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Discoidin Domain Receptors: A promising target in melanoma

Running title: DDR1, a novel target in melanoma

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Abbreviations: DDR1, Discoidin Domain Receptor 1; DDR2, Discoidin Domain Receptor 2; ERK, Extracellular signal-Regulated Kinases; RTKs, Receptor Tyrosine Kinases; siRNA, small interfering RNA; PCR, Polymerase Chain Reaction

ABSTRACT

The Discoidin Domain Receptor 1 (DDR1) is a member of the receptor tyrosine kinase family that signals in response to collagen and that has been implicated in cancer progression. In the present study, we investigated the expression and role of DDR1 in human melanoma progression. Immunohistochemical staining of human melanoma specimens (n = 52) shows high DDR1 expression in melanoma lesions that correlates with poor prognosis. DDR1 expression was associated with the clinical characteristics of Clark level and ulceration and with BRAF mutations. Downregulation of DDR1 by small-interfering RNA (siRNA) *in vitro* inhibited melanoma cells malignant properties, migration, invasion, and survival in several human melanoma cell lines. A DDR tyrosine kinase inhibitor (DDR1-IN-1) significantly inhibited melanoma cell proliferation *in vitro*, and *ex vivo* and in tumor xenografts, underlining the promising potential of DDR1 inhibition in melanoma.

SIGNIFICANCE

The Discoidin Domain Receptor 1 (DDR1) is a receptor tyrosine kinase protein implicated in several cancers. Here, we demonstrate for the first time that high expression of DDR1 in human melanoma lesions strongly correlates with poor patient outcome. We also show that DDR1 can drive *in vitro* and *ex vivo* melanoma cells malignant properties. Moreover, a selective inhibitor of DDR1 inhibits melanoma tumor growth in mice suggesting that DDR1 is a promising therapeutic target in melanoma.

INTRODUCTION

Major recent improvements in the management of advanced melanoma have expanded median overall patient survival from 8 to 24 months (Redman, Gibney, & Atkins, 2016). However, many patients remain in high medical need, therefore discovery and validation of new targets are urgently needed. Receptor tyrosine kinases (RTKs) play an important role in the initiation of signal transduction pathways that promote the development and progression of cancer. A unique set of RTKs known as the Discoidin Domain Receptors (DDR1 and DDR2), are the only members of the RTK family that signal in response to collagen, the major component of extracellular matrices (Fu et al., 2013; Leitinger, 2014; Valiathan, Marco, Leitinger, Kleer, & Fridman, 2012). Therefore, DDRs are major mediators of the cross-talk between tumor cells and their immediate collagenous matrix. Upon collagen binding, DDRs activate intracellular signaling networks, which regulate multiple cellular activities including cell proliferation, cell survival, cell migration, and extracellular matrix invasion (Leitinger, 2014; Valiathan et al., 2012). Altered DDR function has been implicated in the progression of multiple cancers and in the regulation of malignant cellular activities in experimental models of cancer (Rammal et al., 2016). Therefore, DDRs are considered as potential therapeutic targets in cancer. This led to a considerable effort to design specific inhibitors against these receptors (Elkamhawy et al., 2016; Terai et al., 2015).

Several studies investigated the expression and role of DDRs in normal skin and in melanoma cells (Cario, 2018). In normal skin, DDR1 has been shown to mediate the adhesion of melanocytes to collagen IV in the basement membrane, in a process that involves CCN3, another matricellular protein (Fukunaga-Kalabis et al., 2006; Ricard et al., 2012). However, as far as we know there are no studies on the expression of DDR1 in human melanoma tissues and its association with clinico-pathological parameters. Moreover, the roles of DDR1 in experimental models of melanoma cell malignancy have not been investigated (Cario, 2018). DDR2, the other member of the DDR family, has been implicated in metastasis of melanoma. However, the role of DDR2 in this process is unclear. Downregulation of DDR2 in A375 human melanoma cells reduced experimental liver metastases in mice (Badiola, Villacé, Basaldua, & Olaso, 2011). In contrast, a recent study showed no effect of DDR2 downregulation on the ability of mouse B16-F10 melanoma cells to colonize lungs in experimental metastasis assays (Tu et al., 2019). However, a previous study demonstrated that inoculation of B16-F10 melanoma cells into DDR2-deficient mice decreased subcutaneous tumor growth and experimental metastases, suggesting that stromal DDR2 plays a role in melanoma malignancy (Zhang et al., 2014). Thus, there is a significant gap of knowledge on the role of both DDR1 and DDR2 in melanoma. In the present study, we set to investigate the expression and localization of DDR1 in benign naevi and primary melanoma tissues and its association with disease

progression. We have also investigated the role of DDR1 in supporting the malignant behavior of melanoma cells lines *in vitro* and used a specific DDR inhibitor, DDR1-IN-1, displaying selectivity for DDR1 over DDR2 (Kim et al., 2013), to evaluate the role of DDRs in melanoma xenografts.

MATERIALS AND METHODS

Patients and specimens

The research with human specimens was approved by the Ethics Committee of Saint-Louis Hospital (IRB N° 00006477). Written informed consent was obtained from all included patients. Primary or metastatic melanoma specimens, normal skin and benign pigmented cell lesions were collected from 58 patients who underwent surgery between January 2007 and December 2010 in the Department of Dermatology of Saint-Louis Hospital. All cases diagnosed were reviewed by the Department of Pathology. This patient's series consists of: 6 benign nevi, 52 primary melanomas (5 *in situ* melanomas, 5 melanomas with Breslow under 1 mm, 12 melanomas with Breslow between 1-2 mm, 10 melanomas with Breslow between 2-4 mm, and 20 melanomas with Breslow > 4 mm). Melanoma surgical excision and patient's follow-up were performed according to the 2009 AJCC recommendations (Vogel, Gish, Alves, & Pawson, 1997). Clinical records were retrospectively reviewed in standardized forms. The following endpoints were collected January 2014. The clinical data and pathological characteristics of the cohort used here are described in Table S1.

Immunohistochemistry

Immunohistochemical staining was performed in formalin-fixed, paraffin-embedded (FFPE) tissue sections. It was performed by indirect manual immunostaining using the avidin-biotin-peroxidase method. All sections were deparaffinized in xylene and dehydrated through a graduated alcohol series. To prevent endogenous peroxidase activity, the slides were treated with peroxidase 3% H_2O_2 block for 10 min. The slides were then incubated (1 h, room temperature) with 10 µg/ml of an anti-DDR1 polyclonal goat antibody (Catalog #AF2396, R&D). For evaluation of cell proliferation, Ki-67 staining was performed using Ki-67 monoclonal antibody (clone MIB-1, Dako). Negative controls were performed by replacing the primary antibody with PBS-BSA 0.5% alone. Appropriate biotinylated secondary antibody (Vectastain) was incubated with the sections for 30 min at room temperature followed by detection with a streptavidin-peroxidase complex. Peroxidase activity was detected using 3-amino-9-ethylcarbazole for 20 min at room temperature in darkness. Sections were slightly counterstained with haematoxylin and mounted with glycerin jelly. Stained slides were scanned using a Hamamatsu NanoZoomer 2.0-HT digital slide scanner, and images were obtained using the NDPview2 viewing software (Hamamatsu).

Evaluation of immunostaining

Immunohistochemical results were graded independently by two pathologists. Immunohistochemical staining of tumor cells was recorded considering both the intensity of staining and the proportion of tumor cells showing unequivocal positive reaction. Staining was graded as follow: 0) negative, 1) low, 2) moderate, and 3) high expression.

Statistical analyses

Statistical analyses were performed using the R version 3.1.2 statistical software (R-Foundation for Statistical Computing, Vienna/Austria). Using a Cox proportional hazard regression model, univariate analyses were performed to identify the association between the extents of DDR1 expression with overall survival (OS) (survival R Package). Kaplan-Meier curve was plotted with OS as the outcome. Associations between qualitative variables were estimated with the chi square test. P-values of univariate tests were corrected for multiple comparisons by the Bonferroni's correction and considered significant if less than 0.05. GraphPad Prism 5 was used for the statistical analyses (GraphPad Software, USA).

Cell culture

Melan-a cell line, a non-transformed mouse melanocyte cell line that retains many of the characteristics of normal melanocytes (Bennett et al., 1987) was cultured in RPMI medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Invitrogen), 100 nM phorbol 12-myristate 13-acetate (Sigma), and 200 pM cholera toxin (Sigma). Human melanoma cells, M10 ($NRAS^{Q61R}$), established from patient primary nodular melanoma, were maintained in RPMI medium (Gibco, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Human SKMEL5 ($BRAF^{V600E}$) and C8161 ($NRAS^{Q61K}$) (Laugier et al., 2015) melanoma cell lines were obtained from the American Type Culture Collection (Manassas, VA), and maintained in DMEM medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Western blot analyses

Cells were washed with PBS and then lysed in RIPA buffer (50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 1.0% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2.5 mM NaF and 1 mM

Na3VO4, supplemented with Protease Inhibitor Cocktail (Millipore #539134) on ice. After 1 h incubation on ice, the lysates were centrifuged at 13,000 g for 15 min at 4 °C. Protein concentration was determined using the BCA kit (Pierce #23227). Then, 40-50 µg of lysate per lane were resolved by reducing 7.5% SDS-PAGE followed by western blot analyses with either anti-DDR1 rabbit monoclonal antibody (D1G6, Catalog #5583), anti-phosphorylated DDR1b rabbit monoclonal antibody (pDDR1 at Tyr513, Catalog #14531), or anti-DDR2 polyclonal rabbit antibody (Catalog #12133), which were all purchased from Cell Signaling Technology (Danvers, MA). The antigen-antibody complexes were visualized with ECL reagent (Pierce). Finally, blots were re-probed with a monoclonal antibody against β-actin (Sigma, Catalog #A5441) for loading control.

Small interfering RNA (siRNA) transfection

Two siRNAs for DDR1 (IDs: 147251; 5' GCCAAUGCUGUCUGGUUGCtt 3' and 215973; 5' GCUACACAUUGAGAACCUUGtt 3') or scrambled siRNA oligos (Ambion/Applied-Biosystems, France) were transfected into cells using Lipofectamine-2000 (Invitrogen), as described by the manufacturer. The transfected cells were then incubated in complete media for 24 h, and then analyzed for DDR1 expression, cell migration and invasion, and apoptosis.

Real-Time quantitative PCR (qRT-PCR)

Total RNA was extracted from cells using the Trizol reagent (Invitrogen). RNA quantity and quality were assessed using the Nanodrop-ND-1000 (Nanodrop Technologies, Wilmington, VA). First-strand cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied-Biosystems) according to the manufacturer's protocol. DDR1 primers were specifically designed (Eurogentec, Belgium). Transcript levels were measured by qRT-PCR using Perfect Master Mix-Probe (AnyGenes, France) on LightCycler-480 (Roche) according to the manufacturer's protocol. The transcript levels were normalized to the housekeeping β2 microglobulin (B2M) gene transcripts.

***In vitro* migration, invasion and apoptosis assays**

In vitro migration (through uncoated filters) and invasion (through Matrigel (BD Bioscience) coated filters) assays were performed using a modified Boyden chamber fitted with 8-µm pore filter inserts (BD Bioscience), and placed in 24-well plates. Cells transfected with scrambled control or DDR1 siRNA were seeded on the upper side of the chamber in serum-free media at a density of 1.5 $x10^3$ cells/insert. The lower chamber was filled with media supplemented with 1% FBS. After a 24-h incubation period at 37 °C, the cells were fixed, and stained with a solution of 0.5% crystal violet. The cells present in the whole lower side of the filter were counted. Apoptosis was evaluated by measuring

the level of Caspase 3/7 activity using the Caspase-Glo® 3/7 Assay System (Promega), as described by the manufacturer.

Collagen-induced DDR activation, treatment with DDR1-IN-1, and analyses of DDR phosphorylation

C8161 and SKMEL5 cells were cultured in 60-mm tissue culture plates in complete media, as described above. When the cells reached 65-70% confluency, the cell monolayers were washed (2X) with PBS followed by addition of serum-free media. After 18 h at 37 °C, the serum-free media were supplemented with various concentrations (0.1, 0.5 and 1 μM final concentration) of the small molecule DDR kinase inhibitor, DDR1-IN-1, which was purchased from Selleckchem (Catalog #S7498), Munich, Germany. Control cells received 0.125% final amount of DMSO, the vehicle used for making the stock solution of DDR1-IN-1 (in 100% DMSO). After 1-h incubation at 37 °C with DDR1-IN-1, the cells received 10μg/ml (final concentration) of rat-tail collagen type I (BD Biosciences Discovery Labware) diluted in acetic acid. Control cells received an equal volume of 20 mM acetic acid. After 2 h at 37 °C, the cells were washed with cold PBS (2X) and then lysed in RIPA buffer as described above. For detection of DDR1 phosphorylation, the lysates were then processed for western blot analyses using an antibody directed at Tyr513 of phosphorylated DDR1b, as described above.

For detection of DDR2 phosphorylation, lysates of C8161 cells (~400 μg per condition) were incubated (o/n at 4 °C) with 4 μg/ml of anti DDR2 polyclonal antibody (Catalog #AF2358, R&D Systems), followed by the addition of 40 μl of a 50% solution of Protein G agarose beads (Catalog #20398, Pierce). After a 1h-incubation at 4 °C with rotation, the mixtures were centrifuged at 1,000 rpm and the supernatants were collected. The precipitating beads were then washed (three times) with RIPA buffer and the captured immune complexes were released with 2X reducing Laemmli SDS-sample buffer. The samples were boiled and resolved by reducing 7.5% SDS-PAGE, followed by immunoblot analyses using anti-pDDR2 polyclonal antibody recognizing pTyr740 (#MAB25382, R&D Systems. After stripping, total (pulled-down) DDR2 was determined by reprobing the blots with anti-DDR2 polyclonal antibody (Catalog #12133S, CST).

***In vitro* and *ex vivo* proliferation assays**

In vitro cell proliferation assays were conducted with cells treated with or without DDR1-IN-1 inhibitor and in cells transfected with control or DDR1 siRNA. Briefly, cells were seeded in a 96-well plate at a density of 5000 cells/well in media supplemented with 1% FBS. After cell attachment,

DDR1-IN-1 inhibitor (0.1, 0.5 and 1 µM final concentration) dissolved in 1% FBS supplemented media were added to the cells (100 µl/well) in triplicates. Control cells received the same volume of media without inhibitor. In the case of DDR1 downregulation, the cells were transfected with scrambled control or DDR1 siRNA and then plated in 96-well plates, as described above. Cell proliferation was measured using the CellTiter-Glo® assay (Promega, France) 72 h after seeding, in the kinase inhibitor studies, or 24 h after seeding, when using cells transfected with siRNA, as described by the manufacturer. All conditions were evaluated in triplicate**s**.

For the *ex vivo* proliferation assays, we utilized tumor fragments derived from C8161 and SKMEL5 xenografts. Briefly, female 5-weeks-old nude/c mice (Janvier Labs, France) were injected subcutaneously with either 2 x10^6 C8161 or 4 x10^6 of SKMEL5 cells, per mice respectively. Mice were euthanized when their tumor reached a volume of approximately 1-1.5 cm^3, mice were euthanized and tumors were excised. Then, the tumors were cut into fragments and a representative tumor fragment was washed with PBS and sliced. Tumor sections were then placed on gelatin (Sigma-Aldrich, USA)-coated wells in 24-well plates, in quadruplicates. The tumor sections were incubated for 8 days in 250 µl of complete media at 37 °C supplemented with various concentrations (5, 10, and 15 µM final concentrations) of DDR1-IN-1 inhibitor dissolved in ethanol. As controls, some wells received an equivalent volume of ethanol. The medium was replenished every two days. At the end of the incubation period, cell proliferation was measured using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega, France), as described by the manufacturer.

Inhibitor treatment of melanoma xenografts

Animals were housed under controlled conditions of temperature, humidity and light cycle (12h/12h) and were maintained under pathogen free conditions and handled under stringent sterile conditions. Methods were performed in accordance with the guidelines and regulations of Directive 2010/63/EU and all protocols were approved by the Committee on the Ethics of Animal Experiments of the French Ministry of Agriculture (Permit Number: B75-10-2014). Female five-weeks old nude/c mice (Janvier Labs, France) were injected subcutaneously with either 2 ×10^6 C8161 (n=15) or with 4 ×10^6 SKMEL5 cells (n=15). When tumors were visible, approximately 5-7 days after tumor cell inoculation, mice were randomized and divided in three groups (n=5 each) for treatment with either vehicle or DDR1-IN-1 (5 or 7.5 mg/Kg per mouse). For these studies, stock solutions of DDR1-IN-1 (10 mM final concentration) were prepared by diluting the compound in warmed ethanol. Control mice received 5% ethanol in PBS (vehicle). Vehicle and DDR1-IN-1 were administered 5 days out of 7 via a peri-tumoral injection for a period of 16 days. Because the pharmacokinetics and bioavailability of DDR1-IN-1 has not been reported, we choose this method of inhibitor inoculation to ensure compound

availability within the tumor microenvironment. Tumor growth was measured independently by two technicians with a digital caliper and tumor volume was calculated using the formula: Length x Width^2/2. Mice were euthanized when their tumor reached a volume of ~1-1.5 cm^3 (after 16 and 18 days of treatment for C8161 and SKMEL5, respectively). Statistical analyses were performed using Prism software.

RESULTS

DDR1 expression in human melanoma tissues is associated with poor prognosis

We conducted an immunohistochemical study to evaluate the expression of DDR1 in normal skin and in a series of 52 surgically resected primary melanoma samples and 6 benign naevi (Figure 1). In normal skin, DDR1 displays a heterogeneous profile of expression (Figure 1A-D). Specifically, DDR1 was found to be weakly expressed in the cytoplasm of basal and suprabasal keratinocytes of the epidermis. However, DDR1 immunoreactivity was not detected in the granular and horny layers of the epidermis (Figure 1A). A weak staining of DDR1 was also detected in smooth muscle cells of the vessel's media (Figure 1B) and was moderately expressed by mature sebaceous cells in sebaceous glands, but not by hair follicle root sheets (Figure 1C). In contrast, secretory and excretory portions of eccrine sweat glands displayed strong positivity for DDR1 staining (Figure 1D). Consistent with DDR1 being mostly an epithelial protein, fibroblasts, endothelial cells, and adipocytes of the dermis showed no detectable DDR1 staining (Figure 1A).

Figure 1E-M shows the expression DDR1 in benign naevi and in melanoma tissues with various Breslow indexes. Benign naevi displayed no immunoreactivity with the DDR1 antibodies (Figure 1E and 1F). All *in situ* melanoma lesions and melanomas with <1 mm thickness had relatively low levels of DDR1. In contrast, 75% (9/12) of melanomas with a thickness (Breslow index) between 1 and 2 mm, exhibited a moderate expression of DDR1 with 17% (2/12) showing low expression and 8% (1/12) displaying stronger staining (Figure 1G and 1H). In these cases, DDR1 immunoreactivity was readily found in the cytoplasm and membrane of the tumor cells. On the other hand, the stroma and normal epidermis within those samples were negative for DDR1 immunoreactivity (Figure 1G and 1H). Fifty percent (5/10), 20% (2/10) and 30% (3/10) of melanomas with Breslow index between 2 and 4 mm displayed moderate, low, and high expression of DDR1, respectively (Figure 1I and 1J). In contrast, 70% (14/20) of melanomas with a Breslow index >4 mm, indicative of deeper tumor cell invasion, exhibited high DDR1 expression whereas 30% (6/20) had moderate expression (Figure 1K and 1L). Thus, expression of DDR1 in this patient cohort was correlated with the extent of melanoma invasion ($r = 0.47$ $p = 0.0004$) (Figure 2A). We also found that the intensity of DDR1 staining

significantly correlated with Clark level (r = 0.68 p <0.0001), ulceration (r = 0.48 p < 0.0001) and *BRAF* mutation status (r = 0.30 p = 0.03). Next, we evaluated the prognostic impact of DDR1 protein expression on all 52 melanoma patients using available clinico-pathological parameters (Table S1). Analyses of these data showed that DDR1 expression was also associated with melanoma subtype (p=0.006) and ulceration (p=0.0007). Indeed, the superficial spreading melanoma (SSM) subtype was often negative for DDR1 expression when compared to other melanoma subtypes, while nodular melanomas were strongly positive (8/15 tumors had a score of 2 and 6/15 tumors had a score of 1). In addition, 13/26 melanomas with ulceration scored 2, while 13/26 tumors without ulceration were negative. Moreover, DDR1 expression was associated with the locoregional recurrence of tumors (p=0.001). Indeed, tumors with no recurrence were often negative for DDR1 whereas those with locoregional recurrence all displayed a score of 1 or 2.

Our data also showed that DDR1 expression in primary melanomas also tended to be associated with *BRAF* (7/11 tumors scored 2 and 3/11 tumors scored 1) or *NRAS* mutated genotype (4/14 tumors scored 2 and 8/14 tumors scored 1). Univariate analysis showed significantly reduced overall survival in patients with high DDR1 expression in the tumor cells (p=0.011). Indeed, 50% (9/18) of melanomas from deceased patients displayed high DDR1 expression. Kaplan–Meier survival analyses showed that patients with moderate to high levels of DDR1 expression had a shorter survival time when compared to those with low DDR1 expression (Figure 2B), consistent with an association between DDR1 expression levels and poor disease outcome in melanoma patients. Although multivariate analyses of all clinical and biological variables revealed only ulceration as a significant parameter of survival, a stepwise multivariate regression analysis revealed that DDR1 effect was independent of and stronger than the effect of Breslow index, melanoma type, and *BRAF* and *NRAS* genotype. Hence, DDR1 could potentially be used as an additional prognostic factor to complement clinical parameters currently in use in routine practice.

DDR1 is required for *in vitro* melanoma cell migration, invasion, survival and proliferation

To investigate the roles of DDR1 in melanoma cells, we selected several human melanoma cell lines including, BLM and M10 harboring a $NRAS^{Q61R}$ mutation, C8161 with a $NRAS^{Q61k}$ mutation, and SKMEL5 and SKMEL28 displaying an activating BRAF mutation (V600E). The cell lines were analyzed for DDR1 and DDR2 expression by western blot analyses. As shown in Figure 3, under steady state conditions, DDR1 was readily detectable (Figure 3A), albeit at different levels, in SKMEL5, C8161, BLM and M10 cells but not in SKMEL28 cells. SKMEL5, C8161, and BLM cells, but not SKMEL28 cells, also expressed readily detectable levels of DDR2 (Figure 3B). In this study we set our focus on evaluating the role of DDR1 in various *in vitro* assays of cell proliferation,

migration and invasion. To this end, we used siRNA to downregulate the expression of DDR1 in SKMEL5, C8161, and M10 cells. Transient transfection of DDR1 siRNA significantly inhibited DDR1 mRNA expression and consequently resulted in a strong decrease of DDR1 protein levels when compared to cells transfected with scrambled control siRNA (Supplementary Figure 1). Next, the transfected cells were examined for their ability to migrate and invade through Matrigel-coated filters. These studies showed that knockdown of DDR1 expression inhibited the migration and invasion of M10, C8161 and SKMEL5 cells by 46% and 34%, 74% and 43%, and 33% and 82%, respectively, when compared to cells transfected with scrambled siRNA (Figure 4A and 4B). Downregulation of DDR1 expression in C8161, SKMEL5, and M10 cells by siRNA also reduced cell proliferation by 32%, 38% and 25%, respectively (Figure 4C), which was associated with increased apoptosis (90% for SKMEL5 cells, and 39% and 48% for M10 and C8161 cells, respectively), as determined by Caspase 3/7 activity (Figure 4D). Thus, under these conditions, DDR1 appears to be essential for cell survival.

Because DDR1 is highly expressed in melanoma tissues and downregulation of DDR1 reduced cell proliferation in these melanoma cell lines, we wished to test its role in melanoma tumor growth *in vivo*. To this end, we used the available DDR kinase inhibitor, DDR1-IN-1, a type II kinase inhibitor, which has been shown to inhibit both DDR1 and DDR2 activation with a reported IC_{50} of 105 and 413 nM, respectively (Kim et al., 2013). As shown in Figure 5A and 5B, treatment of SKMEL5 and C8161 cells with DDR1-IN-1 (0-1 μM) inhibited dose-dependently the phosphorylation of DDR1 in response to collagen I stimulation in both SKMEL5 (Figure 5A) and C8161 (Figure 5B) cells, as revealed with an antibody directed against phosphorylated Tyr513 of DDR1b. As expected, DDR1-IN-1 also reduced the collagen I-dependent phosphorylation of DDR2 in both C8161 (Figure 5C) and SKMEL5 (data not shown) cells, which is consistent with this inhibitor targeting both DDRs (Canning et al., 2014; Kim et al., 2013).

Next, we examined the effect of DDR1-IN-1 on melanoma cell proliferation *in vitro*. These studies showed that DDR1-IN-1 caused a significant decrease in cell proliferation, in a dose-dependent manner, both in *BRAF* mutated SKMEL5 cells and *NRAS* mutated M10 and C8161 cells (Figure 6A). Because normal melanocytes were shown to express DDR1 (Ricard et al., 2012), we treated Melan-A cells, a murine melanocyte cell line (Bennett, Cooper, & Hart, 1987) with DDR1-IN-1 and examined its effect on cell migration and proliferation. As shown in Supplementary Figure 2, treatment with DDR1-IN-1 inhibited the proliferation and migration of Melan-A cells, suggesting that DDR1 in implicated in normal melanocyte proliferation *in vitro*.

Next, we examined the effects of DDR1-IN-1 in an *ex vivo* histoculture drug response assay in which isolated fragments of human melanoma tumor xenografts are cultured to conduct studies of drug sensitivity (Delyon et al., 2016). Briefly, isolated fragments of C8161 and SKMEL5 tumor xenografts were cultured for 8 days in the presence of various concentrations of DDR1-IN-1 or vehicle control and then analyzed for cell proliferation. These studies showed that DDR1-IN-1 dose-dependently inhibited cell proliferation and at 15 μM it caused a 96 and 68%, inhibition of cell growth in C8161 and SKMEL5 tumor fragments respectively, when compared to the untreated fragments (Figure 6B). Collectively, these results suggest a role for DDR1 in melanoma proliferation, migration and invasion.

DDR1-IN-1 reduces melanoma tumor growth in xenograft models

To evaluate the therapeutic potential of DDR1-IN-1 in melanoma tumors, we generated xenografts of C8161 and SKMEL5 cells in mice. Mice with established tumors were then treated with DDR1-IN-1 for various times and size of tumors was evaluated as described in the Methods section. As shown in Figure 7, these studies demonstrated a significant inhibitory effect of DDR1-IN-1 in both C8161 and SKMEL5 xenografts with a mean reduction in tumor size at sacrifice of 72% and 73% in C8161 and SKMEL5 tumors, respectively, in mice receiving 5 mg/kg of DDR1-IN-1. At doses of 7.5 mg/Kg, DDR1-IN-1 caused a reduction in tumor size at sacrifice of 91% in C8161 tumors and of 79% in SKMEL5 tumors when compared to mice receiving vehicle. Reduction in tumor size was associated with a decrease in tumor cell proliferation as evaluated by Ki-67 immunostaining (Supplementary Figure 3). Thus, in these xenograft models, DDR1-IN-1 is a potent inhibitor of melanoma tumor growth. Collectively, these data suggest that DDRs are key players in regulation of melanoma malignant cell behavior and that targeting DDRs in human melanoma cells harboring BRAF or NRAS mutations may provide therapeutic value.

DISCUSSION

Accumulating evidence suggest that DDRs are major players in cancer progression in multiple cancer types (Jing, Song, & Zheng, 2018; Rammal et al., 2016; Valiathan et al., 2012). However, there is a paucity of information on the expression and roles of DDRs in melanoma (Cario, 2018). Here we focused mostly on DDR1, a member of the DDR family, and evaluated its expression in samples of human melanoma tissues, which, as far as we know has not been investigated. Analyses of DDR1 expression in our cohort of melanoma tissues by IHC demonstrated that higher levels of DDR1 were significantly associated with poor patient survival. Previous studies showed that DDR1 is expressed in the epidermis of normal skin (Ricard et al., 2012). In our samples, we also observed low DDR1 expression in normal skin, with a heterogeneous distribution. DDR1 expression was also low in

benign nevi but increased as a function of Breslow index, which represents the extent of melanoma invasion into the skin and is generally used as a prognostic factor in melanoma. Therefore, our data suggest that higher expression of DDR1 in melanoma cells is associated with the depth of tumor invasion, and consequently with poor patient survival. Analyses of the data in our patient samples revealed that high DDR1 expression significantly correlated with mutated *BRAF*, a frequent genotype in melanoma (Akbani et al., 2015). In the melanoma cell lines analyzed here, however, there was no association between DDR profile and expression of *NRAS* or *BRAF* mutations. Nevertheless, our data from the tumor xenografts showed that DDR1-IN-1, a kinase inhibitor with DDR1 selectivity (Kim et al., 2013), was highly effective in reducing tumor growth of SKMEL5 xenografts expressing the BRAF V600E–activating mutation. Although the molecular and functional relationship between mutated *BRAF* and DDR1 remains to be elucidated, this observation suggests that DDR1 may represent a new target in tumors that develop resistance to BRAF inhibitors. Indeed, resistance to BRAF inhibitors has been shown to involve, in part, reactivation of RTKs, leading to resistance (Manzano et al., 2016). Previous studies in breast cancer tissues implicated DDR1 as one of the RTKs that are induced upon resistance to MEK inhibition, and consistently, DDR1 silencing restored MEK inhibitor sensitivity (Duncan et al., 2012). Since both BRAF and RAS are upstream of MEK, it will be worth exploring whether DDR1 inhibition may restores sensitivity of BRAF inhibitor-resistant melanomas. Likewise, targeting DDR1 in *RAS* mutated melanomas is also worth exploring since this type of melanoma, which represents ~20% of melanoma genotypes (Jakob et al., 2012; Kwong et al., 2012), is still in need of effective treatments. Recent studies in KRAS-driven lung adenocarcinomas in transgenic mice demonstrated upregulation of DDR1, suggesting that this kinase is part of the network of genes activated by KRAS signaling in lung cancer. Importantly, in the mouse model, targeting DDR1 in high expressing DDR1 lung tumors produced the most effective anti-tumor effect (Ambrogio et al., 2016). Together, these findings and our observations in the melanoma tissues provide an impetus to test DDR1 as a target in melanomas with mutated RAS oncogenes.

While our study focused on DDR1, it is important to note that the melanoma cell lines used here also express DDR2 and that the inhibitor DDR1-IN-1 also displays inhibitory activity against this receptor (Kim et al., 2013). *In vivo* at concentrations above the IC_{50}, DDR1-IN-1 is likely to target both DDRs. Therefore, at this junction, we cannot assert whether the inhibitory effect of DDR1-IN-1 in the melanoma xenografts was due to inhibition of both DDRs or a single receptor. In regards to DDR2, currently, there is a significant lack of data on the expression of DDR2 in melanoma tissues and only a few studies on the role of melanoma-expressed DDR2 in tumor cell behavior have been published (Cario, 2018). Moreover, the role of tumor-derived DDR2 in experimental metastases of melanoma cells remains unclear (Badiola et al., 2011; Tu et al., 2019). However, stromal DDR2 has been shown to support the establishment of experimental lung metastases of melanoma cells in mice (Zhang et al.,

2014). Thus, at present, a role for stromal DDR2 in mediating some of the inhibitory effects of DDR1-IN-1 in our mice xenograft studies cannot be ruled out. More studies are warranted to define the role of DDR2 in our experimental models of melanoma. Regardless, our xenograft studies with DDR1-IN-1 in two melanoma cell lines, supports the notion that targeting both receptors may elicit potent anti-tumor activity in melanoma. The importance of DDR1, in particular, as a therapeutic target in melanoma is supported by the following observations: (i) DDR1 is highly expressed in melanoma cells of human tumors, and its expression is associated with the extent of tumor invasiveness and poor patient survival, and (ii) DDR1 plays a key role in melanoma cell proliferation, migration, and invasion. Together, these findings provide a strong rational for targeting DDR1 in melanoma. Consistent with this proposition, we have also found that downregulation of DDR1 expression in melanoma cells was associated with a reduced AKT and ERK activation (data not shown), suggesting that DDR1 regulates these signaling pathways in melanoma cells. It is worth mentioning that both the AKT and ERK pathways are the most frequently activated pathways in human melanomas (Crowell, Steele, & Fay, 2007), and both pathways are implicated in melanoma initiation and resistance to targeted therapies (Davies, 2012; Dhomen & Marais, 2009). Thus, therapies targeting these pathways in combination with DDR1 inhibitors may improve therapeutic outcomes. However, considering that DDR1 is also expressed in normal epidermis, it is important to note that future studies in humans with selective DDR1 inhibitors will need to be aware of potential adverse dermatological effects. In summary, our study provides novel information on the expression of DDR1 in human melanoma and uncovers a key role for DDR1 in the promotion of malignant properties in human melanoma cell lines with both BRAF and RAS activating mutations. Collectively, these results provide a strong rationale for additional studies with selective DDR1 inhibitors for the treatment of melanoma.

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Figures legends

Figure 1. DDR1 expression in human normal skin and in melanoma. (A-D**)** Expression of DDR1 in normal skin (200X magnification). **(**E-L**)** DDR1 expression in cutaneous melanocytic lesions: benign common nevus with no DDR1 expression (E, 100X magnification; F, 400X magnification); superficial spreading melanoma (SSM) with less than 2 mm Breslow index showing low DDR1 expression (G, 100X magnification; H, 400X magnification); SSM of 3 mm Breslow index showing intermediate DDR1 expression (I, 100X magnification; J, 400X magnification); SSM of >4 mm Breslow index with strong DDR1 expression (K, 100X magnification; L, 400X magnification).

Figure 2. Association of DDR1 expression with invasive depth and patient survival. (A**)** DDR1 expression in melanoma as a function of Breslow index. The intensity of staining was scored as high, moderate and low. **(**B**)** Kaplan-Meier survival curve for overall survival according to DDR1 expression. DDR1 protein expression is significantly associated with shorter tumor-specific survival in melanoma. Log rank p=0.011. Green line: low DDR1 expression; black line: moderate DDR1 expression; red line: high DDR1 expression.

Figure 3. Expression of DDR1 and DDR2 in melanoma cell lines. Melanoma cells were lysed in RIPA buffer and equal protein concentrations were resolved by reducing 7.5% SDS-PAGE followed by western blot analyses using antibodies to DDR1 (A) or DDR2 (B), as described in the Materials and Methods section β-actin was used as loading control.

Figure 4. DDR1 modulates malignant properties of melanoma cells. (A,B). M10, C8161 and SKMEL5 melanoma cells were transfected with scrambled (control) or DDR1 siRNA and evaluated for effects on cell migration (A) and invasion (B). After a 24-h incubation, the cells in the lower side of the filters were fixed, stained with crystal violet, and counted under a microscope. Columns indicate means of three independent experiments carried out in triplicate; and bars, SD; *$p < 0.05$, ** $p<0.005$, *** $p<0.0001$. (C) M10, C8161 and SKMEL5 melanoma cells transfected with control or DDR1 siRNA were seeded onto 96-well plates for 24 h. Cell proliferation was then determined using the CellTiter 96® MTT assay. Results are presented as the mean of three independent experiments carried out in triplicate; bars, ± SD. * $p<0.05$, ** $p<0.005$, *** $p<0.0001$. (D) M10, C8161 and SKMEL5 cells transfected with control or DDR1 siRNA were seeded onto 96-well plates for 24 h. Apo-one homogeneous Caspase 3/7 buffer containing Z-DEVD-R110 substrate was then added to the

cells and incubated at room temperature for 4 h. Caspase-3/7 activity was measured using a spectrofluorometer. The data represent means of at least three different experiments; bars, ± SD. * p<0.05, ** p<0.005, *** p<0.0001.

Figure 5. Collagen-dependent activation of DDRs in SKMEL5 and C8161 melanoma cell lines and inhibition by DDR1-IN-1. Serum-starved SKMEL5 (A) and C8161 (B,C) cells were treated (1 h, 37 °C) without (vehicle) or with various concentrations (0.1, 0.5 and 1 μM final concentration) of DDR1-IN-1, followed by addition of 10 μg/ml (final concentration) of rat-tail collagen type I diluted in acetic acid. Control cells received an equal volume of 20 mM acetic acid. After 2 h at 37 °C, the cells were processed for western blot analyses of total and phosphorylated DDR1 (A,B) or for DDR2 immunoprecipitation followed by western blot analyses of total and phosphorylated DDR2 (C) using specific antibodies, as described in Materials and Methods. β-actin was used as loading control in A and B.

Figure 6. Effect of DDR inhibition on melanoma cell proliferation. (A) *In vitro* cell proliferation: C8161, SKMEL5 and M10 melanoma cells were treated with vehicle control or DDR1 inhibitor DDR1-IN-1 (0.1, 0.5, 1 or 5 μM) for 72 h. Cell proliferation was then determined using the CellTiter 96® MTT assay. Results are presented as the mean of three independent experiments carried out in triplicate; bars; SD. *, p<0.05, ** p<0.005, *** p<0.0001. **(**B**)** *Ex vivo* cell proliferation: DDR1-IN-1 (5, 10 and 15 μM) or vehicle control were added to histocultures of C8161 and SKMEL5 tumors for 8 days. Cell proliferation was then determined using the CellTiter 96® MTT assay. Results are presented as the mean of three independent experiments carried out in triplicate; bars; SD. Ns, Not significant, *, p<0.05, ** p<0.005.

Figure 7. DDRI-IN-1 inhibitor reduces tumor growth of C8161 and SKMEL5 xenografts. (A) Mice inoculated with C8161 or SKMEL5 cells were treated 5 days out of 7 with peri-tumoral injections of 5 or 7.5 mg/kg of DDR1-IN-1 or with vehicle control (5% of ethanol in saline). Results represent two independent experiments with 5 mice in each treatment group. Columns, means of tumor volume; bars; SD. *, p<0.05, ** p<0.005, *** p<0.0001. (B) Representative images of mice harboring C8161 tumors, after 16 days of treatment.

Suppl. Figure 1. siRNA transfection efficacy in melanoma cell lines. Upper panel: qRT-PCR of DDR1 expression using *β2 microglobulin (B2M),* as a reference. Results represent mean ± SD; *, p <0.05. Lower panel: Western blot analyses of DDR1 expression in control- and DDR1-siRNA melanoma transfected cells (M10, C8161 and SKMEL5 cells). Equal loading of proteins was assessed by probing for β-actin.

Suppl. Figure 2. Effect of DDR1-IN-1 inhibitor on Melan-A melanocytes. (A) *In vitro* cell proliferation: Melan-A normal melanocytes were treated with vehicle control or DDR1-IN-1 (0.1, 0.5, or 1 μM) for 72 h (left). (B) *In vitro* cell migration: Melan-A normal melanocytes were treated with vehicle control or DDR1 inhibitor DDR1-IN-1 (0.1, 0.5 or 1 μM). After a 24-h incubation, the cells in the lower side of the filters were fixed, stained with crystal violet, and counted under a microscope. Columns indicate means of three independent experiments carried out in triplicate; and bars, SD *p < 0.05 (right)

Suppl. Figure 3. Effect of DDR1-IN-1 inhibitor on Ki-67 expression. (A) Representative photographs of Ki-67 staining in tissue sections from C8161 and SKMEL5 melanoma xenografts untreated (control) or treated with DDR1-IN-1 inhibitor. (B) Quantitation of Ki-67 nuclear immunostaining in untreated and treated (7.5 mg/kg dose) C8161 and SKMEL5 melanoma xenografts.

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Figure 1

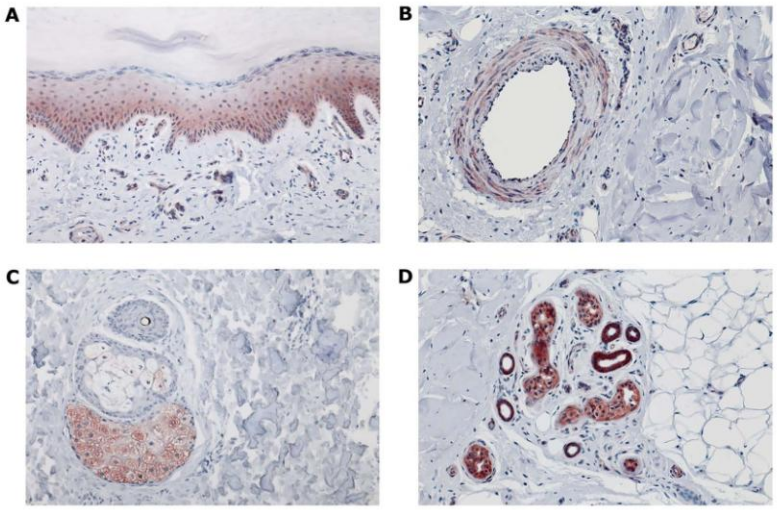

Figure 1

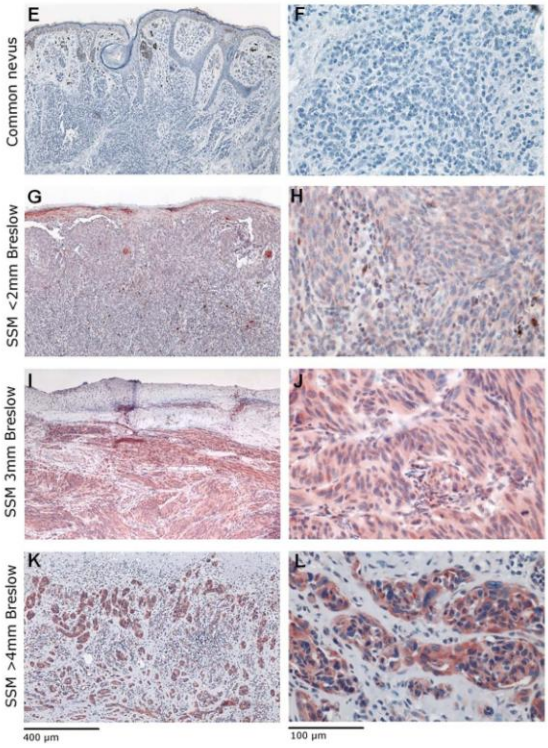

Figure 2

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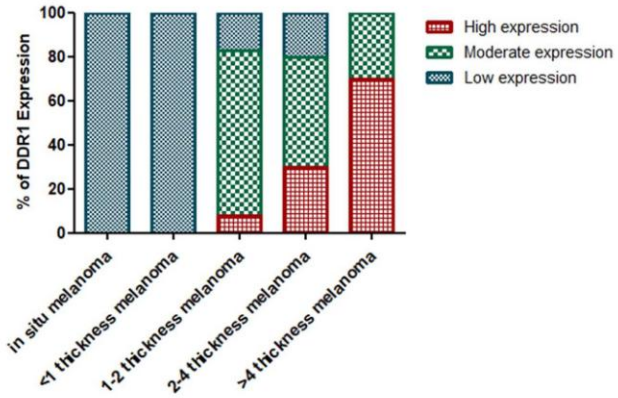

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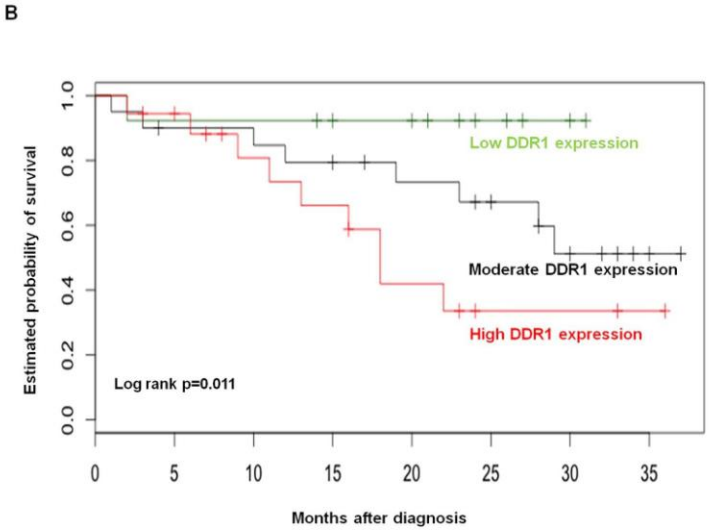

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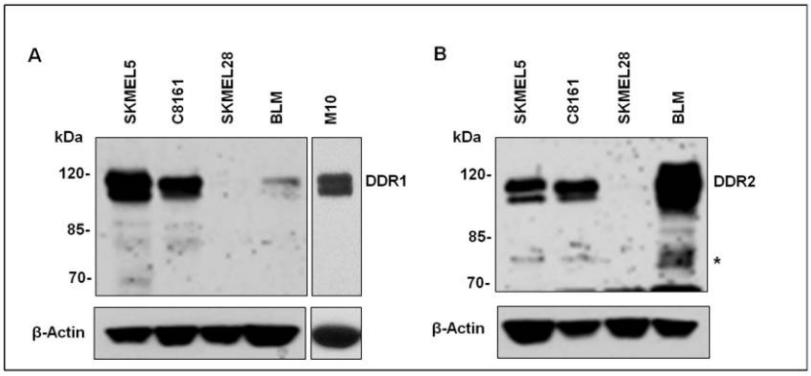

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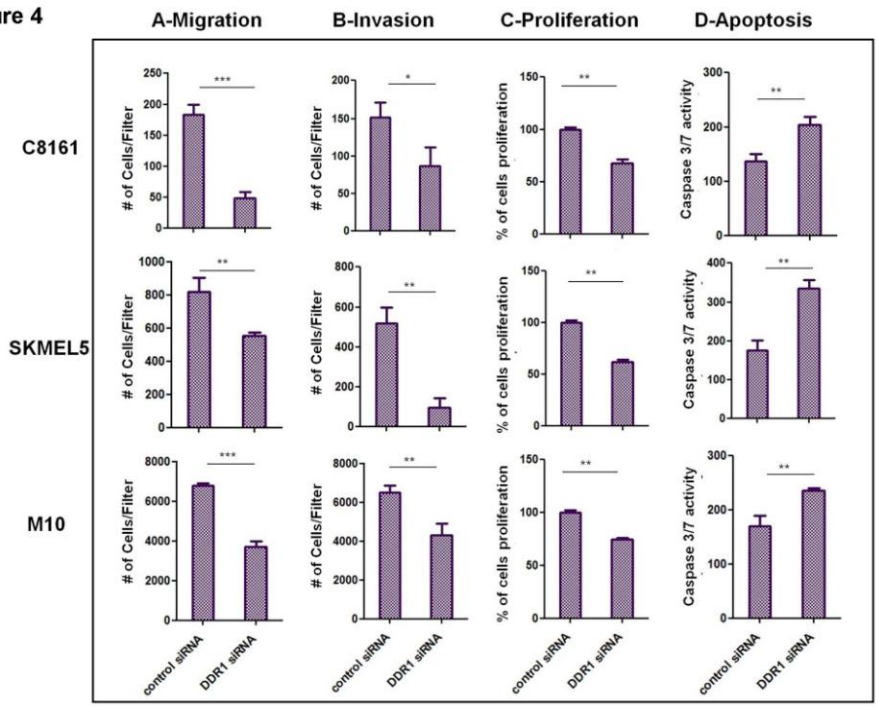

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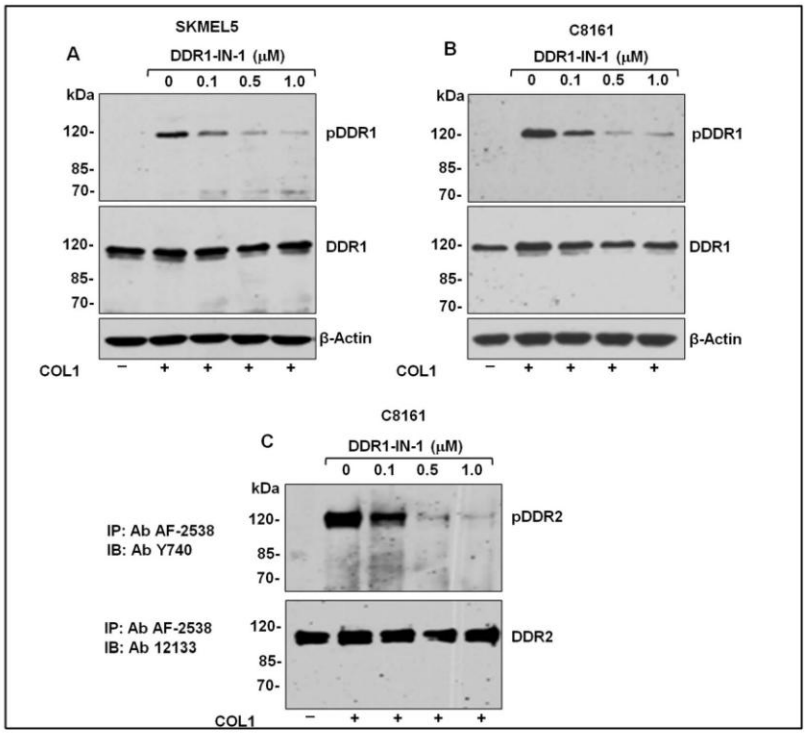

Figure 6

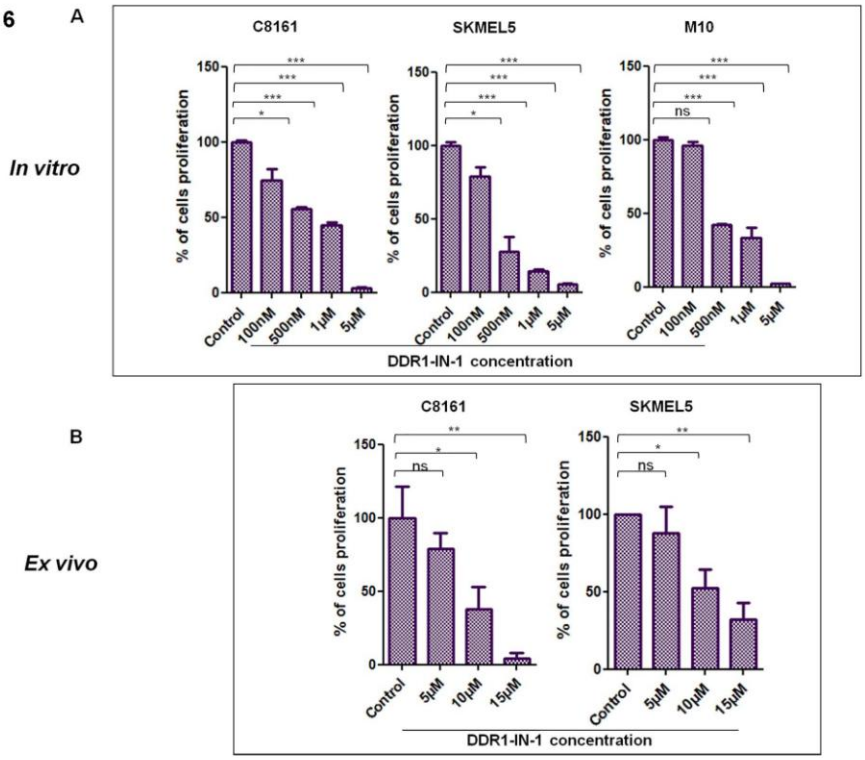

Figure 7