

Coupling of Mutated Met Variants to DNA Repair via Abl and Rad51

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Abstract

Abnormal activation of DNA repair pathways by deregulated signaling of receptor tyrosine kinase systems is a compelling likelihood with significant implications in both cancer biology and treatment. Here, we show that due to a potential substrate switch, mutated variants of the receptor for hepatocyte growth factor Met, but not the wild-type form of the receptor, directly couple to the Abl tyrosine kinase and the Rad51 recombinase, two key signaling elements of homologous recombination-based DNA repair. Treatment of cells that express the mutated receptor variants with the Met inhibitor SU11274 leads, in a mutant-dependent manner, to a reduction of tyrosine phosphorylated levels of Abl and Rad51, impairs radiation-induced nuclear translocation of Rad51, and acts as a radiosensitizer together with the p53 inhibitor pifithrin- α by increasing cellular double-strand DNA break levels following exposure to ionizing radiation. Finally, we propose that in order to overcome a mutation-dependent resistance to SU11274, this aberrant molecular axis may alternatively be targeted with the Abl inhibitor, nilotinib. [Cancer Res 2008;68(14):5769–77]

Introduction

The key molecular feature of activating point mutations in the kinase domain of a receptor tyrosine kinase (RTK) is constitutive ligand-independent activation. Interestingly, a study from the late 1990s using mutated variants of the hepatocyte growth factor (HGF) receptor Met pointed to an additional, yet less explored, phenomenon whereby the substrate specificity of a mutated Met variant was switched from that of the wild-type (wt) receptor (1). In this respect, it was shown that Met mutants were capable of phosphorylating peptide substrates of the cytosolic tyrosine kinase Abl, whereas wt Met was not. Of the diverse functions attributed to Abl, its role in mediating a cellular response to DNA damage and its link to DNA repair via homologous recombination (HR), has attracted our interest and is the background for the present study (2, 3). In this context, two significant studies have recently shed light on the nature of the interactions between Abl and the Rad51 recombinase, the eukaryotic homologue of bacterial RecA which is a key player in HR-dependent DNA double-strand break (DSB) repair (4, 5). Notably, the work by Slupianek and colleagues suggested that the oncogenic form of Abl, Bcr-Abl, affects Rad51 activity via three different mechanisms: (a) by increasing Rad51

gene expression through the STAT5B transcription factor that requires phosphorylation by Bcr-Abl for transactivation of the *RAD51* promoter, (b) by stabilization of Rad51 cellular levels through inhibition of caspase-3 proteolytic activity, and (c) through direct phosphorylation of Rad51 on tyrosine 315, a key regulatory residue, which is probably crucial for Rad51 interactions with other HR components such as Rad52 and hence plays a role in cytoplasmic to nuclear translocation of Rad51 upon generation of DNA lesions (4, 6). Thus, the substrate switch caused by mutated Met signaling, which potentially activates Abl-dependent pathways and the emerging link between Abl and Rad51, prompted us to speculate whether transduction triggered by mutated Met variants might be linked to the activation of a DNA repair pathway.

Here, we report that contrary to cells that express wt Met, only cells that express Met-mutated variants exhibit constitutive levels of tyrosine phosphorylated forms of both Abl and Rad51, which could be reduced by the Met inhibitor SU11274 in a mutant-dependent manner. Furthermore, we report that Met mutants exist in stable signaling complexes with Abl and Rad51. As to a functional effect, we show that ionizing radiation (IR)-induced Rad51 nuclear translocation is significantly reduced by SU11274 only in cells expressing the drug-sensitive Met variant, M1268T. The current study emphasizes the potential benefit as well as the complexity associated with the use of anti-Met small molecule inhibitors for radiosensitization. We show that using an inhibitor such as SU11274, which causes a strong G₁ arrest and hence exerts cytostatic effects, may prove to counteract its main property, which is the interest of this study, e.g., the interfering capability with HR-dependent DNA repair. Thus, in order to unmask the radiosensitization capacity of SU11274, a more complex protocol, which includes the inhibition of p53, was designed. Finally, as Abl plays a central role in the Met/Abl/Rad51 axis identified in this work, we propose that utilization of the Abl small molecule nilotinib may serve as an alternative approach to inhibit this pathway for circumventing the resistance of Met mutations such as Y1248H to inhibitors like SU11274.

Materials and Methods

Cell culture. NIH3T3 lines, stably expressing the Met variants M1268T, Y1248H, and wt Met were provided by Dr. Laura Schmidt (National Cancer Institute, Fredrick, MD). Cells were maintained in DMEM (Life Technologies, Invitrogen) with 10% FCS (Sigma-Fluka), antibiotic-antimycotic (penicillin 100 units/mL, streptomycin sulfate 100 units/mL, amphotericin B as Fungizone 0.25 μ g/mL; Life Technologies) and 0.5 mg/mL of Geneticin (Life Technologies).

Small molecules. SU11274 [(3Z)-N-(3-chlorophenyl)-3-((3,5-dimethyl-4-((4-methylpiperazin-1-yl)carbonyl)-1H-pyrrol-2-yl)methylene)-N-methyl-2-oxindoline-5-sulfonamide] was from SUGEN, Inc. Nilotinib (NVP-AMN107-NX; 4-methyl-N-[3-(4-methyl-1H-imidazol-5-(trifluoro-methyl) phenyl)]-3-[[3-(pyridinyl)-2-pyridinyl]amino]-benzamide) was from Novartis Pharma AG. Dilutions were in DMSO.

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Antibodies. Phospho-Met (Tyr^{1234/1235}) and phospho-Abl (Tyr²⁴⁵) antibodies were from Cell Signaling, γ H2AX antibody was from Upstate. Met (SP260), p21(c-19), and SP1 antibodies were from Santa Cruz Biotechnology. Abl and β -actin antibodies were from Sigma. Rad51 and PY20 antibodies were from Calbiochem and BD Transduction Lab, respectively.

Western blotting and immunoprecipitation. Following treatment for 16 h with SU11274, cells were lysed in a buffer containing 1% Triton X-100, 0.5% NP40, 1 mmol/L of EGTA, 1 mmol/L of EDTA, 150 mmol/L of NaCl, 10 mmol/L of Tris-HCl, 1 mmol/L of Na₃VO₄, 10 mmol/L of NaF, 1 mmol/L of ZnCl₂, 50 μ mol/L of Na₂MoO₄, and a protease inhibitor cocktail (Roche). Protein concentrations were determined (Bio-Rad) and 100 μ g of lysate was used for loading onto SDS-PAGE. For immunoprecipitations, protein lysates (1 mg) were incubated with the indicated antibodies overnight at 4°C. Next day, protein G Sepharose/Fast Flow beads (Amersham Biosciences) or μ MACS protein G Microbeads (Miltenyi Biotec) were incubated for 2 h at 4°C. Immunoprecipitated complexes were washed (20 mmol/L Hepes, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 10 mmol/L NaF, 5 mmol/L EDTA, 1 mmol/L Na₃VO₄, and protease inhibitor cocktail) and separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, following by probing with primary antibodies and detecting by an enhanced chemiluminescence kit (Amersham).

Extraction of cytoplasmic and nuclear fractions. Cells were harvested following 16 h of treatment with 2 μ mol/L of SU11274, 8 h after exposure to 10 Gy of radiation by CLINAC linear accelerator with 6 MV photons. Nuclear and cytoplasmic fractions were obtained using the Nuclear Extraction kit following the manufacturer's instructions (Active Motif). Fifty-microgram protein extracts were processed for Western blotting analysis. β -Actin and SP1 were used as loading controls.

Flow cytometry. Cells were pretreated with 20 μ mol/L of pifithrin- α for 60 min, exposed to 16 h of treatment with SU11274 (2 μ mol/L) followed by irradiation with 10 Gy and fixed in 70% ethanol. Cells were rinsed in TBS (pH 7.4), centrifuged and suspended in TBS containing 4% fetal bovine serum and 0.1% Triton X-100. Cells were rehydrated, centrifuged, and

suspended in 200 μ L antiphosphohistone H2AX antibody. After rinsing, pellets were suspended in 200 μ L of Alexa 488 goat anti-mouse IgG(H+L)F(ab')₂ fragment conjugate (Molecular Probes) for 1 h with agitation. Cells were rinsed and suspended in TBS containing 1 μ g/mL of 4',6-diamidino-2-phenylindole (DAPI; Sigma). Samples were analyzed with Becton Dickinson LSR11 flow cytometry and gated on DAPI for DNA content. Data was analyzed by FlowJo software. Average γ H2AX stainings were calculated based on mean fluorescence. Results are the mean of three independent experiments.

Confocal microscopy analysis. Cells were grown on chamber slides (Nunc) and transfected with an EGFP-Rad51 expression vector (kindly provided by Dr. Roland Kanaar, Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, The Netherlands). Cells were PBS-washed and fixed with 4% paraformaldehyde. After washings, slides were mounted in PBS/glycerol (2:1) containing 170 mg/mL of Mowiol 4-88 (Calbiochem). A Zeiss LSM510Meta with an inverted microscope (Axiovert 200M, laser:Ar 488 nm) was used. Optical sections were taken with 63 \times /1.4 plan-Apochromat objective. Visualization was done using IMARIS software (Bitplane AG).

Results

Constitutive high levels of Met-dependent tyrosine-phosphorylated Abl and Rad51 in cells that express Met-mutated variants, but not in cells with expression of wt Met. As mutated Met variants have been previously reported to phosphorylate peptide substrates which are not in the repertoire of the wild-type receptor, such as substrates of the cytosolic tyrosine kinase Abl (1), and because Rad51 is a substrate of Abl (4), our first object was to determine Rad51 tyrosine phosphorylation in NIH3T3 cells expressing Met variants. Because a major tool in the present work was the small molecule Met inhibitor SU11274, we

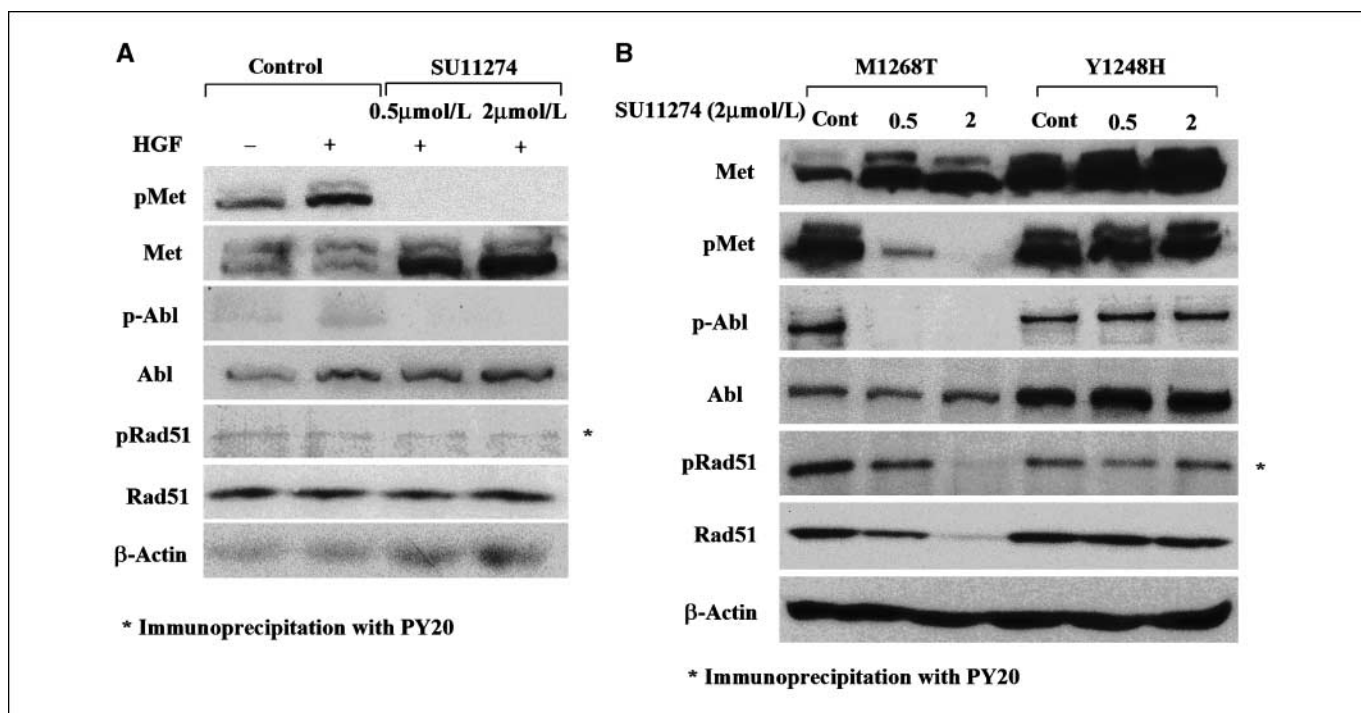


Figure 1. Status of Abl and Rad51 in NIH3T3 cells that express wt Met and the mutated variants M1268T and Y1248H. Cells expressing wt Met (A) were serum-starved overnight, then pretreated with SU11274 (0.5 and 2 μ mol/L) for 2 h and stimulated with HGF (100 ng/mL) for 7 min. M1268T and Y1248H cells (B) were both treated with SU11274. Total protein lysates were fractionated by SDS-PAGE following transfer to PVDF membranes. Phosphorylation of Met (Tyr^{1252/1253}) and Abl (Tyr²⁴⁵) was detected with specific anti-phospho-Met and anti-p-Abl antibodies. To detect tyrosine-phosphorylated Rad51, immunoprecipitation was done with the PY20 anti-phosphotyrosine antibody and membranes were subsequently probed with a total anti-Rad51 antibody. To determine the levels of total proteins, membranes were stripped and re-probed with anti-Met, anti-Abl antibodies. The experiments were repeated independently thrice and β -actin was used as a protein loading control.

focused our studies on two Met variants, M1268T and Y1248H, whose sensitivity and resistance, respectively, towards SU11274 have previously been detailed (7).

Figure 1A shows the status of Met, Abl, and Rad51 in wt Met cells. There is a low basal level of Met tyrosine autophosphorylation which increased following stimulation by HGF. Treatment with SU11274 at a concentration of 0.5 $\mu\text{mol/L}$ completely abolishes Met autophosphorylation. A basal, but weak HGF-independent level of tyrosine-phosphorylated Abl is also present in cells expressing wt Met. Finally, Met stimulation by HGF had no effect on either the total levels of Rad51 or on its tyrosine-phosphorylated status. Similar results were obtained with cell lines that naturally express either HGF-dependent or constitutively activated wt Met such as HepG2 as well as GTL16 and Kat-4, respectively (data not shown). These results exclude the possibility of a cross-talk between wt Met, Abl, and Rad51. Interestingly, the basal HGF-independent levels of tyrosine-phosphorylated Abl in wt Met cells seems to be sensitive to SU11274. A potential explanation is the off-target activity of SU11274, which is independent of its specific interaction with Met. In Fig. 1B, the state of total and phosphorylated levels of Met, Abl, and Rad51 in cells expressing the M1268T and Y1248H variants is depicted. Both cell lines exhibit high constitutive levels of phospho-Met that reach maximal inhibition in M1268T cells with 2 $\mu\text{mol/L}$ of SU11274, whereas the mutated variant Y1248H remains resistant. In contrary to cells with wt Met expression, substantial levels of constitutive tyrosine phosphorylated Rad51 levels were detected in Met M1268T and Y1248H cells. To demonstrate that Rad51 phosphorylation is Met-dependent, we show a dose-dependent reduction in Rad51 phosphorylation in M1268T cells treated with SU11274. To further substantiate that Rad51 phosphorylation is Met-dependent, we show that SU11274 has no effect in Y1248H cells which express the resistant variant. Additionally, Fig. 1B shows a dose-dependent reduction of Rad51 total cellular levels in M1268T cells, but not in Y1248H cells. This finding is in line with the above-described mode of Abl-based Rad51 expression via transcriptional regulation.

Among the most interesting findings in this part of the study concern Abl itself. The study by Bardelli and colleagues first suggested a molecular switch for Met mutants in so much that these variants are capable of phosphorylating Abl substrates themselves (1). However, the effect of Met mutants on Abl itself had not been addressed. The results presented here strongly advocate that the substrate switch initiated by Met mutants leads initially to Abl tyrosine phosphorylation, which is subsequently propagated to Rad51 activity. Thus, the findings in Fig. 1B show that like Rad51, Abl tyrosine autophosphorylation is significantly elevated in both M1268T and Y1248H cells, and that this phosphorylation is fully Met-dependent. Constitutive tyrosine Rad51 phosphorylation has been already previously reported in cells expressing Bcr-Abl, therefore accentuating the link between aberrant Abl activity and potential deregulated Rad51 function (5).

Met-mutated variants are physically associated with both Abl and Rad51. In the next part of the work, we examined the interactions between the components that form the aberrant axis Met, Abl, and Rad51. As shown in Fig. 2A, HGF did not induce an association between wt Met, Abl, or Rad51, whereas in both M1268T and Y1248H cells, Met, Abl, and Rad51 constitute a signaling complex (Fig. 2B). Moreover, these complexes are dependent on Met signaling, as Met inhibition leads to a mutant-dependent disruption of the components from the complex only in M1268T cells. Thus, the results from this and the previous chapter suggest that the Met/Abl/Rad51 pathway is uniquely activated in cells expressing Met mutants.

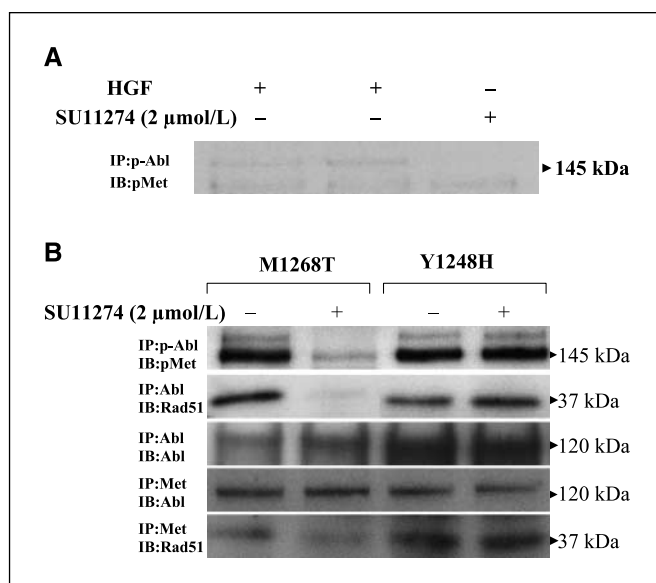


Figure 2. Physical association between Met, Abl, and Rad51 in cells expressing wt Met and the mutated variants M1268T and Y1248H. Following 16 h of starvation in medium with 0.1% FCS, wt Met cells (A) were treated with 2 $\mu\text{mol/L}$ of SU11274 for 2 h and stimulated with HGF (100 ng/mL) for 7 min. M1268T and Y1248H (B) were both treated with the Met inhibitor for 16 h. Protein extracts were isolated for coimmunoprecipitation analysis as described in Materials and Methods. Portions of total protein lysates (1 mg) were subjected for coimmunoprecipitation, fractionated on SDS gels, and blotted onto PVDF membranes as described in Materials and Methods. Coimmunoprecipitations and membrane hybridizations were performed with the same set of antibodies that are mentioned in Fig. 1.

Met inhibition results in impaired nuclear translocation of Rad51 following IR administration. A functional characteristic of Rad51 is its nuclear translocation and formation of typical clusters with additional HR components such as Rad52 and Rad54, presumably at sites of DNA damage in the form of DSBs (8). As mentioned earlier, it has been speculated that one role for Rad51 tyrosine phosphorylation by Abl is to facilitate its nuclear translocation. Therefore, we monitored changes in endogenous Rad51 levels in cytoplasmic and nuclear fractions of M1268T, Y1248H, as well as wt Met cells that were exposed to single or combined treatments with SU11274 and a single dose of 10 Gy. In the absence of IR, M1268T cells display high cytoplasmic and moderate nuclear levels of Rad51 (Fig. 3A). Treatment for 16 hours with 2 $\mu\text{mol/L}$ of SU11274 significantly reduced both cytoplasmic and nuclear Rad51 expression levels. Following IR, the nuclear content of Rad51 in M1268T cells was increased, in accordance with the notion that this protein is recruited to damaged DNA. Also shown in Fig. 3A is that in cells concurrently treated with SU11274 and irradiation, Rad51 translocation to the nucleus is impaired, thus supporting the upstream role of Met in this function. This assumption is further strengthened by the comparable results obtained with the SU11274-resistant variant Y1248H. Blocking Met only had a marginal inhibitory effect on the cytoplasmic levels of Rad51 in nonirradiated cells, whereas no changes were observed on nuclear levels following administration of IR, confirming the Met-dependent nature of the aberrant pathway. Consistent with the results in Figs. 1 and 2, which suggest that Abl and Rad51 do not reside downstream of wt Met; here, we show that nuclear levels of Rad51 in wt Met cells increase postirradiation and SU11274 does not have any inhibitory effect on this translocation.

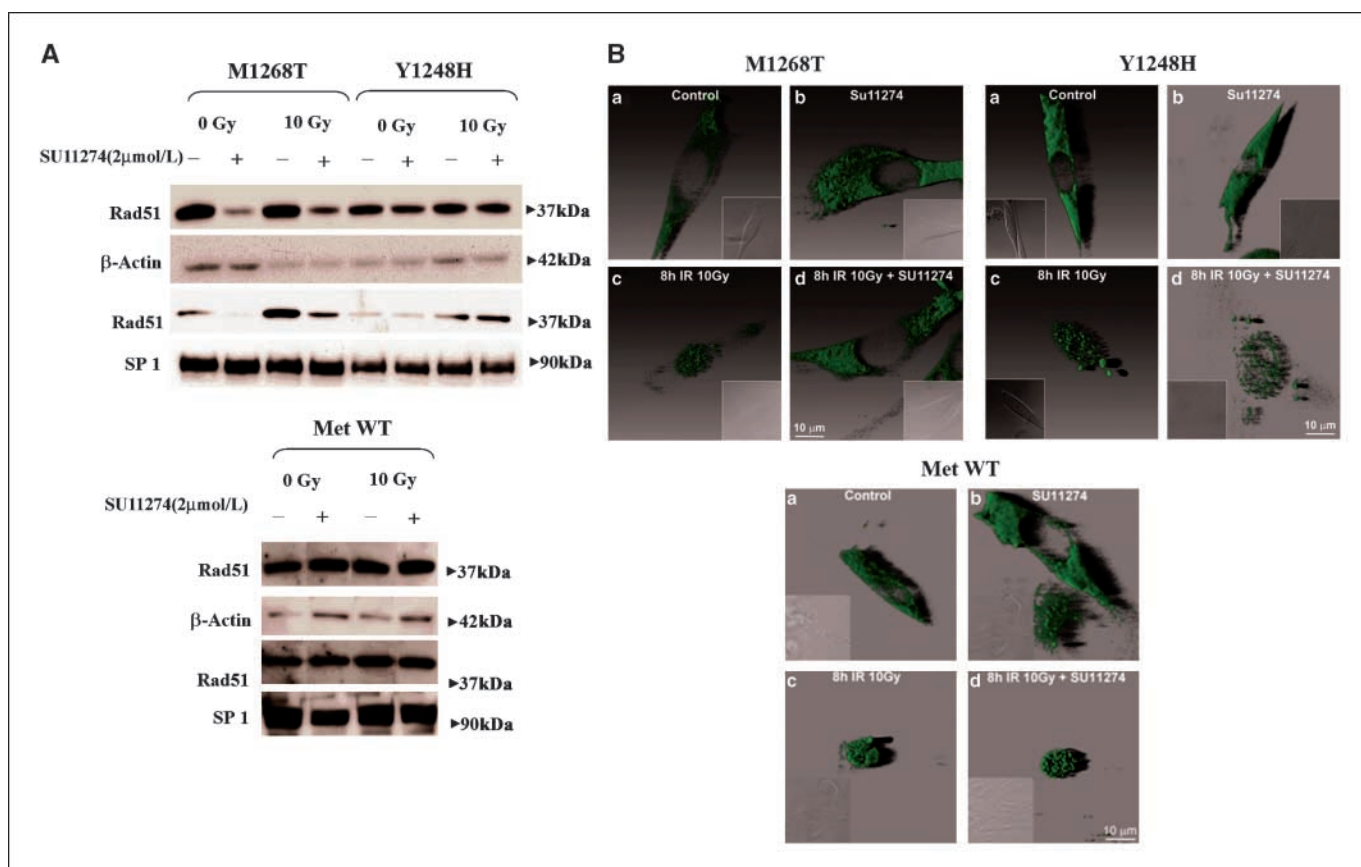


Figure 3. Effect of SU11274 on radiation-induced Rad51 expression and nuclear translocation. **A**, Western blot analysis of cytoplasmic and nuclear fractions from SU11274-treated and irradiated M1268T, Y1248H, and wt Met cells. Cells were pretreated with SU11274 (2 μ mol/L) for 16 h and exposed to a single dose of 10 Gy of radiation. Cells were collected 8 h postirradiation, lysates were prepared with an Active Motif Extraction kit according to the supplier's instructions, and endogenous Rad51 levels were determined as described in Fig. 1. β -Actin and SP1 served as protein loading controls for cytoplasmic and nuclear fractions, respectively. For cells that express wt Met, treatment with 50 ng/mL of HGF for 16 h preceded irradiation. **B**, SU11274 inhibits IR-induced nuclear translocation and foci formation, in a mutant-dependent manner, of a GFP-Rad51 chimeric protein. M1268T, Y1248H, and wt Met cells were transfected by a GFP-Rad51 expression vector as described in Materials and Methods. Transfected cultures were exposed to SU11274 (2 μ mol/L) for 16 h, irradiated by a single dose of 10 Gy, and then fixed for foci evaluation by confocal microscopy 8 h later. The images represent three-dimensional reconstructions. *Insets*, light transmission images. (a) Control cells, (b) 2 μ mol/L of SU11274 alone, (c) IR 10 Gy, (d) combination of 2 μ mol/L of SU11274 and 10 Gy of IR. Cells expressing wt Met were treated with 50 ng/mL of HGF 16 h before IR.

To support these observations, we performed immunofluorescence studies using a chimeric GFP-Rad51 construct that was transiently transfected into M1268T, Y1248H, and wt Met cells. Transfected cells were incubated with SU11274 for 16 hours followed by irradiation with a single dose of 10 Gy, before being analyzed 8 hours postirradiation by confocal microscopy (Fig. 3B). Without irradiation, the subcellular distribution of GFP-Rad51 fusion protein was primarily cytoplasmic and very similar in both M1268T and Y1248H cells. Following irradiation, most of the GFP-Rad51 fluorescence is found in the form of green foci in the nuclei of both M1268T and Y1248H cells. However, and as illustrated in Fig. 3B, a substantial difference is found between the two mutant cell lines when treated with the Met inhibitor prior to irradiation. In the case of M1268T cells, the pattern of fluorescence indicates that almost no GFP-Rad51 was translocated to the nucleus, whereas in Y1248H cells, almost all of the chimeric protein is localized to the nucleus. These results strongly indicate that changes following IR in the subcellular localization of both endogenous and GFP-Rad51 fusion protein are Met-dependent, but only in cells that express mutated Met variants.

Marginal effect of Met inhibition on DNA damage accumulation following treatment with IR. Several studies have previously argued that Met might be involved in tumor-associated

radioresistance (9–11). Here, we aimed to explore whether the above aforementioned results were associated with an augmentation of IR-induced DNA damage, which would suggest a radiation sensitization effect. To that end, we measured the phosphorylation of histone H2AX (γ H2AX), which is considered as a marker for DSBs (12). To evaluate maximal DNA damage in response to IR and its subsequent potential repair, measurements were performed 1 and 24 hours postirradiation, respectively. Because the highest cellular levels of γ H2AX usually manifest within 1 hour following the insult which causes the DSBs, we have used the maximal levels at 1 hour postirradiation as the 100% value. The gray columns in Fig. 4A, B, and C represent γ H2AX levels in M1268T, Y1248H, and wt Met cells, respectively. The data suggests that nonirradiated controls from the mutated Met cell lines and the wt Met cells have basal γ H2AX levels which correspond to 20% and 40% of the irradiated cells, respectively. Similarly, treatment with SU11274 alone did not produce significant changes in γ H2AX status, whereas as already mentioned, the highest levels of γ H2AX appear 1 hour postirradiation. Previous studies have already shown that the extent of γ H2AX which decreases after the DNA-damaging insult parallels the extent of DNA repair, and in this respect, it is considered that most of the possible DNA repair is executed within the first 24 hours following irradiation (13). In the present case, the

results in Fig. 4A and B indicate a drop of ~60% in γ H2AX levels in both M1268T and Y1248H cell lines. As shown in Fig. 4C, in the case of wt Met, a drop of 40% in levels of γ H2AX has been observed 24 hours postirradiation. To address the question of whether the

inhibition of Met will compromise the capacity of the cells to repair their damaged DNA, thereby resulting in higher and sustained levels of γ H2AX 24 hours post-IR, cells were exposed for 16 hours to 2 μ mol/L of SU11274 prior to IR and then evaluated for γ H2AX content. As Met inhibition by SU11274 has been shown to negatively affect HR-related cellular components, we expected that the observations described earlier could be ultimately translated to a sensitization effect in the form of significantly higher levels of DNA damage following an IR insult in cells expressing the SU11274-sensitive variant M1268T. Nevertheless, as Fig. 4A indicates, pretreatment by the Met inhibitor proved to have a marginal effect on γ H2AX levels in irradiated M1268T, as compared with cells, which were only irradiated. Thus, although γ H2AX levels in irradiated M1268T cells dropped by 60% after 24 hours, cells that were exposed to SU11274 prior to IR had a parallel reduction in γ H2AX of ~50%. Yet, this moderate effect by SU11274 is likely to be Met-dependent, as in the case of the SU11274-resistant Met variant, Y1248H, no difference in γ H2AX drop has been observed between cell populations that were exposed to IR only or those exposed to a combination of IR and SU11274. In the case of cells that express wt Met, no substantial differences in levels of γ H2AX are seen 24 hours after irradiation following treatment with SU11274.

Interestingly, the fact that SU11274-treated M1268T cells exhibit a reduction in γ H2AX levels indicates that despite the depletion of the HR-associated proteins, Abl and Rad51, these cells are still capable of repairing their IR-induced damaged DNA to a similar extent as untreated control cells (Fig. 4A). A potential explanation for this observation is that nonhomologous end-joining (NHEJ) repair may be responsible for the drop in γ H2AX and the elimination of DNA DSBs. This hypothesis is primarily based on the assumption that DSBs in replicating eukaryotic cells are mainly controlled through HR, whereas DSBs occurring in resting cells are usually repaired through NHEJ (8). It would therefore be appealing to speculate that once growth arrest is achieved in cells that express responsive Met variants such as M1268T, the balance or even competition between the two major repair machineries is shifted towards NHEJ.

SU11274-induced G₁ arrest is p53-dependent and can be circumvented by pifithrin- α . We speculated that the underlying mechanism by which SU11274 reduces the effects of IR on M1268T cells might be linked to an additional control it wields on cell cycle and on effectors involved in HR-dependent DNA repair, particularly Rad51. We have previously shown that Met inhibition with SU11274 induces a strong G₁ cell cycle arrest in cells expressing SU11274-sensitive Met variants, such as M1268T and H1112Y (7). Therefore, we postulated that in order to obtain the depleting effects of SU11274 on Abl and Rad51 in cells that harbor DSBs induced by IR, the G₁ arrest imposed by the Met inhibitor should be released to allow the compromised Abl/Rad51 cargo to progress to post-DNA replication phases, in which major repair is occurring via HR. To address whether SU11274-dependent G₁ arrest is p53-dependent, we exposed M1268T, Y1248H, and HGF-treated wt Met cells to 5 μ mol/L of SU11274 for 16 hours and looked at the expression of the p53 target, the G₁ regulator p21 (14). The results in Fig. 5A clearly show that following treatment by SU11274, significant up-regulation of p21 occurs only in cells expressing the M1268T-sensitive variant and not in cells expressing Y1248H. This observation prompted the conclusion that SU11274-induced G₁ arrest is p53-dependent. Interestingly, under similar conditions, no induction of p21 could be observed in wt Met cells.

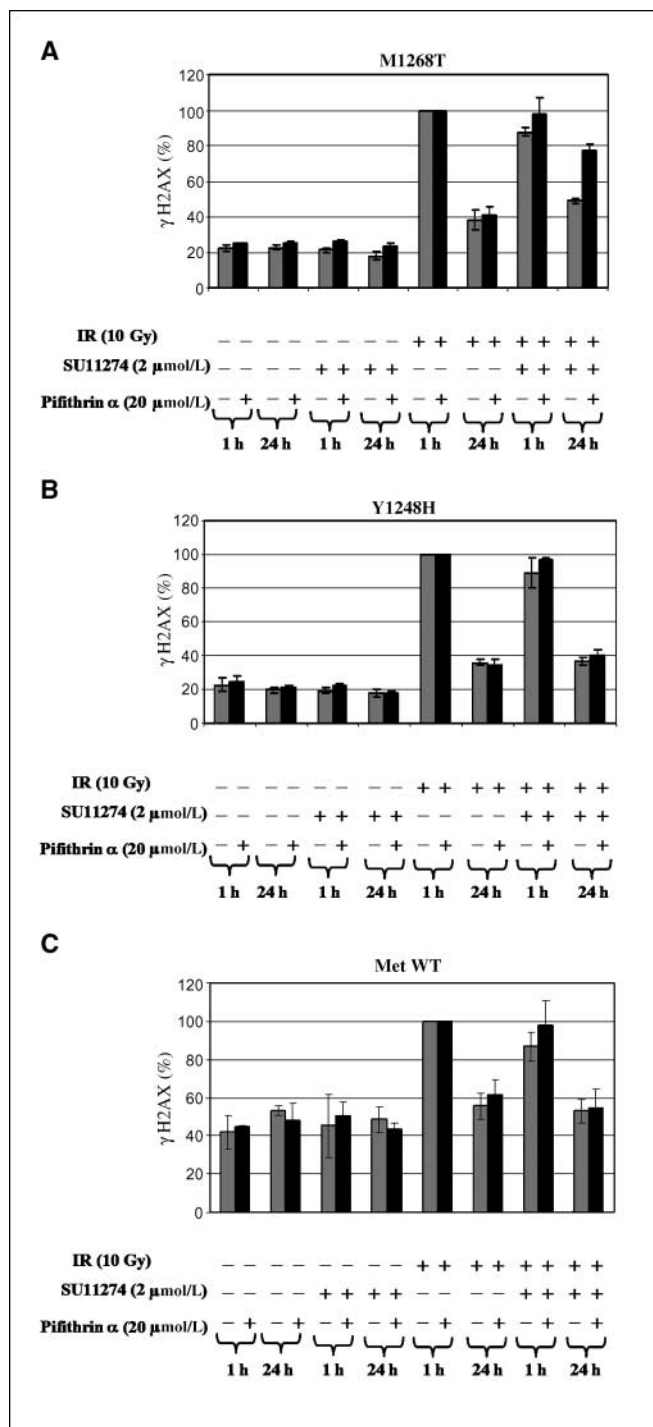


Figure 4. Pifithrin- α increases DNA damage when combined with SU11274 and IR only in M1268T cells. Treatment of cells with 2 μ mol/L of SU11274, 10 Gy and 20 μ mol/L of pifithrin- α was as described above. M1268T (A), Y1248H (B), and wt Met (25 ng/mL HGF, 16 h prior to IR; C) cells were fixed by 70% ethanol and stained with a γ H2AX-specific antibody followed by an Alexa 488 goat anti-mouse IgG (H+L)F(ab)₂ fragment conjugate. Flow cytometry analysis was done using FlowJo software. Average of γ H2AX antibody staining relative to the untreated controls was calculated based on mean fluorescence. Columns, mean of three independent experiments.

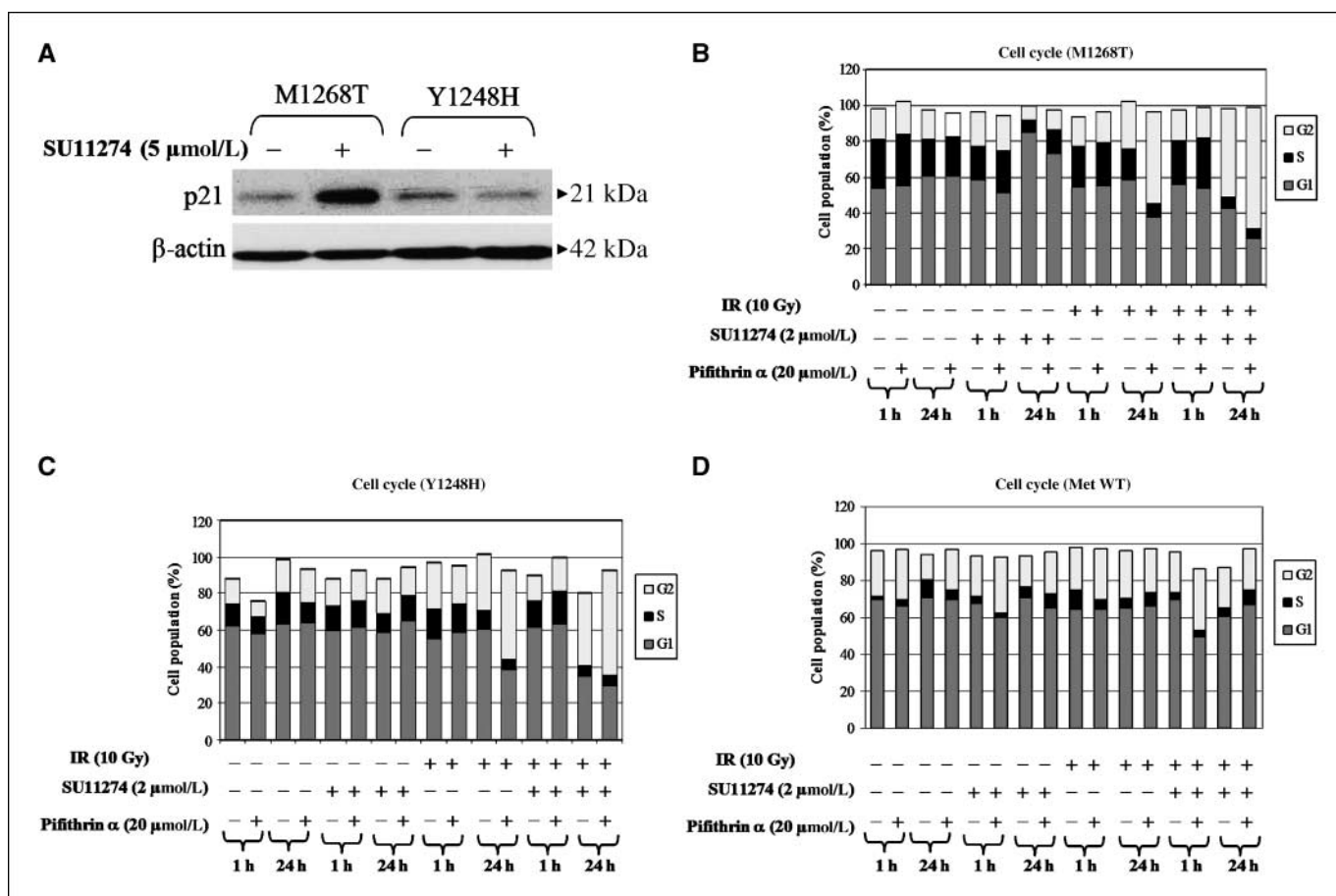


Figure 5. SU11274-induced p21 up-regulation and cell cycle redistribution by the p53 inhibitor pifithrin- α in M1268T and Y1248H cells. **A**, SU11274 induces up-regulation of p21 in a mutant-dependent manner. Expression of p21 following 6-h exposure of M1268T and Y1248H cells to 5 μ mol/L of SU11274 was done by Western blotting (see Materials and Methods). β -Actin was used as a loading control. **B**, **C**, and **D**, cell cycle redistribution following treatment with the p53 inhibitor pifithrin- α . M1268T (**B**), Y1248H (**C**), and wt Met (**D**) cells were exposed to triple treatment by 2 μ mol/L of SU11274, 10 Gy and 20 μ mol/L of pifithrin- α (see Materials and Methods) and wt Met cells treatment by HGF was as described in Fig. 4. Cell fixation was followed by treatment with an anti- γ H2AX antibody and DAPI. Following analysis of γ H2AX staining intensity, samples were gated on DAPI for DNA content of the cell cycle. Results shown are from one out of three independent experiments that were performed.

To assess the effect of Rad51 depletion on DNA repair through HR in cycling M1268T cells, we studied the effect of p53 inhibitor pifithrin- α (15) on the cell cycle. Cell cycle distribution was analyzed by flow cytometry and performed on subpopulations of cells that were positive to γ H2AX. As shown in Fig. 5B and C, pifithrin- α had no significant effect on cell cycle distribution of control M1268T and Y1248H cells, or on cells treated with SU11274 or IR for 1 hour. The results reiterate the strong G₁ block that SU11274 imposes on M1268T cells only in comparison to the untreated culture (85% and 53%, respectively) and show that the major effect of IR alone on both cell lines is a relative moderate increase in G₂ contents. In combination, pifithrin- α moderately reduced the SU11274-induced G₁ arrest in M1268T cells from 85% to 74%, with a concomitant increase in S and G₂ contents. When pifithrin- α was combined with IR alone, both M1268T and Y1248H cells showed substantial changes in cell cycle distribution 24 hours postirradiation. These changes are in agreement with the report from Komarov and colleagues (15), in which significant reductions in the percentage of cells found in G₁ and S phase, with concomitant increases in G₂, were observed. However, in our study, the most striking effects concerning redistribution of the cell cycle were observed in cells that received a triple combined

treatment of IR, SU11274, and pifithrin- α 24 hours postirradiation. In this respect, M1268T cells show a reduction of 65% in G₁ content (from 43% to 26%) and a dramatic associated increase from 49% to 69% in G₂ content.

As pifithrin- α has already been shown to increase the G₂ fraction in both M1268T and Y1248H cells exposed to irradiation, and as this effect is most likely SU11274-independent, it is not surprising that a significant increase in G₂ (from 40% to 58%) is also seen for Y1248H treated by the triple combination pifithrin- α /IR/SU11274. Based on S phase values, the basal proliferation rate of wt Met cells is considerably low and a maximal effect can be seen after exposure to HGF for 24 hours (2–10%; Fig. 5D). We therefore speculate that the reason for the slight differences between the various treated samples of wt Met cells is that exposure of cells to agents, which affect cell cycle distribution, will have a significant effect mainly on cells with a higher proliferation rate as the cells, which express the mutated variants of Met.

Increase of DNA damage by a combination of the Met inhibitor SU11274 and the p53 inhibitor pifithrin- α . As pifithrin- α significantly increased the G₂ contents of cells treated with both SU11274 and IR (Fig. 5), we aimed to check our hypothesis as to whether this approach could unveil a latent and

more effective radiosensitization capability of SU11274. As shown in Fig. 4, pifithrin- α had no effect on basal γ H2AX levels in M1268T and Y1248H that were not exposed to IR. The p53 inhibitor also didn't have any effect on basal DNA damage in cells that were treated by SU11274 alone, and slight, pifithrin- α -sensitive reductions in γ H2AX levels were seen in both M1268T and Y1248H cells treated by SU11274 1 hour postirradiation. However, striking results were obtained with the triple treatment of SU11274, IR, and pifithrin- α at 24 hours postirradiation. As shown by the black columns in Fig. 4A, the addition of pifithrin- α to M1268T cells treated with both SU11274 and IR results in a doubling of γ H2AX levels 24 hours postirradiation, as compared with control cells unexposed to the Met inhibitor (74% and 38%, respectively). The fact that this can be attributed to Met inhibition is supported by the observation that exposing cells expressing the SU11274-resistant variant Y1248H (Fig. 4B) to the same treatment modality resulted in a negligible increase of γ H2AX levels at the same time point as compared with control cells (40% and 36%, respectively). Furthermore, as seen in Fig. 4C, the combined treatment of wt Met cells did not alter the decrease of γ H2AX 24 hours after irradiation. Taken together, these observations strongly support the working hypothesis that mutated Met variants activate a DNA repair axis distinct from the wild-type form of the receptor.

Use of the Abl inhibitor nilotinib as an alternative approach for targeting the Met/Abl/Rad51 axis as a means to circumvent Y1248H resistance to SU11274. The next experiments were performed to evaluate whether an alternative approach for inhibiting the Met/Abl/Rad51 pathway could circumvent Met resistance to SU11274, similar to that displayed by the mutated variant Y1248H. Because Abl seems to be a critical signaling effector in the aberrant cascade initiated by mutated Met forms, we reasoned that its inhibition might provide similar results as SU11274, but with an additional potential benefit in circumventing the resistance to Met inhibition to SU11274 as displayed by the Y1248H variant. In Fig. 6, we show the effect of nilotinib in comparison to SU11274 on the status of Abl and Rad51. Treatment with 10 μ mol/L of nilotinib lead to a significant reduction or complete depletion of tyrosine-phosphorylated Abl levels in M1268T and Y1248H cells, respectively, whereas SU11274 was active only in M1268T cells. Similar results were obtained both with phosphorylated and total levels of Rad51. As expected, no effect on the levels of phosphorylated forms of both Abl and Rad51 as well as on total Rad51 levels were observed in cells expressing wt Met. These results suggest that targeting Abl could have potential use in the treatment of cancer. Whether nilotinib can function as a radiosensitizer in the particular aberrant Met-driven pathway described here is beyond the scope of this work and remains to yet be determined.

Discussion

Activating point mutations in RTK systems are classically associated with a constitutive, ligand-independent function of the affected receptor. However, the report by Bardelli and colleagues, provided clues that point mutations can lead to a more dire behavior of cancer cells (1). To our knowledge, we explore here, for the first time, the phenomenon of a substrate switch whereby a mutation located in the kinase domain of RTK can change the downstream signaling capacity of mutated variants as compared with the wild-type receptor. In addition to shedding light on the molecular machinery that ties Met to DNA repair, the concept that

emerges from this work relates to the principle of oncogenic gain-of-function in cancer biology. Thus, whereas the phenomenon of gain-of-function typically reflects consequences arising from enzymatic hyperactivation of a network of effectors downstream of a key activator, such as the RTK system, the Met mutants used in this study show the capability to extend their signaling to additional effectors, leading to the acquisition of new properties which can contribute to a more aggressively transforming phenotype. In the particular case described in this work, there are several levels by which the activation of an additional DNA repair pathway might translate into practical benefit in a cancer cell. First, due to a high proliferation rate, an excess of naturally occurring DSBs, most of which probably result from stalled replication forks, is generated during the accelerated DNA replication. These DSBs could become an Achilles' heel, which unless properly repaired, will eventually kill the cells due to the accumulation of genomic instability. Similarly, the elevated

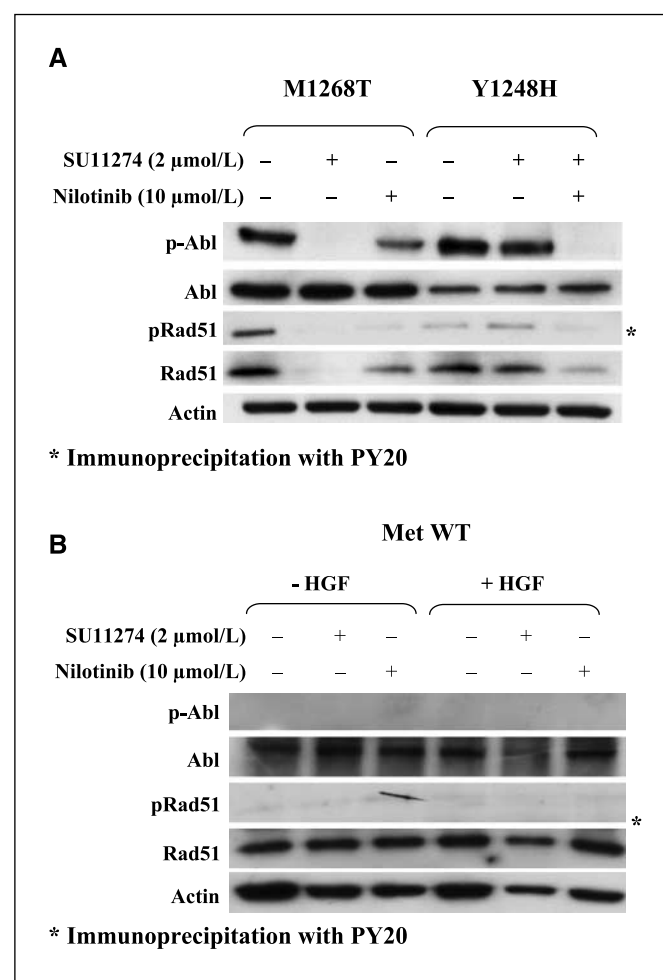


Figure 6. Effect of treatment by the Abl inhibitor nilotinib on Rad51 tyrosine phosphorylation in cells with aberrant Met activity. M1268T, Y1248H (A) and wt Met (20 ng/mL HGF, 1 h before lysis; B) cells were pretreated with 2 μ mol/L of SU11274 and 10 μ mol/L of nilotinib for 16 h. Cell lysates were prepared as described in Materials and Methods. Western blot analysis was performed to detect the phosphorylation status of Abl (Ty²⁴⁵). To detect phosphorylated Rad51 levels, immunoprecipitation was conducted with PY20 and blotted with anti-Rad51 antibody. To determine total Abl levels, membranes were stripped and reprobed with anti-Abl antibody. Rad51 was detected with anti-Rad51 antibody. Experiments were repeated thrice independently and β -actin was used as a protein loading control.

metabolism of cancer cells is usually associated with the increased production of reactive oxygen species (16, 17), the adverse effects of which strongly damage DNA mainly in the form of DSBs. Finally, the damage induced by IR and various chemotherapeutic agents eventually leads to DSBs. The high-efficiency repair capabilities displayed by malignant cells may therefore present a resistance to the mentioned modalities. Taken together, the activation of a DNA repair pathway may turn out to be a cardinal mechanism for cancer cells to survive frequent endogenous and exogenous stimuli that generate harmful DNA lesions.

As the identification of small molecule inhibitors of growth factor receptors increases, it is anticipated that combination studies with established modalities such as radiation therapy will be further examined for cancer therapy. A major goal in the present study was to implement the findings in which Met drives a molecular pathway that eventually leads to aberrant DNA repair and develop a working protocol combining radiosensitization with the Met inhibitor, SU11274. However, the strong G₁ arrest induced by SU11274 competes and eventually obscures its radiosensitizing activity due to the down-regulation of the HR-associated proteins Abl and Rad51. The proof of this principle has been provided by the integration of the p53 inhibitor pifithrin- α , which probably released the G₁ arrest and enabled the Abl/Rad51-depleted cells to proceed to the post-DNA replication stages of the cell cycle, in which HR is the dominant repair mode. This led to a dramatic increase in DNA damage in cells treated with SU11274, IR, and pifithrin- α compared with cells that were exposed only to IR and the p53 inhibitor, thus revealing the strong and anticipated radiosensitizing capacity of the Met inhibitor. It has been previously suggested that Met inhibition can boost tumor-killing by IR in animal models (18, 19). However, these studies have made use of anti-Met gene therapy-based approaches, the development of which for implementation in human cancer treatment may not be realized in the near future. Thus, the present study is the first to successfully use a small molecule Met inhibitor for this purpose. Therefore, it is reasonable to predict that new targeting agents with cytostatic activity via cell cycle arrest at G₁, similar to SU11274, will eventually enter clinical use. If it is desired to enhance the cytotoxicity of such agents by combination with other modalities, such as IR or radiomimetic drugs whose main mode of action is the generation of DNA DSBs, similar phenomena as described in this work are to be expected and the development of practical combination protocols will be necessary. Although pifithrin- α was originally used *in vivo*, the translation of the triple treatment protocol described here into an *in vivo* model is beyond the scope of the present study.

Point mutations emerge as the rate-limiting step in targeting therapies that use small molecule inhibitors. The efficacy of Gleevec in the treatment of chronic myelogenous leukemia is

limited in advanced disease by relapses, primarily caused by the emergence of resistant clones that harbor point mutations in Bcr-Abl (20). Similar observations have been made in gastrointestinal stromal tumors and mastocytomas, in which Kit serves as the target of Gleevec (21). Although anti-Met inhibitors still have not yet entered clinical trials, it is reasonable to anticipate a high rate of molecular resistance to such an inhibitor, due to the numerous point mutations in this RTK system and results already reported with SU11274 (7, 22). Thus, new inhibitors for a given target or the adaptation of already existing agents to newly discovered pathways are likely to be part of the solution for resistant mutation barriers. In this respect, new small molecule Abl inhibitors which inhibit most of the Bcr-Abl mutations resistant to Gleevec have been reported (23, 24). In the present work, we have used one of these agents, nilotinib, as an alternative agent for the inhibition of the aberrant Met/Abl/Rad51 axis. The importance of the anti-Met-dependent Abl activity that nilotinib exhibited in the present study may be translated on four levels. Primarily, this is the first study to point towards a beneficial effect of inhibiting the aberrant activity of Abl itself and, to our knowledge, it is also the first time that inhibition of deregulated Abl activity, driven by an upstream effector is presented, thus potentially extending the utility of targeted Abl inhibitors. Second, compounds such as nilotinib may already be considered for clinical studies to target Met-driven aberrant Abl activity. Third, although the present study couples Met via Abl and Rad51 to aberrant DNA repair, we cannot exclude that the abnormal Abl activity triggered by Met may be responsible for the activation of other oncogenic pathways involving different DNA repair mechanisms, therefore adding an additional dimension for the use of Abl inhibitors in such cases. Fourth, the successful use of nilotinib in the case of the SU11274-resistant Met variant Y1248H exemplifies the use of alternative approaches to overcome resistance of a particular target to a given inhibitor.

Disclosure of Potential Conflicts of Interest

P.W. Manley: Employment, Novartis Pharma. The other authors disclosed no potential conflicts of interest.

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