

Biology Contribution

In Vitro Evaluation of Notch Inhibition to Enhance Efficacy of Radiation Therapy in Melanoma



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Abstract

Purpose: The scope of radiation therapy is limited in melanoma. Using in vitro melanoma models, we investigated a Notch signaling inhibitor as a radiosensitizer to explore its potential to improve the efficacy of radiation therapy to widen the clinical application of radiation therapy in melanoma.

Methods and Materials: Melanoma cell lines A375, SKMEL28, and G361 were grown using standard tissue culture methods. Radiation was delivered with a clinical x-ray unit, and a gamma secretase inhibitor RO4929097 was used to inhibit Notch signaling. Cell viability signal was used to calculate Loewe's combination index to assess the interaction between radiation and RO4929097 and also the effect of scheduling of radiation and RO4929097 on synergy. Clonogenic assays were used to assess the clonogenic potential. An in vitro 3-dimensional culture model, γ -H2AX, and notch intracellular domain assays were used to interrogate potential underlying biological mechanisms of this approach. Scratch and transwell migration assays were used to assess cell migration.

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Results: A375 and SKMEL28 cell lines showed consistent synergy for most single radiation doses examined, with a tendency for better synergy with the radiation-first schedule (irradiation performed 24 hours before RO4929097 exposure). Clonogenic assays showed dose-dependent reduction in colony numbers. Both radiation and RO4929097 reduced the size of melanospheres grown in 3-dimensional culture in vitro, where RO4929097 demonstrated a significant effect on the size of A375 and SKMEL28 melanospheres, indicating potential modulation of stem cell phenotype. Radiation induced γ -H2AX foci signal levels were reduced after exposure to RO4929097 with a tendency toward reduction in notch intracellular domain levels for all 3 cell lines. RO4929097 impaired both de novo and radiation-enhanced cell migration.

Conclusions: We demonstrate Notch signaling inhibition with RO4929097 as a promising strategy to potentially improve the efficacy of radiation therapy in melanoma. This strategy warrants further validation in vivo.

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Introduction

Despite advances in systemic treatment, with biological agents and immune check point inhibitors playing a key role in the management of melanoma, radiation therapy is still used as a definitive treatment for cutaneous melanoma for patients unfit for surgery and in *lentigo maligna*, as an adjuvant treatment, to alleviate symptoms and increasingly as a life prolonging treatment in the form of stereotactic radiation therapy.¹ Melanoma is traditionally viewed as a radioresistant tumor, which may explain the limited role of radiation therapy in melanoma compared with many other tumors. However, improving radiosensitivity may widen the clinical utility of this important therapeutic modality in melanoma. Combining radiation with immune modulators could help with this,² and there are other alternative strategies to achieve this goal.

Notch signaling is an embryonically conserved developmental cell signaling pathway that has 4 transmembrane cell surface receptors (Notch 1-4) and 5 associated ligands (Jag 1 & 2 and Delta-like ligands, DLL1, 3, and 4). After ligand-receptor interaction, a cascade of molecular events results in the transcription of several target Notch genes by notch intracellular domain (NICD) that are critical for homeostasis of cells and organogenesis.^{3,4} Although this pathway has been known for more than a century, it has recently gained more attention because of its role in carcinogenesis but more importantly due to the opportunity to develop Notch inhibition as a cancer treatment strategy.^{5,6}

Notch signaling is active in melanoma, driving carcinogenesis and influencing its molecular biology.⁷ It also plays a crucial part in the migration of melanoblasts and melanocytes. Notch signaling is also involved in the transformation of melanocytes, and there is accumulating evidence supporting this signaling as a molecular driver⁸ and for promoting an aggressive phenotype in melanoma.⁹

There is a strong biological rationale for combining Notch inhibition with radiation.¹⁰ Deregulation of Notch signaling can lead to treatment resistance,⁴ and therefore

its inhibition can potentially help to overcome cellular resistance to radiation therapy. The role of Notch signaling in epithelial mesenchymal transition (EMT)¹¹ makes it an attractive target to minimize the risk of development of distant metastases.

We hypothesized Notch inhibition as a potential radiosensitizer in melanoma. Using in vitro melanoma models we examined the effects of combining Notch inhibition with radiation on radiosensitivity and clonogenic potential, explored potential biological mechanisms, and studied the effects on melanoma cell migration.

Methods and Materials

Cell lines and cell culture

Melanoma cell lines A375 (American Type Culture Collection, Manassas), SKMEL28, and G361 (Sigma-Aldrich, Dorset, UK) were grown using standard tissue culture methods. Periodic mycoplasma testing ensured no infected cell lines were used for the experimental work.

Irradiation technique

A 250 KV (2.7 mm copper filter) x-ray beam of a clinical x-ray generator (Gulmay; Xstrahl) was used for cell irradiation. Multiwell tissue culture plates were placed over a solid epoxy resin water equivalent phantom. An appropriate size applicator was used to deliver a range of radiation doses, 1, 2, 4, 6, 8, and 16 Gy, as required by the experimental design using appropriate monitor unit settings. Monitor units were accurately calculated using the radiation output factor and applying the inverse square law correction to account for the distance between the x-ray applicator and the surface of the media in the multiwell tissue culture plates.

Inhibition of Notch signaling

Notch signaling is a complex and diverse pathway¹² that can be targeted by various agents. The gamma

secretase cleavage releases NICD from the transmembrane receptor complex. RO4929097 (Selleck Chemicals) is a gamma secretase inhibitor that inhibits the gamma secretase cleavage, therefore the release of NICD. RO4929097 has been used in several tumors including melanoma to successfully inhibit Notch signaling,^{13,14} and this compound was used in this work.

Cell viability assay

For synergy experiments, A375 and SKMEL28 cell lines were irradiated to 1, 2, 4, 6, 8, and 16 Gy 1 hour after they were exposed to 1, 3, 10, 30, and 100 μM RO4929097 in 96-well tissue culture plates. Additionally, to study the potential effect of radiation and RO4929097 scheduling on their interaction, scheduling experiments were designed where cells were irradiated either 24 hours before or after they were exposed to RO4929097, with the same combination of radiation and RO4929097 as described above.

For both synergy and scheduling experiments, cells were grown for a further 5 to 7 days to achieve 80% to 90% confluence. Cell viability signal was assessed with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium colorimetric cell viability assay (CellTiter 96 AQueous One; Promega, Madison, WI). Cell viability signal at 490 nm was calculated using a colorimetric plate reader (VARIOSSKAN FLASH; Thermo Scientific) and analytical software (SkanIt Software 2.4.5). Loewe's combination index (CI) values were calculated.¹⁵

Combination index (CI) calculation

The Loewe's CI was calculated using equation (1).

$$CI = \frac{C_{A,X}}{IC_{X,A}} + \frac{C_{B,X}}{IC_{X,B}} \quad (1)$$

Where $C_{A,X}$ and $C_{B,X}$ denote concentrations of drug and radiation doses, respectively, used in combination to achieve effect X . $IC_{X,A}$ and $IC_{X,B}$ represent concentrations of drug and radiation doses, respectively, when they are used alone to achieve the same effect X . CI of <1 or >1 indicates synergy or antagonism, respectively. Normalized cell viability signal was calculated for each radiation and RO4929097 combination. The IC_{50} for RO4929097 was obtained first, followed by $\log IC_{50}$ using nonlinear regression modeling. The same procedure was repeated for radiation to calculate CI values for each radiation and RO4929097 combination. Microsoft Excel and GraphPad Prism were used for this calculation.

Clonogenic assay

The cells were seeded in 6-well tissue culture plates and allowed to settle. Cells were then exposed to multiple combinations of 1, 3, 10, and 30 μM RO4929097 and 1, 2, 4, 8 Gy radiation (cells were irradiated soon after drug exposure), then allowed to grow to form colonies that were monitored frequently with light field microscopy. A group of 50 cells was defined as a colony, which was determined with light field microscopy. Clonogenic assays¹⁶ were performed and assay plates were scanned with ChemiDoc-It2 imager (UVP, CA). Colonies were counted using ImageJ software.

3-dimensional cell culture

The cells were exposed to 0, 1, 3, 10, and 30 μM RO4929097 and irradiated to 2 and 4 Gy, then allowed to grow in ultralow attachment T75 culture flasks (Corning; Sigma-Aldrich) to form melanospheres. Melanospheres were grown in Dulbecco's modified Eagle medium/F12 medium (Sigma-Aldrich), supplemented with 10 ng/mL b-fibroblast growth factor (Sigma-Aldrich), 2% B27 supplement (Invitrogen, ThermoFisher), 20 ng/mL epidermal growth factor (Sigma-Aldrich),¹⁷ and 1% penicillin/streptomycin. Once formed, melanospheres were imaged using an inverted microscope (Nikon ECLIPSE TE 2000-S) and melanosphere sizes were measured from 3 randomly selected light field microscope images using the ImageJ software and compared.

γ -H2AX assay

The cells were exposed to 1, 3, 10, 30, and 100 μM RO4929097 and irradiated to 2 and 8 Gy in 96-well tissue culture plates. γ -H2AX assays were done after 1 hour, which was determined as the optimal time point based on preliminary experiments. A quantitative method, In-Cell Western assay (LI-COR Biotechnology) was used. Phosphorylated γ -H2AX foci were taken as a surrogate marker for the function of the DNA damage repair (DDR) pathway. After irradiation and exposure to RO4929097, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were stained for phosphorylated γ -H2AX foci with phosphohistone γ -H2AX (Ser 139) rabbit McAb primary antibody (Cell Signalling Technology, MA) and goat anti-rabbit IRDye secondary antibody (LI-COR Biotechnology). Nuclear and cytoplasmic cell staining was performed with Cell-Tag700 (LI-COR Biotechnology) for the normalization of phosphorylated γ -H2AX foci to the number of cells within each well. The plates were imaged with Odyssey CLx Imager (LI-COR Biotechnology), and the results were analyzed with Image Studio software (LI-COR Biotechnology).

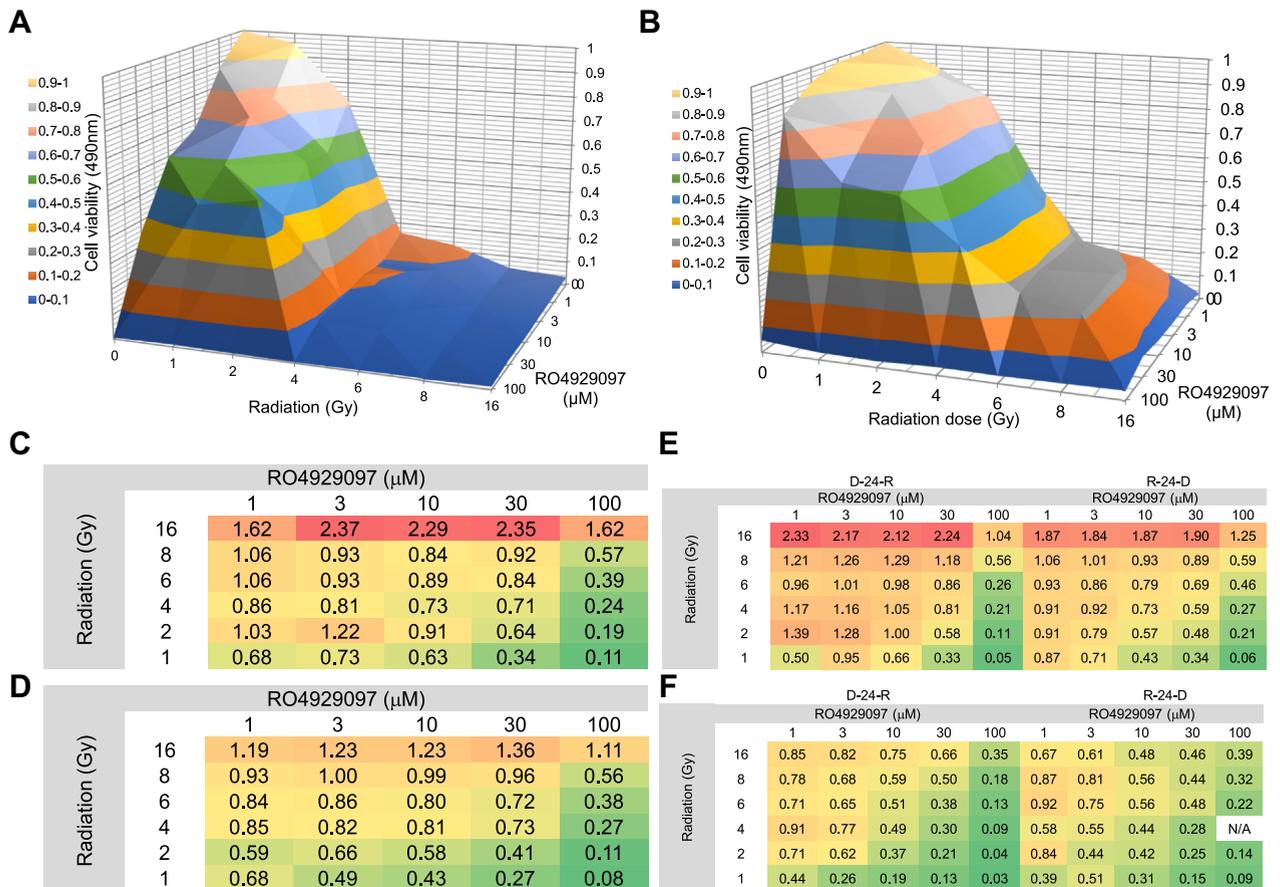


Figure 1 (A) Representative radiation and RO4929097 dose response surface map for A375 cell line; radiation $IC_{50} = 2.5$ Gy and RO4929097 $IC_{50} = 10.8$ μM. (B) Representative radiation and RO4929097 dose response surface map for SKMEL28 cell line; radiation $IC_{50} = 3.4$ Gy and RO4929097 $IC_{50} = 46.97$ μM. (C) A375 synergy: Loewe's combination index (CI) values. (D) SKMEL28 synergy: Loewe's CI values. (E) Loewe's CI values of A375 schedule experiments. (F) Loewe's CI values of SKMEL28 schedule experiments. Loewe's CI values are shown in colour grading with CI <1 indicating synergism and >1 indicating antagonism. Experimental schedules: D-24-R, drug treatment 24 hours prior to radiation; R-24-D, radiation 24 hours prior to drug treatment. Abbreviations: D = RO4929097; N/A = data not available; R = radiation. (A color version of this figure is available at <https://doi.org/10.1016/j.adro.2020.11.007>.)

NICD assay

The A375 cell line was exposed to various radiation doses and RO4929097 concentrations, and the NICD assay was performed. The same technique used for the γ-H2AX assay was applied except for the anti-Notch1 intracellular domain antibody (ab83232, Abcam plc), which was used as the primary antibody.

Scratch assay

A375 and SKMEL28 cell lines grown in 6-well tissue culture plates were exposed to 10 and 100 μM RO4929097 and irradiated to 2 and 8 Gy. A scratch was made at the bottom of each well once cells reached appropriate confluence and daily light field microscope images were taken to assess cell migration into the space created by the scratch.

Transwell migration assay

A375, SKMEL28, and G361 cell suspension (100 μL of 1×10^6 /mL) were allowed to settle onto the membrane of transwell inserts (Corning Costar Transwell) placed in 24-well tissue culture plates. The cells were exposed to 10 and 100 μM RO4929097 and irradiated to 2 and 8 Gy and allowed to migrate through the membranes. Migrated cells attached to the under surface of the membranes were fixed with 70% ethanol. The transwell inserts containing the membranes were allowed to dry for 10 to 15 minutes and membranes were stained with 0.2% crystal violet (Sigma-Aldrich). Cells were viewed using an inverted microscope and counted in different fields of view to get an average sum of cells that had migrated through and attached on the underside of the membrane.

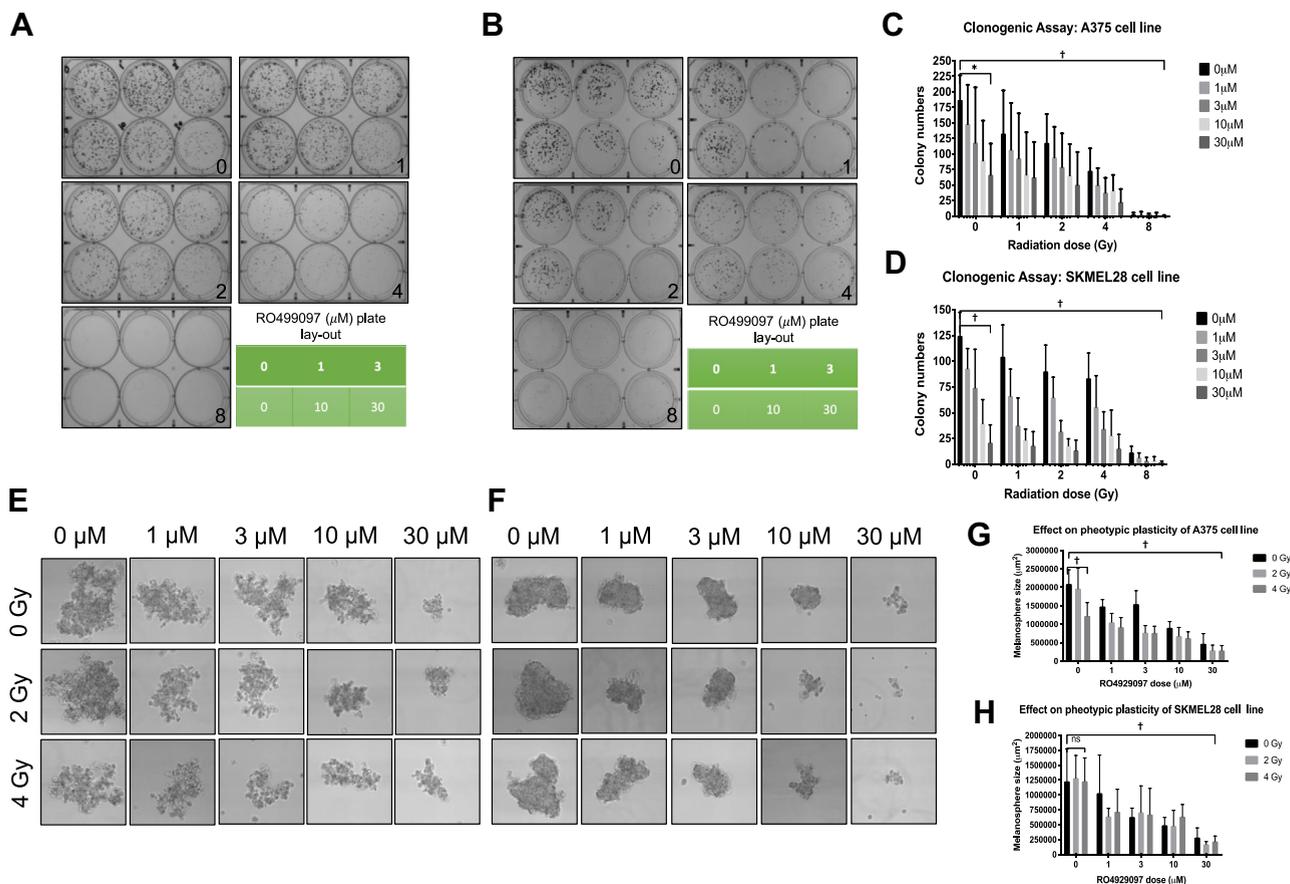


Figure 2 Representative examples of (A) A375 and (B) SKMEL28 cell line clonogenic assays. Radiation delivered to whole 6 multiwell tissue culture plates (number in each well indicates radiation dose delivered to cells in that particular well). RO4929097 treatment scheme is given in the accompanying table. (C) A375 and (D) SKMEL28 clonogenic assay results showing significant reduction in colony numbers for both radiation and RO4929097. Results shown are average from 4 independent biological experiments. Error bars denote standard deviation. * $P = .0015$; † $P < .0001$. (E) A375 and (F) SKMEL28 melanospheres following exposure to various combinations of radiation doses and RO4929097 concentrations. Representative light microscope images at 20 \times magnification are shown. Evaluation of (G) A375 and (H) SKMEL28 melanosphere sizes. Results shown are average from 2 biologically independent experiments. Error bars indicate standard deviation. † $P < .0001$. *Abbreviation:* ns = not significant.

Results

Evaluation of radiation and RO4929097 dose response, synergy, and effect of scheduling on synergy and clonogenic potential

Dose response surface maps are shown [Figure 1A,B](#). For the A375 cell line, radiation IC_{50} was 2.5 Gy and RO4929097 IC_{50} was 10.8 μM . The SKMEL28 cell line showed a more resistant phenotype to both radiation and RO4929097, with an IC_{50} of 3.4 Gy and 46.97 μM , respectively.

The Loewe's CI results are shown in [Figure 1C,D](#). CI analysis reproducibly showed strong synergy for both A375 and SKMEL28 cell lines when radiation doses of 1, 2, 4, 6, and 8 Gy were combined with 100 μM of

RO4929097. A trend toward mild synergy was observed with lower doses of radiation and higher doses of RO4929097. However, with 16 Gy, an antagonistic effect was seen with all concentrations of RO4929097.

CI results for the scheduling experiments are shown in [Figure 1E,F](#). For the A375 cell line, radiation and RO4929097 scheduling experiments showed a similar pattern of interaction regardless of the scheduling used. A high antagonistic effect was observed when cells were irradiated to 16 Gy. High synergy levels were seen with low radiation and high RO4929097 doses. With the SKMEL28 cell line, a different pattern of interaction was seen. No antagonistic effect was observed with any radiation-drug combination. Once again, the strongest synergy was seen with low radiation doses and high RO4929097 concentrations. Although there was a tendency favoring the radiation-first schedule for better

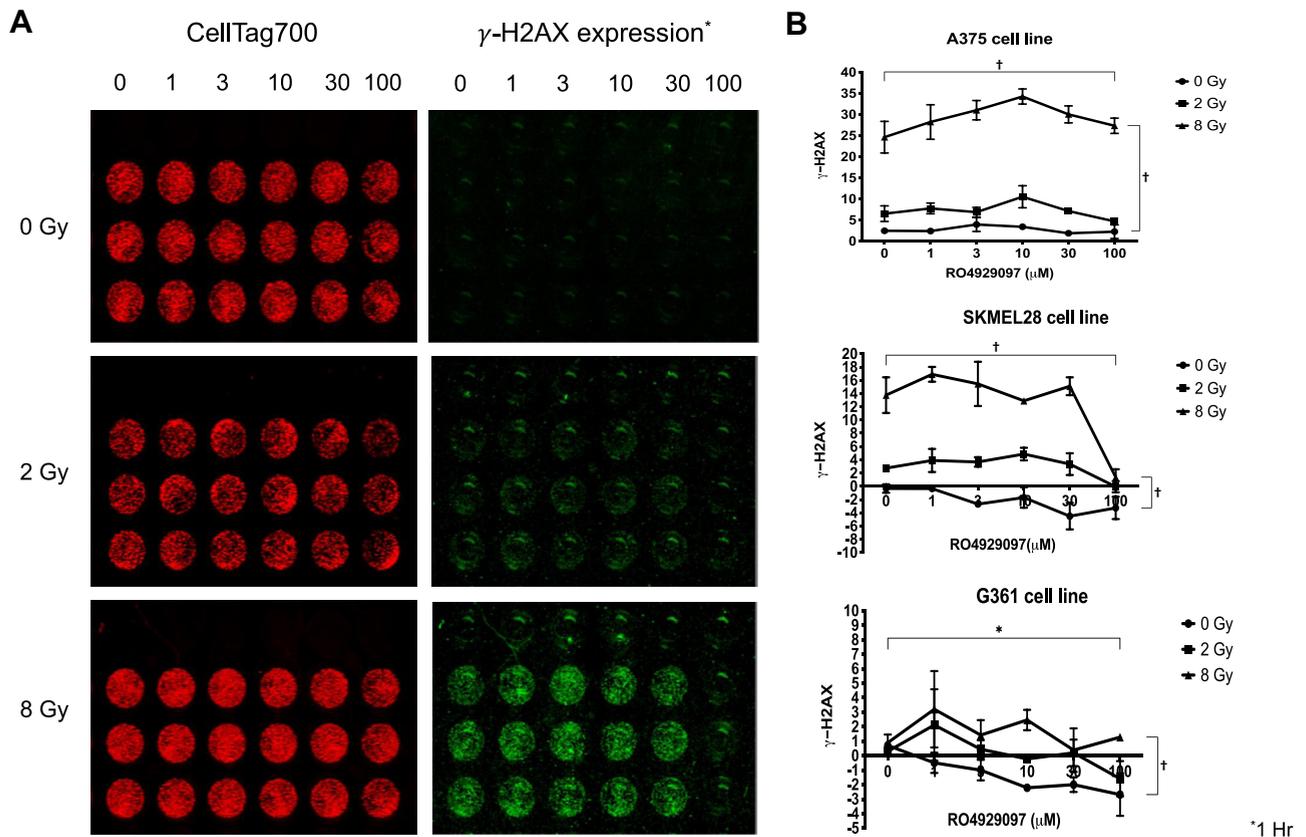


Figure 3 (A) Representative example of an SKMEL28 cell line γ -H2AX assay. Cells were treated with various combinations of radiation doses and RO4929097 concentrations. CellTag700 stain was used to stain cells (nucleus and cytoplasm), shown in red. γ -H2AX foci, normalised to cell number within each well are shown in green. (B) Results of γ -H2AX assay. Negative values indicate background signal higher than measured γ -H2AX. Error bars indicate standard deviation. * $P = .0008$; † $P < .0001$. (A color version of this figure is available at <https://doi.org/10.1016/j.adro.2020.11.007>.)

synergy, this did not reach statistical significance ($P = .06$, general linear model, univariate analysis, IBM SPSS v23).

As shown in Figure 2A-D, a radiation dose-dependent reduction in colony numbers was found for A375 cell line colonies ($P < .0001$, 2-way analysis of variance [ANOVA]), and similar effect was seen with increasing concentrations of RO4929097 ($P = .0015$, 2-way ANOVA). Similarly, for the SKMEL28 cell line, both radiation and RO4929097 reduced colony numbers in a dose-dependent fashion ($P < .0001$ for both radiation and RO4929097).

Evaluation of phenotypic plasticity

As shown in Figure 2E-H, for both A375 and SKMEL28 cell lines, the ability to form melanospheres was impaired following irradiation and also after exposure to RO4929097, as evidenced by significantly smaller melanosphere sizes for A375 cells ($P < .0001$ for both effects) and for SKMEL28 cells ($P = .77$ for radiation effect and $P < .0001$ for RO4929097 effect, 2-way ANOVA).

Evaluation of effect on DDR pathway and inhibition of Notch signaling

γ -H2AX assay results are shown in Figure 3. A375 cell line experiments demonstrated that radiation-induced γ -H2AX foci signal levels were reduced with higher concentrations of RO4929097 ($P < .0001$, 2-way ANOVA), although there was an increase in the γ -H2AX foci signal level after 10 μ M RO4929097 at 8 Gy. Further γ -H2AX evaluation using SKMEL28 and G361 cell lines showed similar results. For the SKMEL cell line, there was a significant reduction in the γ -H2AX foci signal level following exposure to 100 μ M RO4929097, particularly for 8 Gy ($P < .0001$, 2-way ANOVA). For the G361 cell line, there was significant reduction in γ -H2AX foci signal level, particularly with 2 Gy ($P = .0008$, 2-way ANOVA).

There was a reduction in NICD levels following exposure to RO4929097 for the A375 cell line, as shown in Table E1. Similar results were observed after radiation for the SKMEL28 and G361 cell lines; however, there was some degree of rebound in NICD levels following higher doses of RO4929097, as shown in Figure 4.

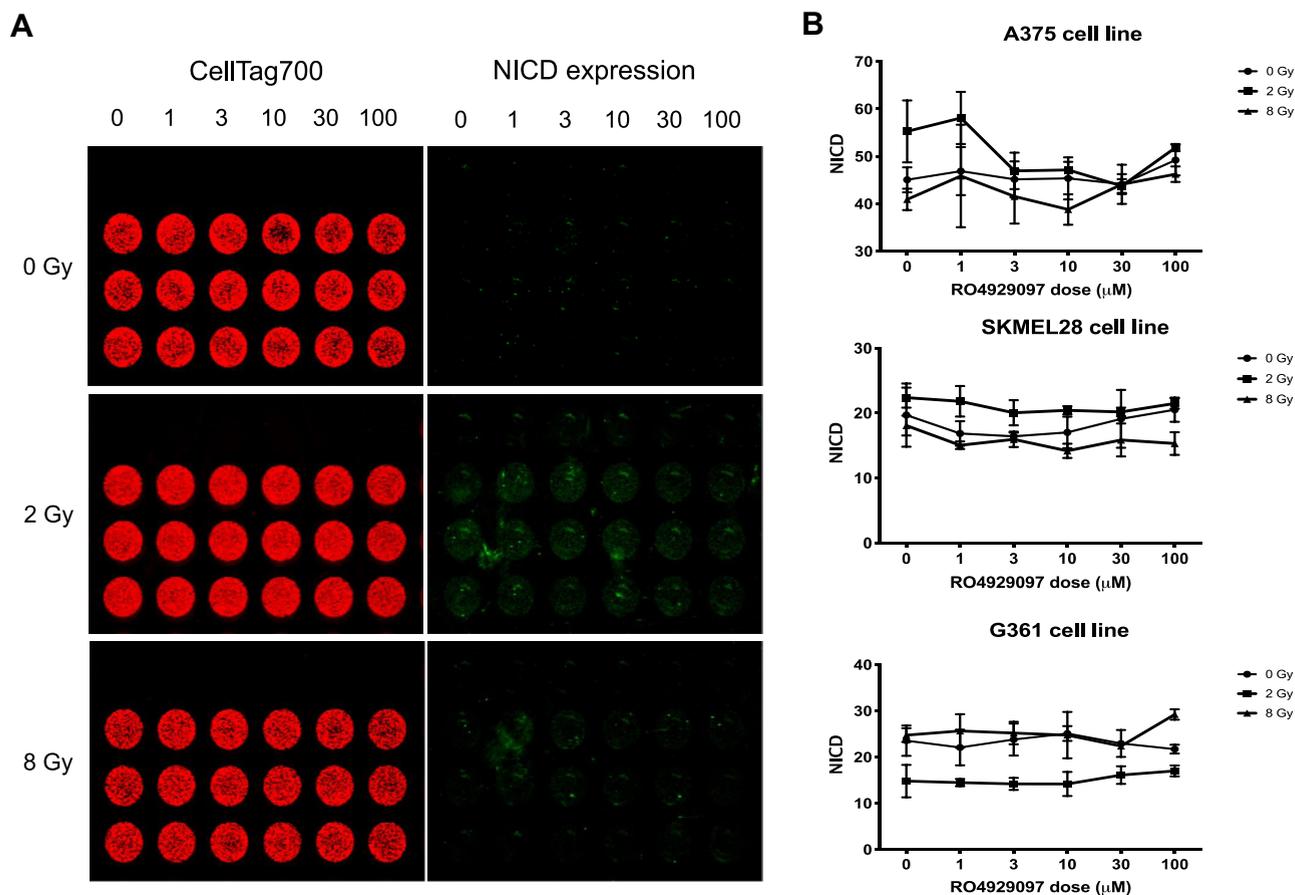


Figure 4 (A) Representative example of an A375 cell line Notch intra cellular domain (NICD) assay. Cells were treated with various combinations of radiation doses and RO4929097 concentrations. CellTag700 stain was used to stain cells (nucleus and cytoplasm), shown in red. NICD, normalised to cell number within each well, shown in green. (B) NICD assay results. Error bars indicate standard deviation. (A color version of this figure is available at <https://doi.org/10.1016/j.adro.2020.11.007>.)

Evaluation of effect on cell migration

Scratch assay results are shown in Figure 5. A375 and SKMEL28 cell migration was inhibited following exposure to 10 and 100 μM RO4929097, and this effect was more pronounced at 100 μM. Similar effects were seen when radiation was combined with RO4929097. Radiation alone did not have a noticeable effect on cell migration but superadded RO4929097 impaired cell migration.

Transwell migration assay results are shown in Figure 6. RO4929097, at both 10 and 100 μM concentrations, impaired cell migration. Radiation led to an increase in cell migration, particularly at 8 Gy, but this enhanced cell migration was reversed with the addition of RO4929097 at both concentrations. Impairment of cell migration reached statistical significance for all 3 melanoma cell lines examined (for A375, SKMEL28, and G361, $P < .0001$ for RO4929097 effect, and $P = .62$, $P = .49$, $P = .51$ for radiation effect, respectively, 2-way ANOVA).

Discussion

Melanoma, where radiation therapy is underutilized, is an ideal tumor type for exploring novel radiosensitization strategies. Targeting Notch signaling is an attractive radiosensitizing strategy, as this developmental cell signaling pathway is aberrant or overexpressed in melanoma, driving carcinogenesis and tumor progression, and it may be accountable for aggressive clinical behavior in some melanoma cases.

We demonstrated a reproducible radiosensitization for up to 8 Gy single doses using RO4929097. In line with our results, radiosensitivity of Notch-expressing lung cancer cells was enhanced with Notch inhibition,^{18,19} and similar results were seen in nasopharyngeal cancer cells.²⁰ We also showed that Notch inhibition significantly reduced the clonogenic potential in melanoma in vitro, similar to the observation noted in glial tumor cell lines.²¹

We showed a tendency toward better synergy with a radiation-first schedule. Similar results were observed in lung cancer cell lines.¹⁸ Although the mechanistic basis

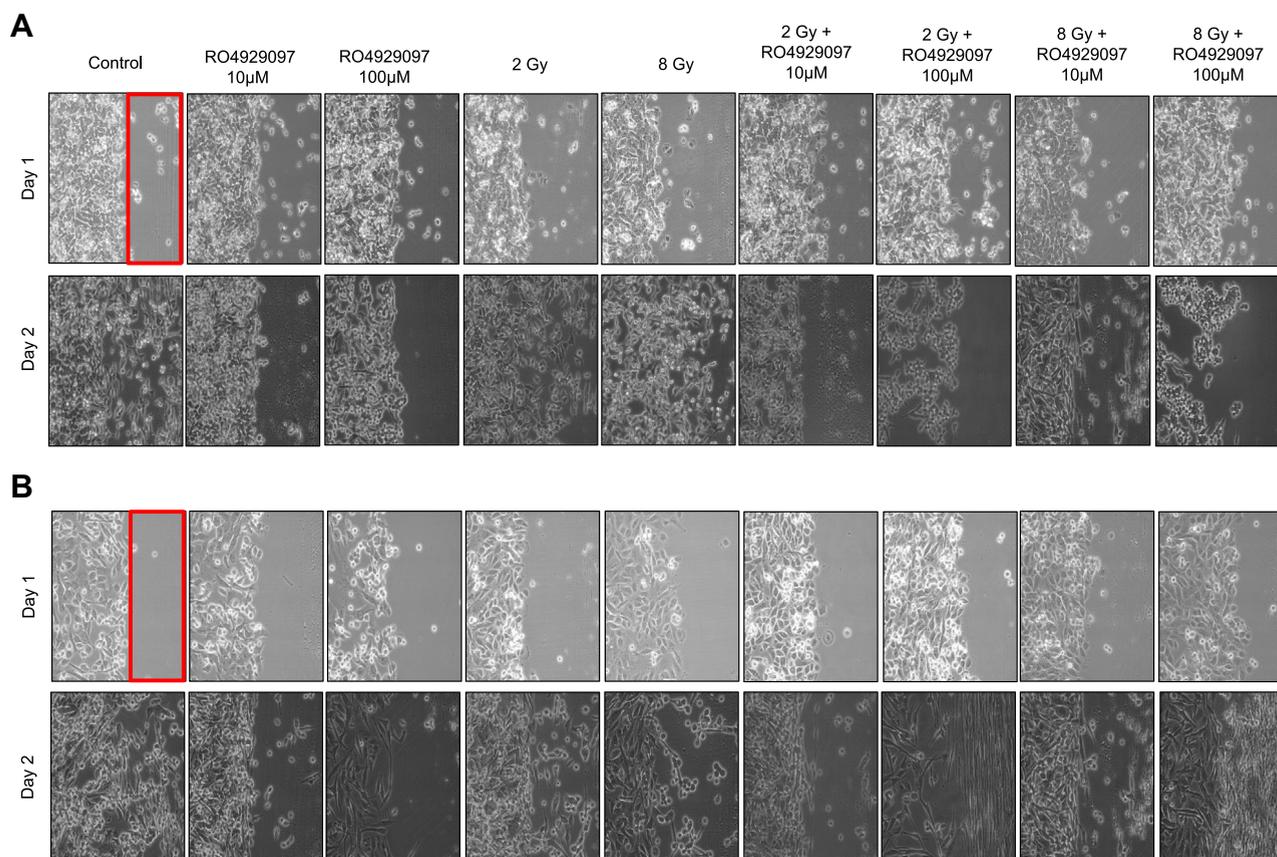


Figure 5 Light field microscopy images (20 \times magnification) of representative (A) A375 and (B) SKMEL28 scratch assays. Scratch areas are highlighted by a red box. Images show obliteration of scratch with cell migration in control wells and wells that received radiation alone but not in wells that were exposed to RO4929097. (A color version of this figure is available at <https://doi.org/10.1016/j.adro.2020.11.007>.)

for this observation was not explored in detail, we did see inhibition of the DDR pathway when radiation was combined with RO4929097. This would suggest impairment of repair of the DNA damage induced by radiation-first schedules by subsequent Notch inhibition as a plausible biological explanation.

Eradication of cancer stem cells (CSCs) is vital to the success of radiation therapy, and Notch signaling is critical for their homeostasis.^{22,23} Activation of Notch signaling may be an important mechanism by which CSCs develop into a radioresistant phenotype.²⁴ Notch inhibition increases the radiosensitivity by interacting with CSCs and by reducing their numbers.²⁵⁻²⁷ Ability of cancer cells to form tumor spheroids may indicate “stemness” of cancer cells, and melanospheres do exhibit this phenotype.²⁸ With Notch inhibition, as shown in Figure 2, we demonstrated a potential reduction in “stemness” of melanoma cell lines, consistent with a study in glial tumors where inhibition of neurosphere growth and suppression of stem cell markers were observed *in vitro*.²⁹

We consistently showed a reduction in γ -H2AX foci signal levels, which suggests that Notch inhibition

may impair function of the DDR pathways. Notch signaling modulates function of a DDR protein, ATM.³⁰ Notch inhibition enhances ATM-dependent apoptosis; therefore, it is plausible that this interaction between Notch and the DDR pathways could explain another potential mechanistic basis for our approach. Demonstration of decrease in NICD levels would be a strong indication for Notch signaling inhibition. Although we did not prove this unequivocally, our results demonstrated a reduction in NICD levels with RO4929097.

Melanoma has a high potential for metastatic spread, which makes it a difficult tumor to eradicate. Over-expression of Dll1 increases melanoma cell adhesion and metastasis.³¹ This observation is supported by a key role that Notch signaling plays in the induction of EMT.^{11,32,33} Based on our promising *in vitro* results, supported by other studies,³⁴ it is plausible that our approach may play a part in interrupting the molecular process leading to the development of metastasis.

Several preclinical studies raised concern of the potential role of radiation in metastasis development. Following radiation, cells acquire a mesenchymal

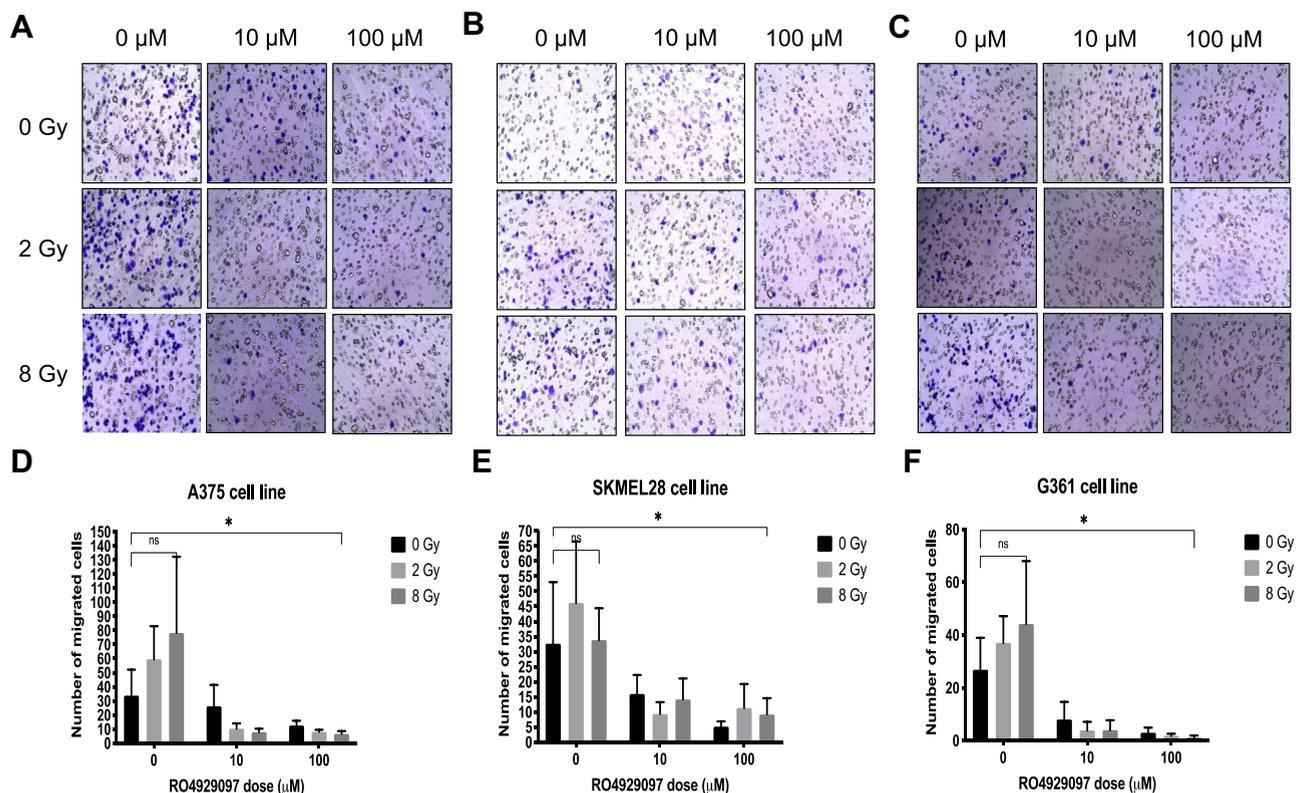


Figure 6 Transwell migration assays. Representative example of light field microscopy images (20× magnification) of (A) A375, (B) SKMEL28, and (C) G361 assays. Results of assays: (D) A375, (E) SKMEL28, and (F) G361. In addition to impairment of cell migration with RO4929097, RO4929097 also reversed radiation driven increase in cell migration. Results shown are average from 2 independent biological experiments. Error bars denote standard deviation. * $P < .0001$. Abbreviation: ns = not significant.

phenotype, which is one of the earlier changes seen in EMT. In addition, radiation alters tumor microenvironment by modulating the expression of various matrix metalloproteinases.³⁵ The Notch signaling pathway, which is upregulated following radiation,³⁴ may also partly explain enhanced cell migration and EMT that occurs after radiation. This supports our observation that Notch inhibition not only reduces de novo cell migration but also abates radiation-driven enhancement in cell migration.

Addition of Notch inhibition to radiation therapy has been successfully and safely translated into an early phase human clinical study. The addition of RO4929097 to a chemo-radiation therapy regimen in glioblastoma was well tolerated.³⁶ This study showed that it would be feasible to take forward this combined approach into human clinical studies. Intracranial drug level was an issue in this proof-of-concept study, but more potent and highly tumor-selective gamma secretase inhibitor may help to overcome this challenge.

Conclusions

We demonstrate that combining Notch inhibition with radiation in melanoma reverses its relative

radioresistant phenotype in vitro. Potential biological mechanisms for this effect may include modification of stem cell phenotype expressed by some melanoma cells and inhibition of the DDR pathway. Moreover, Notch inhibition reduces cell migration and also reverses radiation-induced increase in cell migration. Therefore, combining radiation with Notch inhibition may prove to be a promising strategy to improve radiosensitivity and widen the application of radiation therapy in melanoma.

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Supplementary Materials

Supplementary material for this article can be found at <https://doi.org/10.1016/j.adro.2020.11.007>.

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