAF-I resistance still remain not convincing as well as the PDGFRβ mediated resistance and the lack of efficacy of PDGFRβ inhibitor in overcoming this resistance. Lastly mechanisms in the MAPK pathways need to be better elucidated such as the lower ability of MEK-I as compared to BRAF-I in inhibiting melanoma growth when it is driven by MAPK pathway.

2. AIMS OF THE STUDY

The discovery of BRAF mutation and the development of targeted agents to inhibits mutant BRAF has changed the approach to the treatment of melanoma patients. However the existence of many potential resistance mechanisms to BRAF-I has demonstrated the plasticity of melanoma in intrinsic and acquired resistance. These observations have led to the development of novel combinatorial strategies which have demonstrated the importance of targeting multiple signaling pathways to achieve a sustainable therapeutic response. Nevertheless more effort is need to discover the unknown mechanisms of intrinsic and acquired resistance to develop strategies which are effective in counteracting the mechanisms of BRAF-I resistance of melanoma cells. It is likely that the existence of so many potential resistance mechanisms will require patient-specific approaches to the management of therapeutic escape and the further personalization of melanoma therapy.

Therefore the aims of this study were to identify novel potential mechanisms of BRAF-I resistance and to validate novel combinatorial strategies for overcoming BRAF-I resistance. We have identified PDGFRα upregulation as a new mechanism involved in the intrinsic and acquired BRAF-I resistance in BRAF V600E melanoma. Melanoma cell lines with acquired BRAF-I resistance provided a model to study melanoma patients who develop BRAF-I resistance. To validate our cell line results demonstrating PDGFRα upregulation, we tested melanoma lesions harvested from patients who developed BRAF-I resistance. Lastly we demonstrate that both *in vitro* and *in vivo* the PDGFRα mediated BRAF-I resistance can be overcome by combining a BRAF-I, vemurafenib, with a PDGFRα inhibitors such as sunitinib or imatinib.

3. MATERIALS AND METHODS

3.1. Cell cultures.

The parental BRAF V600E melanoma cell lines Colo38 and M21 were cultured in RPMI 1640 medium (Mediatech, Inc. Herndon, VA) supplemented with 2 mmol/L L-glutamine (Mediatech) and 10% fetal calf serum (FCS; Atlanta Biologicals, Lawrenceville, GA). The BRAF V600E melanoma cell line TPF-10-741 was cultured in DMEM (Mediatech) supplemented with 2 mmol/L L-glutamine and 10% FCS. This cell line was started from a cutaneous metastasis of the melanoma patient TPF-10-741 who had developed BRAF-I resistance following treatment with vemurafenib. Melanoma cell lines with acquired vemurafenib resistance (Colo38R and M21R) were generated by propagating parental Colo38 and M21 cells in increasing concentrations of BRAF-I (up to 2 μM) to achieve chronic selection. Colo38R and M21R were cultured in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 10% FCS and 500 nM vemurafenib. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

3.2. Chemical reagents, antibodies and shRNAs.

Vemurafenib (PLX4032), was purchased from ChemieTek (Indianapolis, IN). Sunitinib and imatinib were purchased from Selleck Chemicals LLC (Houston, TX). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO). Phospho (p)-AKT (Ser473)-, AKT-, p-PI3K p85 (γ458)-, p-CRAF (S289/296/301)-, p-MEK 1/2 (S217/221)-, p-ERK 1/2 (Thr202/Tyr204)-, ERK1/2-, PTEN-, PDFGRβ-, p-PDGFRα-, PDGFRα-, VEGFR2-, Cleaved Caspase-3 (Asp175)-, p-Histone H3 (Ser10)- and β-actin-specific monoclonal antibodies (mAbs) were purchased from Cell Signaling Technology (Danvers, MA). The calnexin-specific mAb TO-5 was developed and characterized as described [88]. PDGFRα-specific short hairpin RNA (shRNA) and

GFP-shRNA were provided by the- Vector Core Facility of the University of Pittsburgh Cancer Institute (Pittsburgh, PA).

3.3. Cell proliferation and MTT assay.

Cells were plated in triplicate in 96-well microtiter plates at the density of 2.5 x 10^3 per well in 100ul in RPMI 1640 or DMEM medium supplemented with 2% FCS and treated with vemurafenib and/or PDGFRα inhibitor (sunitinib or imatinib). DMSO (vehicle of the drugs) concentration was maintained at 0.02% in all wells. Cell proliferation was evaluated by MTT assay at indicated time points. Briefly, 10 uL of MTT was added into each well, and the mixture incubated for approximately 3–4 h at 37°C. Metabolically active, viable cells convert MTT into a purple-colored formazan product that can be measured using a spectrophotometric microplate reader (MTX Lab System, Inc, Vienna, VA) at 540 nm following solubilization. Data were expressed as percent of inhibition or percent of proliferation of treated cells compared with the untreated control cells. All experiments were performed three independent times in triplicates.

3.4. Western blot analysis.

For samples preparation from cell lines, cells were seeded at the density of 1 x 10^5 per well in a 6-well plate in medium supplemented with 2% FCS and treated with vemurafenib (1 μM), sunitinib (3 μM), imatinib (20 μM) and vemurafenib plus sunitinib or imatinib at 37 °C in a 5% CO2 atmosphere for the indicated time points. The DMSO (vehicle of the drugs) concentration was maintained at 0.02% in all wells. Untreated cells were used as a control. Cells were collected and lysed in lysis buffer [10 mM Tris–HCl (pH 8.2), 1% NP40, 1 mM EDTA, 0.1% BSA, 150 mM NaCl) containing 1/50 (vol/vol) of protease inhibitor cocktail (Calbiochem, La Jolla, CA). For sample preparation from tumor xenografts, tumors were extracted at the time of killing, harvested and stored at -80°C.

Proteins were extracted by homogenization in the presence of 2 to 5 ml lysis buffer. After incubation on ice for 20 to 30 minutes, the lysates were centrifuged at 10,000 rpm for 30 minutes. The protein concentration of the lysate was determined. Equal amounts of proteins (80 μg per well) for cell lysates and for tumor lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 lm pore size) (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk plus 2% BSA at room temperature for 2 h and then incubated overnight at 4°C with the indicated mAbs. Corresponding peroxidase-conjugated secondary mAbs (Cell signaling technology) were then added to respective blots and incubations continued at room temperature for an additional 1 h. Blots were washed 5 times for 5 min with 0.01 M tris-buffered saline (pH 7.2), 0.05% tween-20 (TBST) between all steps. Blots were developed using the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and bands visualized using the FOTO/Analyst Investigator Eclipse System (Fotodyne Incorporate, Hartland, WI). β-actin or Calnexin were used as the protein loading control.

3.5. Transduction of melanoma cells with Lentiviral vectors encoding shRNA.

Colo38R, M21R and TPF-10-741 cells were seeded at the density of 6 $x10^4$ per well in a 6-well plate and incubated in culture medium for 24 h at 37ºC in a 5% CO2 atmosphere prior to viral infection. Cells were transduced with PDGFRα-specific shRNAs [Target sequence: CCAGCCTCATATAAGAAGAAA (#1), CCAGCTTTCATTACCCTCTAT (#2), CGGTGAAAGACAGTGGAGAT (#3), CCCAACTTTCTTATCCAACTT (#4), CAATGGACTTACCCTGGAGAA (#5)] or GFP-shRNA, used as a control, lentiviral particles (1 x 10^6 per well) in presence of polybrene (2 μg/ml) as described elsewhere [89]. Following an 18 h incubation at 37ºC, culture medium was removed and replaced with fresh culture medium.

Following an additional incubation for up to 72 h at 37ºC, cells were analyzed for GFP expression under the microscope, split, enriched for infected cells by selection with puromycin (2.5 ug/ml) and collected for further analysis.

3.6. Flow Cytometry.

Cells were seeded in 6-well plates in triplicate at the density of 1 x 10^5 per well and treated with vemurafenib and/or sunitinib. Following a 24 h treatment apoptotic cells were identified by staining with Annexin -V and PI (BD Bioscience, San Jose, CA) for 15 min at room temperature. Flow cytometry data were analyzed using Summit v4.3 software (DAKO, Carpinteria, CA).

3.7. *In vivo* studies.

C.B-17 severe combined immunodeficient (SCID) female mice (8–10 week old) were purchased from Taconic Farms, Inc. (Hudson, NY). Parental and BRAF-I resistant cell lines M21 and M21R (1 x 10^6 cells/mouse) were implanted subcutaneously in the right lateral flank of mice. A total of 20 SCID mice was challenged with each cell line. Body weight and tumor volume were measured twice per week. Tumor volume was measured by vernier caliper. Treatment was initiated 10 days after cell inoculation when the tumor developed and had a diameter of around 0.4 cm. Mice were randomly divided into 4 groups of 5 mice each. Mice in Group 1 were treated with vemurafenib (12.5 or 25 mg/kg/twice per day)[50], those in Group 2 with sunitinib (20mg/kg/day)[90] or imatinib (100mg/kg/twice)[91] and those in Group 3 with vemurafenib (12.5 or 25mg /kg/twice per day) plus sunitinib (20mg/kg/day) or imatinib (100mg/kg/twice). Mice in Group 4 were left untreated as a reference for the natural course of the disease. Drugs were administered by oral gavage. When tumor diameter from untreated mice reached 2.0 cm all mice were sacrificed. Primary tumors and

organs were collected for further analysis. Animal studies have been approved by the Institutional Animal Care and Use Committee.

3.8. Patient Samples.

Patients with metastatic melanoma harboring the BRAF V600E mutation (confirmed by genotyping) were enrolled in clinical trials with the BRAF-I (vemurafenib) or with BRAF-I (dabrafenib) and MEK inhibitor (MEK-I) (trametinib). Patients were consented for tissue acquisition per IRB-approved protocol. Tumor biopsies were performed pre-treatment (day 0), at 10-14 days on treatment, and/or at the time of disease progression [as defined by Response Evaluation Criteria In Solid Tumors (RECIST)] if applicable. Formalin-fixed tissue was analyzed to confirm that viable tumor was present via hematoxylin and eosin (H&E) staining.

3.9. Immunohistochemistry.

Patient biopsies and tumors generated in mice were formalin fixed and paraffin embedded and then used as substrates in immunohistochemical reactions. Five-um thick xenograft tissue sections were fixed on silane-coated glass slides, deparaffinized, and subjected to antigen retrieval (Target retrieval solution, DAKO). Following blocking, slides from mice were incubated with Cleaved Caspase-3 (Asp175) and p-Histone H3 (Ser10) –specific mAbs overnight. Eight-um thick sections from patient-derived samples were incubated with PDGFRα-specific mAb (sc-338, Santa Cruz) (1:400) overnight. Sections were then washed with PBS, and the primary antibody was amplified using the VECTASTAIN ABC Kit (Peroxidase rabbit IgG, Vector Laboratories, PK-4001). The detection of this antibody was performed with the DAB Peroxidase Substrate Kit from DAKO and the sections were counterstained with H&E. PDGFRα expression, as measured by its staining intensity, in tumors harvested from BRAF-I treated patients either on

treatment or at the time of disease progression was compared to that the staining in pretreatment tumors. Scores were recorded semiquantitatively as 1+, 2+, 3+ and 4+, when , 1–25%, 26–50%, 51-75% and >75% of nuclei were stained, respectively. Mitotic and apoptotic tumor cells in the sections of primary tumors harvested from mice were detected by staining p-Histone H3 (Ser10) and Cleaved Caspase-3 proteins, respectively, and quantified by counting 5 random fields per section (magnification ×200). Data were expressed as the mean number of mitotic or apoptotic tumor cells in each group. The number of mitotic or apoptotic tumor cells was counted by an investigator who was blinded to the type of treatment received by the mice from which tumors had been harvested.

3.10. Statistical analysis.

Averages, standard deviations, and unpaired t-test were calculated using MS-Excel. Data showed the mean ± SD of the results obtained in at least three independent experiments. Time of disease progression (time to progression) of BRAF-I treated patients was calculated using the Kaplan-Meier methods. Differences between groups were considered significant when the P value was < 0.05. The asterisk (*) indicates $P < 0.05$.

4. RESULTS AND DISCUSSION

4.1. ERK reactivation, AKT activation and PDGFRα upregulation in Colo38R, M21R and TPF-10-741 melanoma cell lines with acquired vemurafenib resistance.

Colo38 and M21 melanoma cell lines which are driven by mutant BRAF V600E are exquisitely sensitive to the anti-proliferative activity of vemurafenib. To select cells with acquired BRAF-I resistance, Colo38 and M21 cells were grown in the presence of serially increasing concentrations of vemurafenib up to a dose of 2 μM. At the end of 3 months, resistant cells were isolated from each of the two cell lines. These cell lines were named Colo38R and M21R. Additionally a novel cell line TPF-10-741 was developed from a cutaneous metastasis from a melanoma patient who had developed BRAF-I resistance following treatment with vemurafenib, and that harbors the BRAF V600E mutation. MTT assays demonstrated that Colo38R and M21R cells had acquired resistance to the growth inhibitory effects of vemurafenib, while the TPF-10-741 cell line displayed an intermediate sensitivity to vemurafenib **(Fig. 5).**

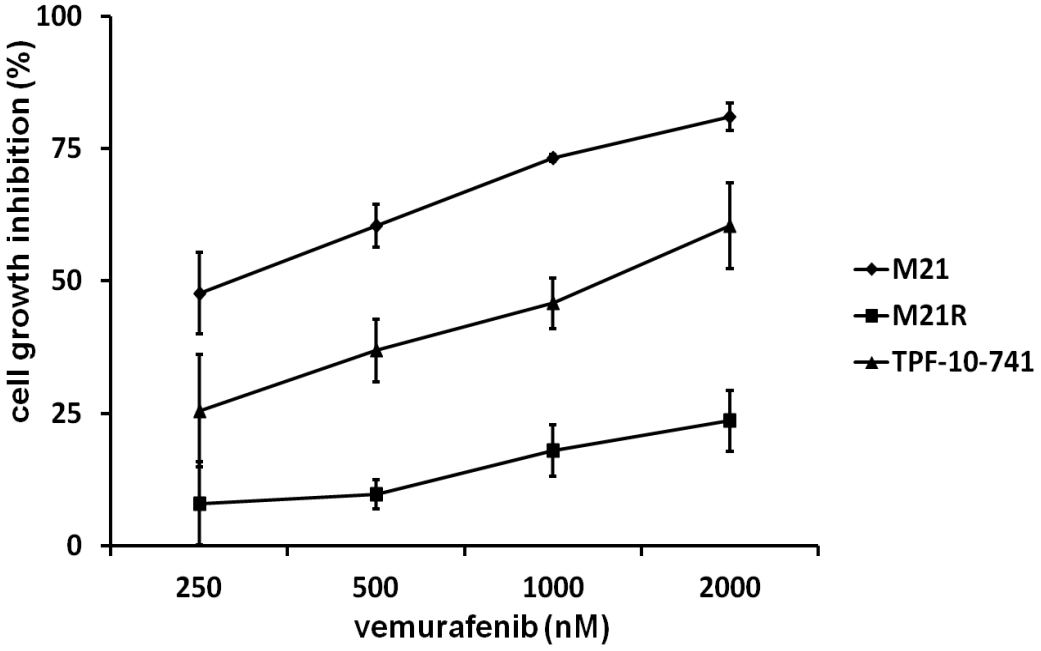


Figure 5. BRAF V600E melanoma cell lines, autologous cells made resistant to BRAF-I by continuous exposure to increasing doses of vemurafenib and a melanoma cell line isolated from a patient who acquired BRAF-I resistance. M21, M21R and TPF-10-741 cells were treated with the indicated concentrations of vemurafenib. Cell growth inhibition was determined by MTT assay following a five day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells at each vemurafenib dose. Data are expressed as the mean ± SD of the results obtained in three independent experiments.

This acquired resistance model was then used to investigate the molecular mechanisms underlying disease progression after an initial response to vemurafenib.

Growth inhibitory effects of vemurafenib are mediated by inhibiting the RAF/MEK/ERK pathway [92]. Since acquired vemurafenib resistance can be mediated by reactivation of the RAF/MEK/ERK pathway or activation of alternative pathways like PI3K/AKT, signaling through these pathways were evaluated in both parental and resistant cell lines **(Fig. 6).** Western blot analysis demonstrated that following a 1, 24 and 48 h incubation with vemurafenib p-ERK

levels were reduced in both Colo38 and M21 cells, but there was no detectable effect on p-ERK levels in Colo38R and M21R cells, and a limited effect on TPF-10-741 cells. p-AKT levels were increased in vemurafenib-resistant cells (Colo38R and M21R) compared to vemurafenib-sensitive cells (Colo38 and M21), and in TPF-10-741 cells as well as in the M21 sensitive cells after treatment with BRAF-I. Thus the decreased sensitivity to the inhibition of the RAF/MEK/ERK pathway by BRAF-I was associated with the reactivation of the RAF/MEK/ERK pathway and with activation of AKT.

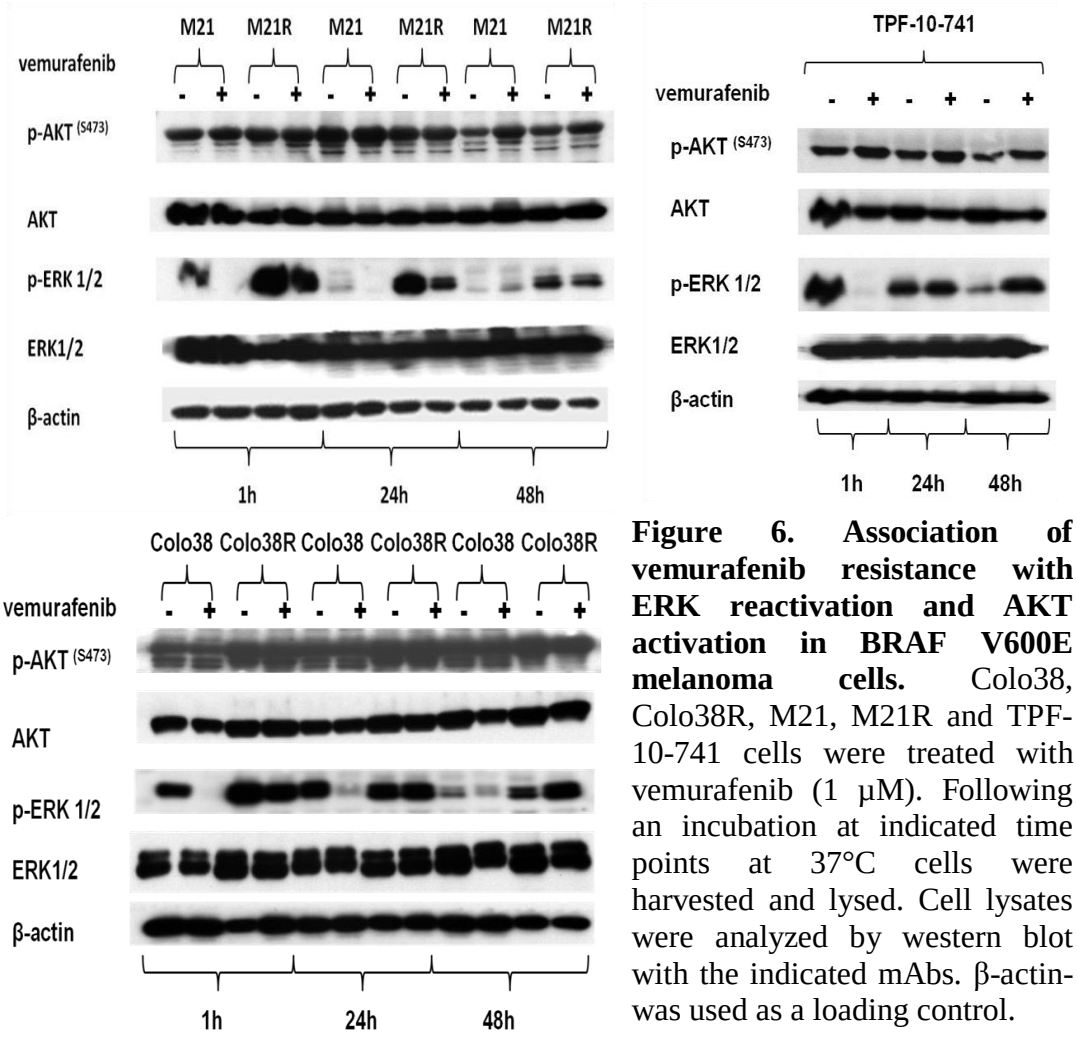


Figure 6. Association of vemurafenib resistance with ERK reactivation and AKT activation in BRAF V600E melanoma cells. Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 μM). Following an incubation at indicated time points at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β-actin-was used as a loading control.

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Acquired vemurafenib resistance through activation of the PI3K/AKT pathway can be mediated by the upregulation of PDGFRβ or other growth factor receptors like IGFR [65]. Therefore, to investigate the role of growth factor receptors in acquired vemurafenib resistance, we evaluated the expression levels of PDGFRα, PDGFRβ and VEGFR2 in Colo38, Colo38R, M21, M21R, and TPF-10-741 cell lines after treatment with vemurafenib. Western blot analysis demonstrated that treatment with vemurafenib enhanced PDGFRα expression and activation in the two resistant cell lines as compared to the BRAF-I sensitive cell lines. PDGFRα was also expressed and activated in the partially BRAF-I resistant TPF-10-741 cell line both under basal conditions and following treatment with vemurafenib (**Fig. 7**).

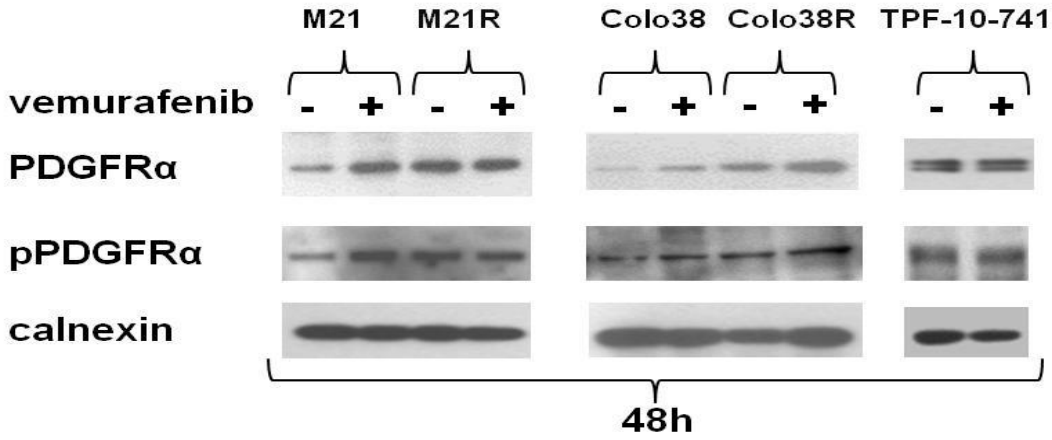


Figure 7. Association of vemurafenib resistance with PDGFRα upregulation in BRAF V600E melanoma cells. Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 μM). Following a 48 h incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. Calnexin was used as a loading control. The results presented are representative of the results obtained in three independent experiments.

PDGFRβ was found to be upregulated on TPF-10-741 cells after treatment with vemurafenib, but not in Colo38, Colo38R, M21 and M21R cells. VEGFR2

expression was not detected in any of the cell lines (Colo38, Colo38R, M21, M21R and TPF-10-741) before or after treatment with vemurafenib. Further analysis of components of the RAF/MEK/ERK pathway and PI3K/AKT pathway demonstrated that PDGFRα upregulation was associated with PI3K, CRAF and MEK activation in Colo38R, M21R, and TPF-10-741 cells. Lasltly PTEN mutation was demonstared in TPF-10-741 cells but not in Colo38, Colo38R, M21 and M21R. Based on these observations, we investigated whether PDGFRα upregulation was causally related to vemurafenib resistance (**Fig. 8**)

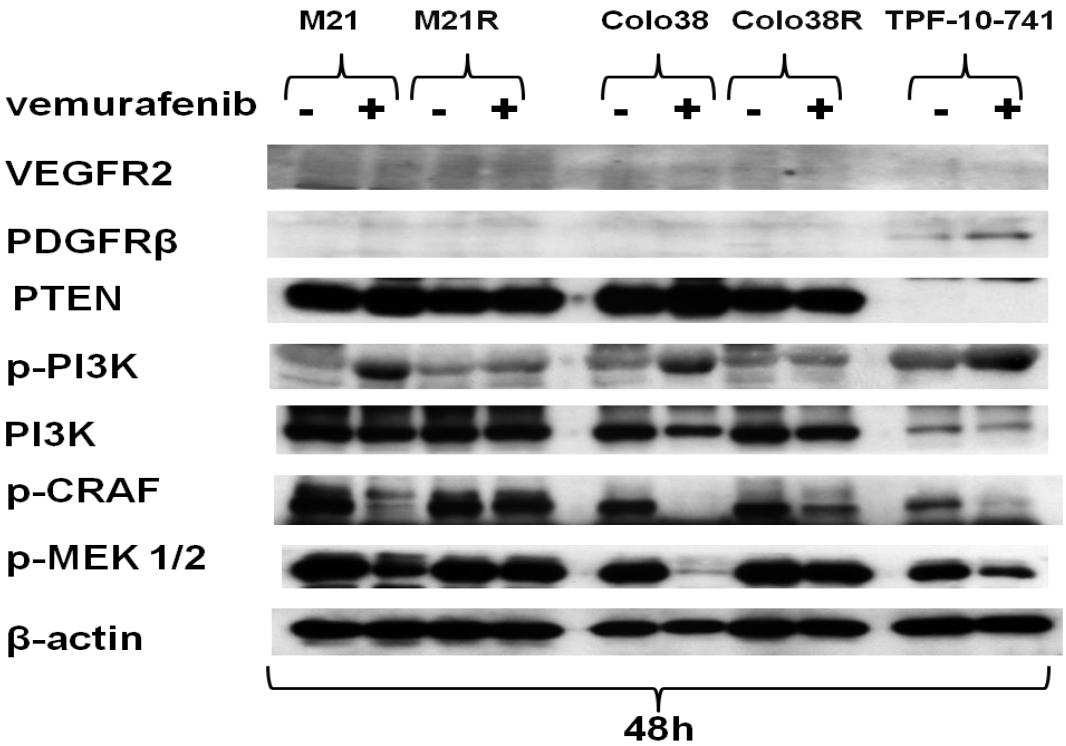


Figure 8. Analysis of RTK, MAPK and PI3K/AKT pathway components in BRAF V600E melanoma cells. Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 μM). Following a 48 h incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. B-actin was used as a loading control. The results presented are representative of the results obtained in three independent experiments.

4.2. PDGFRα mediates acquired vemurafenib resistance of Colo38R, M21R, and TPF-10-741 melanoma cells by reactivation of the RAS/RAF/ERK pathway and activation of the PI3K/AKT pathway.

To test whether increased PDGFRα expression caused the vemurafenib resistance of Colo38R, M21R and TPF-10-741, PDGFRα was knocked down in the three cell lines using 5 PDGFRα-specific shRNAs. As shown in **Fig. 9**, lentiviral transduction of M21R cells with a PDGFRα-specific shRNA (#4) construct knocked down PDGFRα protein expression. PDGFRα downregulation was associated with decreased p-ERK and p-AKT.

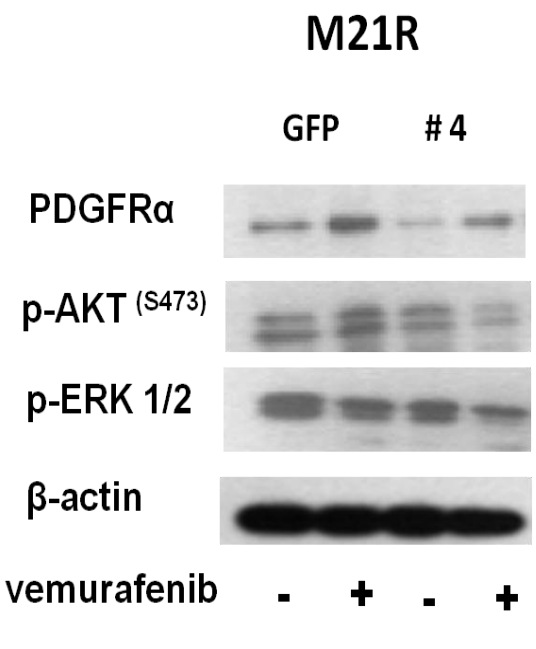


Figure 9. Restoration by PDGFRα downregulation of vemurafenib signaling pathway inhibtion of BRAF V600E melanoma cells with acquired BRAF-I resistance. M21R and TPF-10-741 cells were transduced with PDGFRα-specific shRNA (#4) or with GFP-shRNA, used as a control, lentiviral particles. Transduced cells were treated with vemurafenib (1μM). Following a three day incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β-actin was used as a loading control. The results presented are representative of the results obtained in three independent experiments.

Additionally, the M21R and TPF-10-741 cells transduced with the PDGFRα-specific shRNA (#4) displayed a significantly increased sensitivity to vemurafenib, as compared to the autologous cells transduced with a GFP-shRNA ($P<0.01$) (**Fig. 10**). Lastly, western blot analysis demonstrated that the PDGFRα downregulation caused by the PDGFRα-specific shRNA (#4) in combination with vemurafenib

treatment dramatically decreased the levels of p-ERK and p-AKT in M21R cells (**Fig. 9**). These findings demonstrate that PDGFRα mainly induces both ERK and AKT activation in the cells that are completely or partially resistant to vemurafenib, and suggests that PDGFRα inhibition could overcome this acquired resistance.

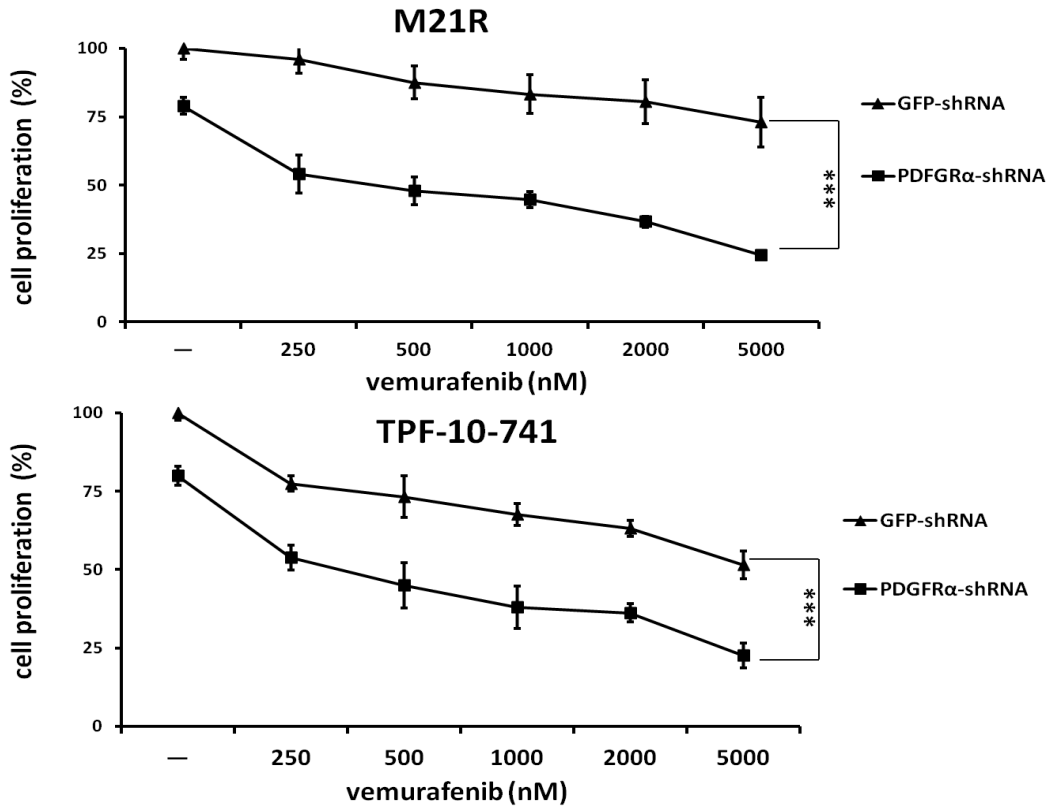
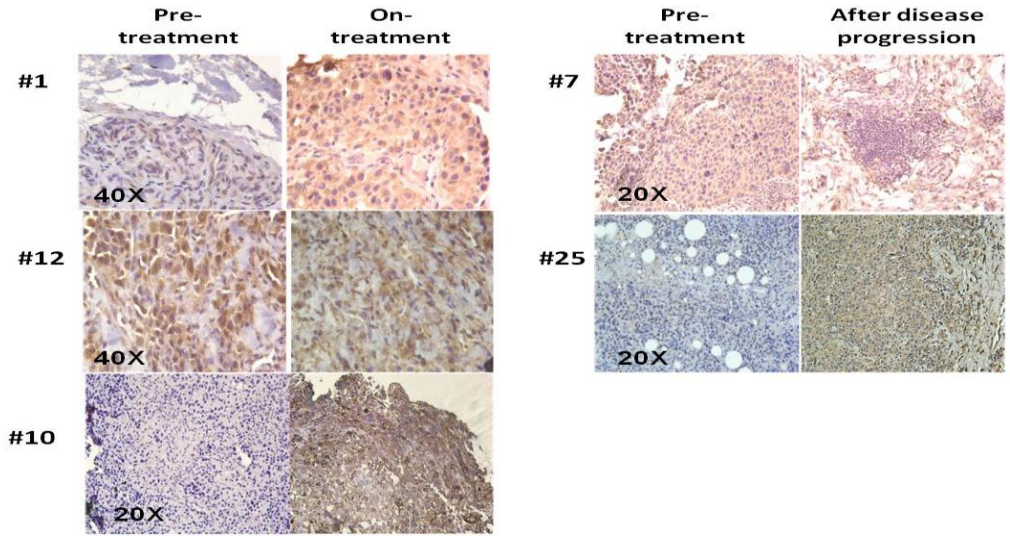


Figure 10. Restoration by PDGFRα downregulation of vemurafenib cell growth inhibtion of BRAF V600E melanoma cells with acquired BRAF-I resistance. PDGFRα-specific shRNA (#4) transduced M21R and TPF-10-741 cells were treated with the indicated vemurafenib concentrations. GFP-shRNA transduced M21R and TPF-10-741 cells were used as controls. Cell proliferation was determined by MTT assay following a three day incubation at 37°C. Percentage of cell proliferation was calculated as the ratio of treated cells to untreated GFP-shRNA transduced cells. Data are expressed as mean ± SD of the results obtained in three independent experiments. *** indicates $P < 0.01$.

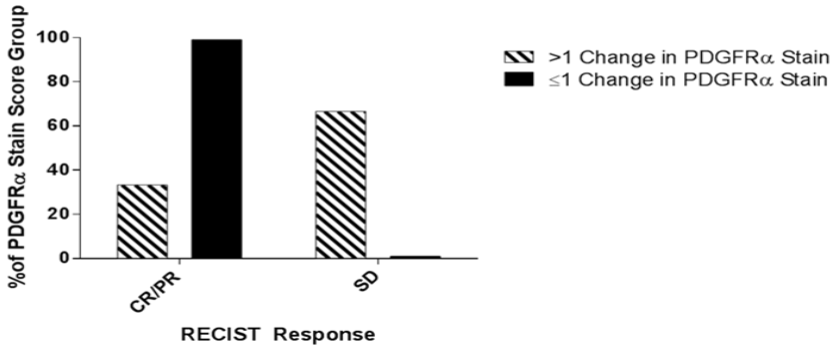
4.3. Association of PDGFRα upregulation in melanoma patient derived biopsies with vemurafenib resistance.

To validate our in vitro findings we compared PDGFRα expression in biopsies obtained from 9 melanoma patients treated with BRAF-I or with BRAF-I and MEK-I (**Table 1**). Tumor biopsies were performed before treatment, on treatment, and at the time of disease progression. IHC staining demonstrated PDGFRα upregulation in 5 out of 9 patients following treatment with vemurafenib or with dabrafenib and trametinib (**Fig. 11A**). Interestingly, in 3 of the 5 patients a significant increase in PDGFRα expression (>1) was observed after treatment. Patients with a significant (>1) increase in PDGFRα expression after treatment with BRAF-I +/- MEK-I had less tumor regression based on RECIST criteria (**Fig. 11B**) and a shorter time to disease progression (**Fig. 11C**) when compared to patients who had no change or a small change in expression (≤1). These results demonstrate an association between PDGFRα overexpression and vemurafenib resistance in patients with melanoma.

A.



B. **Change in PDGFRα Stain after BRAF inhibition association with RECIST Response**



C. **Progression Free Response Based on Change in PDGFRα Staining after BRAF inhibitor**

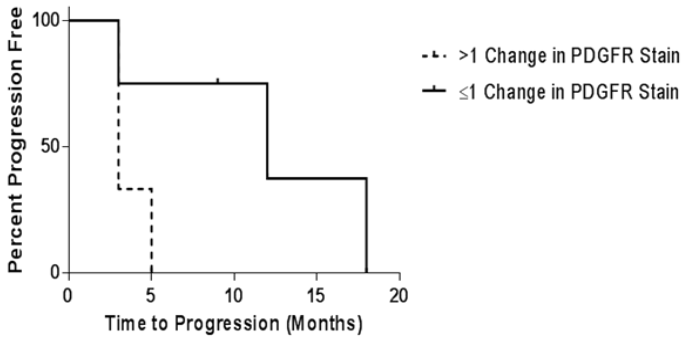


Figure 11. PDGFRα expression in melanoma tumors obtained from patients who acquired BRAF-I resistance. Tumor biopsies of melanoma removed from patients were performed pre-treatment (day 0), at 10-14 days on treatment, and/or at the time of disease progression following treatment with BRAF-I or with BRAF-I and MEK-I. Frozen sections were fixed with 4% PFA and stained with H&E and PDGFRα-specific rabbit antibody. Patients were divided in two groups based on change of PDGFRα expression at IHC after treatment: those whose PDGFRα staining score had no or 1 point increase after treatment (≤1) and those for whose PDGFRα staining score increased 2 or more points after treatment (>1). **A**. Representative IHC staining of PDGFRα expression in melanoma patients before treatment, on treatment and at the time of disease progression in 5 out of 9 tumor biopsies. Magnification is indicated. **B**. Two groups of patients were graphed based upon RECIST criteria and compared as a percent of the total population of the PDGFRα stain score group. **C.** Two groups of patients were graphed based upon the time to disesase progression utilizing Kaplan–Meier methods.

4.4. Inhibition of PDGFRα increases the anti-tumor activity of vemurafenib in BRAF-I sensitive and resistant melanoma cell lines.

To investigate whether the anti-tumor activity of BRAF-I could be enhanced by PDGFRα inhibition in both BRAF-I sensitive and resistant melanoma cell lines, the BRAF-I sensitive Colo38 and M21 cell lines and the BRAF-I resistant Colo38R, M21R and TPF-10-741 cell lines were treated with vemurafenib and/or PDGFRα inhibitors sunitinib[90] and imatinib [91]. Both multitargeted tyrosine kinase inhibitors (TKIs) markedly inhibit both PDGFRα and PDGFRβ. Sunitinib also inhibits VEGFR2 and c-Kit receptor kinases. A dose titration experiment established the dose of PDGFR inhibitors sunitinib and imatinib to be combined with vemurafenib in the 5 cell lines. The IC50 dose of sunitinib and imanitinib were found to be 2 and 15 µM, respectively (**Fig. 12**).

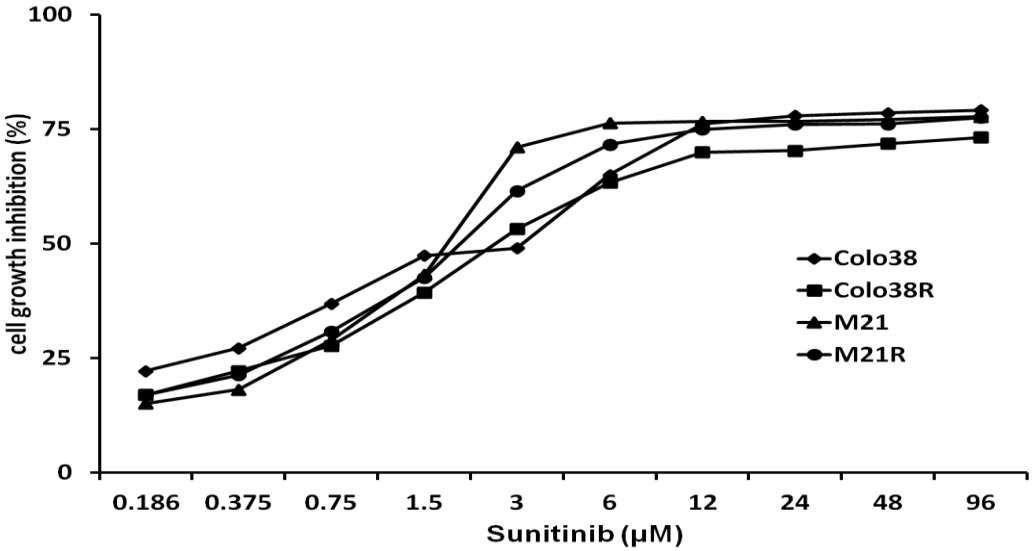
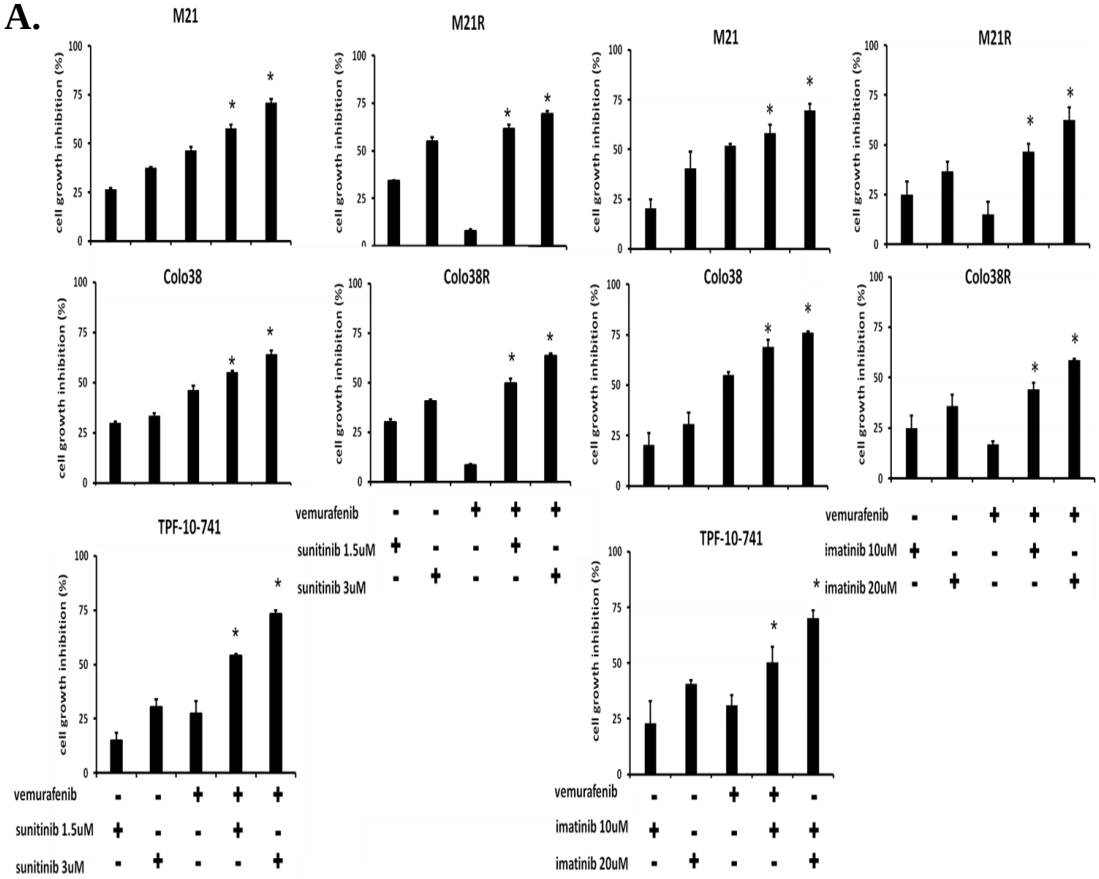


Figure 12. Dose dependent effect of sunitinib on the *in vitro* proliferation of BRAF-I sensitive and resistant melanoma cells harboring BRAF V600E. Colo38, Colo38R, M21and M21R cells were treated with the indicated concentrations of sunitinib. Cell growth inhibition was determined by MTT assay following a five day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells at each sunitinib dose. Data are expressed as mean ± SD of the results obtained in three independent experiments.

The dose of 1.5 μM and 3 μM for sunitinib and the dose of 10 μM and 20 μM for imatinib were chosen to be tested in combination with vemurafenib for their effect on cell growth and survival. Cell growth inhibition of Colo38 and M21 by the MTT assay demonstrated that following a three and five day treatment of vemurafenib in combination with PDGFRα inhibitors sunitinib or imatinib significantly inhibited proliferation ($p<0.05$) when compared to each agent alone (**Fig. 13**). Furthermore, both sunitinib and imatinib synergized ($p<0.05$) with vemurafenib to overcome BRAF-I resistance, increasing the growth inhibition of BRAF-I resistant Colo38R and M21R cells, and the partially BRAF-I resistant TPF-10-741 cells.



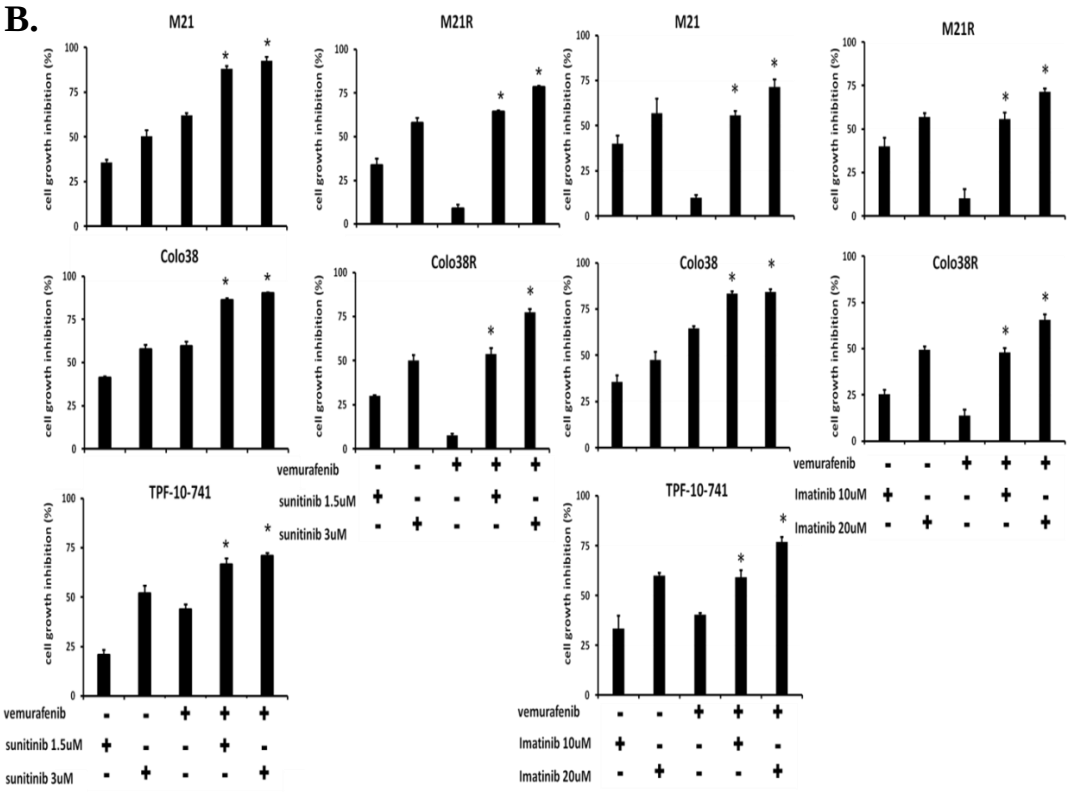


Figure 13. Enhancement by PDGFR*α* inhibition of the *in vitro* anti-proliferative activity of vemurafenib in BRAF-I sensitive and resistant melanoma cells harboring BRAF V600E. Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with the indicated concentrations of vemurafenib and/or sunitinib or imatinib. Cell growth inhibition was determined by MTT assay following a three days (**A**) and five (**B**) of treatment. Percentage of cell growth inhibition was calculated as ratio of treated to untreated cells at each treatment. Data are expressed as mean ± SD of the results obtained in three independent experiments. The asterisk (*) indicates $P < 0.05$.

Analysis of apoptosis by the Annexin-V expression assay (**Fig. 14**) demonstrated that following a 24 h treatment of vemurafenib and sunitinib induced apoptosis in a significantly ($p<0.05$) higher percentage of cells than each agent alone in both BRAF-I sensitive and resistant cell lines. When tested individually, vemurafenib induced apoptosis in a smaller number of BRAF-I sensitive and resistant cells than sunitinib ($p<0.05$).

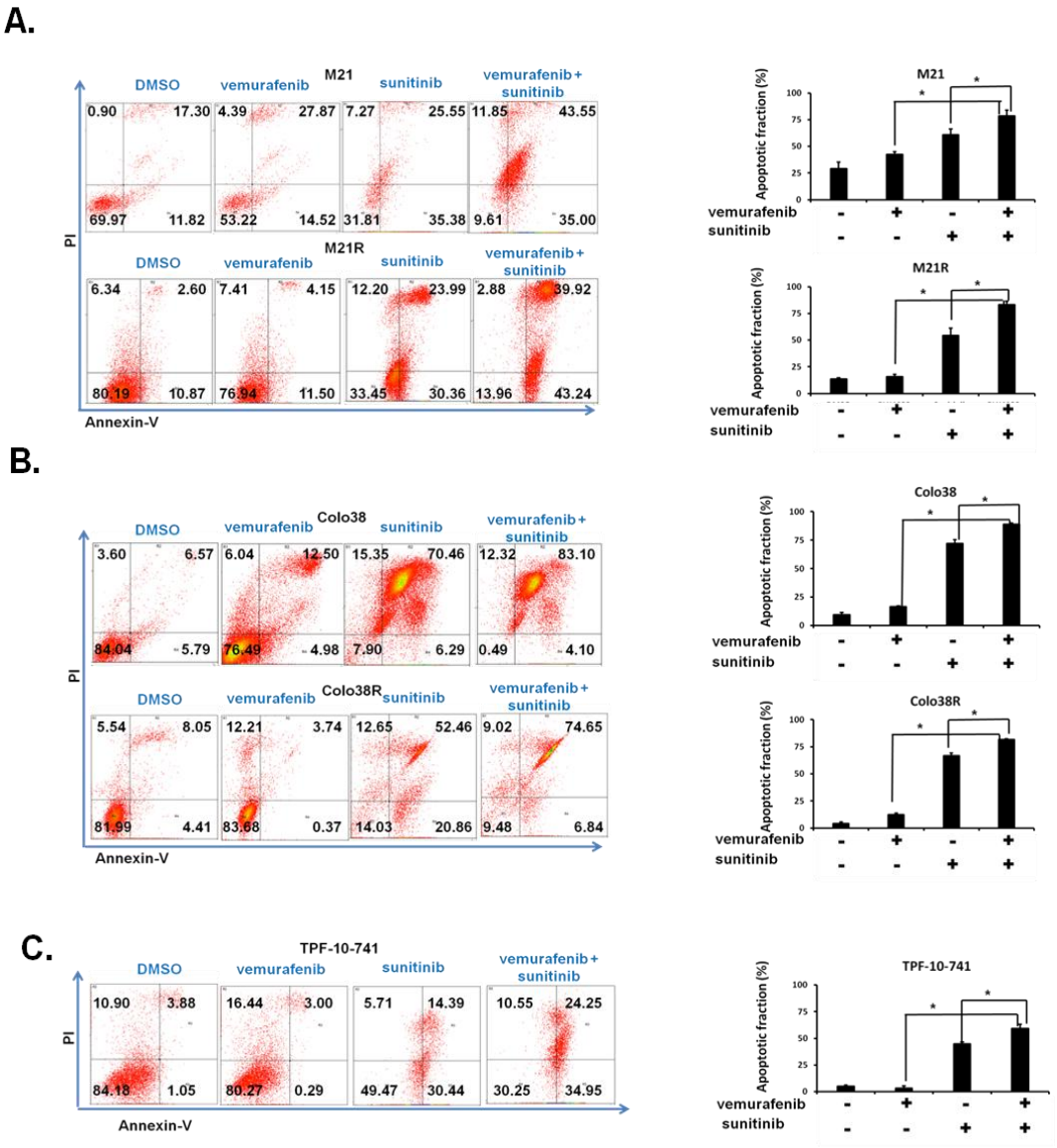


Figure 14. Enhancement by PDGFRα inhibition of apoptosis induction by vemurafenib in melanoma cells harboring BRAF V600E. M21 and M21R cells (**A**), Colo38 and Colo38R cells (**B**), and TPF-10-741 cells (**C**) were treated with vemurafenib (500 nM) and/or sunitinib (3 µM). Following a 24 h incubation at 37°C cells were harvested and stained with Annexin V and PI. The data presented are representative of the staining obtained in three independent experiments (**left panel**). The levels of apoptosis are plotted and expressed as mean fraction of apoptotic cells ± SD of the results obtained in three independent experiments (**right panel**). The asterisk (*) indicates $P < 0.05$.

4.5. BRAF and PDGFRα inhibition leads to ERK and AKT kinase signaling pathway inhibition.

The extent of PDGFRα depletion by specific shRNA in BRAF-I resistant cell lines was correlated with the degree of inhibition of ERK reactivation and AKT activation. Thus, additional experiments tested whether vemurafenib in combination with the PDGFRα inhibitors sunitinib or imatinib could inhibit the ERK and AKT kinase signaling pathways in BRAF-I sensitive and resistant cells. Western blot analysis (**Fig. 15**) demonstrated that following a 1, 24, and 72 h incubation p-ERK levels were decreased when BRAF-I sensitive Colo38 and M21 cells were treated with vemurafenib and to a lesser extent with sunitinib. In addition, p-AKT levels were increased when M21 cells were treated with vemurafenib and reduced when Colo38 and M21 cells were treated with sunitinib. However, vemurafenib in combination with sunitinib strongly inhibited the levels of both p-ERK and p-AKT in both BRAF-I sensitive cell lines. On the other hand, p-ERK levels were not inhibited by the treatment with vemurafenib in BRAF-I resistant Colo38R and M21R cells or the partially resistant TPF-10-741 cells. Treatment with sunitinib minimally inhibited p-ERK levels in Colo38R, M21R and TPF-10-741 cells, but reduced p-AKT levels in Colo38R, M21R and TPF-10-741 cells. However, vemurafenib in combination with sunitinib inhibited both p-ERK and p-AKT levels to a greater extent than each single agent in the all BRAF-I resistant cell lines. Similar results were obtained with vemurafenib in combination with imatinib. These data demonstrate that vemurafenib in combination with a PDGFRα inhibitor is an effective strategy to inhibit ERK reactivation and AKT activation caused by PDGFRα mediated BRAF-I resistance. Furthermore, this provides the mechanism for the anti-proliferative and pro-apoptotic effects seen in the BRAF-I sensitive and resistant cell lines treated with vemurafenib in combination with a PDGFRα inhibitor.

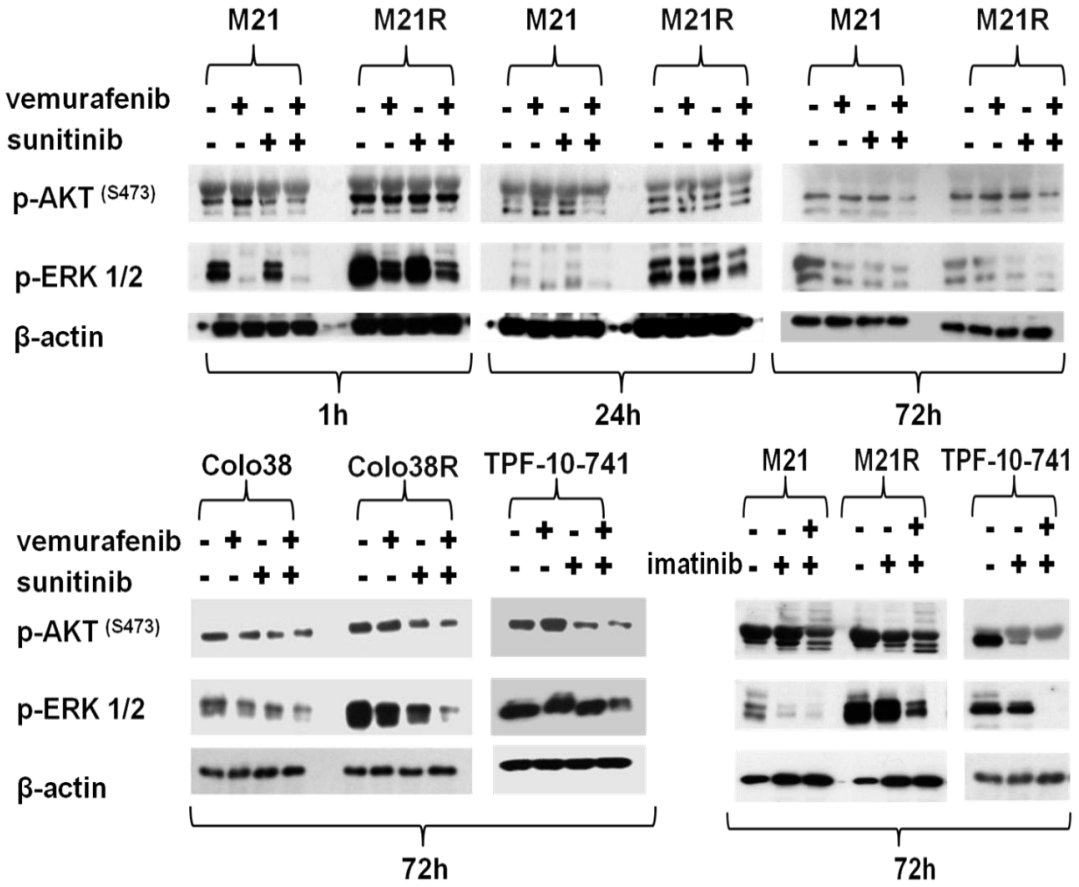


Figure 15. Enhancement by PDGFRα inhibition of the signaling pathway inhibition by vemurafenib in melanoma cells harboring BRAF V600E. Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 µM) and/or sunitinib (3 µM) and/or imatinib (20 µM). Following an incubation at indicated time points at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β-actin was used as a loading control. The results presented are representative of the results obtained in three independent experiments.

4.6. Effective melanoma xenograft growth inhibition by the combination of vemurafenib and a PDGFRα inhibitor.

To assess the *in vivo* relevance of the described *in vitro* results, the combination of vemurafenib and the PDGFRα inhibitors sunitinib and imatinib were tested for its

ability to inhibit the growth of BRAF-I sensitive M21 cells and BRAF-I resistant M21R cells in SCID mice. The oral administration of the drugs was well tolerated and caused no side effects (**data not shown**). In the mice grafted with the BRAF-I sensitive human melanoma cells M21 (**Fig. 16**) treatment with vemurafenib or sunitinib significantly ($p<0.001$) inhibited tumor growth as compared to untreated mice. However, vemurafenib in combination with sunitinib inhibited tumor growth to a significantly ($p<0.001$) greater extent than each single agent. The mean tumor volume at day 38 was 241.33, 674.37 and 539.28 cm3 in the group of mice treated with vemurafenib and sunitinib, with vemurafenib alone or sunitinib alone, respectively. The tumor volume in the group of untreated mice was 3544.95 cm^3.

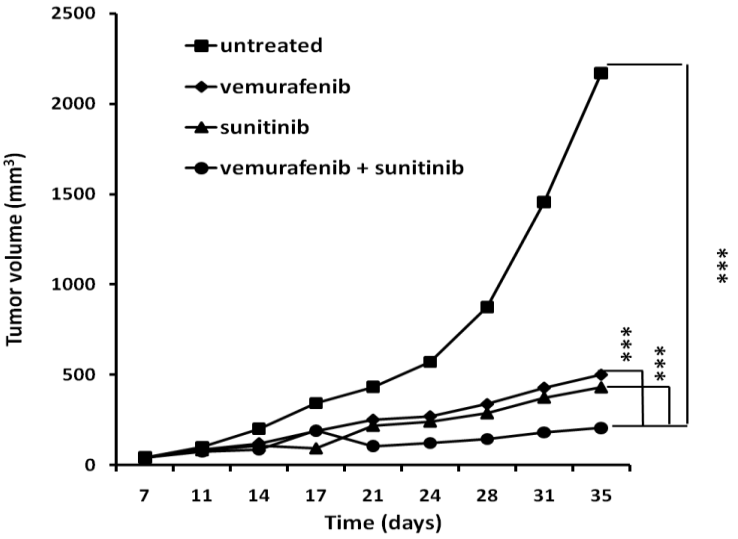


Figure 16. Enhancement by PDGFRα inhibition of the growth inhibition by vemurafenib of BRAF V600E melanoma M21 cells grafted in immunodeficient mice. M21 cells were implanted subcutaneously in 20 SCID mice. When tumors became palpable, mice were randomly divided into 4 groups (5 mice/group). One group was treated with vemurafenib (12.5 mg/kg, twice daily), one with sunitinib (20 mg/kg, once each day) and one with the vemurafenib (12.5 mg/kg, twice daily) in combination with sunitinib (20 mg/kg, once each day). One group of mice was left untreated as a reference for the natural course of the disease. Efficacy data are plotted as mean tumor volume (in mm^3) ± SD. The asterisk (***) indicates $P < 0.001$

Similar results were obtained combining vemurafenib (25 mg/kg twice daily) with imatinib (100 mg/kg each day) (**Fig. 17**).

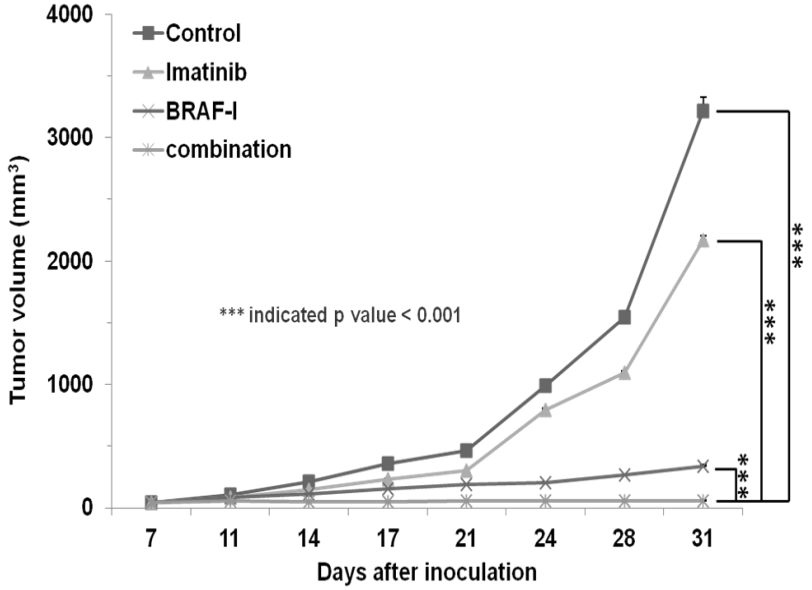


Figure 17. Enhancement by PDGFRα inhibition of the growth inhibition by vemurafenib of BRAF V600E melanoma M21 cells grafted in immunodeficient mice. M21 cells were implanted subcutaneously in 20 SCID mice. When tumors became palpable, mice were randomly divided into 4 groups (5 mice/group). One group was treated with vemurafenib (25 mg/kg, twice daily), one with imatinib (100 mg/kg, once each day) and one with the vemurafenib (25 mg/kg, twice daily) in combination with imatinib (100 mg/kg, once each day). One group of mice was left untreated as a reference for the natural course of the disease. Efficacy data are plotted as mean tumor volume (in mm^3) ± SD. The asterisk (***) indicates $P < 0.001$.

Western blot analysis of the tumors lysates removed from treated and untreated mice (**Fig. 17**) demonstrated that vemurafenib decreased p-ERK levels but increased p-AKT levels, while sunitinib decreased p-ERK and p-AKT levels. This effect was more marked in tumors from mice treated with vemurafenib in combination with sunitinib.

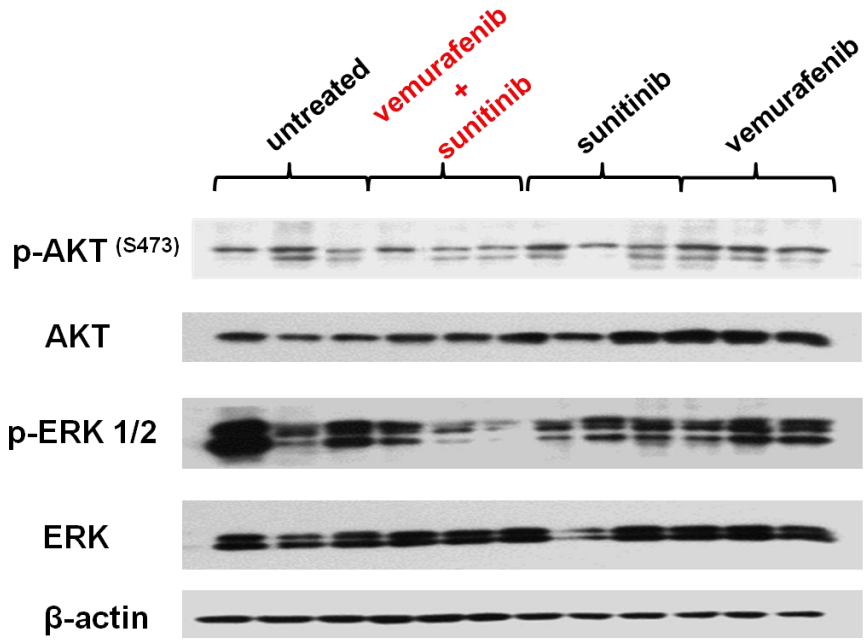


Figure 17. Enhancement by PDGFRα inhibition of signaling pathway inhibition by vemurafenib of BRAF V600E melanoma M21 cells grafted in immunodeficient mice. Tumors harvested from untreated and treated mice (three for each group) were lysed and analyzed for expression and activation of the indicated signaling pathways components. β-actin was used as a loading control.

Primary tumors were also evaluated by IHC for the rate of tumor cell proliferation and for induction of apoptosis using the surrogate markers p-Histone H3 and Cleaved Caspase-3. Both vemurafenib and sunitinib significantly decreased the number of mitotic cells in tumors as compared to tumors from untreated mice. Vemurafenib in combination with sunitinib (**Fig. 18A**) reduced the number of mitotic cells in tumors by 80% and 50%, respectively, when compared to tumors from untreated mice ($p < 0.001$) or mice treated with the single agents ($p<0.001$). The number of apoptotic cells (**Fig 18B**) in tumors from mice treated with vemurafenib in and sunitinib was approximately 40%, 29% and 26% higher than in tumors from untreated mice or from mice treated with vemurafenib or sunitinib individually ($p<0.001$). Sunitinib, but not vemurafenib resulted in a significantly

higher number of apoptotic cells in the tumors when compared to untreated mice ($p<0.001$).

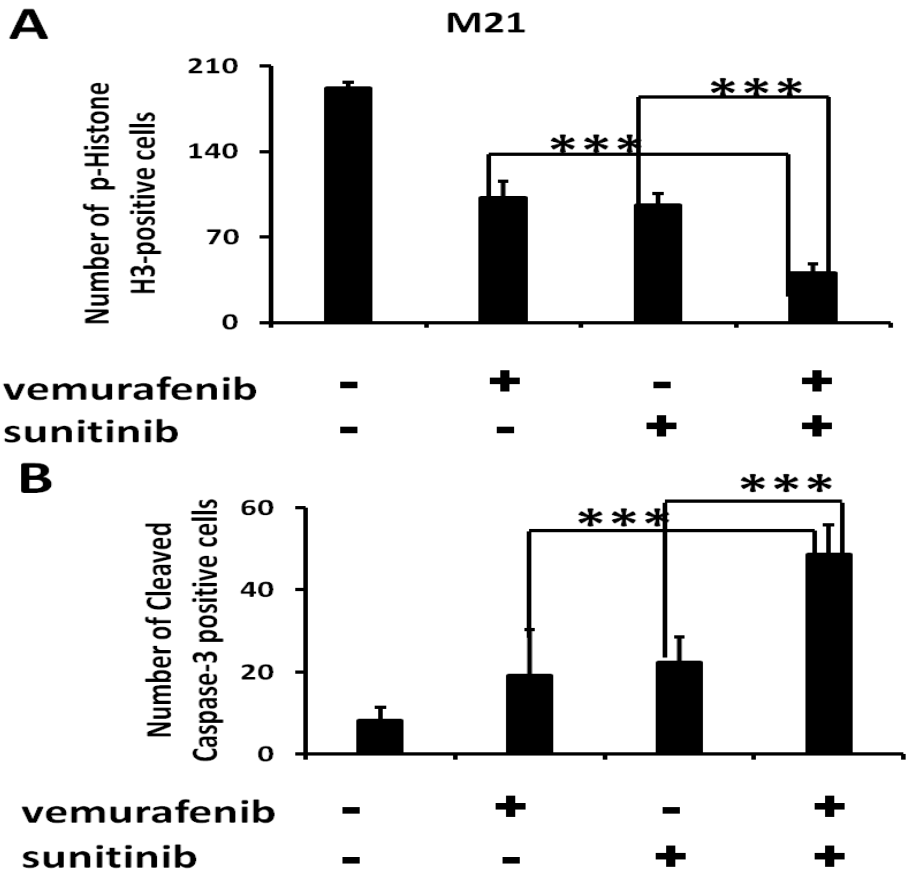


Figure 18. Enhancement by PDGFRα inhibition of the mitotic cell inhibtion and induction of the apoptotic cells by vemurafenib of BRAF V600E melanoma M21 cells grafted in immunodeficient mice. Tumor tissue sections were analyzed for the content of mitotic cells by staining with p-Histone H3 (Ser10) protein-specific antibodies. Mitotic tumor cells were quantified by counting 5 randomly selected high-power fields per section (magnification ×200) (**A**). Tumor tissue sections were analyzed for the content of apoptotic cells by staining with Cleaved Caspase-3 (Asp175)-specific antibodies. Apoptotic tumor cells were quantified by counting 5 randomly selected high-power fields per section (magnification ×200) (**B**). Data are presented as means ± SD. *** indicates $P < 0.001$.

Mice engrafted with the BRAF-I resistant human melanoma cells M21R (**Fig. 19**) had no significant inhibition of tumor growth when treated with vemurafenib as compared to untreated mice. In contrast, treatment with sunitinib caused a statistically significant inhibition of tumor growth compared to untreated mice ($p<0.001$). However vemurafenib in combination with sunitinib inhibited tumor growth to a significantly ($P<0.001$) greater extent than sunitinib alone.

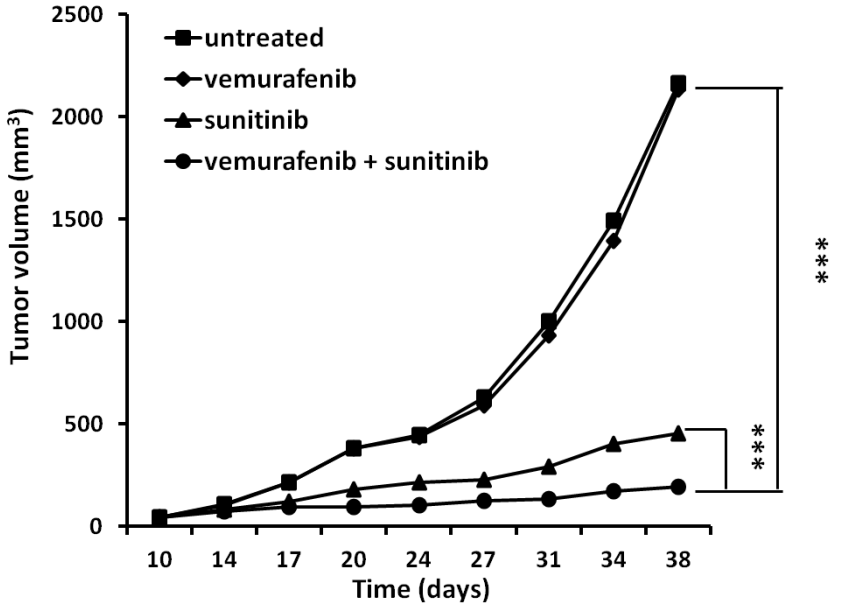


Figure 19. Enhancement by PDGFRα inhibition of the growth inhibition by vemurafenib of BRAF V600E melanoma M21R cells grafted in immunodeficient mice. M21R cells were implanted subcutaneously in 20 SCID mice. When tumors became palpable, mice were randomly divided into 4 groups (5 mice/group). One group was treated with vemurafenib (12.5 mg/kg, twice daily), one with sunitinib (20 mg/kg, once each day) and one with the vemurafenib (12.5 mg/kg, twice daily) in combination with sunitinib (20 mg/kg, once each day). One group of mice was left untreated as a reference for the natural course of the disease. Efficacy data are plotted as mean tumor volume (in mm^3) ± SD. The asterisk (***) indicates $P < 0.001$

Western blot analysis of tumor lysates (**Fig. 20**) demonstrated that sunitinib inhibited p-AKT and p-ERK levels. This effect was more marked in tumors from mice treated with vemurafenib in combination with sunitinib. As expected the levels of p-ERK and p-AKT were not affected by treatment with vemurafenib as a single agent.

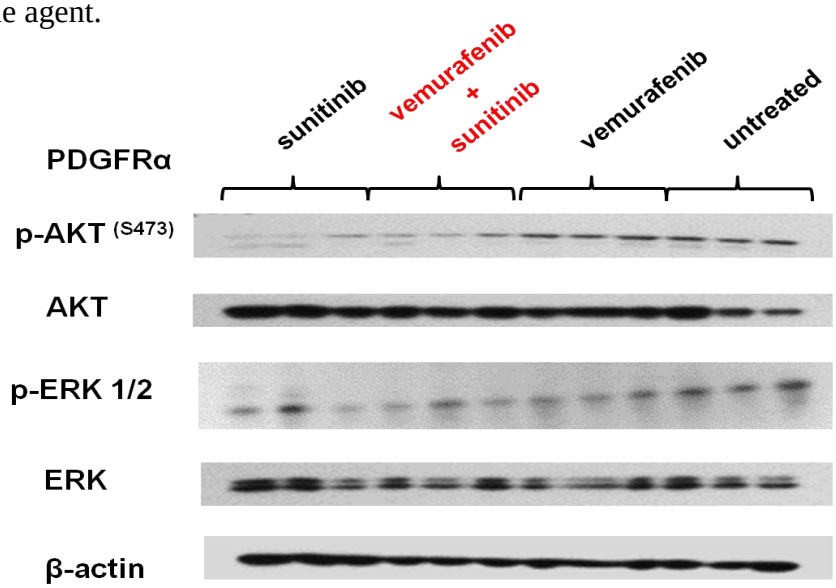


Figure 20. Enhancement by PDGFRα inhibition of signaling pathway inhibition by vemurafenib of BRAF V600E melanoma M21R cells grafted in immunodeficient mice. Tumors harvested from untreated and treated mice (three for each group) were lysed and analyzed for expression and activation of the indicated signaling pathways components. β-actin was used as a loading control

IHC staining (**Fig. 21A**) revealed that treatment with sunitinib decreased the number of mitotic cells in tumors by 46% and 43% as compared to that in tumors from vemurafenib or untreated mice ($p<0.001$). In addition, treatment with sunitinib caused a strong increase in the number of apoptotic cells in tumors as compared to tumors from vemurafenib treated or untreated mice (Fig. **21B**). However treatment with vemurafenib in combination with sunitinib decreased the

number of mitotic cells and increased the number of apoptotic cells to a significantly ($p<0.001$) greater extent than treatment with sunitinib alone.

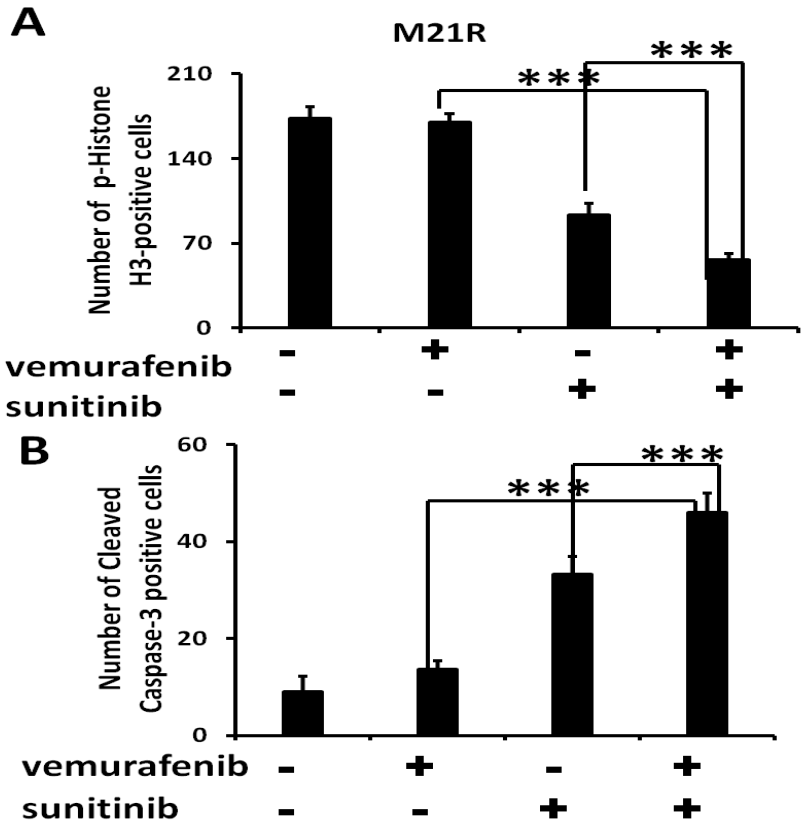


Figure 21. Enhancement by PDGFRα inhibition of the mitotic cell inhibtion and induction of the apoptotic cells by vemurafenib of BRAF V600E melanoma M21R cells grafted in immunodeficient mice. Tumor tissue sections were analyzed for the content of mitotic cells by staining with p-Histone H3 (Ser10) protein-specific antibodies. Mitotic tumor cells were quantified by counting 5 randomly selected high-power fields per section (magnification ×200) (**A**). Tumor tissue sections were analyzed for the content of apoptotic cells by staining with Cleaved Caspase-3 (Asp175)-specific antibodies. Apoptotic tumor cells were quantified by counting 5 randomly selected high-power fields per section (magnification ×200) (**B**). Data are presented as means ± SD. *** indicates $P < 0.001$.

These findings corroborate the *in vitro* results and demonstrate that the oral administration of vemurafenib in combination with a PDGFRα inhibitor is more effective than either agent alone in inhibiting the growth of BRAF-I sensitive and resistant melanoma cells if PDGFRα is mediating the BRAF-I resistance. Furthermore, they demonstrate that this growth inhibitory effect in both BRAF-I sensitive and resistant melanoma cell lines is mediated by a marked decrease of p-ERK and p-AKT levels. Lastly, they demonstrate that the oral administration of vemurafenib and PDGFRα inhibitors (sunitinib or imatinib) is well tolerated, supporting their further investigation in a clinical setting.

5. CONCLUSIONS

PDGFRα is overexpressed in sarcoma and glioma. It is thought to be involved in tumor growth, metastasis and neoangiogenesis in the tumor microenvironment, as well as in the development of resistance to cytotoxic therapy [93]. These functional properties of PDGFRα are likely to reflect its ability to engage signaling pathways, such as RAS/RAF/MEK/ERK, PI3K/AKT and PLC-γ which play a role in tumor cell proliferation and aggressive tumor phenotypes. The present study demonstrates that PDGFRα is expressed by human melanoma cells both *in vitro* and *in vivo*, and has identified an additional potential clinically relevant property of this growth factor receptor. Specifically, PDGFRα upregulation in human melanoma cells harboring the BRAF V600E mutation has been shown for the first time to be associated with the loss of their sensitivity to the anti-proliferative and pro-apoptotic activity of the BRAF-I vemurafenib both *in vitro* and *in vivo*. The association between PDGFRα upregulation and vemurafenib resistance reflects a cause-effect relationship, since sensitivity to vemurafenib is restored in melanoma cells which downregulate PDGFRα expression following transduction with a PDGFRα-specific shRNA. Association between PDGFRα and BRAF V600E mutation is also observed in wide type PDGFRα gastrointestinal stromal tumors (GIST) which acquire the BRAF (V600E) mutation when they develop resistance to PDGFRα inhibitors [94-97].

Vemurafenib resistance of melanoma cells harboring a BRAF mutation is likely to reflect ERK and AKT activation induced by PDGFRα upregulation, since inhibition of its synthesis by PDGFRα-specific shRNA causes a reduction of ERK and AKT activation and restores sensitivity to BRAF-I. This conclusion is corroborated by the *in vitro* and *in vivo* results obtained by inhibiting the function of PDGFRα with the tyrosine kinase inhibitor sunitinib or imatinib. Vemurafenib in combination with a PDGFRα inhibitor inhibits *in vitro* proliferation and induces apoptosis of melanoma cells with a PDGFRα upregulation mediated BRAF-I

resistance. These results are paralleled by our *in vivo* findings. Vemurafenib in combination with the PDGFRα inhibitor sunitinib inhibited the growth and induced apoptosis in human melanoma cells with PDGFRα upregulation mediated BRAF-I resistance engrafted in immunodeficient mice. These effects are mediated by the inhibition of the RAF/MEK/ERK and PI3K/AKT signaling pathways since the levels of p-ERK and p-AKT were markedly reduced in melanoma cells with PDGFRα upregulation mediated BRAF-I resistance following *in vitro* or *in vivo* treatment with vemurafenib in combination with a PDGFRα inhibitor. It is noteworthy that vemurafenib in combination with a PDGFRα inhibitor (sinitinib or imatinib) has a significantly greater anti-proliferative and pro-apoptotic effect than either agent alone both *in vitro* and *in vivo* also with BRAF-I sensitive human melanoma cells which expresses PDGFRα. These effects are mediated by a more marked inhibition of ERK and AKT activation by vemurafenib in combination with a PDGFRα inhibitor than by the individual agents. Therefore our results suggest that the combinatorial strategy we have developed may overcome not only the acquired, but also the intrinsic BRAF-I resistance if PDGFRα is expressed. Furthermore they confirm that simultaneous inhibition of both the AKT and ERK signaling pathways is more effectively in suppressing tumor cell proliferation and in inducing apoptosis in both BRAF-I sensitive and resistant melanoma cells [75, 98-104].

PDGFRα is not the only growth factor receptor which plays a role in BRAF-I resistance. IGFR [75] and PDGFRβ [65, 86] are involved in the acquired BRAF-I resistance of melanoma cell lines chronically exposed to increasing doses of BRAF-I and are upregulated in melanoma cells isolated from patients who developed BRAF-I resistance following treatment. BRAF-I resistance mediated by IGFR and PDGFRβ, similar to PDGFRα, is mediated by ERK and AKT activation. However as reported by Shi et al [80] and as found by us (**data not shown**) the PDGFRα / PDGFRβ inhibitors sunitinib and imatinib are not able to

overcome BRAF-I resistance mediated by PDGFRβ upregulation. The inability of sunitinib to overcome PDGFRβ mediated BRAF-I resistance reflects the lack of inhibition of ERK activation in spite of the inhibition of AKT activation since the inhibition of these two downstream components of the RAF/MEK/ERK and PI3K/AKT signaling pathways by a PDGFRβ specific shRNA restored sensitivity of melanoma cells to vemurafenib.

The potential clinical relevance of our results is suggested by two lines of evidence. First, PDGFRα expression was upregulated in 5 out of the 9 melanoma lesions with a BRAF V600E mutation surgically removed from patients who had developed BRAF-I resistance. Second, the extent of PDGFRα increase in melanoma lesions, as indicated by the IHC staining intensity, was associated with the clinical course of the disease. Specifically a marked increase in PDGFRα was found to be associated with a significantly shorter time to progression and worse objective response based on RECIST criteria. Notably, baseline expression of PDGFRα did not correlate with response or time to progression. In order to utilize the phenomenon we had observed as a method for patient selection, one would need to monitor up regulation of PDGFRα in tumor biopsy specimens in a similar early time point as we observed or develop noninvasive or surrogate methods to detect its up regulation.

These findings provide a strong rationale to translate to a clinical setting the combinatorial strategy which we have shown to be effective in counteracting the BRAF-I PDGFRα-mediated resistance of melanoma cells both *in vitro* and *in vivo*. The translation of this approach is facilitated by the fact that the agents we investigated are already approved for cancer treatment. Whether the side effects caused by the combined use of a BRAF-I and a PDGFRα inhibitor will represent a major obstacle to their clinical use remains to be determined.

6. REFERENCES

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7. TABLE

Table 1

						PDGFRα IHC Expression Score (+)		
ID#	Sex	Age	Treatment	Objective Response	time to progression (months)	Pre-treatment	On-treatment	After Progression of disease
1	M	68	BRAFi	SD (-25%)	5	1	3	-
7	M	56	BRAFi + MEKi	CR (100%)	17	1	-	2
8	M	37	BRAFi + MEKi	PR (-30%)	3	4	4	-
10	F	37	BRAFi + MEKi	SD (-13%)	3	0	4	-
12	F	31	BRAFi + MEKi	PR (-88.9%)	12	3	3	-
13	M	69	BRAFi + MEKi	PR (-57.9%)	9	1	2	-
17	M	74	BRAFi	PR (-71.7)	15	-	-	1
21	M	61	BRAFi + MEKi	PR (-49%)	13	-	-	4
25	M	72	BRAFi + MEKi	PR (-64%)	3	1	-	4

Title: p53 modulates the enhanced cytotoxicity induced by Poly(ADP-Ribose) Polymerase-1 inhibition to Topoisomerase-I inhibitor and radiotherapy in human glioblastoma cell lines.

Running title: p53, PARP inhibitor, topotecan, radiotherapy in glioblastoma cells

Authors:

Francesco Sabbatino[1,4], Celeste Fusciello [1], Antonio Leonardi [2], Roberto Pacelli[3], Ravin Poudel[3], David Pepin[4], Soldano Ferrone[4], Chiara Carlomagno[1], Stefano Pepe[5]

University of Naples "Federico II", [1]Department of Clinical and Molecular Oncology and Endocrinology, [2]Department of Cellular and Molecular Biology and Pathology, [3]Department of Diagnostic Imaging and Radiation Oncology, Federico II University School of Medicine, Via Sergio Pansini 5, Naples, Italy 80131.

[4]Department of Surgery, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114.

[5]Department of Medicine, University of Salerno and Division of Oncology, San Giovanni di Dio e Ruggi d' Aragona Hospital, Salerno, Italy 84131.

Correspondence should be addressed to Stefano Pepe (spepe@unisa.it)

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Keywords: p53, glioblastoma, PARP inhibitor, radiotherapy, topotecan

Abstract

Purpose. Chemo-radiotherapy still represents the standard of care for glioblastoma (GBM) patients. This combination, although leads to increased overall survival, is effective for limited time and GBM patients continues to carry a poor overall survival. Inhibition of Poly (ADP-Ribose) polymerase-1 (PARP-1) is shown to offer great promise for the treatment of GBM by sensitizing GBM cells to DNA- damaging agents such as radiotherapy or Topoisomerase-1 inhibitors. Nevertheless, there is the need to identify molecular biomarkers which can predict use of PARP inhibitors in combination with TPT and/or RT. **Methods and Materials.** Here, we investigated and compared the cytotoxic effects of combined treatment with the DNA-topoisomerase- 1-inhibitor topotecan (TPT), PARP-1 inhibitor NU1025 and radiotherapy in GBM cells. **Results and Conclusions.** Our results demonstrate that although PARP inhibition enhances the cytotoxicity of TPT and radiotherapy, the extent of this enhanced inhibition is modulated by the activity of p53. Specifically, utilizing cells carrying a different status of p53 and evaluating the growth inhibitory effects, the cell cycle perturbations and the DNA damage induced by the different combinatorial strategies of TPT, NU1025 and RT we demonstrated that in GBM cells carrying a p53 wild type the combination of PARP inhibitor and radiotherapy represents the most effective therapeutic strategy. On the other hand in GBM cells carrying a p53 mutated or silenced the combination of PARP inhibitor and TPT is the most effective therapeutic strategy. Our results can be clinical relevant since they expand the potential application of

PARP inhibitors in combination with TPT or RT for the treatment of GBM based on their p53 status.

Introduction

Gliomas represent the most common solid tumors of the Central Nervous System (CNS). Around 30% of Gliomas are Astrocitomas, which are further divided into four subgroups in relation to their grading: III and IV are named Glioblastomas (GBM). Chemotherapy (temozolomide) given concurrently or after radiation therapy (RT) is the standard of care for patients with newly diagnosed GBM [33]. This combination has been shown to significantly increase the overall survival of newly diagnosed GBM patients when compared with RT alone (12.1 *vs* 14.6 months). However, despite these successes, GBMs continue to carry a dismal two year survival rate of 3.3%[22] .

Disruption of p53, which is widely prevalent in GBM cells, has been shown to sensitize them to chemotherapeutic agents such as inhibitors of DNA topoisomerase I (TOPO I)[39].Topotecan (TPT), an inhibitor of TOPO I, is approved for GBM treatment in the refractory and relapsed setting[31]. *In vitro* and *in vivo* [2,9,12,17,26]evidence demonstrates that TPT exhibits radiosensitization and cell growth inhibition effects on human GBM cells. However, concurrent administration of TPT and radiotherapy in phase II trials demonstrated an increased toxicity with only small benefits in terms of progression-free survival (PFS) for GBM patients [10,18].

Recently, the use of Poly (ADP-ribose) polymerase-1 (PARP-1) inhibitors has shown great promise in the treatment of several cancers, including GBMs [29]. PARP-1, as well as p53, is involved in the DNA repair system. Specifically, it is a nuclear protein involved in sensing and signaling the presence of DNA

damage. Its activation causes the addition of poly (ADP-ribose) branched chains from nicotinamide adenine dinucleotide donors onto several proteins of DNA repair machinery. In this way PARP-1 leads the recruitment of proteins of the base excision repair system [28], single or double stranded break repair pathway [6,7,23], and nuclear proteins such as p53 [40] and TOPO I/II [32].

Different chemical inhibitors of PARP-1 have been shown *in vitro* and *in vivo* to potentiate the cytotoxic effects induced by RT and by chemotherapeutic agents such as temozolomide or TOPO I inhibitors in GBMs [3,5,11,35,36]. Nevertheless the lack of predictive factors of response to different emerging strategies emphasizes the need to identify biomarkers which indicate the most effective strategy to achieve the maximum cytotoxic effect without an increase of the side effects. 8-idrossi-2-metiquinazoline-4-one (NU1025) is a PARP-1 inhibitor that has been shown *in vitro* to inhibit PARP-1 activity without cytotoxicity [5,30].

In this study, using human GBM cell lines with an active or inactive p53, we compared *in vitro* treatments utilizing TPT and/or RT and/or PARP-1 inhibition. Our aim is to define the role of p53 in modulating the potentiated cytotoxicity of TPT and/or RT induced by PARP-1 inhibition and simultaneously to define the most effective strategy for the treatment of GBM. Our data confirm the clear potential of PARP-1 inhibition in potentiating the cytotoxicity of TPT and RT in GBM cells. Furthermore, they demonstrate that p53 activity influences and predicts the response to the enhanced cytotoxicity induced by PARP-1 inhibition. Specifically our data provide a rational basis for treating subgroups of patients

carrying a functional or non-functional p53 with a PARP inhibitor in combination with RT and TPT, respectively.

Materials and Methods

Cell cultures

The human GBM cell line D54, carrying an active p53 (D54p53wt), and the human GBM cell line U251, carrying a mutated (codon 273) and inactive p53 (U251p53mut), were obtained from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixtures F-12 (Ham's) 1:1 (GIBCO/Invitrogen, CA, USA), supplemented with 10% foetal calf serum (FCS, Lonza, CA, USA), 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM L-glutamine (complete medium). Cells were cultured at 37°C in a 5% CO_2 atmosphere.

Chemical reagents and antibodies

Topotecan (TPT) was purchased from Glaxo Smith-Kline (Brentford, United Kingdom). 8-hydroxy-2 methylquinazolinone-4one (NU1025) was purchased from Alexis Biochemicals (San Diego, CA, USA). 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Propidium iodide (PI) and RNAse were purchased from Sigma Chemicals Co (St Louis, MO, USA). 5-bromo-2'-deoxyuridine (BrdU) and Alexa Fluor 488 goat anti-mouse were purchased from Invitrogen (Portland, OR, USA). Anti-BrdU Pure mouse monoclonal antibody was purchased from BD Biosciences (San Giose, CA, USA). Anti-phospho-Histone

γH2AX (Ser139) Fluorescein isothiocyanate (FITC)-conjugate and anti-Poly (ADP-Ribose) mouse monoclonal antibodies were purchased from Millipore (Billerica, MA, USA). FITC-conjugated secondary antibody goat anti-mouse was purchased from Dako (Milan, Italy). p53-, p21- and β-actin-specific monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibody horseradish peroxidase-conjugated was purchased from Amersham-Pharmacia (Buckinghamshire, UK).

Cell proliferation and MTT assay

Cells were plated in triplicate in 96-well microtiter plates at a density of 2.5 x 10^3 per well in 100 ul of complete medium. Following a 24 h incubation at 37°C in a 5% CO_2 cells were treated with TPT, NU1025 and RT. Adherent cells were irradiated in medium with 250 kVp X-rays (dose rate 0.5 Gy/min). For the combined treatments NU1025 was administrated at 10μM. This dose has been shown to inhibit PARP activity in D54p53wt and U251p53mut tumor cells by >90% [5]. For each experiment an untreated control was included. Cell proliferation was evaluated by MTT assay at indicated time points. MTT assay was carried out as reported elsewhere [20]. Absorbance was measured using a spectrophotometric microplate reader (MTX Lab System, Inc, Vienna, VA) at 540 nm. Cells in randomly selected fields per well were photographed at different time points using a Zeiss Inverted Fluorescence Microscope (AxioVision Software, Carl Zeiss Micro-Imaging GmbH, Germany). Data were expressed as

percent inhibition of treated cells compared with the untreated control cells. All experiments were performed three independent times in triplicates.

Cell cycle analysis and BrdU incorporation

Cells were seeded in triplicate in 6-well plates at a density of 3 x 10^5 cells. Following a 24 h incubation at 37°C in a 5% CO_2 cells were treated with TPT, NU1025 and RT. Untreated control cells were included. Cell-cycle analysis was determined by PI staining at indicated time points. Briefly, cells were trypsinized, counted, washed with PBS and stained with PI (50µg/ml; 60 min at RT). Possible double-stranded RNA was removed by incubation with RNAse (0.1 mg/ml). Cell proliferation with possible freezing or block phase in cell cycle was determined by BrdU incorporation and PI staining. After treatment cells were labelled with BrdU (20µM) for 30 min, immediately the medium was removed and fresh medium was added. Cells were then trypsinized at 0, 6 and 12 h and washed with PBS. After centrifugation cell were resuspended in 70% ice-cold ethanol and stored at -20°C until cell cycle analysis. Preparation of samples for FACS analysis was performed as described elsewhere [15]. Cells were analyzed by flow cytometry (FACScan, Becton Dickinson, San Josè, CA, USA) using the CyCLOPS Summit (Cytomation, Fort Collins, CO, USA). Distribution of the cells in the different cell cycle phases was evaluated by Mod Fit 2.0 (Verity Software HOUSe INC., Ranger, ME, USA). For analysis of the BrdU-DNA bivariate graphs, cells were split into the following four categories according to their DNA content and fluorescence intensity level: (1) BrdU-positive cells *(BrdU+)*; (2) G1 phase / BrdU-

positive cells (*G1+*); (3) S phase / BrdU positive *(S+)*; (4) G2/M phase / BrdU-positive cells (*G2/M+*).

DNA damage

Cells were seeded and were treated with TPT, NU1025 and RT as described above. Untreated control cells were included. DNA damage was assessed by flow cytometric characterization of DNA Strand Breaks using phosphorylated Histone γH2AX on Ser-139 and DNA content detection [14]**.** Following 1 and 4 h of treatment cell media was aspirated and cells were trypsinized, washed with TBS, fixed with 70% ice-cold ethanol and stored at -20°C until flow-cytometric analysis. During preparation for FACS analysis cells were permeabilized with 1% paraformaldehyde and 0.25% Triton-X-100 in TBS for 5 min. After centrifugation, cells were suspended in TBS and 3% BSA for 30 min. Then the cells were stained with anti-phosho-Histone H2AX (Ser139)-FITC conjugate antibody with 3% BSA and incubated for 1 hr at room temperature. To correlate DNA damage with cell position in the cell cycle after three washes with TBS, cells were stained with PI 20µg/ml and RNAse 0.1 mg/ml for 60 min at RT. Cells with their distribution in the different cell cycle phases with or without γH2AX expression were analysed by FACScan. Cells were plotted with bivariate distributions (DNA content *versus* γH2AX expression). To evaluate cell analysis distribution untreated and treated cells were split in the following different gates: (1) H2AX-positive cells *($H2AX^+$)*; (2) H2AX-negative cells *($H2AX^-$)*. Mathematic difference (∆) between percentage of levels of γH2AX expression (*H2AX+*) of treated cells

(tr) and untreated cells (untr) was calculated to compare the effects (Δ % = $H2AX^{+}_{tr} - H2AX^{+}_{untr}$).

Immunofluorescent staining

Cells were seeded at a density of 1 x 10^5 cells and were grown as monolayers on cover slips placed in cell culture dishes. Following a 24 h of incubation at 37°C in a 5% CO_2 cells were treated with TPT, NU1025 and RT. Untreated control cells were included. PARP-1 activity was determined by immunofluorescent detection of poly-ADP-ribosylation of DNA as described by Bürkle et al. [4]. Following 4 h of treatment cover slips were gently removed and cells were fixed with 3% paraformaldehyde pH 7 in PBS at room temperature. Cells on cover slips were then washed three times with PBS and were permeabilized with 0.2% Triton-X-100 for 5 min on ice. After washing with PBS and 1% BSA, cells were incubated with anti-Poly (ADP-Ribose) monoclonal antibody at dilution of 1:200 in 1% BSA for 1 h at room temperature. Then cells were washed again with PBS and 1% BSA and stained with FITC-conjugated goat anti-mouse IgG antibody (1:100 in PBS and 1% BSA) for 1 h at room temperature. Lastly cells were stained with PI (5µg/ml) for 30 min as an indicator of nuclear compartment and cover slips were mounted on slides. Slides were examined by Leica DMRXA fluorescent microscope. Images were captured by a QImaging CCD camera and exported in Microsoft PowerPoint.

Cell transfection

D54p53wt cells were seeded at the density of 6 x10^4 per well in a 6-well plate and incubated in culture medium for 24 h at 37ºC in a 5% CO_2 atmosphere prior to viral infection. Cells were transfected with vector encoding for p53-RNAi using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Two synthetic oliginucleotides were designed using the following sequences: 5'-CCGGAATTCCCGACTCCAGTGGTAATCTACttcaagagaGTAGATTACCA-CTGGAGTCTTTTTGGAACTCGAGCGG-3' and 5'-CCGCTCGAGTTCCAAAAAGACTCC-AGTGGTAATCTACtctcttgaaGTAGATTACCACTGGAGTCGGGAATTCCGG-3' [13]. The resulting double stranded oligonucleotides were then cloned into the pcRNAi vector encoding resistance for geneticin (G-418), and derived from the pcDNA3.1 vector (Invitrogen) replacing the viral promoter-cassette with the H1 promoter that is specifically recognized by RNA polymerase III (kindly provided by Prof. Antonio Leonardi). Two days after transfection, geneticin G-418 (GIBCO) was added to cell culture medium (200 µg/ml) and maintained to enrich resistant-transfected clones (D54p53-) (clones named P1 and P2).

Western blot analysis

D54p53wt and D54p53- (P1 and P2) GBM cells were seeded at a density of 1 x 10^5 cells and treated with doxorubicin 2.5 µg/ml at 37 °C in a 5% CO_2 atmosphere for 1 h. Untreated control cells were included. After treatment cells were collected and lysed in lysis buffer [10 mM Tris–HCl (pH 8.2), 1% NP40, 1 mM EDTA, 0.1% BSA, 150 mM NaCl) containing 1/50 (vol/vol) of protease

inhibitor cocktail (Sigma). Protein concentrations were determined with the Protein Assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS-page and transferred to PVDF membranes (Millipore). Nonspecific binding was blocked by incubation with TBST (20mM Tris-HCl pH 7.4, 150mM NaCl and 0.1% Tween-20) plus 5% of non fat dry milk. Membranes were incubated with p53-, p21- and β-actin-specific monoclonal antibodies for 1 h at room temperature. After TBST washing, blots were incubated for 1 h at room temperature with secondary antibody horseradish peroxidase-conjugated and the signals detected diluted 1:5000 in TBST buffer, and then revealed by ECL (Amersham Biosciences).

Statistical methods

Averages, standard deviations, and unpaired t-test were calculated using Excel software (Microsoft). Data showed the mean ± SD of the results obtained in at least three independent experiments. Differences between groups were considered significant when the P value was < 0.05. The asterisk (*) indicates $P < 0.05$.

Results

Effect of NU1025 in Combination with TPT and RT on Tumor Cell Growth

The ability of NU1025 to enhance the growth-inhibitory effects of TPT and/or RT was evaluated using human GBM cell lines D54p53wt and U251p53mut. In order to establish the dose of TPT and RT to be used in the combinatorial treatments

and to confirm the lack of cytotoxicity induced by NU1025, a dose titration experiment was performed in all cell lines utilizing different doses of TPT (0.024-800 nM), RT (1; 2; 4 Gy) and NU1025 (0.170-160µM) **(Fig 1A-C)**. U251p53mut cells were found to be more radioresistant than D54p53wt. The doses of 5nM and 10nM for TPT and those of 2Gy for RT were chosen for further testing in the combinatorial treatments. The dose of 10µM for NU1025 was chosen to combine with TPT and RT based on the data available in the literature that demonstrated 90% inhibition of PARP-1 activity [5]. Analysis of the cell growth inhibition by MTT assay demonstrated that (**Fig. 2**):

1. in the D54p53wt cell line, the inhibition of PARP-1 with NU1025 10µM synergized ($P<0.05$) with TPT (5nM / 10nM) or 2Gy in inhibiting tumor cell growth. However the combination of 2Gy and NU1025 inhibited the proliferation of D54p53wt cells to a significantly ($P<0.05$) greater extent than the combinations of TPT (5nM / 10nM) and NU1025. In addition no statistically significant differences were detected between the combination of 2Gy and NU1025, and the combinations of TPT (5nM / 10nM) and 2Gy;

2. in the U251p53mut cell line, although it is more radioresistant than D54p53wt, the inhibition of PARP-1 with NU1025 10µM synergized ($P<0.05$) with TPT (5nM / 10nM) or 2Gy in inhibiting tumor cell growth. However the combination of TPT 10nM and NU1025 caused the maximum inhibitory effect inhibiting the proliferation of U251p53mut cells to a significantly ($P<0.05$) greater extent than the combination of NU1025 and 2Gy, and TPT (5nM / 10nM) and 2Gy;

3. in both cell lines, use of triple treatments did not increase the cytotoxicity when compared with the more effective double combinations.

These data confirmed the clear potential of the inhibition of Poly (ADP-ribosyl)ation in enhancing the cytotoxicity of TPT and RT in GBM cell lines although its inhibition did not cause direct cytotoxic effects. However in the D54p53wt cell line the combination of RT and NU1025 showed greater activity than the combination of TPT and NU1025, and comparable activity than the potential toxic combination of TPT and RT. On the other hand, in the U251p53mut cell line, which are more radioresistant than D54p53wt cells, the combination of TPT 10nM and NU1025 demonstrated more cytotoxicity than the combination of RT and NU1025, and the combination of TPT and RT.

Effects of PARP-1 Inhibition, TPT and RT on the cell cycle distribution

To evaluate the effects of NU1025, RT and TPT on cell cycle, PI staining was performed in D54p53wt and U251p53mut GBM cell lines. Cell cycle analysis following a 96 h incubation demonstrated no alteration in cell cycle distribution in both cell lines after treatment with NU1025 (10 µM). In D54p53wt cells treatment with 2Gy or TPT 5nM or TPT 10nM alone did not alter cell cycle distribution as compared with untreated cells **(Fig 3A, B)**. Treatment with TPT (5nM or 10nM), 2Gy and NU1025 used as double or triple combinatorial treatment significantly ($p<0.05$) increased the percentage of cells in S-phase. However a significant ($p<0.05$) difference in the percentage of S-phase was found between the cells treated with 2Gy and NU1025 and those treated with TPT (5nM or 10nM) and

NU1025. Furthermore no difference was found between cells treated with 2Gy and NU1025, and those treated with TPT (5nM or 10nM) and 2Gy. In the U251p53mut cell line (**Fig 3C, D**) cell cycle analysis demonstrated that treatment with 2Gy, and 2Gy in combination with NU1025 did not alter cell cycle distribution. Treatment with TPT (5nM and 10nM) alone or in combination with 2Gy significantly ($p<0.05$) increased the percentage of cell death and of cells in S- and G2/M-phase. However the combination of TPT and NU1025 more markedly ($p<0.05$) increased the cell death and accumulation of cells in S- and G2/M-phase when compared to TPT and RT ($p<0.05$) or to NU1025 and RT ($p<0.05$) combinations.

To validate the S-phase block or delay in cell cycle progression, BrdU incorporation and PI staining were performed after treatment with TPT, 2Gy and NU1025 in D54p53wt and in U251p53mut GBM cells. Following a 96 h incubation, flow cytometric analysis demonstrated that in the D54p53wt cells treatment with 2Gy and NU1025 decreased the percentage of cells labeled with BrdU (BrdU+) when compared to untreated cells (**Fig 4A)**. Analysis of cell cycle at different time points defined a delay of cell cycle progression. The cells principally reached G1-phase, but 12 h after BrdU incorporation they were still arrested in G1. Similar results were obtained with the combination of TPT and 2Gy (**data not shown**).

In U251p53mut BrdU incorporation and PI staining confirmed a strong accumulation in S-phase and block in G2/M-phase following 96 h incubation treatment with TPT 10nM and NU1025 (**Fig 4B**). This effect was more evident

gating BrdU-positive cells: at 0 h the cells were in G1-phase and entered in S-phase, but at 6 hrs and at 12 hrs from BrdU incorporation all cells were arrested in G2/M-phase.

These data demonstrate that the inhibition of poly(ADP-ribosyl)ation enhanced cell cycle perturbations caused by TPT or RT, but did not cause alterations when used alone. However in D54p53wt cells the combination of RT and NU1025, which showed comparable activity to RT and TPT combination and greater activity than TPT and NU1025 combination, caused a delay of cell cycle progression consistent with a G1-phase block. In U251p53mut cells the combination of TPT and NU1025, which altered cell cycle progression to a greater extent than the RT and NU1025 combination or the TPT and RT combination, caused complete block in G2/M phase and cell death.

Effects of PARP-1 Inhibition, TPT and RT on DNA Damage

To evaluate the mechanisms underlying the enhanced cytotoxic effects of TPT and RT by PARP-1 inhibition, we determined DSB presence by γH2AX detection and PI staining in the cells treated with TPT, 2Gy and NU1025. Flow cytometric analysis demonstrated that:

1. in D54p53wt cells (**Fig 5A-C**), following a 1 h incubation, treatment of TPT, 2Gy and NU1025 alone or in combination did not increase γH2AX expression in treated cells when compared to untreated cells (Δ). An increase of γH2AX expression was found after 4 h of treatment with TPT or RT. These effects were markedly enhanced ($p<0.05$) by the treatment

with NU1025, which did not increase γH2AX expression when used alone. However, the combination of RT and NU1025 increased γH2AX expression to a greater extent ($p<0.05$) than the combination of TPT and NU1025 and demonstrated comparable effect to the combination of TPT (10nM) and RT, which also strongly increased γH2AX expression in treated cells;

2. in U251p53mut cells (**Fig 5D-E)** following a 1 h and 4 h treatment of TPT or RT increased γH2AX expression in the treated cells when compared to untreated cells. The inhibition of PARP-1 by NU1025, as seen in D54 p54wt, enhanced the DNA damage induced by TPT or RT. Nonetheless in U251p53mut cells the combination of TPT (10nM) and NU1025 increased γH2AX expression to a greater extent than the combination of RT and NU1025 ($p<0.05$) or the combination of TPT (10nM) and RT.

These data are in line with the growth inhibitory effects and with cell cycle perturbations obtained by the treatment with TPT, NU1025 and RT in human GBM cells D54p53wt and U251p53mut. The Inhibition of poly(ADP-ribosyl)ation enhances the cytotoxic effect caused by TPT and RT in both cell lines through an increase of DNA damage. However in D54p53wt cells the combination of RT and NU1025 demonstrate a comparable effect than the combination of TPT and RT but a greater effects than the combination of TPT and NU1025. In U251p53mut cells the combination of TPT and NU1025 demonstrated more cytotoxicity than the combination of RT and NU1025, and the combination of TPT and RT.

Fluorescent microscopy expression of PARP activity in D54p53wt and U251p53mut cells and its inhibition by NU1025

To demonstrate the activation of PARP-1 by TPT or RT treatment and its inhibition by NU1025, we evaluated the poly(ADP-ribosyl)ation expression of DNA damaged sites in D54p53wt and U251p53mut cells. D54p53wt and U251p53mut GBM cell lines were treated with TPT 10nM, 2Gy, NU1025 10µM and stained after 4 h of treatment. Immunofluorescent staining demonstrated that:

1. In D54p53wt (**Fig 6A)** treatment with TPT 10nM or 2Gy increased nuclear activation of PARP-1 when compared to untreated cells. However its activation was strongly decreased by the treatment with NU1025 10µM.
2. In U251p53mut cells (**Fig 6B)** a higher nuclear activity of PARP-1was found compared to D54p53wt in basal conditions. This ‘‘stand alone’’ phenomenon was most likely caused by the lack of functional active p53 as reported in other tumors [21]. Nonetheless treatment with 2Gy and even more with TPT strongly increased PARP-1 activation. This effect was strongly decreased by the treatment with NU1025, as in D54p53wt cells.

These data demonstrated the ability of NU1025 to inhibit PARP-1 activation at 10µM in both GBM cell lines.

p53 activity as a predictive marker of PARP-1 dependent enhancement of RT and TPT inhibition.

Our studies indicated that inhibition of poly(ADP-ribosyl)ation by NU1025 enhances the cytotoxic effects of TPT or RT in both D54p53wt and U251p53mut human GBM cell lines. However these two cell lines showed different sensitivity to the enhanced cytotoxicity of TPT and RT induced by PARP-1 inhibition. To test whether p53 activity may modulate the enhanced cytotoxicity of TPT and RT induced by PARP-1, p53 was knocked down in D54p53wt cells using two specific p53-siRNAs, and the effects of TPT, RT, and NU1025 were studied by cell proliferation assay and cell cycle analysis. Western blot analysis demonstrated that p53 expression was strongly inhibited in both transfected clone (P1 and P2 cells) cells (D54p53-) under basal conditions and after treatment with doxorubicin **(Fig. 7)**. p53 as well as its downstream protein p21 were both down regulated. Irradiation of both GBM cell lines D54p53wt and D54p53- with different doses of RT demonstrated that D54p53- cells were more radioresistant than D54p53wt cells **(Fig 8A).** Furthermore analysis of cells growth inhibition after treatment with TPT 10nM, 2Gy and NU1025 in both cell lines demonstrated that PARP-1 inhibition enhanced the cytotoxicity of TPT and RT also in D54p53- (**Fig. 8B**). However in D54p53- cells the combination of TPT and NU1025 inhibited cell growth to a greater extent than the combination of TPT and RT, and the combination of RT and NU1025. Lastly, cell cycle analysis of D54p53- cells treated with TPT, RT and NU1025 demonstrated an accumulation in S- and G2/M-phase of treated cells **(Fig 8C)** when compared to untreated cells. However this effect was more pronounced with the combination of TPT and

NU1025 than the combination of 2Gy and NU1025 or TPT and 2Gy. These results were in line with the data obtained with U251p53mut cells.

Discussion

In the Era of the targeted therapy current treatment for GBMs still involves a combination of chemotherapy and radiotherapy. So far, the combination of temozolomide and radiotherapy still represents the standard of care for GBM treatment. This combination, although leads to increased overall survival, is effective for limited time and GBM patients continues to carry a poor overall survival. There is evidence that the combination of Topoisomerase-I inhibitors and radiotherapy may be an alternative strategy for the treatment of GBMs. However its use seems to lead to an increased toxicity [10,12,27]. Radioresistance and chemoresistance are the most significant causes for treatment failure and toxicity. Therefore there is a need to identify novel agents that will sensitize resistant cancer cells to traditional cytotoxic therapies. PARP inhibitors have been shown to sensitize tumor cells including GBM cells to radiotherapy (RT) and to different classes of chemotherapeutic agents such as temozolomide and topotecan (TPT). Furthermore use of PARP inhibitors has been demonstrated to not affect the toxicities induced by the treatment and to improve both the therapeutic index and the potential success rate of established cancer therapies [8,16,24,37,38]. Thus PARP inhibition might serve as a target for the development of chemo and radiation modifiers in GBMs. Nevertheless, no evidence identifies molecular biomarkers which can predict use of PARP

inhibitors in combination with TPT or RT and no data compare different strategies which utilize TPT, RT and PARP inhibitors in GBM.

To date many PARP inhibitors have been developed for cancer therapy [24]. In this study we show *in vitro* that NU1025, a PARP-1 inhibitor, selectively inhibits an enhanced PARP activity induced by TPT or RT but its administration, as expected, not induced any cytotoxic effects in GBM cell lines. Furthermore we confirmed the role of PARP inhibitor in potentiating the cytotoxic effects of TPT and RT in GBM cell lines. The increase cytotoxicity induced by PARP inhibitor in combination with TPT or RT is likely to reflect an increase cell growth inhibition, an increase perturbation of the cell cycle and an enhanced induction of DNA damage of GBM cells induced by TPT and RT. It is known that a critical event in determining sensitivity to RT or to TPT is the repair of DNA DSBs. In the data presented here, we demonstrate that inhibition of poly(ADP)ribosilation leads to an increase of DNA DSBs induced by TPT and RT. γH2AX expression a marker of DNA DSBs induced by RT or TPT [1,19,25] is found to be elevated in cells treated with RT or TPT in combination with NU1025 as compared with the cells treated with agents alone. Therefore these findings in line with data available in literature confirm that the effects of NU1025 are related to an inhibition of DNA repair.

p53 is one of the major protein involved in DNA repair and most of the GBM carry a mutated and inactive p53 [34]. The enhanced cell growth inhibition and the increased DNA DSBs by PARP inhibition in combination with TPT or RT is shown to be independent by the integrity of p53. PARP inhibition enhances the

cytotoxicity of TPT and RT in GBM cell lines carrying a p53 wild type (D54p53wt) and in cells carrying a p53 mutated and inactive (U251p53mut and D54p53-). However our data demonstrate that p53 activity modulates the extent of the enhanced cytotoxicity of TPT or RT induced by PARP inhibition and may represent a useful marker to predict the most effective strategies utilizing TPT, RT and PARP inhibition for the treatment of GBM cells. Specifically the loss of p53 appears to be correlated with an increased radioresistance of GBM cells. Therefore, although PARP inhibition synergizes with RT in GBM cells with p53 loss (U251p53mut and D54p53-), the extent of GBM growth inhibition as well as cell cycle perturbations and induction of DNA damage are demonstrated to be less than the combination of PARP inhibition and TPT. Furthermore in these cells the combination of PARP inhibition and TPT demonstrates not only to be better than the combination of PARP inhibition and RT but also to be the most effective strategy, been more cytotoxic than the supposed toxic combination of TPT and RT. On the other hand in cells carrying an active p53 (D54p53wt), although both the combinations of PARP inhibition and RT or TPT appears to be reasonable strategies, PARP inhibition and RT combination demonstrates to be more effective that the combination of PARP inhibition and TPT and shows comparable efficacy than the supposed toxic combination of TPT and RT.

These *in vitro* results although need to be validated *in vivo* are clinical relevant since they expand the potential application of PARP inhibitors in combination with TPT or RT for the treatment of GBM based on their p53 status. Furthermore they might suggest selective treatments for GBM cell lines based on status of

activity of p53 but with a potential more favourable therapeutic index as compared with the more toxic combination of chemotherapy and radiotherapy. Indeed radio- and chemo-sensitizing agents that do not carry side effects such as PARP inhibitors have the great potential to achieve selective modulation of cytotoxic drug action in tumors by increasing their efficacy and dose tolerance.

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Figure Legend

Figure 1. Dose dependent effect of TPT, RT and NU1025 on the *in vitro* proliferation of human GBM cell lines D54p53wt and U251p53mut.

D54p53wt and U251p53mut cells were treated with the indicated concentrations of TPT (**A**), RT (**B**) and NU1025 (**C**). Cell growth inhibition was determined by MTT assay following a five day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells at each concentration point. Data are expressed as mean ± SD of the results obtained in three independent experiments. (**C)** A representative experiment with D54p53wt and U251p53mut cells treated with NU1025 (10µM) is shown.

Figure 2. Enhancement by PARP-1 inhibition with NU1025 of the *in vitro* anti-proliferative activity of TPT or RT in human GBM cell lines D54p53wt and U251p53mut.

D54p53wt and U251p53mut cells were treated with the indicated concentrations of TPT and/or RT and/or NU1025 (10µM). Cell growth inhibition was determined by MTT assay following a five days of treatment. Percentage of cell growth inhibition was calculated as ratio of treated to untreated cells at each treatment. Data are expressed as mean ± SD of the results obtained in three independent experiments.

Figure 3. Enhancement by PARP-1 inhibition with NU1025 of the cell cycle perturbations induced by TPT or RT in human GBM cell lines D54p53wt and U251p53mut.

D54p53wt (**A, B**) and U251p53mut (**C, D**) cells were treated with the indicated concentrations of TPT and/or RT and/or NU1025 (10μM). Cell cycle perturbations were detected by PI staining following a five days of treatment. The percentage of D54p53wt and U251p53mut cells in the different phases of cell cycle is shown. Data presented are representative of three indipendent experiments.

Figure 4. Enhancement by PARP-1 inhibition with NU1025 of the cell cycle block and delay induced by TPT or RT in human GBM cell lines D54p53wt and U251p53mut.

D54p53wt (**A**) and U251p53mut (**B**) cells were treated with the indicated concentrations of TPT and/or RT and/or NU1025 (10μM). Cell cycle perturbations were detected by BrdU incorporation (at the indicated time points) and PI staining following a five days of treatment. The percentage of D54p53wt and U251p53mut BrdU positive cells in the different phases of cell cycle (G1+, S+ and G2/M+) is shown. Data refer to one of two experiments giving similar results.

Figure 5. Enhancement by PARP-1 inhibition with NU1025 of the DNA damage induced by TPT or RT in human GBM cell lines D54p53wt and U251p53mut.

D54p53wt (**A-C**) and U251p53mut (**D-F**) cells were treated with the indicated concentrations of TPT and/or RT and/or NU1025 (10μM). DNA damage was determined by flow cytometric γH2AX expression following the indicated time points of treatment. Relative γH2AX expression was calculated as the

mathematic difference (Δ) between percentage of levels of (*H2AX+*) of treated cells and untreated cells (Δ % = $H2AX^{+}_{tr}$ – $H2AX^{+}_{untr}$). A representative experiment of γH2AX expression in D54p53wt (**A, B**) and in U251p53mut (**D, E**) with or without treatment with TPT, RT and NU1025 is shown. Data are expressed as mean ± SD of the results obtained in three independent experiments (**C, F**).

Figure 6. Inhibtion by NU1025 of PARP-1 activation induced by TPT or RT in human GBM cell lines D54p53wt and U251p53mut.

D54p53wt (**A**) and U251p53mut (**B**) cells were treated with the indicated concentrations of TPT and/or RT and/or NU1025 (10μM). PARP-1 activity was determined by immunofluorescent staining of poly-ADP-ribosylation of DNA following a 4h of treatment. PI staining was used as an indicator of nuclear compartment.

Figure 7. p53 downregulation in human GBM cell line D54p53wt.

D54p53wt were transduced with transfected with vector encoding for p53-RNAi. Resistant-transfected clones (D54p53-) were named P1 and P2. D54p53wt and transduced cells were treated with doxorubicin (2.5 μg/ml). Untreated cells were used as a control group. Following a 12h incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β-actin was used as a loading control. The results presented are representative of the results obtained in three independent experiments.

Figure 8. Modulation by p53 of the enhanced cytotoxicity of TPT or RT induced by PARP-1 inhibition in human GBM cell lines.

A. D54p53wt and D54p53- P1 cells were irradiated with the indicated doses of RT. Cell growth inhibition was determined by MTT assay following a five day incubation at 37°C. **B.** D54p53wt and D54p53- P1 cells were treated with the indicated concentrations of TPT and/or RT and/or NU1025 (10µM). Cell growth inhibition was determined by MTT assay following a three day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells at each concentration point. Data are expressed as mean ± SD of the results obtained in three independent experiments. **C.** Cell cycle perturbations were detected by PI staining following a three days of treatment. The percentage of D54p53wt and D54p53- cells in the different phases of cell cycle is shown. Data presented are representative of three indipendent experiments.

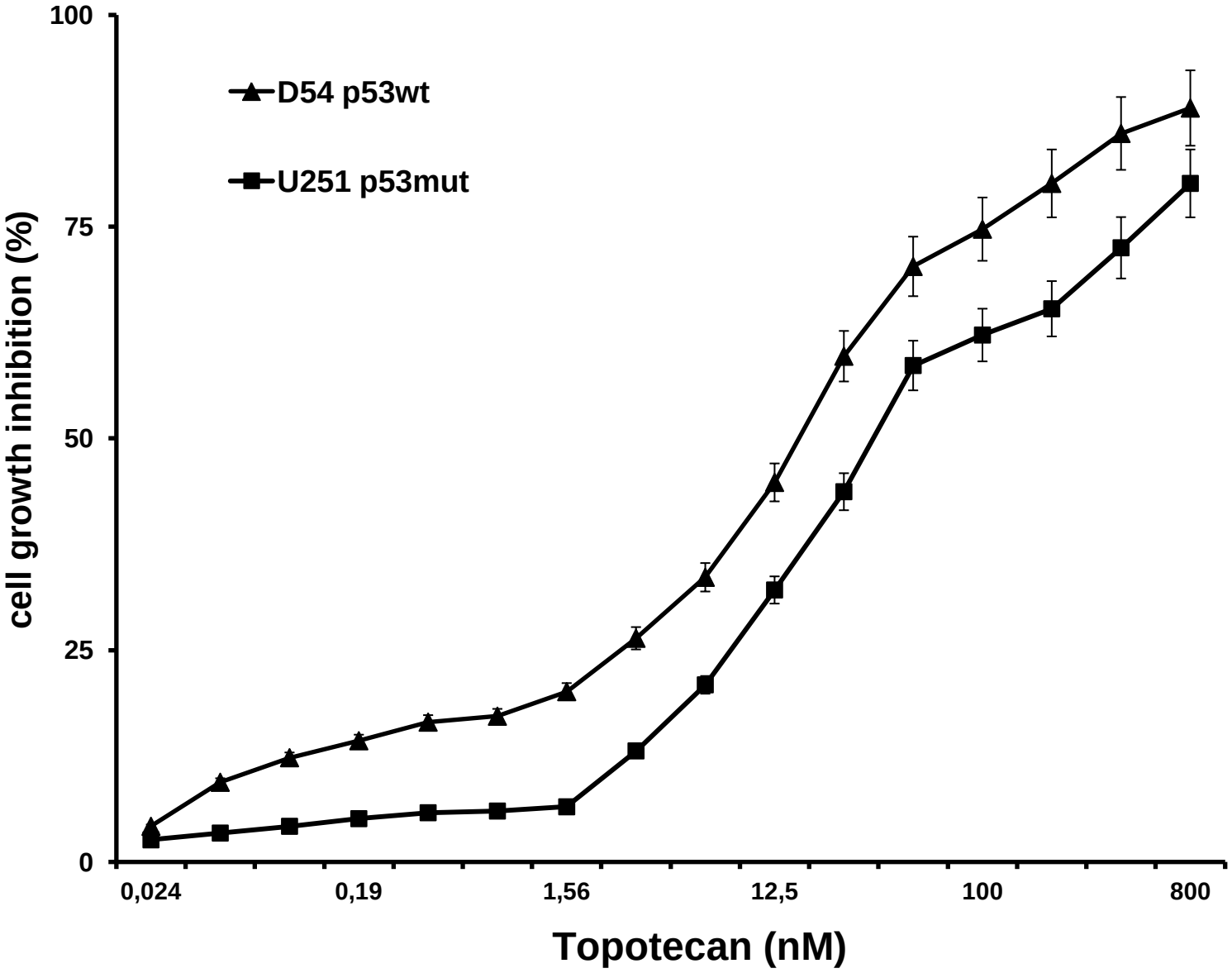

100
75
50
25
0
cell growth inhibition (%)
D54 p53wt
U251 p53mut
0,024
0,19
1,56
12,5
100
800
Topotecan (nM)

Fig. 1A.

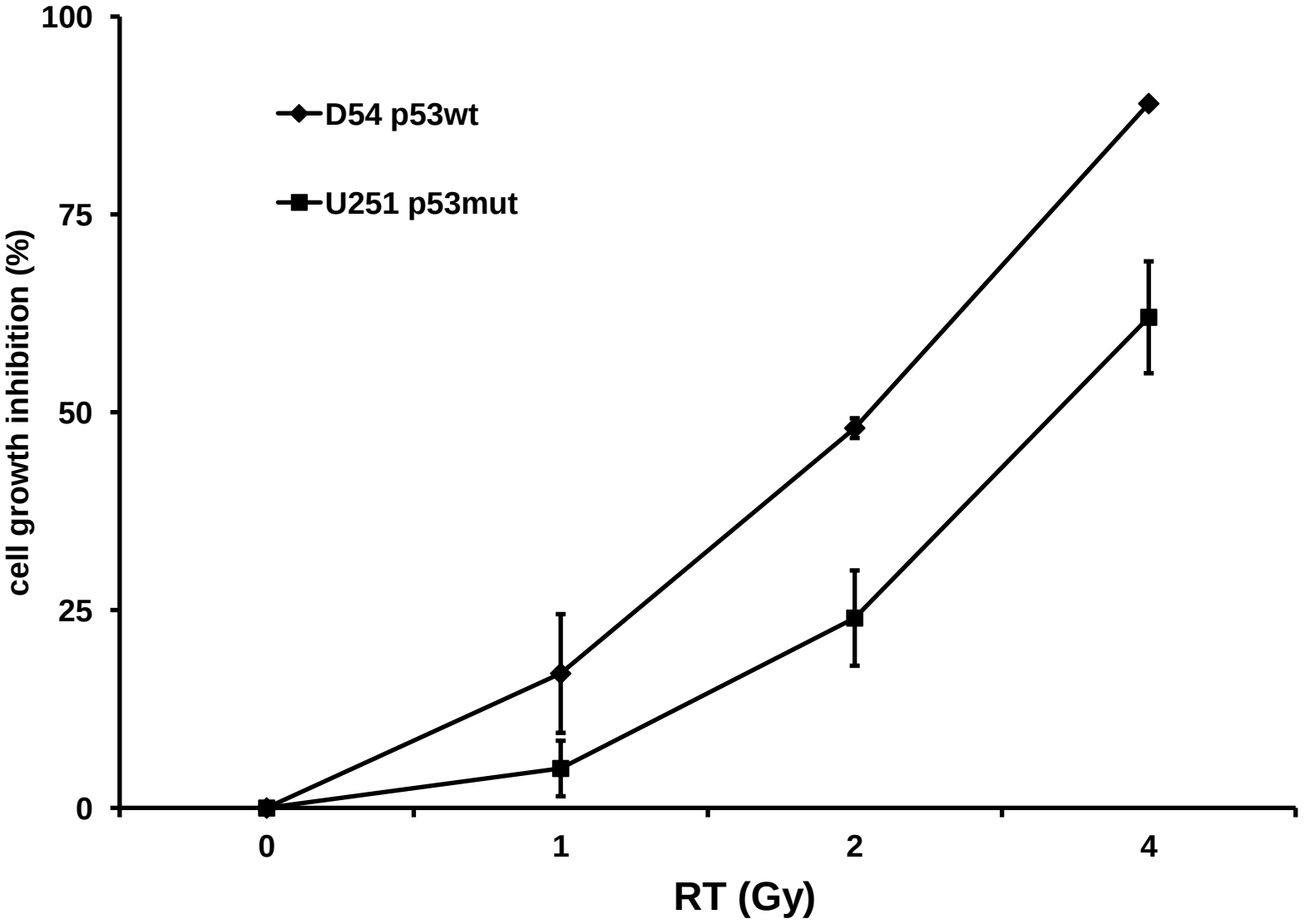
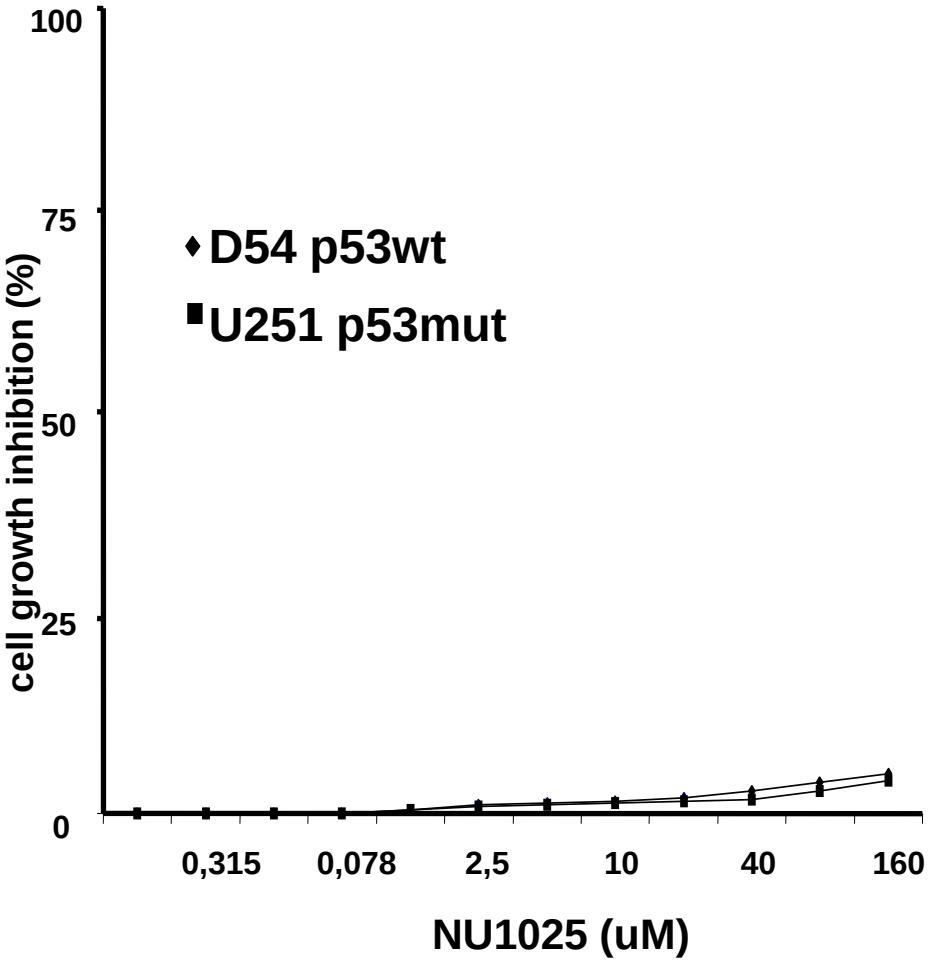

100
75
50
25
0
cell growth inhibition (%)
D54 p53wt
U251 p53mut
0
1
2
4
RT (Gy)

Fig. 1B.


100
75
50
25
0
cell growth inhibition (%)
D54 p53wt
U251 p53mut
0,315
0,078
2,5
10
40
160
NU1025 (uM)

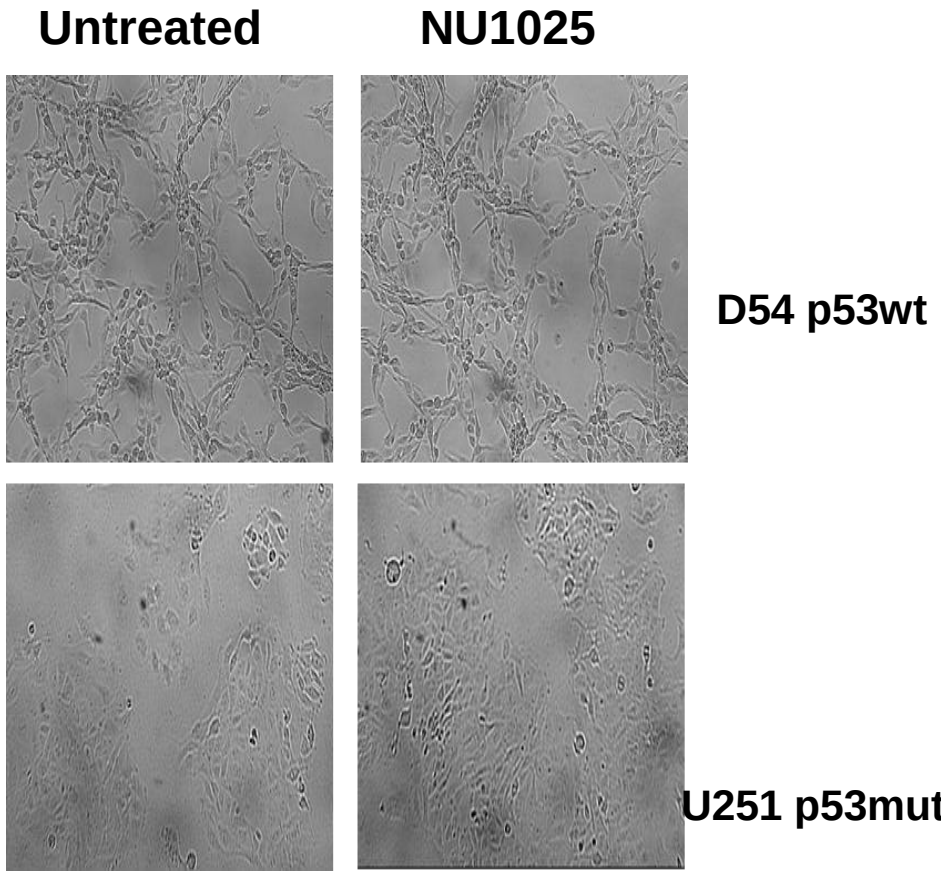

Untreated
NU1025
D54 p53wt
U251 p53mut

Fig. 1C.

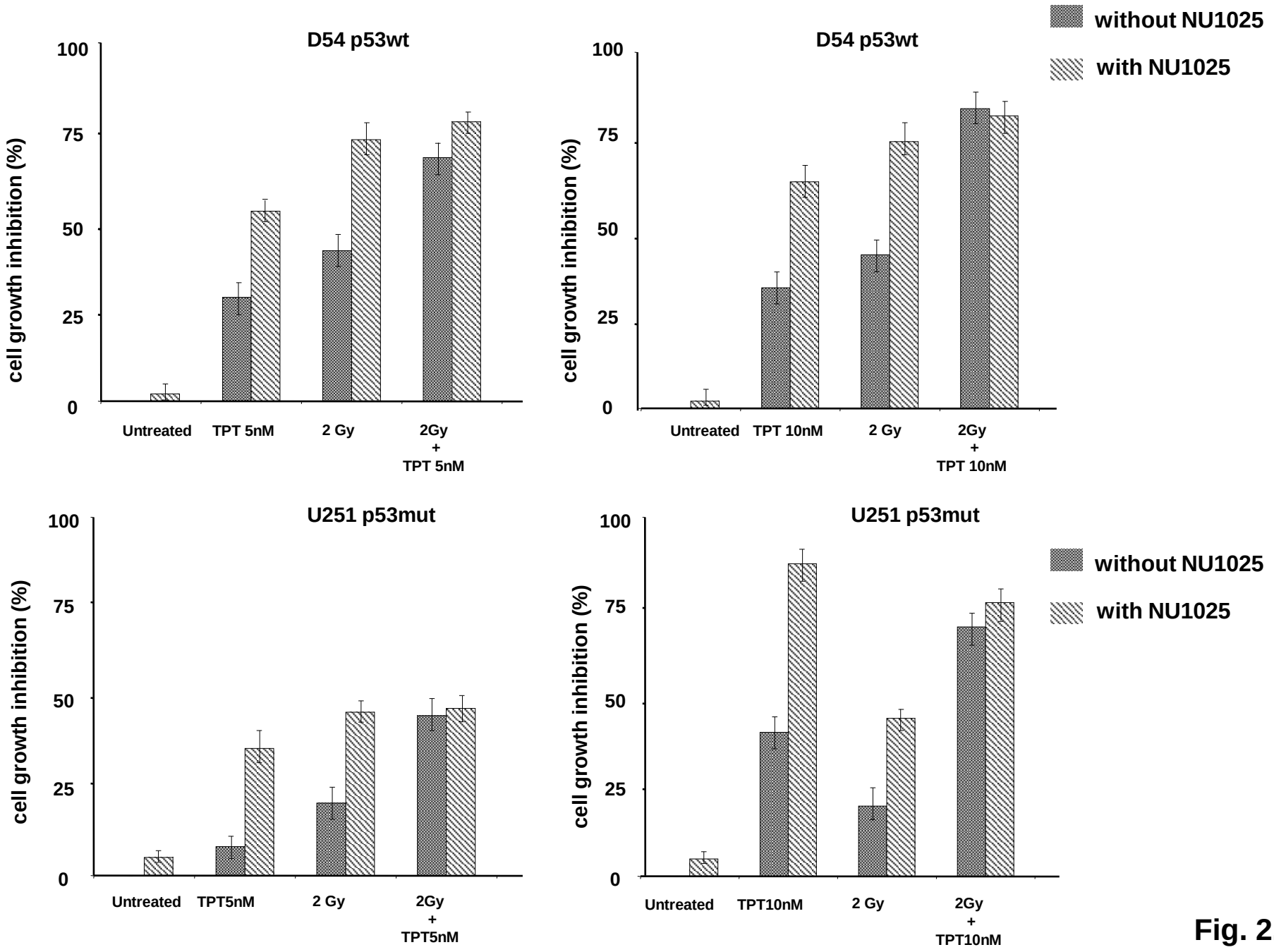
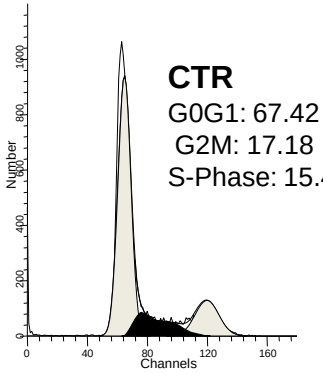
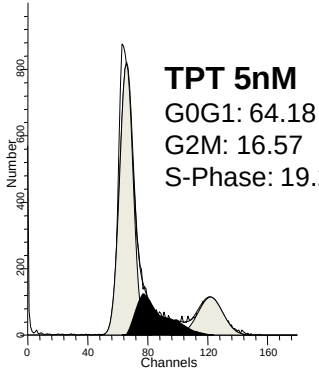

D54 p53wt
cell growth inhibition (%)
100
75
50
25
0
Untreated
TPT 5nM
2 Gy
2Gy + TPT 5nM
D54 p53wt
cell growth inhibition (%)
100
75
50
25
0
Untreated
TPT 10nM
2 Gy
2Gy + TPT 10nM
without NU1025
with NU1025
U251 p53mut
cell growth inhibition (%)
100
75
50
25
0
Untreated
TPT5nM
2 Gy
2Gy + TPT5nM
U251 p53mut
cell growth inhibition (%)
100
75
50
25
0
Untreated
TPT10nM
2 Gy
2Gy + TPT10nM
without NU1025
with NU1025

Fig. 2.

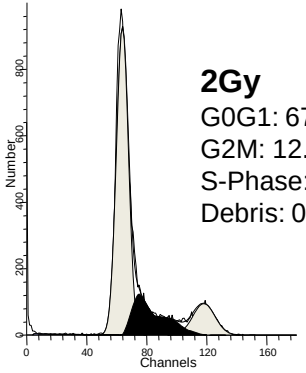
D54 p53wt



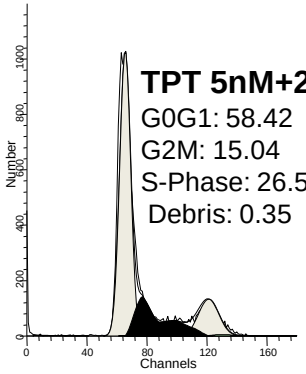
CTR
G0G1: 67.42
G2M: 17.18
S-Phase: 15.40



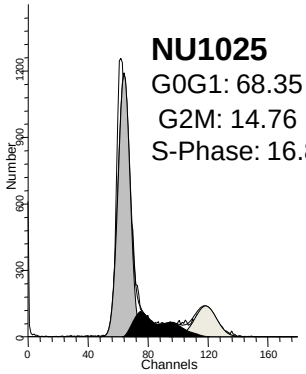
TPT 5nM
G0G1: 64.18
G2M: 16.57
S-Phase: 19.25



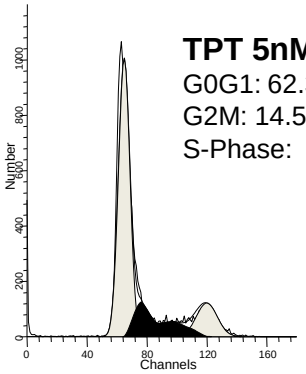
2Gy
G0G1: 67.29
G2M: 12.73
S-Phase: 19.98
Debris: 0.90



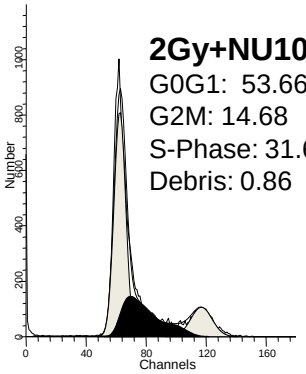
TPT 5nM+2Gy
G0G1: 58.42
G2M: 15.04
S-Phase: 26.53
Debris: 0.35



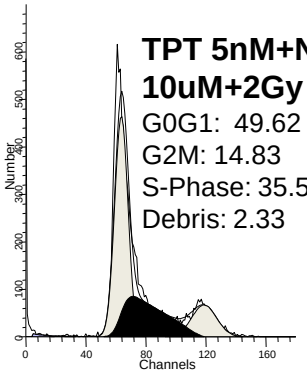
NU1025
G0G1: 68.35
G2M: 14.76
S-Phase: 16.89



TPT 5nM+NU1025
G0G1: 62.33
G2M: 14.58
S-Phase: 23.10



2Gy+NU1025
G0G1: 53.66
G2M: 14.68
S-Phase: 31.66
Debris: 0.86

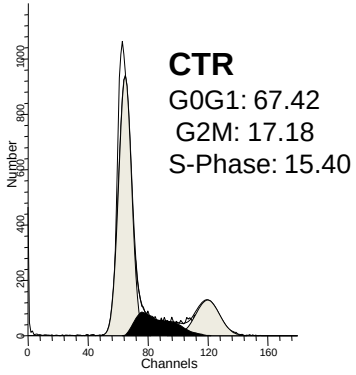


TPT 5nM+NU1025 10uM+2Gy
G0G1: 49.62
G2M: 14.83
S-Phase: 35.55
Debris: 2.33

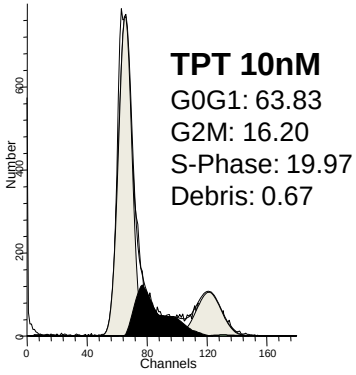
TPT 5nM , RT 2Gy, NU1025 10μM in D54

Fig. 3A.

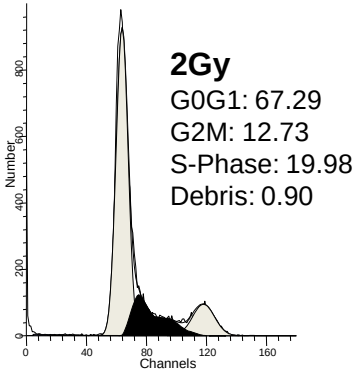
D54 p53wt



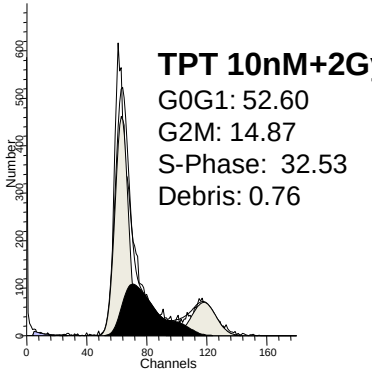
CTR
G0G1: 67.42
G2M: 17.18
S-Phase: 15.40



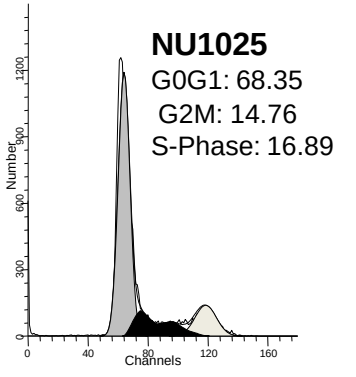
TPT 10nM
G0G1: 63.83
G2M: 16.20
S-Phase: 19.97
Debris: 0.67



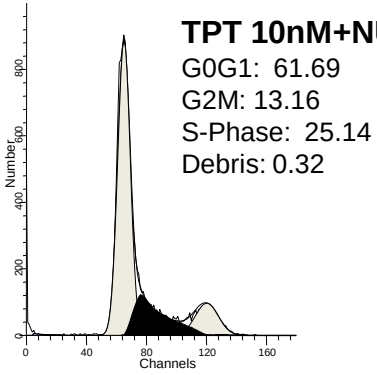
2Gy
G0G1: 67.29
G2M: 12.73
S-Phase: 19.98
Debris: 0.90



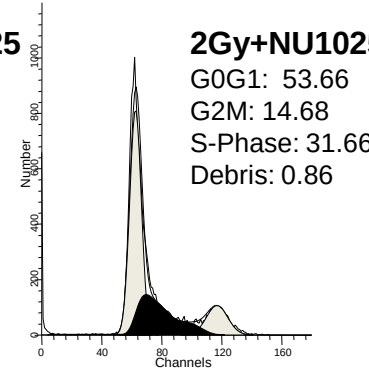
TPT 10nM+2Gy
G0G1: 52.60
G2M: 14.87
S-Phase: 32.53
Debris: 0.76



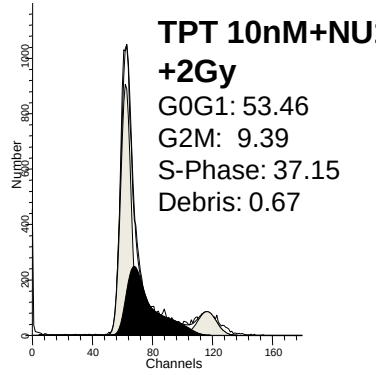
NU1025
G0G1: 68.35
G2M: 14.76
S-Phase: 16.89



TPT 10nM+NU1025
G0G1: 61.69
G2M: 13.16
S-Phase: 25.14
Debris: 0.32



2Gy+NU1025
G0G1: 53.66
G2M: 14.68
S-Phase: 31.66
Debris: 0.86

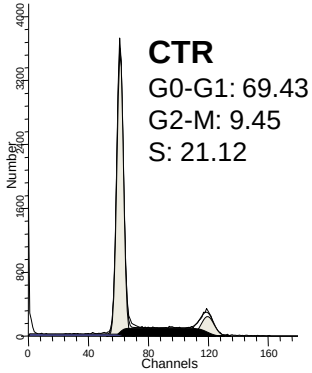


TPT 10nM+NU1025 +2Gy
G0G1: 53.46
G2M: 9.39
S-Phase: 37.15
Debris: 0.67

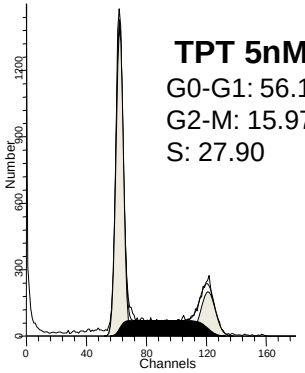
TPT 10nM , RT 2Gy, NU1025 10μM in D54

Fig. 3B.

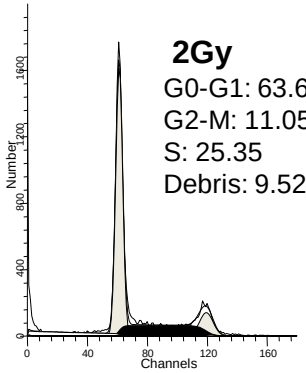
U251 p53mut



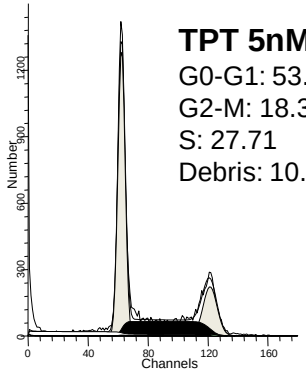
CTR
G0-G1: 69.43
G2-M: 9.45
S: 21.12



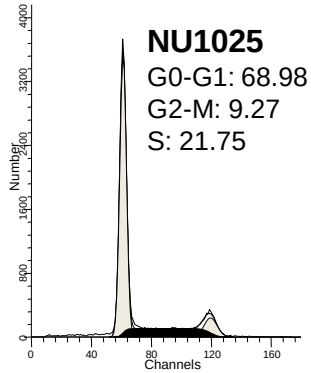
TPT 5nM
G0-G1: 56.13
G2-M: 15.97
S: 27.90



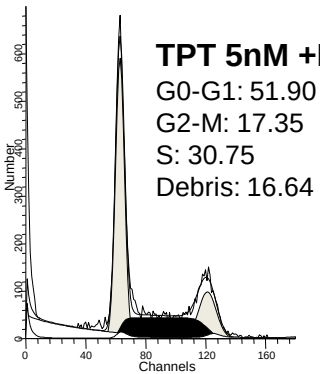
2Gy
G0-G1: 63.60
G2-M: 11.05
S: 25.35
Debris: 9.52



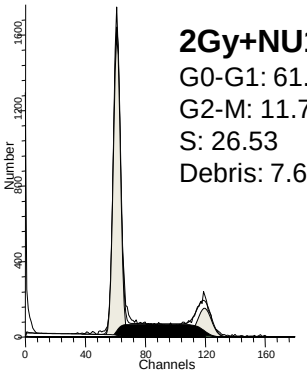
TPT 5nM+2Gy
G0-G1: 53.94
G2-M: 18.35
S: 27.71
Debris: 10.32



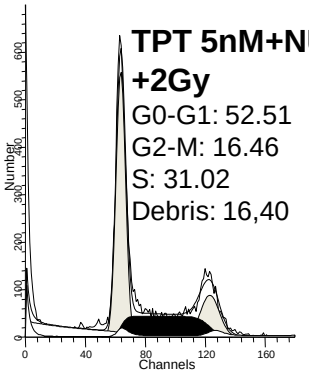
NU1025
G0-G1: 68.98
G2-M: 9.27
S: 21.75



TPT 5nM +NU1025
G0-G1: 51.90
G2-M: 17.35
S: 30.75
Debris: 16.64



2Gy+NU1025
G0-G1: 61.70
G2-M: 11.77
S: 26.53
Debris: 7.69

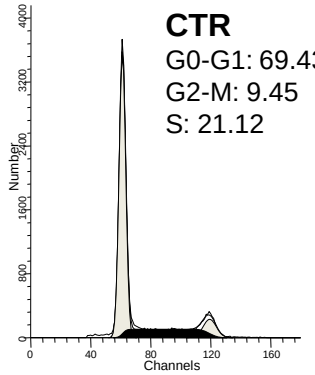


TPT 5nM+NU1025 +2Gy
G0-G1: 52.51
G2-M: 16.46
S: 31.02
Debris: 16,40

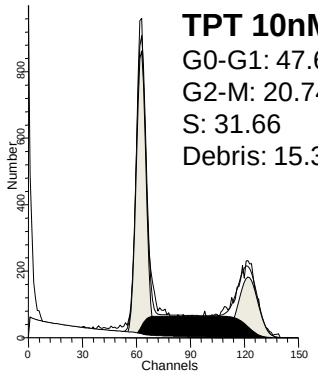
TPT 5nM , RT 2Gy, NU1025 10μM in U251

Fig. 3C.

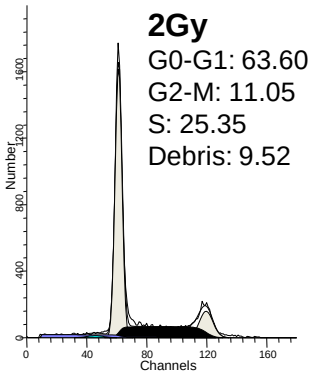
U251 p53mut



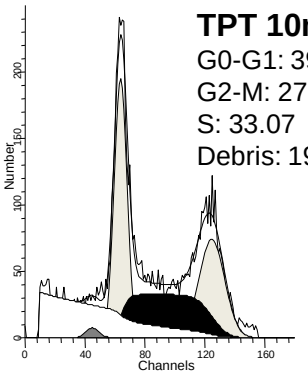
CTR
G0-G1: 69.43
G2-M: 9.45
S: 21.12



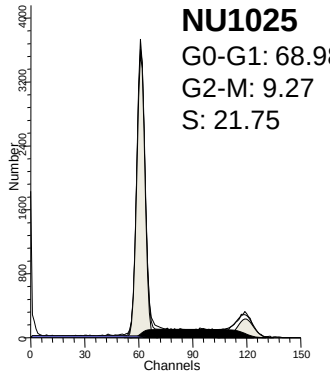
TPT 10nM
G0-G1: 47.60
G2-M: 20.74
S: 31.66
Debris: 15.33



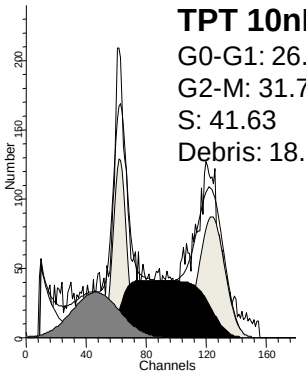
2Gy
G0-G1: 63.60
G2-M: 11.05
S: 25.35
Debris: 9.52



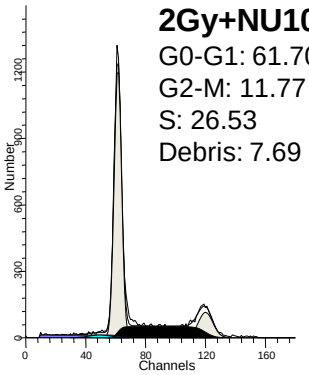
TPT 10nM+2Gy
G0-G1: 39.48
G2-M: 27.45
S: 33.07
Debris: 19.95



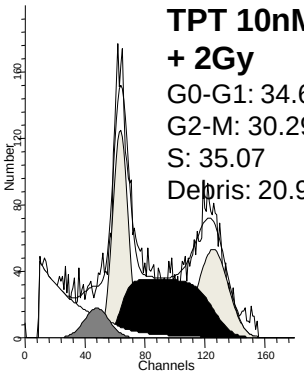
NU1025
G0-G1: 68.98
G2-M: 9.27
S: 21.75



TPT 10nM+NU1025
G0-G1: 26.61
G2-M: 31.76
S: 41.63
Debris: 18.55



2Gy+NU1025
G0-G1: 61.70
G2-M: 11.77
S: 26.53
Debris: 7.69



TPT 10nM+NU1025 + 2Gy
G0-G1: 34.64
G2-M: 30.29
S: 35.07
Debris: 20.95

TPT 10nM , RT 2Gy, NU1025 10μM in U251

Fig. 3D.

D54 p53wt

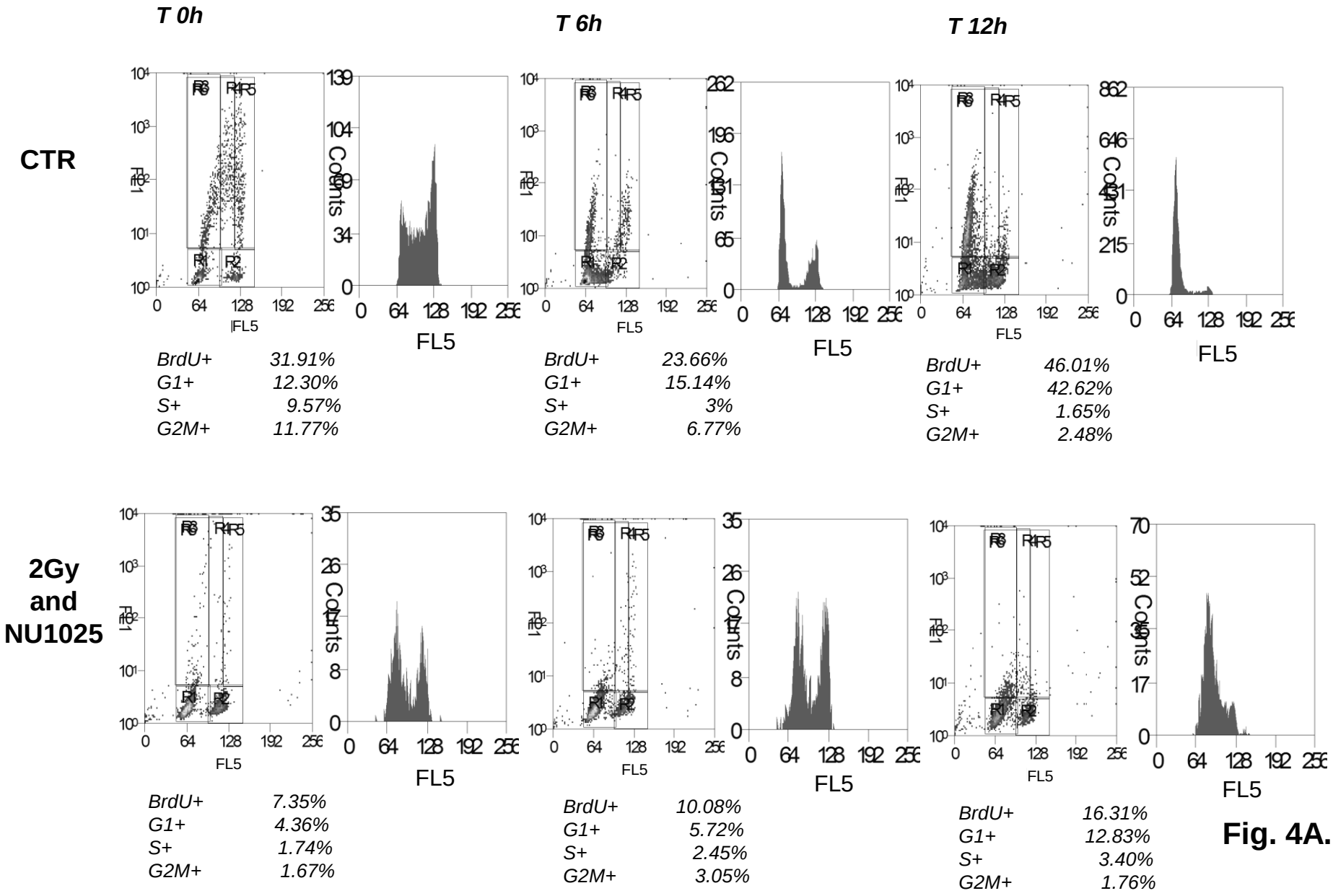


Fig. 4A.

U251 p53mut

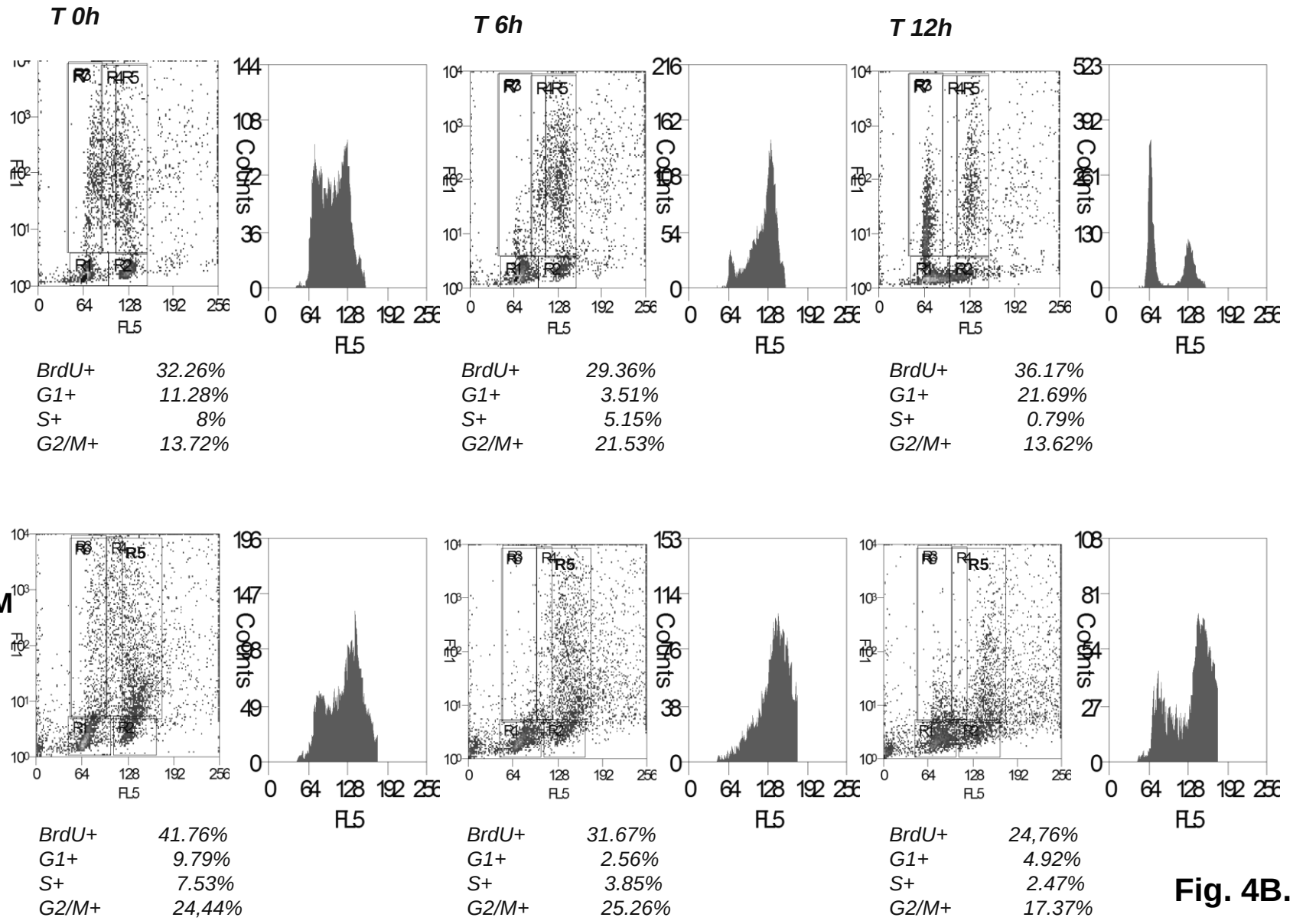
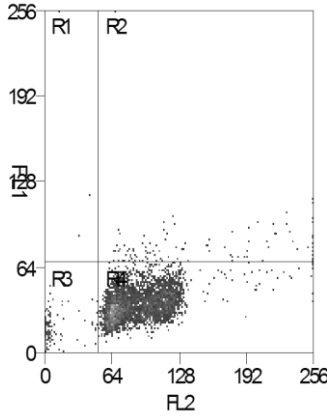


Fig. 4B.

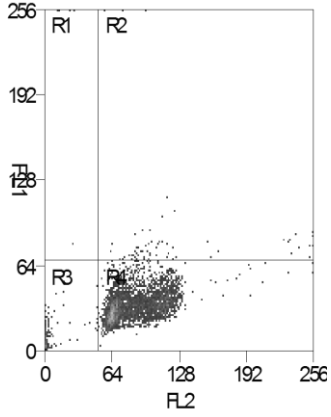
D54 p53wt Histone H2AX after 1h

CTR



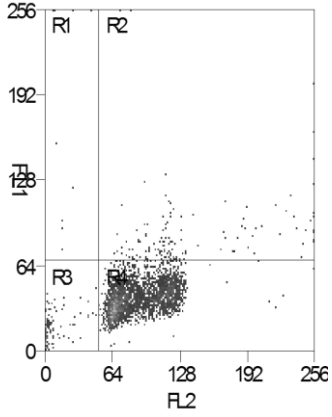
R2=H2AX+ 2.18%
R4=H2AX- 91.20%

NU1025



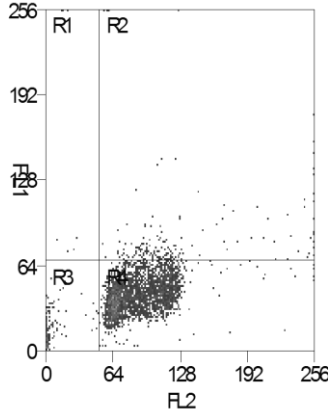
R2=H2AX+ 2.05%
R4=H2AX- 89.27%

TPT 5nM



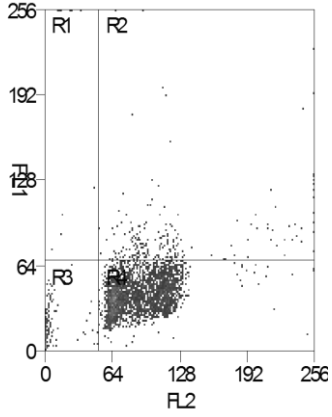
R2=H2AX+ 4.67%
R4=H2AX- 86.31%

TPT 10nM



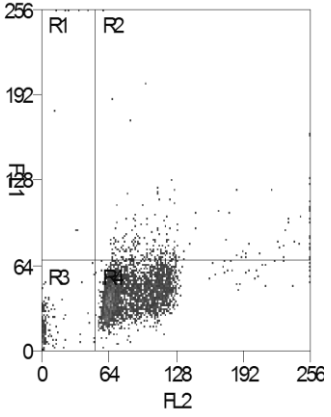
R2=H2AX+ 5.73%
R4=H2AX- 84.79%

TPT 5nM + NU1025



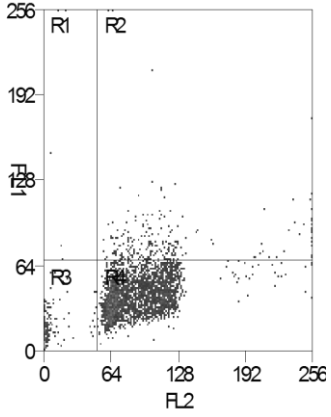
R2=H2AX+ 6.83%
R4=H2AX- 82.62%

TPT 10nM+NU1025



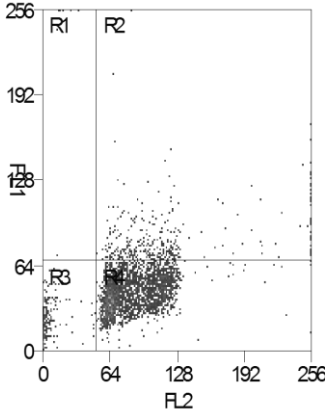
R2=H2AX+ 7.45%
R4=H2AX- 82.53%

2Gy



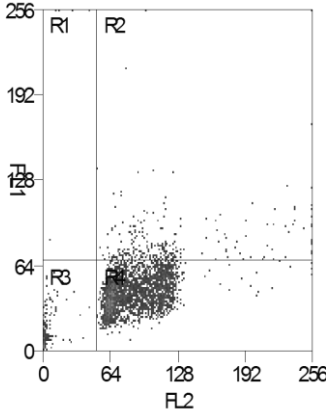
R2=H2AX+ 7.18%
R4=H2AX- 81.99%

2Gy+NU1025



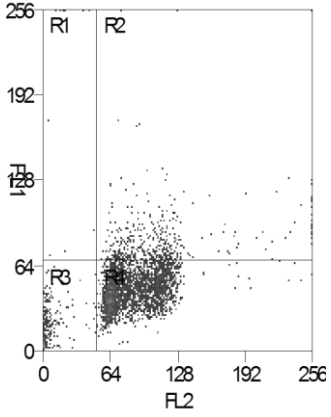
R2=H2AX+ 9.94%
R4=H2AX- 78.71%

2Gy+TPT 5nM



R2=H2AX+ 7.78%
R4=H2AX- 82.39%

2Gy+TPT 10nM

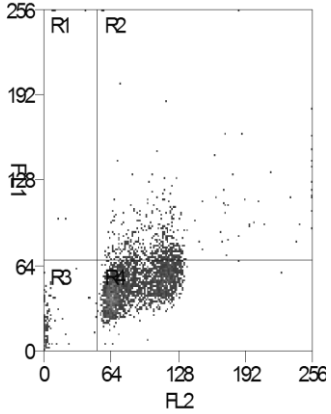


R2=H2AX+ 12.69%
R4=H2AX- 73.87%

Fig. 5A.

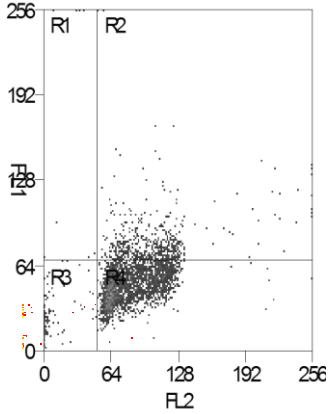
D54 p53wt Histone H2AX after 4h

CTR



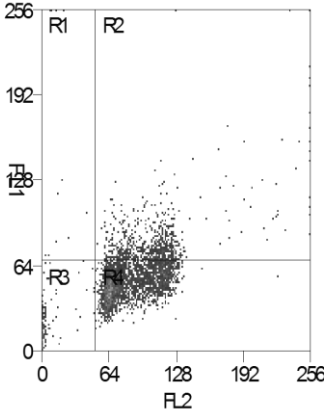
R2=H2AX+ 16.27%
R4=H2AX- 75.88%

NU1025



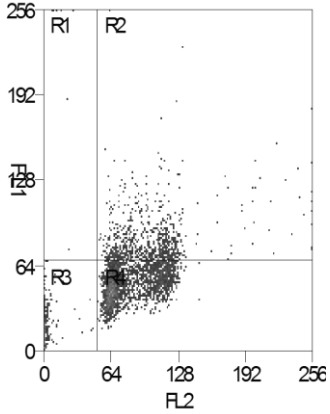
R2=H2AX+ 17.33%
R4=H2AX- 76.17%

TPT 5nM



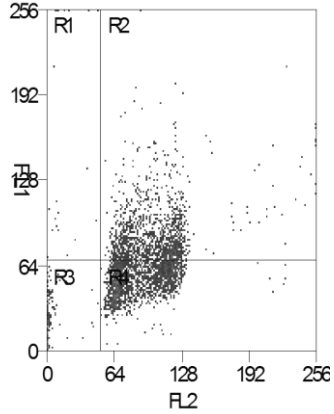
R2=H2AX+ 20.74%
R4=H2AX- 72.40%

TPT 10nM



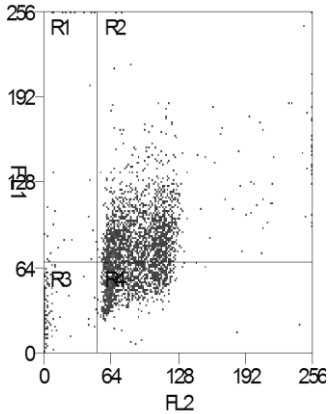
R2=H2AX+ 24.65%
R4=H2AX- 66.00%

TPT 5nM+NU1025



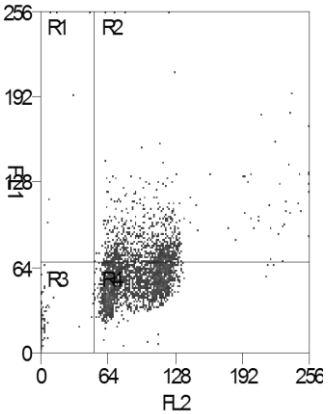
R2=H2AX+ 40.15%
R4=H2AX- 51.48%

TPT 10nM+NU1025



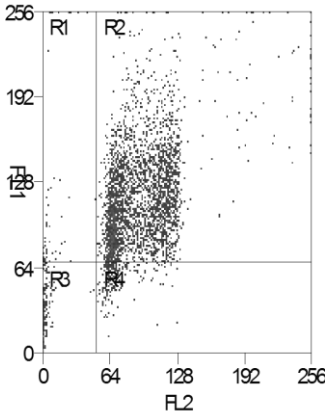
R2=H2AX+ 54.39%
R4=H2AX- 34.59%

2Gy



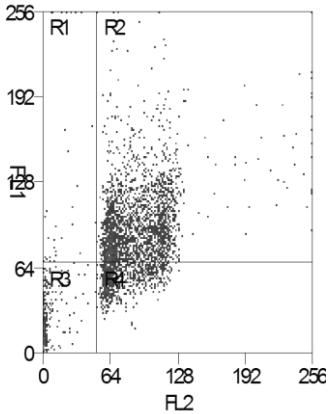
R2=H2AX+ 29.10%
R4=H2AX- 65.01%

2Gy+NU1025



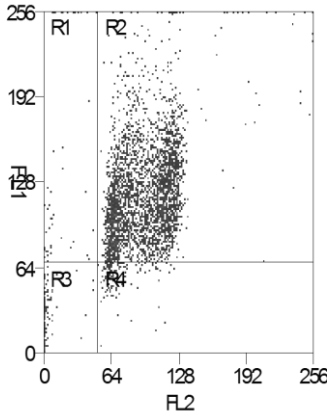
R2=H2AX+ 85.40%
R4=H2AX- 6.36%

2Gy+TPT 5nM



R2=H2AX+ 63.17%
R4=H2AX- 21.55%

2Gy+TPT 10nM



R2=H2AX+ 89.05%
R4=H2AX- 3.87%

Fig. 5B.

D54 p53wt

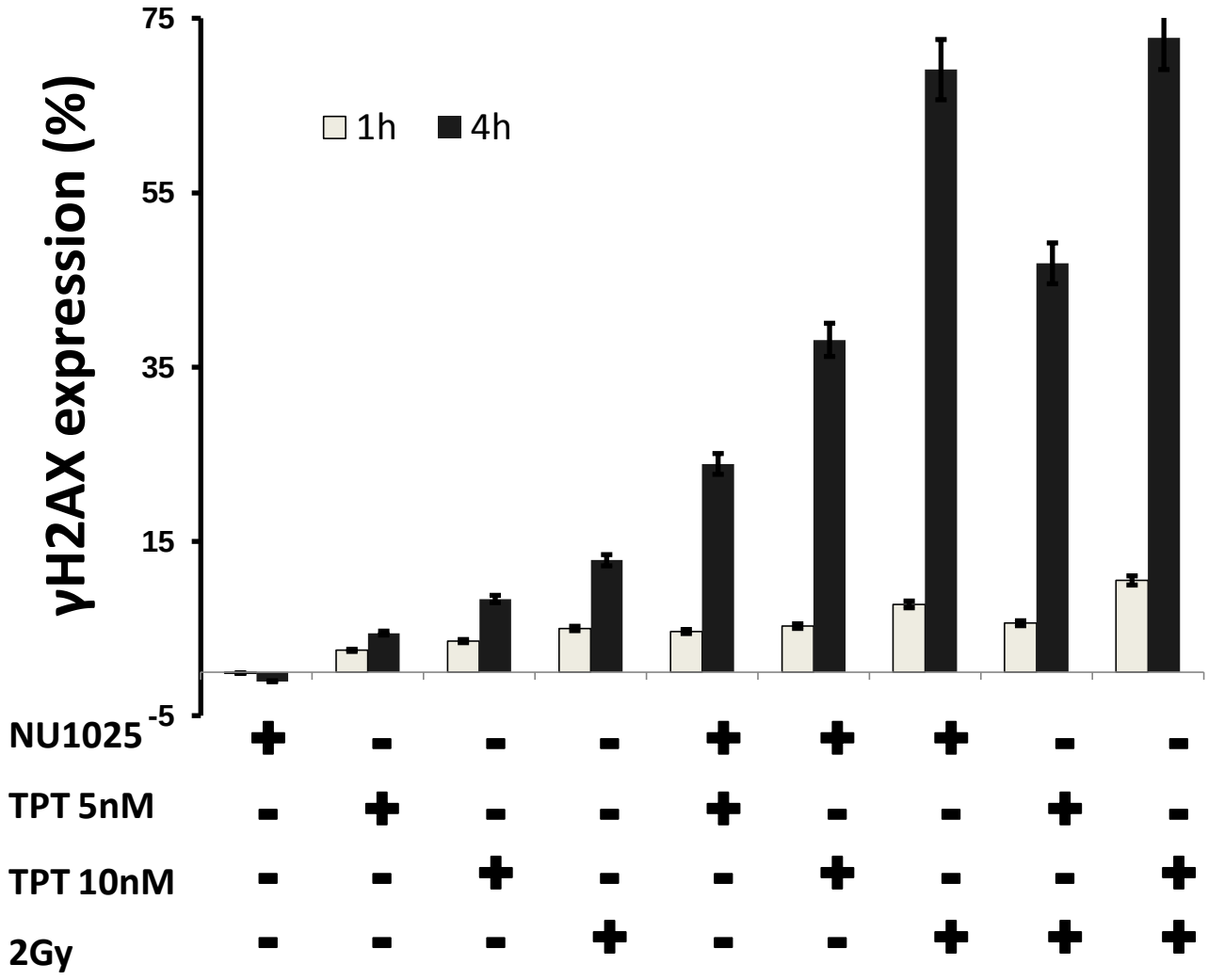


Fig. 5C.

U251 p53mut Histone H2AX after 1h

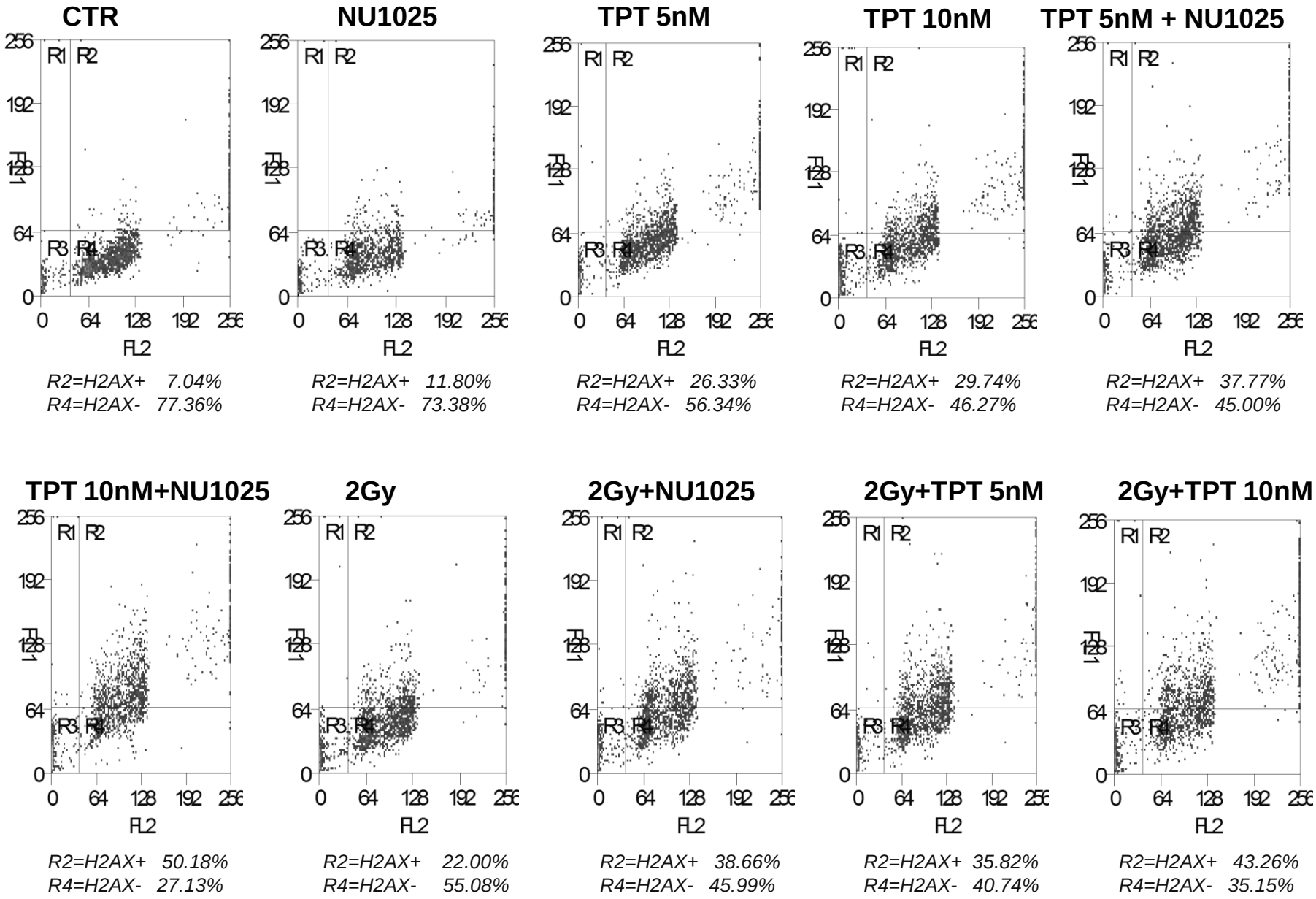
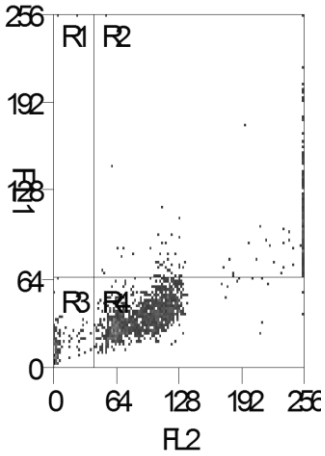


Fig. 5D.

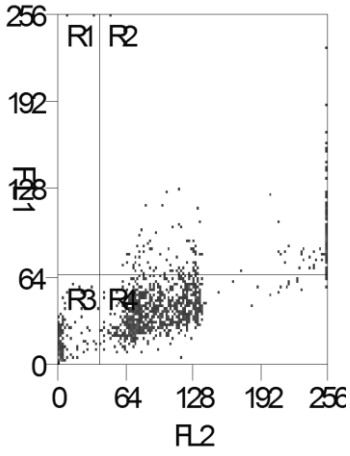
U251 p53mut Histone H2AX after 4h

CTR



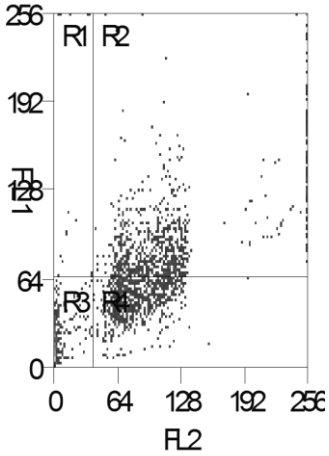
R2=H2AX+ 7.03%
R4=H2AX- 77.25%

NU1025



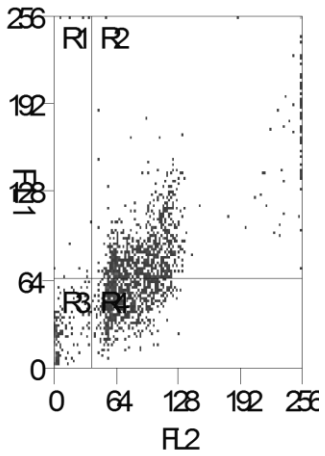
R2=H2AX+ 12.62%
R4=H2AX- 71.66%

TPT 5nM



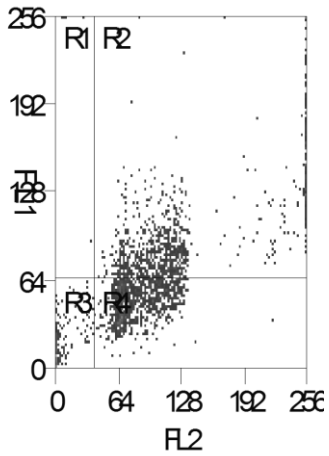
R2=H2AX+ 41.06%
R4=H2AX- 41.80%

TPT10nM



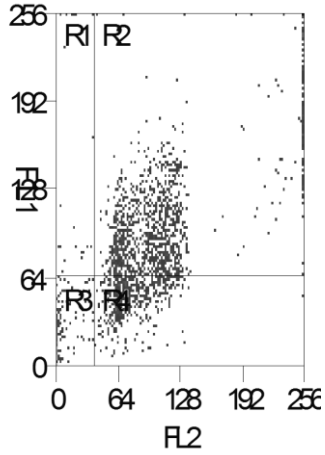
R2=H2AX+ 44.65%
R4=H2AX- 38.56%

TPT5nM+ NU1025



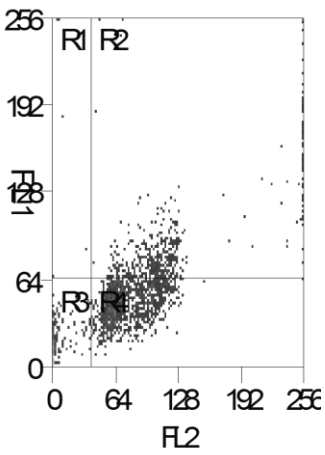
R2=H2AX+ 41.87%
R4=H2AX- 49.14%

TPT 10nM+NU1025



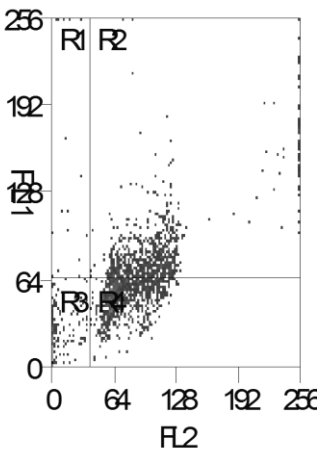
R2=H2AX+ 60.00%
R4=H2AX- 27.18%

2Gy



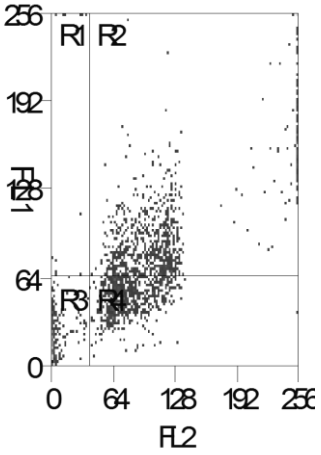
R2=H2AX+ 24.96%
R4=H2AX- 63.39%

2Gy+NU1025



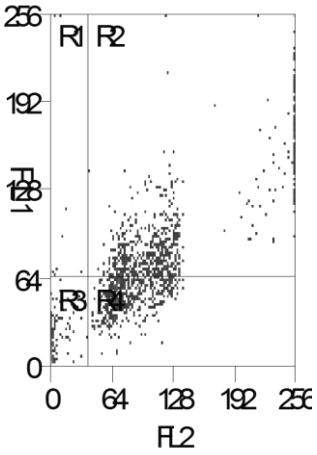
R2=H2AX+ 40.01%
R4=H2AX- 46.02%

2Gy+TPT 5nM



R2=H2AX+ 43.45%
R4=H2AX- 38.98%

2Gy+TPT 10nM



R2=H2AX+ 46.13%
R4=H2AX- 40.83%

Fig. 5E.

U251 p53mut

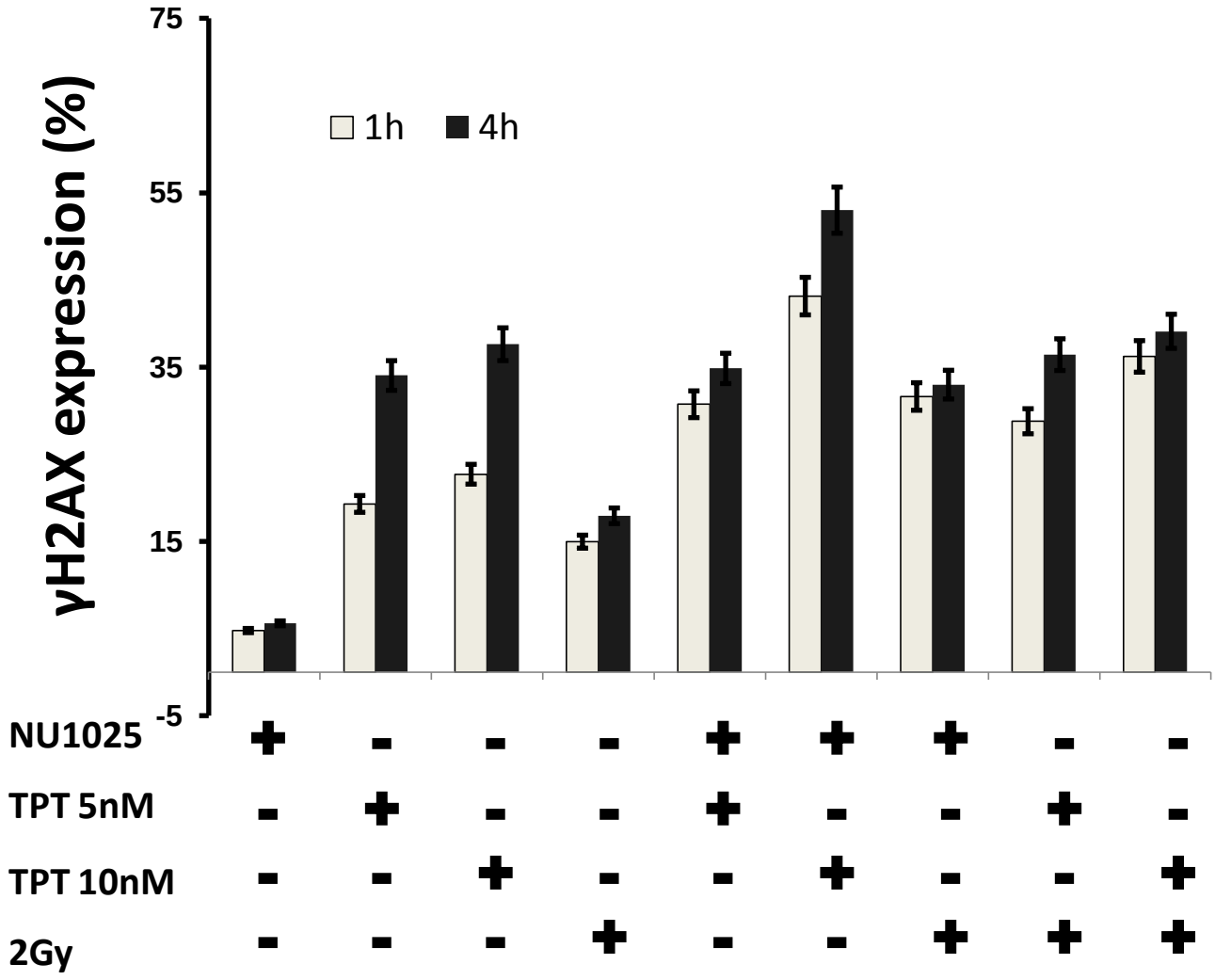


Fig. 5F.

D54 p53wt

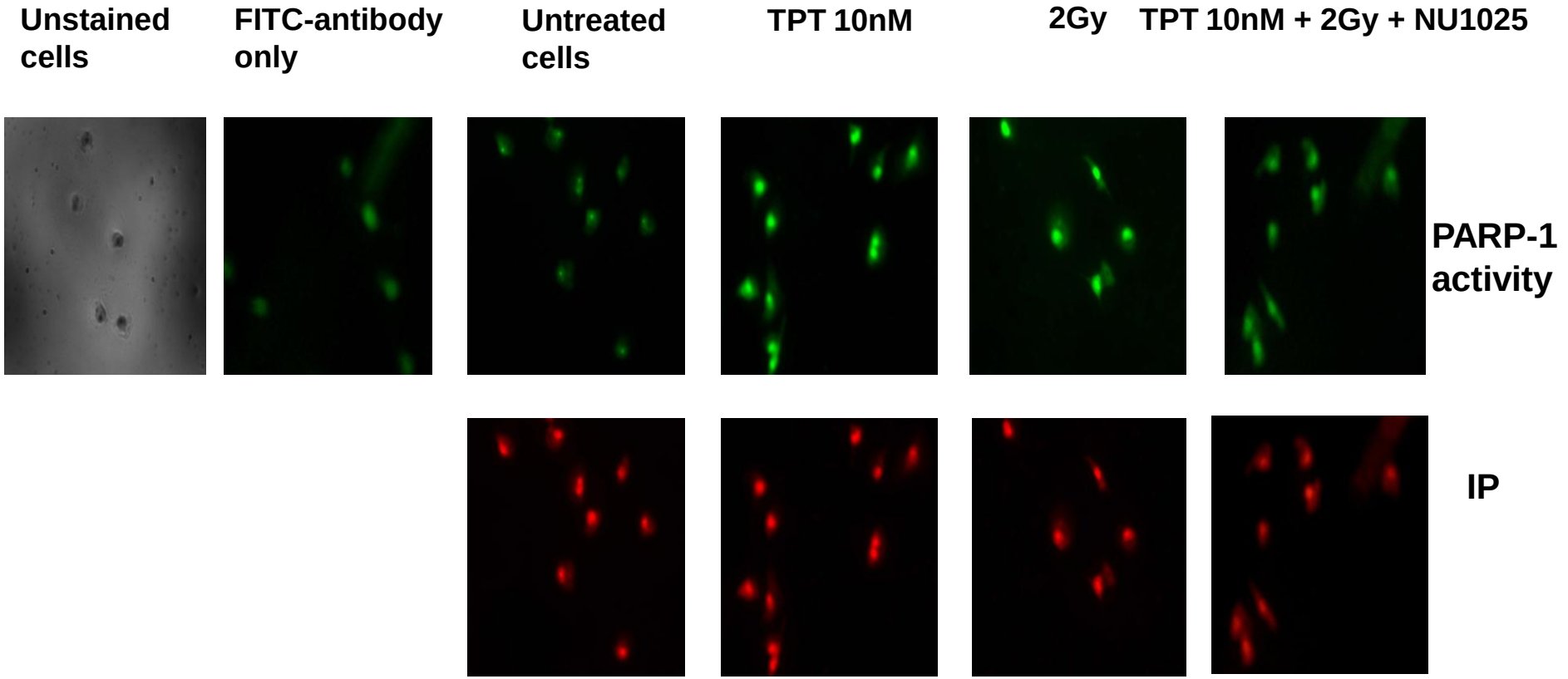


Fig. 6A.

U251p53mut

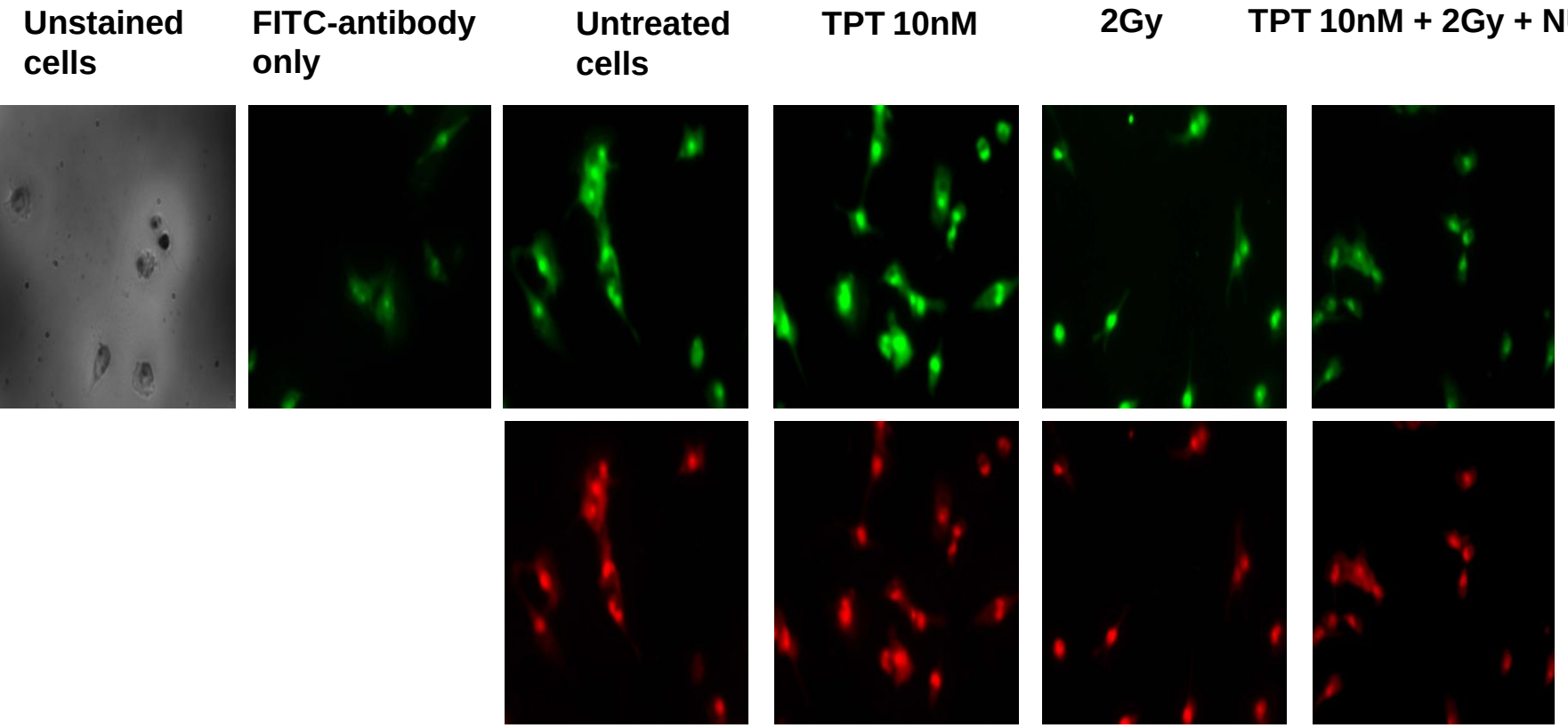


Fig. 6B.

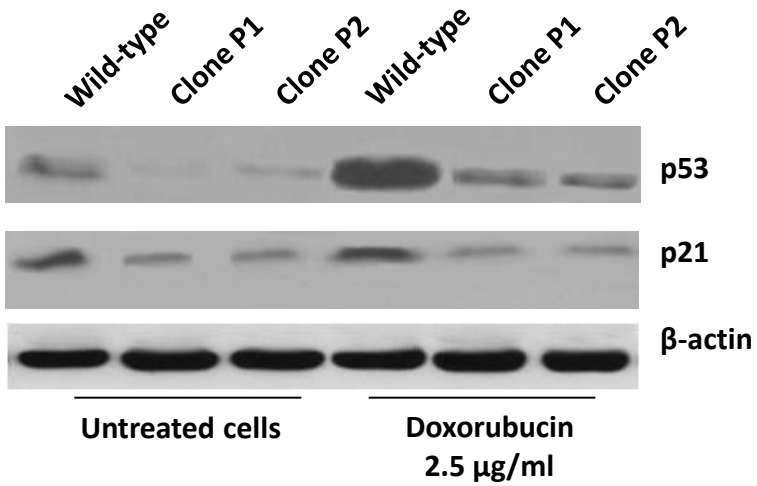

Wild-type
Clone P1
Clone P2
Wild-type
Clone P1
Clone P2
p53
p21
β-actin
Untreated cells
Doxorubucin
2.5 µg/ml

Fig. 7.

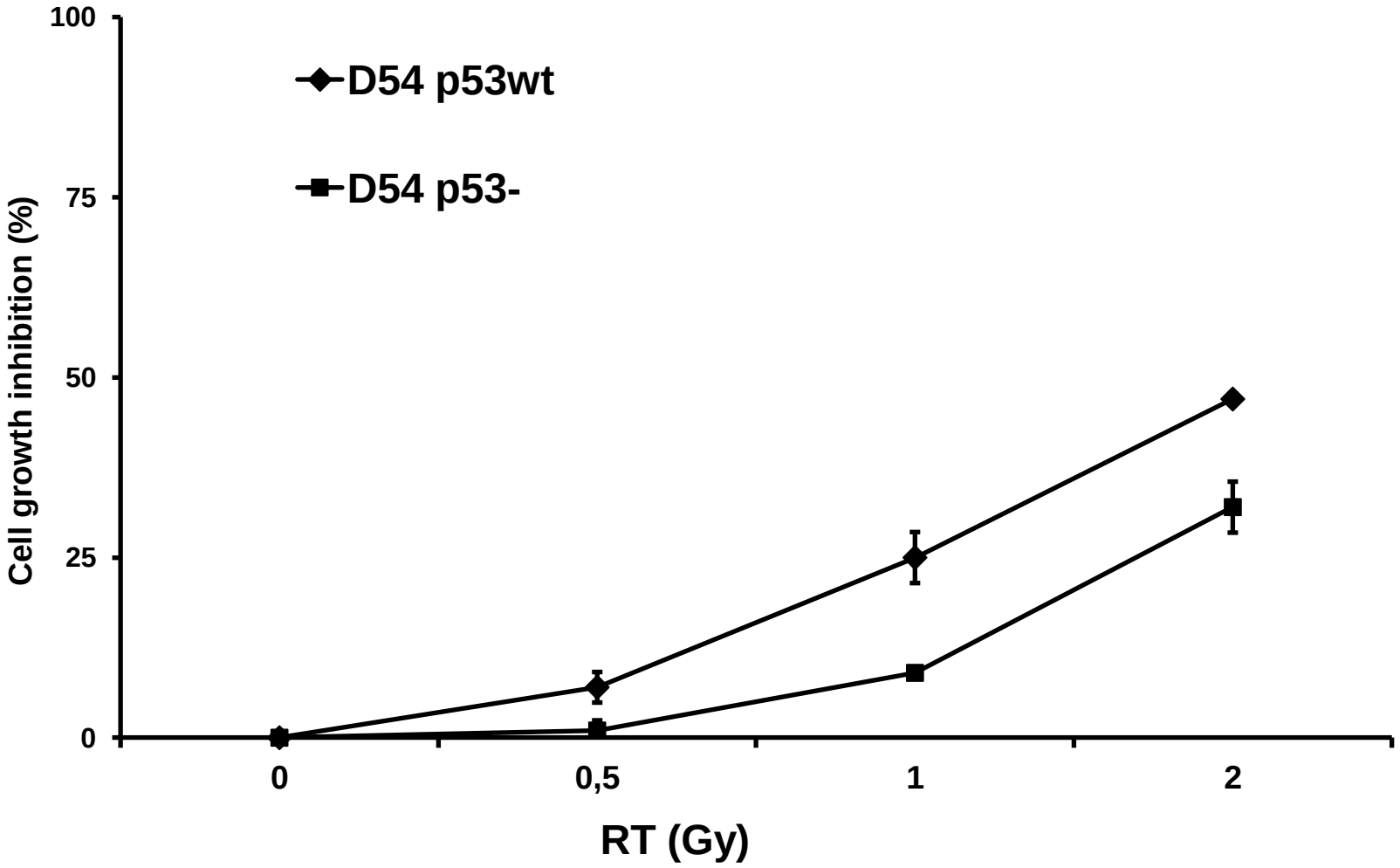

100
75
50
25
0
Cell growth inhibition (%)
D54 p53wt
D54 p53-
0
0,5
1
2
RT (Gy)

Fig. 8A.

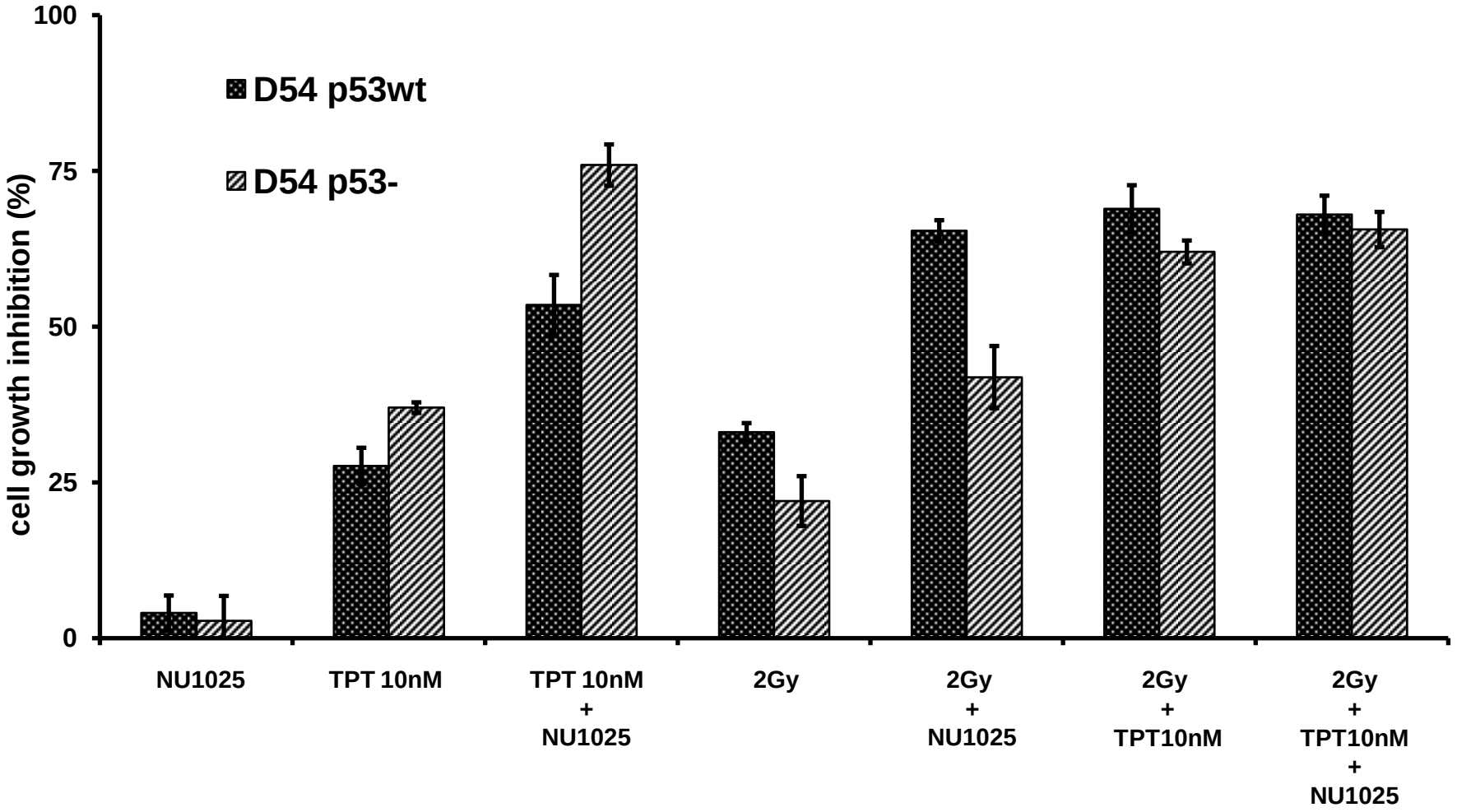
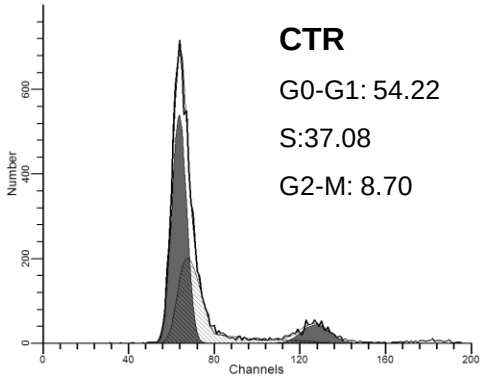
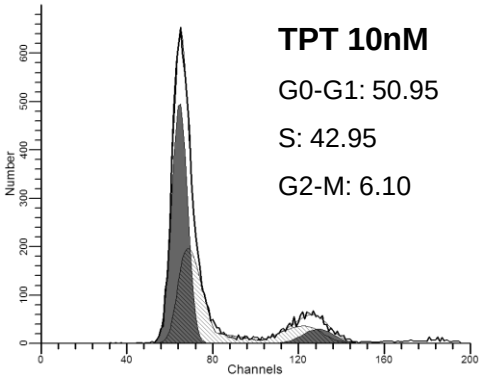
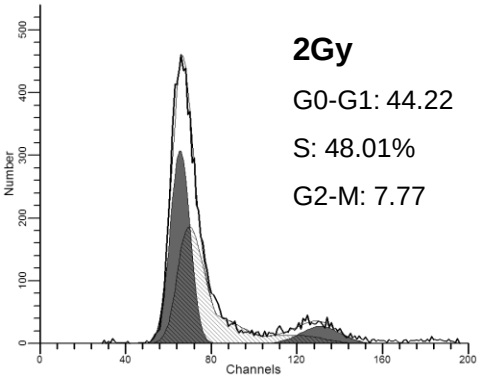

100
75
50
25
0
cell growth inhibition (%)
D54 p53wt
D54 p53-
NU1025
TPT 10nM
TPT 10nM + NU1025
2Gy
2Gy + NU1025
2Gy + TPT10nM
2Gy + TPT10nM + NU1025

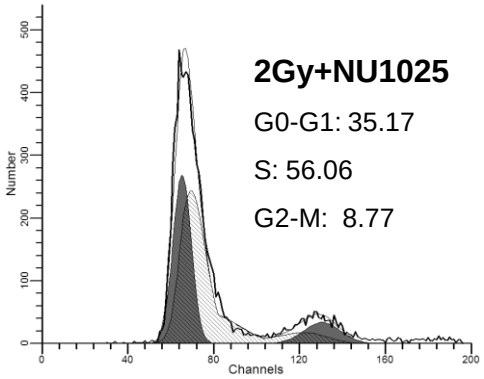
Fig. 8B.

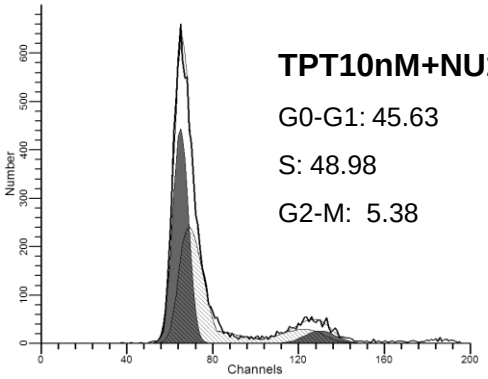
D54 p53wt











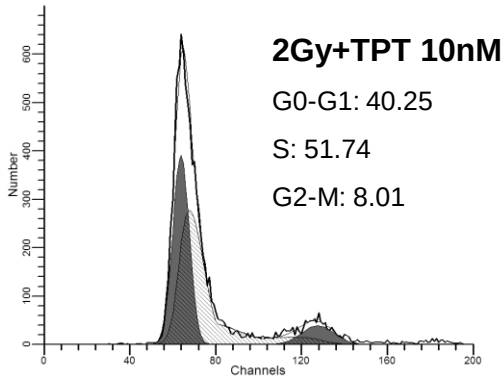
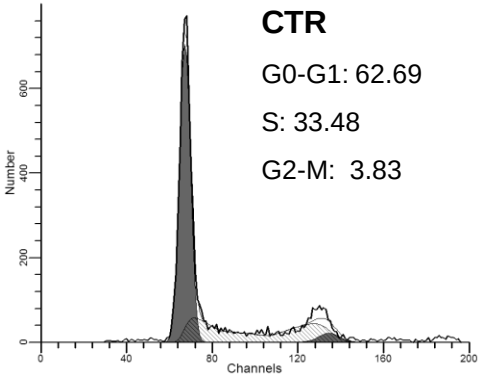
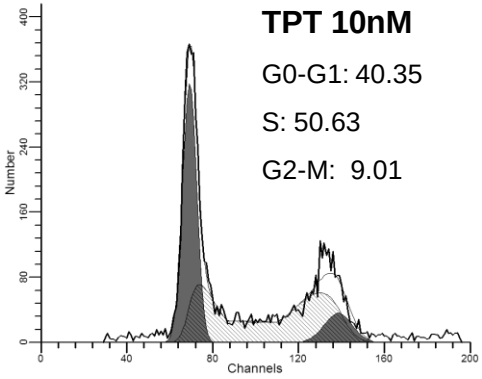
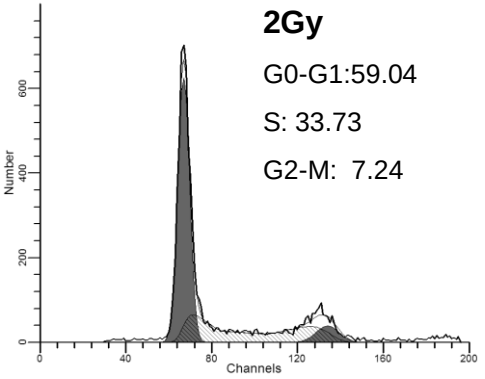


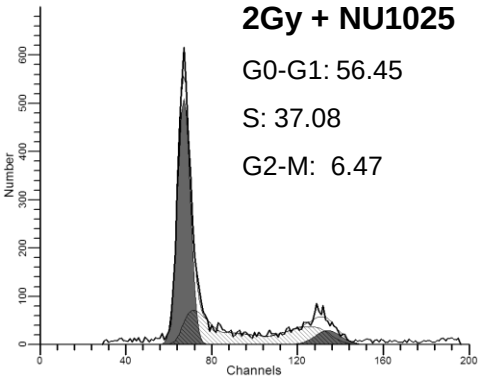
Fig. 8C.

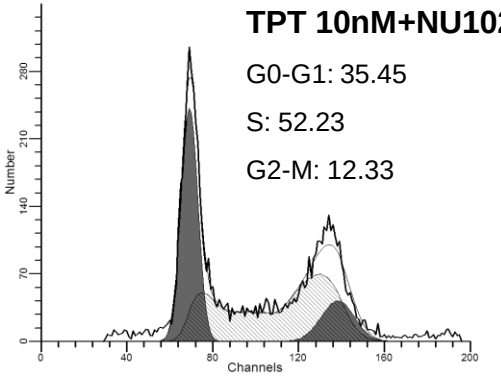
D54 p53-











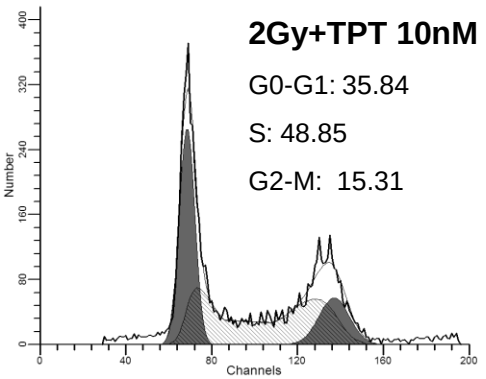


Fig. 8C.

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Multidisciplinary Approach to Patient with Malignant Melanoma

Giuseppina Della Vittoria Scarpati[1], Celeste Fusciello[1,2], Francesco Sabbatino[3], Soldano Ferrone[3], Francesco Caponigro[4], Francesco Perri[4], Chiara Carlomagno[2] and Stefano Pepe[1,*]

[1]Department of Medicine, University of Salerno and Division of Oncology, "San Giovanni di Dio e Ruggi d'Aragona" Hospital, Salerno; [2]Departement of Molecular and Clinical Endocrinology and Oncology, University of Neaples "Federico II"; [3]Departement of Surgery, Massachusetts General Hospital; [4]Head and Neck Oncology Unit, Istituto dei Tumori di Napoli, Fondazione "G.Pascale"

Abstract: The incidence of melanoma is rapidly increasing worldwide and the prognosis of patients with metastatic disease is still poor, with a median survival of 8–9 months and a 3-year overall survival (OS) rate less than 15% [1,2].

A complete surgical excision is the main treatment for primary cutaneous melanoma [3], but controversies about the extension of excision margins still remain [4].

Sentinel lymph node biopsy (SLNB) provides important prognostic and staging data by the identification of regional node-negative patients who would not benefit from a complete nodal dissection. However, there is no consensus in the definition of melanoma thickness to enforce the execution of the SLNB [5].

To date, Interferon-α (IFN-α)is the only approved adjuvant treatment after surgical excision of high-risk melanoma, but its indication remains still controversial [2,6].

Keywords: melanoma, immunotherapy, B-RAF Inibithors, chemotherapy

INTRODUCTION

Dacarbazine (DTIC) has been the standard first-line therapy for patients with metastatic disease for a long time with a reported response rate less than 15% [7]. Recently, two drugs have been approved for treating patients with metastatic melanoma. The first one, ipilimumab, is a monoclonal antibody which targets the cytotoxic T-lymphocyte Antigen 4 (CTLA4). Two phase III clinical trials comparing ipilimumab with DTIC demonstrated an improved OS for patients treated with ipilimumab. Nevertheless, this agent resulted to be effective only in a subset of patients and the related toxicities were considerable.

Vemurafenib is the second drug that has been approved for the treatment of metastatic melanoma. It is an agent that selectively targets BRAF, a component of the MAPK pathway. Approximately 60% of all melanomas harbor activating mutations in BRAF oncogene. Treatment with vemurafenib has shown to improve OS and to induce tumor regression in approximately 50% of treated patients with metastatic melanoma carrying a BRAF V600E mutation that implies the substitution of glutamic acid instead of valine at amino acid 600. However, the responses are rarely complete and the median time to disease progression is less than 7 months due the onset of specific drug resistance. Several mechanisms of resistance have been described and clinical trials are testing new strategies to overcome the intrinsic or acquired BRAF-inhibitors resistance.

DIAGNOSIS

Early detection of cutaneous melanoma and its early removal is the only therapeutic strategy able to increase the percentage of cures. Therefore identification and treatment of suspected skin lesions play the major role in the treatment of melanoma.

Dermoscopy is a non-invasive skin imaging technique which can help in this aim. The technique consists of a magnifier (typically x10), a non-polarized light source, a transparent plate and a liquid medium between the instrument and the skin, and allows inspection of skin lesions unobstructed by skin surface reflections. Modern dermatoscopes avoid the liquid medium and employ instead polarized light to cancel skin surface reflections [8].

Argenziano *et al*. showed that the introduction of dermoscopy leads to a reduction of excised nevi and to an increased number of early diagnosed melanomas [9].

Timing of dermoscopy is an issue of outstanding difficulty and several randomized clinical studies have faced the argument comparing different follow-up schedules [10-12].

Results of these studies suggest two follow-up plan: (a) short-term follow-up of 3 months for patients with familial atypical mole and melanoma syndrome and (b) long-term follow-up of 6-12 months for those with atypical mole syndrome. Patients with multiple common nevi and no additional risk factors were found to have low benefit from sequential digital dermoscopy.

It's now clear that dermoscopy imaging is a useful strategy to avoid missing melanomas while minimizing unnecessary excision of benign lesions.

The informations to be taken early during diagnostic work-up are detailed in Table **1**.

LIMITATED DISEASE

Surgical Treatment

Surgical excision remains the mainstay of treatment for primary cutaneous melanoma. Controversy exists concerning the extension of excision margin for cutaneous melanoma with Breslow ≥ 2 mm.

In the first prospective randomized trial, 584 melanoma patients with tumors thinner than 2.0 mm were included. Results demonstrated that 1 cm of radial margin was a safe strategy for thin melanoma <1.0 mm thick, but not for melanomas >1.1 mm thick. In fact, melanomas with a thickness > 1.1 mm surgically treated with a free margin of 1 cm, recorded a recurrence rate of 4% [13]. Results of other trials have not provided clear features both regarding thin (<1 mm) and deeper (> 1mm) lesions [14-19].

*Address correspondence to this author at the Department of Medicine, University of Salerno and Division of Oncology, "San Giovanni di Dio e Ruggi d'Aragona" Hospital, Salerno; Tel: 089 672538; Fax: 089672539;
E-mail: stepepe@unisa.it

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Table 1. Pathological Main Characteristics of Melanoma

Diagnosis of Melanoma	Important Features to Determinate
Hystotype	in situ melanoma; superficial spreading melanoma; nodular melanoma; malignant lentigo and melanoma developed from malignant lentigo; acral melanoma; desmoplasic melanoma; malignant blu nevus; melanoma developed fromcongenital nevus; metastatic melanoma
Cellular tipology	epithelioid; fusate; nevoid
Growth phase	radial; vertical
Invasion depth according to Breslow	mm
Number of mitosis per mm²	Particularly important for thick lesions (<)1 mm
Ulceration	yes or not
Distance from resection margins	Necessary for staging completion and re-resection performing
Tumor Infiltrating Lymphocytes	present or absent
Microsatellytosis	Necessary for staging completion and indication to perform also radiotherapy (discussed)
Regression	parzial or total; It may indicate a poor prognosis for invasive lesions

At present, the scientific community agrees on the following recommendations:

a) for melanoma ≤ 1 mm, wide excision with 1.0 cm margin (Category 1);

b) for melanomas with thickness > 1mm, wide excision with 2 cm (category 1 for tumors 4 mm or less in thickness; category 2A for melanomas more than 4 mm) [20].

Sentinel lymph node biopsy (SLNB) is a minimally invasive staging method to identify patients with subclinical nodal involvement [21].Vital blue-dye and radio-colloid are used to map the lymphatic drainage from primary cutaneous melanoma to the first tumor-draining regional lymph node (or nodes). Radical limphoadenectomy is avoided in absence of metastasis in sentinel node (nodes). On the other hand, when metastases are detected, regional limphoadenectomy is required since other nodes in the basin might be involved.

Multicenter Selective Lymphadenectomy Trial (MSLT-I) was the most important study designed to evaluate the contribution of SLNB to the outcomes of patients with newly diagnosed melanoma. This randomized study enrolled 1,269 patients with intermediate thickness (1.2 to 3.5 mm) melanomas. Patients were randomized to receive: i) wide excision of primary tumor associated with a postoperative observation and complete lymph node dissection (CLND) only if nodal metastases were clinically detected or, ii) wide excision and SLNB with CLND if metastasis in sentinel lymph node (SLN) was detected. The results of the study did not demonstrate statistically significant differences in OS between patients treated with SNLB and those treated without SNLB. However, a significant improvement in 5-year disease free survival (DFS) in the SLNB group was found [22].

The value of SLNB for thin (< 1mm) and thick (> 4mm) melanomas was not addressed specifically in MSLT-I study. The incidence of occult regional nodal metastasis in patients with thin melanoma (≤1.0 mm) is low and the necessity to perform SLNB in these patients remains controversial. Andtbacka and Gershenwald reviewed literature with the aim to identify sentinel node positivity predictors in patients with thin melanomas [23]. The authors concluded that SLNB can be considered for Breslow ≥0.75mm, in patients with T1b melanomas (i.e. Clark level IV/V and/or ulcerated) or with thin melanomas characterized by high mitotic activity (≥1 mitosis/mm^2). The young age (≤ 40 years) could be an additional parameter to be considered, particularly if the primary tumor is characterized by a high tumor mitotic rate. Tumor regression does not seem to be associated with an increased risk for SLN metastasis.

In conclusion, in the scientific community there is broad agreement in recommending SLNB only for patients with melanoma 1 mm. SLNB should be considered for patients with melanoma thickness ≥ 0.75 mm in presence of adverse features, such as ulceration, mitotic rate ≥ 1/mm^2, positive deep margins, lymphovascular invasion or young patient age [20].

The value of the SNLB is still controversial in patients with melanomas Breslow thickness ≥4 mm. A number of clinical trials have faced this issue, but there are no strong recommendations to perform SNLB in this category of patients and the choice is committed to the clinicians experience and discretion.

Some pathologic melanoma characteristics are prognostics and they should be detailed in the pathology report. The NCCN melanoma panel recommends the inclusion in pathology report of Breslow thickness, ulceration, mitotic rate, margin status (positive or negative), presence of satellitosis, and Clark's levels for non-ulcerated and T1 melanomas [20]. In patients with localized melanoma, Breslow thickness, ulceration and mitotic rate are the most important predictive factors for outcome [24]. Recent studies suggested that mitotic rate is an independent prognostic variable in patients with thin and non ulcerated melanomas [25]. Controversy exists about the prognostic value of the tumor regression in patients with cutaneous melanoma. Tumor regression might be associated with an underestimation of the true Breslow thickness. On the other hand it can indicate recognition of the primary tumor by the immune system. Authors who have faced this argument have discovered that tumors regression cannot be considered a prognostic factor for patients with cutaneous melanoma and it should not be used to guide clinical decision-making for such patients [26].

Several markers have been studied as predictors of SLN involvement in melanoma. Results of clinical trials highlighted that the site of primary melanoma (extremities *vs.* axial locations) and T-lymphocytes infiltrating tumor (TILs) were predictive for lower probability of SLN involvement, while thickness (>4mm *vs.* 0-1mm) was predictive for higher risk of SLN involvement [27].

Adjuvant Therapy

The immune system plays a key role in the onset of tumors, in the regulation of tumor growth and even in response to therapy, mainly by the immunosurveillance mediated by CD8+T and NK cells [28]. Melanoma, as well as other tumors like renal cell carcinoma, is strongly immunogenic. This hypothesis stems from data demonstrating the presence of infiltrating tumor specific CD8+ T lymphocytes in patients with spontaneous regression of

melanoma. The elusion of immunosurveillance has been proposed in 2004 by Schreiber *et al.* as the seventh hallmark of cancer [38] and avoidance of immunosurveillance is a mechanism put in place by the majority of tumors through different mechanisms. Several data support the ability of melanoma cells to suppress the immune system by a specific immunoediting to select non-immunogenic tumor cell clones or by an *in situ* immune suppression driven by the tumor and /or specific cytokines [29]. Advanced melanoma is often associated with an immunosuppressive status driven by the increase of negative regulatory specific T cells (Treg) [30, 31] and the induction of immunosuppressive chemokynes, such as IL-10 [32-36].

Three main immuno therapies have been used in melanoma:

i pleiotropic immunomodulators, such as IL-2 and IFN-α2b.

ii monoclonal antibodies (mAbs) targeting the specific negative immunoregulator receptors CTLA4 and PD1.

iii cancer vaccines (protein-, peptide- and cell based vaccines) and adoptive cell transfer (ACT) therapy.

In the last two decades, several observations led to the evidence that IFN-α, a molecule belonging to the type I IFN family, had antitumor activity towards several cancers, like renal cancer, lymphoma and melanoma [37, 38, 39].

Many trials have been conducted over the last 3 decades and several trials are still ongoing to establish the real benefits deriving from IFN-α2b as adjuvant therapy of melanoma.

Three main clinical trials need to be mentioned: 1) the ECOG Trial E1684 comparing High Dose Interferon (HDI) (IFN-α2b 20 million UI/m^2/day i.v. x 4 weeks followed by 10 million UI/m^2/3times a week s.c. x 48 weeks) versus Observation; 2) Intergroup Trial E1690 comparing HDI and Low Dose Interferon (LDI) versus Observation; 3) Intergroup E1694 comparing GMK vaccine versus HDI.

All these trials showed significant and durable effects of IFN-α2b on recurrence free survival (RFS) and overall survival OS. A meta-analyses pooled data from these three trials and confirmed the benefits deriving from IFN-α2 therapy as adjuvant treatment for patients with highrisk of recurrence [40-42]. On the basis of these data, HDI emerged to be the only effective regimen to be administered.

In 2011 the pegylated preparation of IFN-α2 (Peg-IFN-α2) has been approved by Federal Drug Administration (FDA) as adjuvant treatment of stage III melanoma. Peg-IFN-α2 is administered subcutaneously for up to five years. The approval is justified by data deriving from a randomized phase III trial, the EORTC 18991[43].

However, updated analysis of this trial raised some controversies since its benefits in terms of RFS and OS appeared to be not statistically significant at 7.6 years follow up [44].

Unfortunately, predictive factors of response to IFN are still unknown and, at present, it is not possible to identify those patients who will respond to this specific immunotherapy.

Yurkovetsky *et al.* [45] showed that a multiplexed analysis of pre-treatment serum concentrations of pro-inflammatory cytokines (IL-1β, IL-1α, IL-6, TNF-α, and chemokines MIP-1α and MIP-1β) were significantly higher in patients obtaining longer RFS following an IFN-α2 treatment than patients with low levels of cytokines. Eggermont *et al.* [46] conducted a retrospective analysis of two phase III adjuvant trials (EORTC 18952 and EORTC 18991) and demonstrated that only the subset of melanoma patients characterized by ulcerated primary tumors and/or regional lymph node involvement (stage IIB and III-N1/N2) obtained a significant improvement of RFS and OS by IFN-a treatment. Moreover, Gogas *et al*. demonstrated that the development of clinical and immunological manifestations of autoimmunity during IFN-α2 treatment appeared to correlate with a better clinical outcome in terms of RFS and OS [47].

Different adjuvant treatments for high risk melanoma patient have been explored, such as treatment with antitumor vaccines (CancerVax or GMK), but with negative results. Data from ongoing trials evaluating the role of anti-CTLA-4 mAbs, BRAF and MEK inhibitors in the adjuvant setting are awaited for the next years.

Anticancer vaccine and adoptive cell transfer approaches represent an attractive research topic since both strategies try to kill tumor cells by the engagement of the immune system. Unfortunately, to date only modest clinical benefits have been demonstrated with these approaches in melanoma patients. This is probably due to the huge complexity of the mechanisms underlying an immune response against tumor cells and to the immuno-suppressive state caused by the tumor cells and even associated with advanced-stage cancer patients. Anticancer vaccine use finds its roots on the identification of Tumor Antigens (TAs) which are expressed by tumor cells but not by normal cells. Thus, TAs might be recognized by T cells by their presentation in the context of MHC class I molecules and stimulate a cellular/humoral response able to target cancer cells by the activation of cytotoxic T lymphocytes (CD8+ T cells).

Five classes of TAs can be distinguished: differentiation antigens, mutated antigens, cancer-testis or cancer-germline antigens, overexpressed antigens and viral antigens [48].

Among TAs, several different melanoma-associated antigens (MAGE) have been identified since 1991, when Van der Bruggen *et al.* isolated the first one (MAGE-A1) and discovered that it antigen was recognized by cytotoxic T lymphocytes. Two main classes of MAGEs (MAGE I and MAGE II) have been distinguished according to their structure and expression profile [49].

The first class of MAGEs is widely expressed among various types of malignant tissues, but not in adult normal cells, except for the germ-line cells lacking HLA expression; it appears to be more interesting as immunotherapy target, showing promising results in preliminary trials [50].

In order to enhance the efficacy of a vaccine approach utilizing TAs they can be synthetically engineered (i.e., GP100 melanoma-associated antigen and MELAN-1 peptides) [51] or be associated to adjuvant chemical components (like water-in-oil emulsion, Montanide).

The introduction of toll-like receptor ligands (TLR9L, TLR3L, and TLR4L) to activate NK and APCs [52] also showed promising results.

Adoptive cell transfer is the passive transfer of cells, most commonly immune-derived cells, into a new recipient host with the goal of transferring the immunologic functionality and characteristics into the new host. Clinically, this approach has been exploited to transfer either immune-promoting or tollerogenic cells (often lymphocytes) to patients to either enhance immunity against viruses and cancer or to promote tolerance in the setting of autoimmune disease. Tumor Infiltrating Lymphocytes (TIL) therapy represent a milestone in ACT, although it is an expensive, difficult and time-consuming approach. Autologous TIL are selected from tumor, grown under conditioning presence of stimulating T lymphocyte cytokines, such as IL-2, and re-injected in patients. The adoptive transfer of autologous TILs or genetically re-directed peripheral blood mononuclear cells has been used to successfully treat patients with advanced solid tumors, including melanoma and colorectal carcinoma, as well as patients with CD19-expressing hematologic malignancies. During the last decades several strategies have been proposed and tested to overcome technical difficulties of production and handling of ACT.

Pre-conditioning regimens utilizing non mieloablative chemotherapy or total body irradiation have been successfully

introduced to induce patient lymph depletion before TILs administration in patients with melanoma. It avoids endogenous Treg and myeloid derived suppressor cells (MDSCs) suppression of immune antitumor response [53].

Faster TIL production techniques which utilize the so called 'young TILs' appear to warrantee, combined with patients' lymph depletion, improved persistence and antitumor response *in vivo* [54].

TILs derived from autologous T cells carrying genetically engineered TCRs showed promising preliminary results [55]. These TCRs are directed to some TAs, such as the cancer/testis antigen NY-ESO-1, and allowed to administer ACT therapy even to patients in whose a TIL isolation and sampling might be not performed due to difficult access to the metastatic site.

Dendritic cells (DCs) are professional antigen presenting cells (APCs) which are involved in inducing primary T-cell response. This feature makes them optimal candidates to be used as anticancer immunotherapy [56]. The administration of tumor-antigen loaded DCs showed encouraging results in the treatment of advanced solid tumors, but, unfortunately, these results were often transient. These poor results are probably due to: i) tumor-induced immune suppressive state which prevents a strong and durable immunoresponse by down-regulation of MHC class I antigen; ii) secretion of soluble immunosuppressive cytokines converting DCs into tolerogenic status; iii) induction of Treg cells [57].

Many devices have been studied to improve response to DCs based therapy. Additional activation of DCs by toll-like-receptors ligands (TLRLs) seems to neutralize the immunosuppressive effects induced by Tregs and enhances T cells proliferation [58].

Other approaches to induce stronger and more durable responses to DCs have been proposed. One of them includes DCs maturation under conditioning cytokines cocktails containing IL-1α, TNF-α, IL-6 and PGE2 [59]. The other one is based on adding to the classical type1 polarizing cocktail administration of IFN-α (alpha-type-1-polarized dendritic cells). This approach guarantees a satisfying population of efficient DCs which display high ability in chemotaxis, interleukin12, p70 secretion and the expression of several co-stimulatory molecules. The clinical activity of alpha-1 polarized DCs in melanoma, as well as in other malignancies, is on evaluation [60].

METASTATIC DISEASE

Immunotherapy

The use of immunotherapy in metastatic melanoma has undergone profound changes in the last decade and remains one of the most promising therapeutic strategies in this setting. Historically, the first immunotherapeutic agent registered for the treatment of metastatic melanoma has been the IL-2, a T cell growth factor, which plays a crucial role in immune regulating process and T cell proliferation [61], demonstrated a potent antitumor activity in murine models [62].

In 1992, the FDA approved high dose IL-2 (HD-IL2) for the treatment of patients with metastatic renal cell carcinoma [63] and, in 1998, for the treatment of metastatic melanoma. In metastatic melanoma patients, HD-IL2 treatment induced an interesting overall response rate, around 10-15%, and, in a small proportion of patients (3-5%) a durable complete response (> 5 years).

Predictive factors of response to IL-2 therapy include development of autoimmunity (hypothyroidism), low LDH levels, good ECOG performance status, a low number of metastases in specific sites (lung, lymph-nodes and soft-tissues) and high pre-treatment neutrophil count [64].

Biochemotherapy regimen associating chemokines, such as IL-2 or IFN-α, with chemotherapeutic demonstrated no improvement of OS, a slightly increase of response rates and RFS with a significative increase of toxicity [65].

However, there have been important advances in the understanding of crucial immunoregulatory pathways underlying the regulation of the immune response against tumors which have led to develop novel therapeutic strategies for the treatment of metastatic melanoma. One of the most promising approaches to overcome cancer immune tolerance is the promotion of T cell activation by blocking inhibitory signals arising from receptors like the cytotoxic T lymphocytes-associated antigen-4 (CTLA-4) and Programmed Death-1 (PD-1) receptor [66].

CTLA-4 receptor represents a key molecule able to decrease T-cell activation; its activation is physiologically required to induce tolerance to self antigens avoiding autoimmunity reactions and can be figured as a natural brake of the immune system [67, 68]. CTLA-4 receptor is up regulated on activated T-cells and competes with CD28 for binding to the ligand B7; the binding of CTLA-4 with B7 leads the block of co-stimulatory signals needed for T-cell activation [68]. Specific mAbs blockade of CTLA-4 receptor activity allows CD28 to bind B7.

Two fully human anti-CTLA-4 mAbs are currently in clinical setting: ipilimumab and tremelimumab.

Ipilimumab is a fully human mAb IgG1 which demonstrated activity in several phase II trials on metastatic melanoma and, more recently, in two phase III trials. In the first one, published in 2010, pre-treated melanoma patients were randomized to receive gp100 peptide vaccine and ipilimumab in combination or gp100 vaccine or ipilimumab alone. Data analysis demonstrated a significant OS improvement in ipilimumab arms compared with gp100 vaccine arm (10 *vs.* 6.4 months; HR 0.68 and P<0.001). No statistically significant different in OS was found between ipilimumab and ipilimumab plus gp100 peptide vaccine [69]. The OS among subgroups of patients showed a benefit for all subgroups in ipilimumab and ipilimumab plus gp100 versus gp100 alone. In the second phase III trial (MDX-024), published in 2011, untreated metastatic melanoma patients were randomized to receive DTIC with or without ipilimumab. The study confirmed a significant survival benefit in the ipilimumab/DTIC arm (median OS 11.2vs. 9.1 months). There was no difference in disease control rate and in ORR (15%*vs.* 10%), but the duration of response was longer for ipilimumab/DTIC combination (19.3 *vs.* 8.1 months). Interestingly, several patients obtained long-lasting benefit from ipilimumab despite they did not meet the standard RECIST criteria for evaluation of objective response. The toxicity of the ipilimumab/DTIC combination was considerable and 56% of patients experienced grade 3 or 4 adverse events. Toxicity profile resulted to be different from that seen with ipilimumab alone or in combination with gp100 in the first clinical trials. Patients in the ipilimumab/DTIC arm experienced more hepatic toxicity and slightly less gastrointestinal toxicity mainly caused by the concomitant administration of the chemotherapeutic agents [70]. The FDA has approved ipilimumab for the treatment of metastatic melanoma, although the iindividuation of predictive biomarkers of response for ipilimumab is lacking. The study of tumour microenvironment led to the observation that a pro-inflammatory gene expression profile appears to correlate with favourable clinical outcome in patients treated with ipilimumab [71].

Tremelimumab is the second anti-CTLA-4 mAb which has been tested in melanoma patients, but the single phase III trial, with tremelimumab vs. DTIC or temozolomide in naïve metastatic melanoma, failed to demonstrate a significant increase of OS in patients treated with tremelimumab [72].

PD-1 is a receptor belonging to the CD28 family. It plays a role in tumor associated mechanisms of immune-escape [73]. PD-1 has two ligands: PD-L1 and PD-L2, which act as down-regulators of immune response. PD-L1 and PD-L2 are widely expressed on cell

membrane of macrophages, B lymphocytes, T resting lymphocytes, dendritic cells and cancer cells [74]. Several evidences demonstrate a predominant role of PD-1 in melanoma immune tolerance. It has been shown that engaged of PD-1 receptor by his natural ligands and the up-regulation of Tim-3 is associated with tumor antigen $CD8^+$cell dysfunction in advanced melanomas [75]. Furthermore, an increase of PD-1 expression on a subset of Melan-A-reactive T cells has been shown in metastatic melanoma patients in comparison with those in stage I-II (67). Lastly, PD-1 appears to be a negative regulator of CD8+ T cells by the specific tumor antigen NY-ESO-1 which activity was restored by PD-1/PD-L1 pathway blockade [76].

Taken together all these evidences support the idea that targeting of PD-1 may represent a promising tool to enhance tumor specific Tcell activity. The MDX 1106 mAb, a fully human mAb againstPD-1 receptor, has been recently tested in advanced melanoma patients and preliminary data have been presented at the ASCO 2012. MDX 1106 activity and safety were encouraging, showing durable clinical benefits in patients with advanced melanoma. However, these remarkable results require further evaluations [77, 78].

Target Therapy

Around 60% of patients with advanced melanoma have mutations affecting BRAF, with nearly all of those affecting a single-amino-acid residue within the kinase domain. The most common mutation of BRAF gene is the valine to glutamate substitution at 600 position of kinase domain (V600E). This altered amino acid sequence results in a protein that remains constitutively in the active conformation and has an 800-fold increased kinase activity compared with its wild-type counterpart. Less frequent mutations, between 10 and 30%, have been discovered, such as the variant V600K, which represents 5% to 20% of BRAF mutation [79, 80].

BRAF (V600E) leads to the sustained activation of the MEK1/2→ERK1/2 MAP kinase pathway. This pathway plays a critical role in regulating gene expression, cell proliferation and survival, thereby contributing to the initiation and progression of melanomas [81, 82]. Knockdown of BRAF (V600E) leads to inhibition of cell proliferation *in vitro* by the lack of MEK and ERK activation. Beyond proliferation and apoptosis, knockdown of BRAF (V600E) increased the expression of melanocyte-associated antigens and suppressed the expression of pro-angiogenic cytokines [83].

Several inhibitors have been developed to target BRAF (V600E). The first attempt with a small-molecule inhibitor, sorafenib, a multi-targeted receptor tyrosine kinase (RTK) utilized for its properties to inhibit mutated and wild-type BRAF, produced disappointing results [84].

In contrast, selective BRAF inhibitors (BRAF-I), such as vemurafenib (formerly known as a PLX4032) and dabrafenib (also known as GSK2118436), have been showed to largely mimic the effects of selective BRAF (V600E)-specific siRNA knockdown [85]. In fact, inhibition of proliferation and induction of apoptosis is limited to BRAF mutant cancer cells and these effects are mediated by inhibition of MEK and ERK phosphorylation.

Vemurafenib and dabrafenib are orally available drugs and have been both validated in clinical setting. Phase I, II and III trials all have shown that both BRAF-I can induce ORR in approximately 60% of patients with metastatic melanoma harboring the BRAF (V600E) oncogene [86].

The IC50 of vemurafenib for mutated BRAF (V600E) is 31 nM while for wild-type BRAF is about 100 nM. Other kinases, such as CRAF, ACK1, KHS1, and SRMS appear to be inhibited at concentrations less than 100 nM [87].

Recommended dose of vemurafenib was determined to be 960 mg twice daily. The common toxicities observed at this dose are rash, photosensitivity, arthralgia, and the appearance of well-differentiated cutaneous squamous cell carcinomas or keratoacanthomas during the first 3 months of therapy. Rash, photosensitivity and arthralgia are typically mild to moderate and do not require dose modification. However, approximately 30% of patients require at least a temporary dose reduction to 720 mg twice daily because of these toxicities [86]. In the context of clinical trials cutaneous squamous cell carcinomas and keratoacanthomas have been successfully excised and patients have been permitted to continue the treatment without interruption. To date, no other type of malignancy beyond these cutaneous tumors has been observed [88].

In phase III clinical trial, 675 patients with previously untreated metastatic melanoma with BRAF mutations (V600E or V600K) were randomly assigned to receive vemurafenib (960 mg orally twice daily) or DTIC (1000 mg/m^2 intravenously every 3 weeks). In the interim analysis for OS after 6 months, the hazard ratio for death in the vemurafenib group was 0.37 ($P < .001$). Thus the trial was modified so that patients assigned to DTIC could immediately switch to vemurafenib. Data analysis showed that OS was 84% in the vemurafenib group and 64% in the DTIC group. PFS was 5.3 months in the vemurafenib group versus only 1.6 months in the DTIC arm. The ORR for patients receiving vemurafenib was 48% versus only 5% in the DTIC group ($P< .001$). Interestingly, tumor response occurred early in the vemurafenib group, approximately 6 weeks. Anyway, a 15% of patients appeared to have primary refractory disease and, although the likelihood of initial response was high, there was a high degree of variability in the duration of responses. vemurafenib have been approved by FDA in 2011 for the treatment of patients affected by advanced melanoma harboring BRAF V600E mutation [89].

Dabrafenib (GSK2118436) is a highly potent and selective ATP competitive BRAF (V600E) inhibitor. The phase I trial identified the dose of 150 mg twice daily for further studies. The most common toxicities observed were pyrexia, fatigue, rash, and headache. Arthralgia was observed, as it had been with vemurafenib, but photosensitivity was not described. Cutaneous squamous cell carcinomas and keratoacanthomas were observed in fewer than 10% of patients treated at the highest dose level. Among the V600E BRAF mutant patients enrolled at the highest dose of dabrafenib (150 mg twice daily), 25 of 26 patients demonstrated evidence of tumor regression, with an ORR in 77% of cases. Results of a multicenter phase III trial have been recently published. Briefly, 250 BRAF (V600E)-mutated metastatic melanoma patients were randomly assigned to receive either dabrafenib or DTIC. Data analyses showed a significant improvement of PFS (5.1 months for dabrafenib arm vs. 2.7 months for DTIC; $p<0.0001$; HR 0.30) [90].

However, complete response is infrequently seen and these drugs are effective for a short time before the occurrence of resistance to BRAF kinase inhibitors.

The mechanisms of resistance to BRAF-I can be divided in two groups: a) those mediated by ERK signaling reactivation, which can be caused by point mutation in MEK1 [91, 92], amplification of mutated BRAF [93], elevated CRAF activity [94], activating NRAS mutation [95], increased levels of COT/Tpl2 [96], FGFR3 [97] and/or aberrantly spliced BRAF (V600E) [98]; b) those mediated by an increase of receptor tyrosine kinase (RTK) driven signaling *via* PDGFRβ over expression [99], IGF1R activation [100, 101], AXL [102], ERBB4 [103], stromal cell secretion of HGF [104], and PTEN loss with possibly elevated activation of PI3K/AKT [105], which is a major pro-survival signaling of RTKs.

From a therapeutic perspective, a recent phase 1/2 study combined the oral MEK1/2 inhibitor GSK1120212 (trametinib) with the BRAF inhibitor GSK2118436 (dabrafenib). One hundred and sixty-two metastatic melanoma patients with BRAF (V600)

mutations were randomized to receive combination treatment with dabrafenib plus trametinib or dabrafenib alone. The study demonstrated a significant median PFS increase for combination arm (9.4 vs 5.8 months; p<0.001; HR 0.39) and a significant improvement of ORR [106].

Chemotherapy

Chemotherapy is an accepted palliative therapy for stage IV metastatic disease and DTIC is the most widely used single chemotherapeutic agent for the treatment of metastatic melanoma. For symptomatic patients, or patients who are not eligible for investigational trials, chemotherapy remains a reasonable palliative option; for novel agents being tested in clinical trials, DTIC based chemotherapy is the accepted comparator. DTIC originally reported objective responses in up to 25% of patients in older phase II trials, but current trials have shown response rates of 5% –12%. Unfortunately, most responses to DTIC are transient; in fact, only 1%–2% of patients achieve a durable long-term response. Temozolomide (TMZ), an oral prodrug with the same active intermediate (3-methyl-[triazen-1-yl]imidazole-4-carboxamide) as DTIC, has been demonstrated to be as effective as DTIC in phase III studies. EORTC Melanoma Group randomized a total of 859 patients to receive oral TMZ at 150 mg/m(2)/day for seven consecutive days every 2 weeks or DTIC, administered as intravenous infusion, 1000 mg/m(2)/day on day 1, every 3 weeks. The primary endpoint was OS. Only ORR was higher in the TMZ arm, but there were no differences between TMZ and DTIC arms in PFS and OS. Moreover, the median duration of response was longer for DTIC [107].

Fotemustine, a chloroethyl nitrosourea, have shown to be effective as single agent against metastatic melanoma with an ORR of 24% in a large phase II trial including 153 patients [108]. The efficacy of fotemustine was confirmed in international phase II studies with an ORR ranging from 12% to 47% and median duration of responses ranging from 18 to 26 weeks [109-111]. In a phase III study was evaluated ORR, as primary end-point, and OS, duration of responses, time to progression, time to occurrence of brain metastases (BM), safety and quality of life in patients with metastatic cutaneous melanoma, treated with fotemustine compared to DTIC. ORR was higher in the fotemustine arm compared to the DTIC arm. A trend in favor of fotemustine in terms of OS and time to development of BM was seen [112].

In a French dermato-oncology retrospective study, the outcome of patients with metastatic melanoma who received two lines or more of cytotoxic treatments has been analyzed. Most of these patients received DTIC for the first line of chemotherapy and fotemustine for the second line of chemotherapy. A clinical benefit was observed in 24.1 % of the patients and OS was 4.1 months after the second-line treatment. The presence of more than two sites of metastasis and an M1c staging represented negative predictive factors of clinical benefit. This study shows a modest benefit of a second line of cytotoxic chemotherapy in a non-selected population [113].

The efficacy and tolerability of fotemustine, cisplatin, IFNα and IL-2 biochemotherapy was evaluated in advanced melanoma patients. The schedule consisted of fotemustine (100 mg/m) and cisplatin (75 mg/m) intravenous on day 1, followed by subcutaneous IL-2 (4.5 MUI) on days 3-5 and 8-12 and IFNα (3 MUI) three times/week, every 3 weeks for six cycles. Sixty patients were evaluated for tumor response, 12 of whom had brain metastases. One patient with BM showed a complete response and partial responses were observed in 10 patients. Disease control (ORR and stable disease) was 58.4% in all patients and 75% in patients with BM. Median time to progression was 3.2 months (4.2 months in BM patients). Median OS was 8.9 months (7.6 months in BM patients). This combination was well tolerated and showed acceptable clinical activity, especially in BM patients [114].

In another phase II trial, patients with advanced melanoma were enrolled to receive induction treatment of 10 mg/kg intravenous ipilimumab every 3 weeks to a total of four doses, and 100 mg/m^2 intravenous fotemustine weekly for 3 weeks and then every 3 weeks from week 9 to week 24. Patients with a confirmed clinical response received maintenance treatment from week 24, with ipilimumab every 12 weeks and fotemustine every 3 weeks. This combination was clinically active. It was reported a significantly disease control also in patients with brain metastases [115].

In a retrospective analysis, 36 consecutive patients with hepatic metastases from ocular or cutaneous melanoma were assigned for surgical hepatic port-catheter implantation. Fotemustine was delivered weekly for a 4-week period, followed by a 5-week rest and a maintenance period every 3 weeks until progression. OS, ORR and toxicity were analyzed and compared. After port-catheter implantation 30/36 patients were finally treated. Nine out of 30 patients achieved partial remission, 10/30 stable disease; 11/30 patients were progressive. Median OS was 14 months. Serum LDH was a significant predictor of both response and survival. Treatment was well tolerated [116]. Similarly, Meldola *et al* conducted a retrospective study on patients with hepatic melanoma metastases (from both cutaneous and uveal melanoma) which had been treated with intra-hepatic arterial chemotherapy with fotemustine or carboplatin. Treatment was well tolerated. The ORR and disease control rate was 16.7% and 38.9% in patients with uveal melanoma. Median time to progression was 6.2 months and median OS was 21 months. The authors concluded that intra-hepatic arterial chemotherapy was a valid choice for patients with hepatic metastases [117].

Polychemotherapy regimen was tested in a phase III trials (Dartmouth regimen: cisplatin/vinblastine/dacarbazine/tamoxifen). However the trial failed to demonstrate a benefit in OS compared with a monochemotherapy with DTIC alone [118].

Several investigators have attempted to combine different strategies with the aim to improve treatment response and patients survival. Immunotherapy regimens with IFN-α and/or IL-2 in combination with chemotherapeutic agents have been explored too. Combinations of IL-2–based immunotherapy and cisplatin- and DTIC-based chemotherapy in patients with metastatic melanoma have been reported to induce 50% of response rate. The median survival has been reported between 11 and 12 months [119-124]. Two meta-analyses performed suggested that this approach induced higher response rate and a potentially longer median survival than chemotherapy or IL-2 alone [125,126].

Two systematic reviews of the literature including 18 trials and more than 2,600 patients compared administration of immunotherapy with IFN-α, IL-2, or IL-2 plus IFN-α in combination with chemotherapeutic agents was compared with chemotherapy alone. They reported higher response rates for combined strategy but no significant difference in OS [127,128]. This data were further confirmed by Atkins *et al*., in a phase III trial. In consideration of the severe toxicity reported in the combination, the authors concluded that concurrent administration of immunotherapy and chemotherapy regimen cannot be recommended for patients with metastatic melanoma [129].

FUTURE PERSPECTIVES

Some investigators have attempted to evaluate the efficacy and safety of concurrent administration of sorafenib and DTIC in patients with advanced melanoma. Sorafenib is an orally available multi-kinase inhibitor that inhibits tumor proliferation by targeting multiple kinases including the vascular endothelial growth factor receptors VEGFR1, VEGFR2, VEGFR3, the platelet-derived growth factor receptor PDGFR, and targets tumor progression by inhibiting FLT3, C-Kit and BRAF. A randomized multicenter study enrolled chemo-naıve patients with advanced melanoma to receive placebo plus DTIC (n 50) or sorafenib plus DTIC (n 51). The

primary end point was progression PFS by independent assessment. Secondary and tertiary end points included time to progression (TTP), ORR, and OS. Median PFS in the sorafenib plus DTIC arm was 21.1 weeks versus 11.7 weeks in the placebo plus DTIC arm. There were statistically significant improvements in PFS rates and in TTP at 6 and 9 months in sorafenib and DTIC arm. However no difference in OS was observed [130].

In a phase II trial investigation, conducted in chemotherapy-naive patients with advanced melanoma, 93 patients were randomized to receive TMZ plus bevacizumab or nab-paclitaxel plus bevacizumab and carboplatin. The addition of bevacizumab to nab-paclitaxel and carboplatin shows promising activity despite tolerability issues, with a significant improvement of median PFS [131]. Bevacizumab was tested also in association with fotemustine [132], as first line treatment, in a multicenter, single-arm, open-label, phase II study, in which serum cytokines, angiogenesis, and lymphangiogenesis factors were monitored by multiplex arrays and by *in vitro* angiogenesis assays. The study demonstrated a clinical activity of this association and found that this combination promoted suppression of some soluble factors involved in angiogenesis and lymphangiogenesis. Aflibercept is a soluble decoy VEGF receptor and angiogenesis inhibitor with potent preclinical antitumor activity in melanoma. Tarhini *et al.* conducted a multicenter phase II study in chemotherapy-naïve patients with advanced skin and uveal melanoma. Aflibercept was intravenously administered at 4 mg/kg every 2 weeks. Response rate and progression-free survival rate was evaluated after 4 months. These results were promising and a significant association between severity of hypertension following aflibercept treatment and survival improvement was found [133]. C-kit, also known as CD117, is a receptor tyrosine kinase that is mutated in approximately 20% of acral, mucosal, and chronically sun-damaged skin melanomas. The ligand for c-kit is the stem cell factor (SCF) and binding of SCF to c-kit induces activation of downstream signaling pathways that are involved in mediating growth and survival signals within the cell, including the P13K-AKT-mTOR pathway and the RAS-RAF-MEK-ERK pathway. Melanomas c-kit mutations are usually point activating mutations in the juxta-membrane domain at exon 11 and exon 13, or in the kinase domain at exon 17. Imatinib, an oral multi-tyrosine kinase inhibitor, which inhibits c-kit activation, has been evaluated in a phase II trial in metastatic melanoma patients expressing at least one of the target protein of imatininb (c-kit, platelet-derived growth factor receptors, c-abl, or abl-related gene). Imatinib induced a dramatic response after 6 weeks of treatment and a partial response lasting 12.8 months was reported [134]. Another phase II trial tested imatinib [135] in 33 patients with metastatic melanoma harboring mutations and/or gene copy number amplification of c-Kit. The study demonstrated a significant antitumor activity of imatinib with an ORR of 23.3%. Escalation of imatinib to 800 mg have been tested to restore disease control after tumor progression, as seen in treatment of gastro-intestinal stromal tumors carrying c-kit mutation, but the result was disappointing. Nilotinib, a new tyrosine kinase inhibitor which inhibits c-Kit more than imatinib, was also tested in several clinical studies [136]. It has demonstrated to be a promising agent in the treatment of metastatic melanoma harboring the c-Kit mutation. A multicenter randomized phase III trial (Tasigna Efficacy in Advanced Melanoma) enrolling patients with locally advanced or metastatic mucosal melanoma, acral melanoma or solar melanoma is ongoing and results are still pending.

Palliative Treatments

It is of paramount importance to manage patients with multi metastatic disease, especially in presence of metastatic sites which may result difficult to treat. Nineteen percent of metastatic melanoma patients developed skin metastases. Skin metastasis can seriously affect the quality of life of melanoma patients. During tumor progression skin lesions increase in number and size, are often ulcerated and associated with bleeding and severe pain.

Electro-chemotherapy (ECT) is a therapeutic approach based on the local application of short and intense electric pulses that transiently permeabilize cell membrane, thus allowing transport of molecules otherwise not permitted by a cellular membrane. Applications for treatment of cutaneous and subcutaneous tumors have reached clinical use (antitumor electro-chemotherapy using bleomycin or cisplatin). A typical protocol for ECT of solid tumors consists of an intratumoral injection of the chemotherapeutic drug (bleomycin or cisplatin) or systemic intravenous administration of bleomycin, followed by delivery of a sequence of (typically eight) square monopolar electric pulses to the tumor. Electric pulses can be delivered either noninvasively, using a pair of parallel plate electrodes, or invasively, using several needle-shaped electrodes arranged in various configurations with predefined distances between the electrodes. Electric pulses are delivered at the time when the maximum extracellular concentration of the chemo-therapeutic drug is expected. Typically, the optimum effect is obtained 3 min after systemic intravenous injection or immediately after intratumoral injection. Since drugs are adminstred at lower dose than systemic treatment, ECT treatment of skin lesions does not induce systemic side effects [137]. Several studies had demonstrated the efficacy of ECT in improving the local disease control and the quality of life of melanoma patients. [138-142].

Isolated limb perfusion is a therapeutic approach employed for treating cutaneous isolated melanoma metastases of the extremities. In this procedure, the major vascular structures are isolated, cannulated, and then attached to a bypass device so that therapeutic agents can be given to the extremity at high doses [143]. Several retrospective studies demonstrated that isolated limb perfusion using IFN-α, melphalan and TNF-α as single agent or in combination, can induce complete response rates ranging from 26% to 69% and additional partial responses from 25% to 43% [144,145]. Unfortunately, objective responses appear to be transient and PFS ranges from 9 to 12.4 months [146]. Furthermore, long-term toxicities such as lymphedema, abnormal limb function, muscle atrophy or fibrosis, neuropathy, persistent pain, and recurrent infection are associated with isolated limb perfusion [147].

The role of radiotherapy in the management of melanoma is still controversial. This reflects the extreme radioresistance of melanoma. Several clinical studies have shown no benefits from radiotherapy in melanoma patients either as adjuvant treatment after surgical resection or definitive treatment [148]. However recent results suggest a role of the radiotherapy in adjuvant setting for patients with high risk of local and regional recurrence. Specifically radiotherapy can be useful to reduce local recurrence rate in patients with desmoplastic histology, positive margins, recurrent disease, Breslow > 4.0 mm, ulceration and satellitosis. Similarly, patients with ≥4 lymph nodes involvement, extracapsular node extension, lymph node size ≥ 3 cm, cervical lymph node involvement, SLN involvement without complete lymph node dissection, and node recurrent disease may also benefit from radiotherapy [149]. In a phase II study of adjuvant radiation therapy, an impressive regional control rate was shown. The study enrolled 234 patients from 3 nodal basins (head and neck, axilla/ supraclavicular, and ilio-inguinal). The authors observed a low local recurrence rate (7%), a low adjacent relapse rate (14%), and an impressive 5 year regional control rate (91%) [150].

In a multicenter phase III trial, which included post-lymphadenectomy patients with isolated regional recurrence who were at high risk for further regional recurrence (≥1 parotid lymph node, ≥2 cervical or axillary lymph node, ≥ 3 groin nodes, any extra nodal spread of melanoma, or maximum metastatic node diameter ≥ 3 cm in neck or axilla, or ≥ 4 cm node in the groin), 250 patients were randomly assigned to observation versus regional radiation

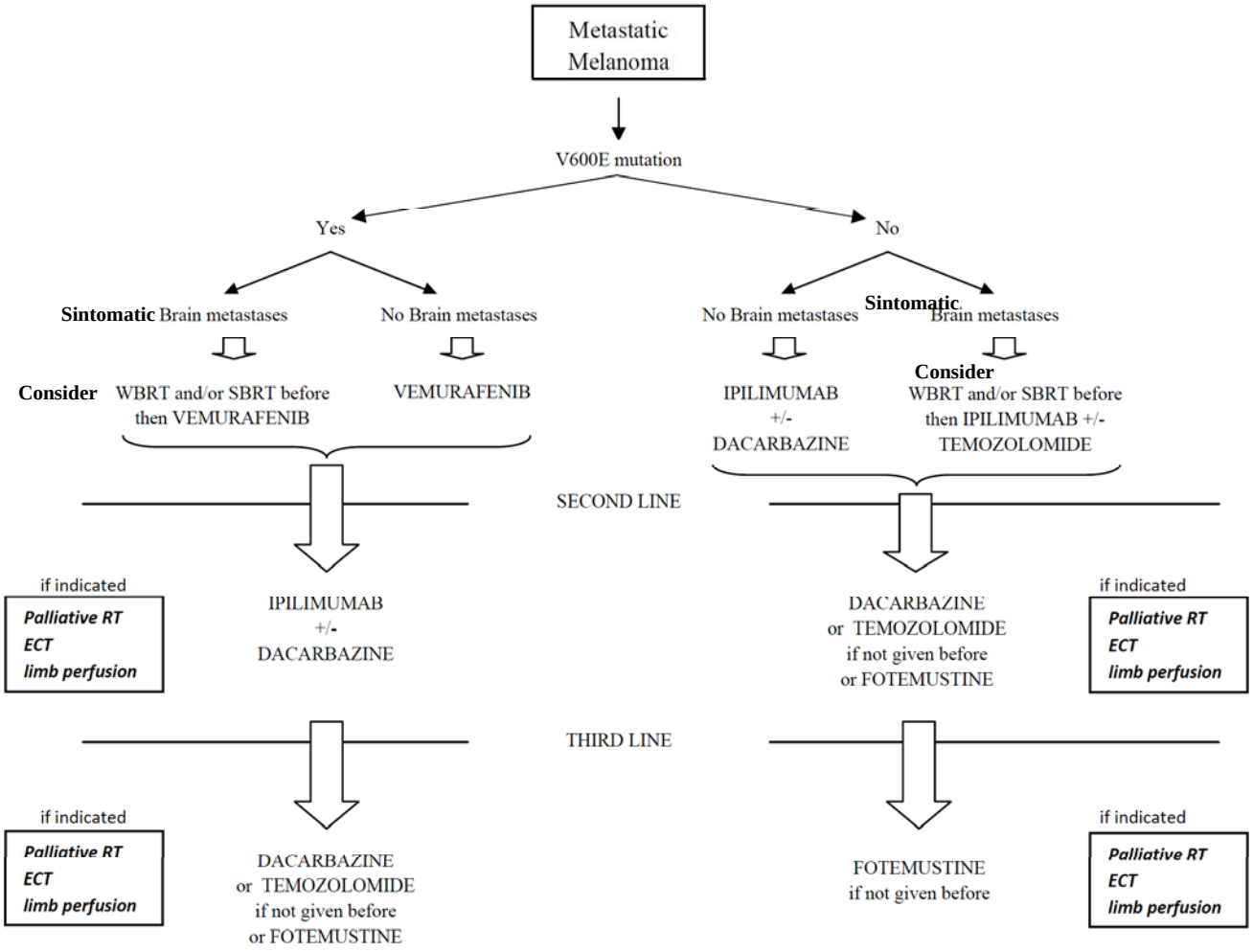

Fig. (1). Therapeutic alghorytm proposal for treating metastatic disease.

therapy. Total dose performed was 48 Gy in 20 fractions delivered at 2.4 Gy per fraction. As result, postoperative radiation significantly improved DFS (HR 1.77, p=0.041) [151]. These results were further confirmed by other two clinical trials [152,153]. Therefore, adjuvant radiotherapy after therapeutic lymphadenectomy for lymph node-metastatic melanoma can be an appropriate treatment in selected patients.

Radiotherapy is still one of the available treatments to control brain metastases in melanoma patients. Brain is one of most common site of melanoma metastases, contributing to 20%–54% of all deaths for melanoma. Patients with brain metastases have generally a poor prognosis and systemic treatments are almost ineffective. Surgical resection, usually followed by radiotherapy, can be used to treat brain metastases in selected patients. Patients which undergo to surgical resection have usually solitary and superficial lesion. In surgically unresectable metastasis, whole brain radiotherapy (WBRT, generally 3000 cGy in 10 fractions) has been employed as palliative treatment. Median OS of patients with melanoma brain metastases treated with WBRT has been reported to range from 3.6 to 4.8 months. Fife *et al* recently showed a survival benefits from WBRT compared with supportive care (OS, 3.4 vs. 2.1 months) [154]. Recent reports have suggested high local control rates for patients with 1 to 3 brain metastases by stereotactic radio surgery (SRS). SRS can be performed by linear accelerator (Linac) or Gamma-Knife-based approaches. Samlowski *et al*. reported their clinical experience utilizing SRS in patients with up to 5 brain metastases. SRS was followed by planned systemic therapy. Results suggest that an aggressive approach, including SRS and systemic treatment, prolong overall survival (11.1 months, 95% CI: 8.2–14.9 from diagnosis). SRS demonstrated a high local control rate and the median duration of response in treated lesions was 10.2 months. Based on their results, they propose a new treatment algorithm for patients with brain metastases. Treatment includes SRS when metastasis are up to 5 and re-irradiation with SRS or WBRT when less or more than 5 metastasis are respectively detected [155].

Fig. (**1**) illustrates a possible approach to the patients with metastatic melanoma on the basis of the disease localization, hystopatologic and biomolecular findings.

CONCLUSION

The treatment of melanoma has been for decades a source of frustration for clinical oncologists. In fact, surgery was the cornerstone of treatment, not only as a primary treatment, but also to treat metastatic lesions if that were possible. Over the past five years, we have seen the advent of new therapeutic strategies that have sparked new hope in the treatment of this deadly disease. In the area of immunotherapy, the introduction into clinical practice of specific monoclonal antibodies able to overcome cancer immune tolerance by blocking inhibitory signals arising from receptors like the cytotoxic T lymphocytes-associated antigen (CTLA) -4 and Programmed Death-1 (PD-1) receptor have been shown to prolong significantly the survival of patients with advanced melanoma. Furthermore, specific inhibitors of BRAF(V600) have shown

extraordinary activity against melanoma characterized by this specific mutation. It is possible that the combination of treatments to target specific molecular and immunotherapy may lead, in the near future, to a significant survival increase of patients with this disease.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Clinical Cancer Research

CCR Translations

See related article by Gerdes et al., p. 1126

Can the "Right" EGFR-Specific mAb Dramatically Improve EGFR-Targeted Therapy?

Francesco Sabbatino and Soldano Ferrone

EGF receptor (EGFR)–specific monoclonal antibodies (mAb) display limited therapeutic efficacy in EGFR-positive solid tumors. To overcome this limitation, the significant improvement of the antibody-dependent cell-mediated cytotoxicity–mediated antitumor activity of a novel EGFR-specific mAb is described. Its potential impact on the efficacy of immunotherapy for EGFR-positive solid tumors is discussed. *Clin Cancer Res; 19(5); 958–60. ©2013 AACR.*

In this issue of *Clinical Cancer Research*, Gerdes and colleagues (1) describe the development and characterization of the functional properties of the novel EGF receptor (EGFR)–specific monoclonal antibody (mAb) GA201. The latter immunoglobulin G (IgG)1 mAb was generated by humanization of the EGFR-specific rat mAb ICR62 and glycoengineering of its Fc portion to enhance its binding to FcγRIIIA expressed on effector cells.

EGFR has been shown to be expressed and activated in several epithelial malignancies, including colorectal cancer (CRC), squamous cell carcinoma of the head and neck (HNSCC), and carcinoma of the pancreas, lung, cervix, renal cell, prostate, bladder, and breast (2). Like other growth factor receptors, EGFR can mediate oncogenic signals involved in proliferation and survival of tumor cells. This background information has provided the rationale to develop EGFR-targeted therapies, with small-molecule EGFR tyrosine kinase inhibitors (TKI) and with EGFR-specific mAbs (3).

Several lines of evidence have convincingly shown that both TKIs and mAbs can blockade proliferative and/or antiapoptotic pathways in tumor cells and that these mechanisms play a major role in their therapeutic activity. However, the EGFR-TKIs and the EGFR-specific mAbs inhibit EGFR activation through different mechanisms; the latter block the EGF binding to EGFR (4), whereas the former inhibit its autophosphorylation (5).

In addition to inhibiting EGFR-activated signaling, IgG1 EGFR-specific mAbs may display antitumor activity through an antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism, that is, by mediating the lysis of target cells by effector cells such as monocytes, macrophages, and natural killer (NK) cells. This effect is influenced by the binding affinity of the mAb to the Fcγ receptors (FcγR) expressed by effector cells, as indicated by the association between polymorphism of FcγRIIIA and extent of lysis of target cells in ADCC (ref. 6; Fig. 1).

Some EGFR-TKIs (erlotinib and gefitinib) and EGFR-specific mAbs (chimeric IgG1 cetuximab and humanized IgG2 panitumumab) have received U.S. Food and Drug Administration (FDA) approval for treatment of various types of cancer either as single agents or in combination with chemotherapy or radiotherapy. In general, EGFR-TKIs have been poorly effective in the treatment of malignancies with an EGFR pathogenesis, except for those which selectively target EGFR abnormalities responsible for the oncogenic signal. This is exemplified by the significant therapeutic efficacy of erlotinib and gefitinib in patients with lung adenocarcinoma harboring activating mutations in the EGFR tyrosine kinase domain (7). Modest clinical efficacy has also been reported for the FDA-approved EGFR-specific mAbs cetuximab and panitumumab (8).

The antitumor activity mediated by EGFR-TKIs can be bypassed by mutations in molecules, which activate oncogenic signals downstream EGFR blockade. These mutations seem to counteract also the immune-mediated antitumor activity of the available EGFR-specific mAbs. This is exemplified by the poor therapeutic efficacy of the EGFR-specific mAbs, cetuximab and panitumumab, in patients with KRAS-mutated CRC (8). These findings are surprising as no mechanism is readily available to explain why signaling activation downstream EGFR blockade can be associated, if not cause the resistance of CRC cells harboring KRAS mutations to the immune attack mediated by the IgG1 EGFR-specific mAbs used.

In this issue of *Clinical Cancer Research*, Gerdes and colleagues (1) postulate that these surprising findings reflect the poor ADCC activity of the presently FDA-approved EGFR-specific mAbs. This possibility is supported by the results Gerdes and colleagues (1) have obtained with their own newly developed mAb GA201. Comparison of the binding characteristics and of the functional properties of the latter mAb with the mAb cetuximab in *in vitro* assays and

Authors' Affiliation: Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

Corresponding Author: Soldano Ferrone, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114. Phone: 617-726-6087; Fax: 617-726-8623; E-mail: sferrone@partners.org

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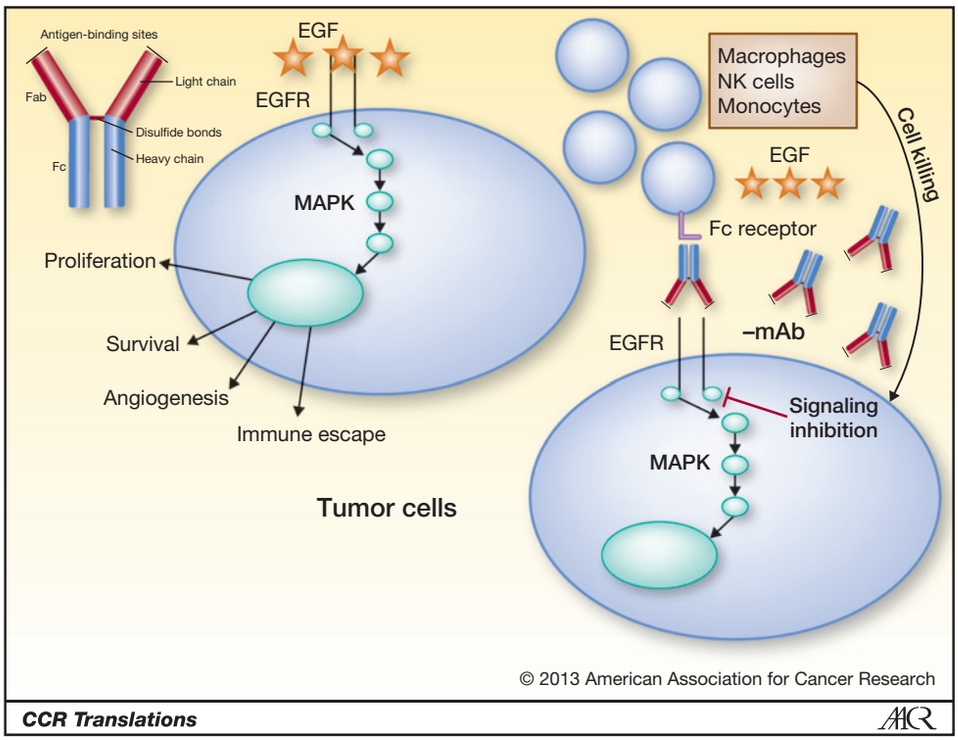

Figure 1. EGFR-specific mAb can mediate antitumor effect by inhibiting EGFR activation and mediating cell-dependent lysis of tumor cells in ADCC.

in animal model systems has shown that these 2 IgG1 mAbs recognize distinct and spatially distant EGFR epitopes. Furthermore, mAb GA201 displays a lower affinity for EGFR than cetuximab. Nevertheless, the 2 mAbs do not differ in their ability to inhibit tumor cell proliferation and to induce apoptosis *in vitro*. Both mAbs exert these effects by inhibiting EGFR/HER2 heterodimerization and downstream signaling. However, mAb GA201 displays a significantly higher activity than cetuximab in ADCC assays conducted with several types of effector cells and with target cells expressing different EGFR levels. Whether this difference reflects at least in part the distinct characteristics of the EGFR epitopes recognized by the 2 mAbs remains to be determined. Furthermore, at variance with cetuximab, the ADCC activity of mAb GA201 is not influenced by its affinity for FcγRIIA and FcγRIIIA as the extent of lysis of target cells mediated by mAb GA201 is similar when FcγRIIIA high- and low-affinity human NK cells are used as effectors. It is noteworthy that differences in affinity of FcγRIIIA, which reflect its polymorphism, seem to have clinical significance, as an association between FcγRIIIA polymorphism and clinical response to cetuximab in patients with CRC has been reported (9). This association is not unique of cetuximab as it has been described also in patients with follicular lymphoma and in patients with breast cancer treated with the CD20-specific mAb rituximab (10) and with the HER2-specific mAb trastuzumab (11), respectively. Finally, at variance with cetuximab, mAb GA201 is not affected in its ADCC activity by the presence of KRAS mutation in target cells. mAb GA201 mediates lysis of target cells even when they express low EGFR level and the human NK cells used as effectors express a low-affinity FcγRIIIA.

The conclusions derived from the described *in vitro* experiments have been corroborated by those derived from *in vivo* experiments. Using various types of human tumor cell lines grafted in immunodeficient mice, Gerdes and colleagues (1) have convincingly shown that mAb GA201 is significantly more effective than cetuximab in controlling tumor growth, both as a single agent and in combination with chemotherapy. More importantly, the *in vivo* antitumor activity of mAb GA201 does not seem to be affected by variables such as level of EGFR expression and/or presence of KRAS mutations, which abrogate the cetuximab antitumor activity.

In view of the potential clinical relevance of Gerdes and colleagues' results (1), it is noteworthy that the mAb GA201 broadens the patient population who may be treated with EGFR-targeted immunotherapy. Specifically, the patients to be treated with mAb GA201 will include also those with low-affinity FcγRIIIA as well as those with KRAS-mutated tumors.

The comparison of the properties of mAb GA201 and cetuximab would have benefited from the identification of the normal tissue(s) with an EGFR expression level sufficient to trigger an ADCC by mAb GA201. Are the likely side effects caused by this mechanism a major obstacle to the clinical use of mAb GA201? Furthermore, does mAb GA201, like other tumor antigen–specific mAbs (12), trigger a tumor antigen–specific T-cell response? Finally, in view of the postulated role of cancer-initiating cells in disease recurrence and metastatic spread, does mAb GA201 either

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as a single agent or in combination with chemotherapeutic agent(s) and/or inhibitor(s) of core stem cell pathways (Notch, Sonic Hedgehog, Wnt) target cancer-initiating cells? Nevertheless, Gerdes and colleagues' compelling results emphasize the urgency to translate to a clinical setting the strategies developed with mAb GA201, once its potential toxicity has been better defined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Sabbatino, S. Ferrone
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Sabbatino, S. Ferrone
Writing, review, and/or revision of the manuscript: F. Sabbatino, S. Ferrone

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