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A Novel Anti-Cancer Agent, 1-(3,5-Dimethoxyphenyl)-4-[(6-Fluoro-2-Methoxyquinoxalin-3-yl)Aminocarbonyl] Piperazine (RX-5902), Interferes With β-Catenin Function Through Y593 Phospho-p68 RNA Helicase

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ABSTRACT

1-(3,5-Dimethoxyphenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (RX-5902) exhibits strong growth inhibition in various human cancer cell lines with IC_{50} values ranging between 10 and 20 nM. In this study, we demonstrate that p68 RNA helicase is a cellular target of RX-5902 by the drug affinity responsive target stability (DARTS) method, and confirmed the direct binding of ^{3}H-labeled RX-5902 to Y593 phospho-p68 RNA helicase. We further demonstrated RX-5902 inhibited the β-catenin dependent ATPase activity of p68 RNA helicase in an in vitro system. Furthermore, we showed that treatment of cancer cells with RX-5902 resulted in the downregulation of the expression of certain genes, which are known to be regulated by the β-catenin pathway, such as c-Myc, cyclin D1 and p-c-Jun. Therefore, our study indicates that the inhibition of Y593 phospho-p68 helicase - β-catenin interaction by direct binding of RX-5902 to Y593 phospho-p68 RNA helicase may contribute to the anti-cancer activity of this compound. J. Cell. Biochem. 116: 1595–1601, 2015.

KEY WORDS: ANTI-CANCER; p68 RNA HELICASE; PHOSPHO-p68; RX-5902

Previously in our laboratory, a series of novel quinoxalinyl-piperazine compounds were synthesized and their effects on proliferation and apoptosis were examined [Lee et al., 2010; Lee et al., 2012]. Among them, one compound (RX-5902) demonstrated potent inhibition of cancer cell growth and induction of apoptosis, with IC_{50} between 10 and 20 nM [Lee et al., 2010]. Additionally, RX-5902 compound displays good pharmacokinetic properties in rats [Lee et al., 2012], has a high level of oral bioavailability (F; 83%) and half-life (*T*1/2; 7.9 h in oral), indicating the potential of RX-5902 as an orally available, anti-cancer agent.

A number of heterocyclic N-substituted piperazine derivatives and other piperazine derivatives have anti-viral activities and have been used for treatment of nervous system disorders [Blair et al., 2000; Jordan et al., 2002]. Several anti-cancer compounds containing a quinoxaline ring have been reported as well [Shoemaker, 1986; Rigas et al., 1992; Monge et al., 1995; Miller et al., 1997; Corbett et al., 1998; Gali-Muhtasib et al., 2001; Diab-Assef et al., 2002; Gao et al., 2003; Undevia et al., 2008; Kakodkar et al., 2011; Noolvi et al., 2011]. Although there are reports suggesting unsubstituted quinoxaline compounds containing piperazine exhibit microtubule-inhibiting activity [Cho et al., 2004; Yi et al., 2004], the molecular mechanisms and cellular targets of these compounds are not well studied.

In our previous studies, we demonstrated that treatment of cancer cells with our synthesized quinoxalinyl-piperazine derivative

Abbreviations: DARTS, drug affinity responsive target stability; RX-5902, 1-(3,5-dimethoxyphenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine; phospho-p68, phosphorylated p68; EMT, epithelial-mesenchymal transition; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Kd, dissociation constant.

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RX-5902 led to downregulation of cellular levels of Bcl-2 and cell cycle arrest at G2/M phases, which consequently resulted in cell death [Lee et al., 2010]. However, the molecular target(s) by which RX-5902 exerts its effects leading to cell cycle arrest and apoptosis is not clear.

The nuclear p68 RNA helicase is a prototypical DEAD box family of RNA helicases [Lane and Hoeffler, 1980; Crawford et al., 1982]. A number of studies have shown that p68 RNA helicase is involved in cancer progression and cell proliferation/survival [Shin et al., 2007; Davis et al., 2008; Dey and Liu, 2012; Nicol et al., 2013; Wang et al., 2013; Yang et al., 2005a]. Experiments from our laboratory demonstrate that p68 RNA helicase is phosphorylated at multiple amino acid residues, and phosphorylation of p68 at tyrosine residues in particular closely correlates with cancer progression [Yang et al., 2005a]. Specifically, phosphorylation of p68 at Y593 mediates the effects of growth factors in promoting epithelial-mesenchymal transition (EMT) [Yang et al., 2006]. Phospho-p68 also plays a critical role in up-regulating expression of Snail1, a gene product that plays an important role in EMT and cancer progression [Carter et al., 2010]. These studies suggest that phospho-p68 may have a very important function in tumor development, cancer progression and metastasis. Thus, phospho-p68 may constitute an important target for development of anti-cancer therapies.

We report here that RX-5902 interacts with Y593 phospho-p68 RNA helicase and may result in inhibition of β-catenin dependent ATPase activity with no effect on p68 RNA-dependent ATPase activity. Furthermore, cancer cells treated with RX-5902 display downregulation of several proliferation-associated genes that are known to be regulated by phospho-p68-β-catenin interaction. Our studies indicate that interaction between RX-5902 and Y593 phospho-p68 may contribute to the anti-tumor activity of RX-5902.

MATERIALS AND METHODS

CELL CULTURE AND ANTIBODIES

MDA-MB-231, SK-MEL-28, and WI-38 cells were obtained from ATCC (Manassas, VA, USA) and were cultured according to the vendor's instructions. Anti-p68 antibody and anti-Y593-p68 antibody were purchased from Cell Signaling (Danvers, MA) and Abcam (Cambridge, MA), respectively. Antibodies against β-actin, cyclin D1, p-c-Jun and c-myc, were purchased from Santa Cruz (Dallas, TX). Anti-phospho-tyrosine antibody and HRP conjugated GAPDH antibody were obtained from Cell Signaling (Danvers, MA).

RECOMBINANT PROTEINS

Recombinant β-catenin and p68 protein were purchased from Creative Biomart (Shirley, NY) and Origene (Rockville, MD), respectively. Recombinant β-catenin was used without further treatment whereas recombinant p68 protein was either used as p68 or phosphorylated by c-Abl for filter binding assay. Recombinant c-Abl was obtained from Abcam (Cambridge, MA).

DRUG TREATMENT

RX-5902 was dissolved in DMSO to prepare a stock solution of 2 mM. The stock solution was stored at −20°C and diluted with medium to prepare working concentrations.

IDENTIFICATION OF RX-5902 BINDING PROTEINS

MDA-MB-231 cells were plated onto 6-well plates and treated with RX-5902 at various concentrations (0, 0.1, 1, and 10 μM) for 1 h. Cells were lysed with p-MER buffer containing protease/phosphatase inhibitors on ice. Cell lysates were treated with thermolysin protease (1:1,500 ratio) for 10 min at RT and the reaction was stopped by addition of 0.5 M EDTA solution. The reaction mixtures were separated on a 10% SDS-PAGE visualized by Coomassie staining. After identifying several candidate proteins from mass spectrometry sequencing analysis, we confirmed the protein, which may interact with RX-5902 by western blot analysis.

FILTER BINDING ANALYSES

Filter binding studies have been previously described elsewhere [Coombs and Pearson, 1978]. Briefly, recombinant p68 RNA helicase with/without tyrosine phosphorylation was added to the ^{3}H-labeled RX-5902 (10 Ci/mmol) with PBS. ^{3}H-labeled RX-5902 was synthesized from Quotient Bioresearch (Cardiff, UK). After incubation at room temperature for 30 min, the binding mixtures were loaded onto a nitrocellulose membrane. The membrane was washed five times with PBS, and then dried by vacuum. The amounts of RX-5902 bound to p68 with/without phosphorylation of p68 were determined by ^{3}H scintillation counting. The same procedure was done with ^{3}H-labeled RX-5902 alone without addition of p68 RNA helicase and sample p68

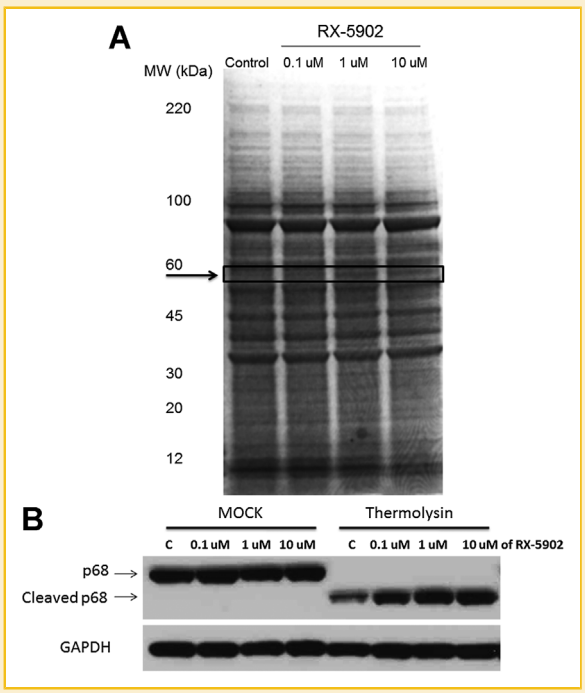

Fig. 1. RX-5902 interacts with p68 RNA helicase. MDA-MB-231 cells were treated with RX-5902 at various concentrations (0, 0.1, 1, and 10 μM) for an hour, and cell lysates were prepared. The extracts were treated by thermolysin (1:1,500 ratio) for 10 min. The reaction mixtures were loaded and separated onto SDS-PAGE gel visualized by Coomassie staining (A). The reaction mixtures were separated onto SDS-PAGE gel and probed with anti-p68 antibody along with GAPDH as a control (B).

RNA helicase alone without addition of the ^{3}H-labeled RX-5902 as background ^{3}H scintillation counting. The binding percentages of p68 to the compound were calculated and plotted against concentrations. The dissociation constant (Kd) was estimated by the concentration at 50% of p68 bound to RX-5902 and calculated by linear regression analysis.

ATPASE ASSAY

ATPase activity was determined by measuring the released inorganic phosphate during ATP hydrolysis using a direct colorimetric assay [Yang et al., 2006; Shin et al., 2007]. The method is based on the change in absorbance (A_{623nm}) of malachite green-molybdenum complex in the presence and absence of inorganic phosphate. A typical ATPase assay was carried out in 50 μl reaction volumes, containing 20 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT, ~1 – 2 μg of appropriate substrate, 4 mM ATP, and 10 μl of helicase. The ATPase reactions were incubated at 37°C for 30 min. After incubation, 1 ml of malachite green-molybdenum reagent was added to the reaction mixture, and reactions were further incubated at room temperature for exactly 5 min. The absorption (A) at 630 nm was then measured. The concentrations of inorganic phosphate were determined by matching the A_{630nm} in a standard curve of A_{630nm} versus known phosphate concentrations. The proteins, p68 or phospho-p68, used for this assay were prepared in-house similar to the procedure reported previously [Yang and Liu, 2004]. The percentage of inhibition by defining the ATPase activity of phospho-p68 without RX-5902 as zero percent inhibition was calculated and IC_{50} of RX-5902 was calculated by non-linear regression analysis using Kaledia Graph software program (Synergy Software, Reading, PA).

WESTERN BLOTTING

Protein mix or cell lysates were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked by blocking buffer (1X TBST containing 5% BSA) at room temperature for 1 h. After a brief wash, the membrane was incubated with primary antibody in blocking buffer at 4°C overnight. After incubation in primary antibody, the membrane was washed with 1X TBST three times and subsequently incubated with HRP-conjugated secondary antibody in blocking buffer at room temperature for 1 h. The membrane was again washed three times and visualized by ECL system (Thermo Scientific, Rockford, IL).

RESULTS

We previously reported the synthesis and evaluation of the anti-cancer activities of a series of quinoxalinyl-piperazine derivatives [Lee et al., 2010]. Among them, RX-5902 showed a potent anti-cancer effect and here, we focused on determining the cellular targets of this compound and what role these targets play in inhibiting cell proliferation and inducing apoptosis. To do this, we employed the well-established target identification method DARTS assay [Lomenick et al., 2009], to find target proteins that would interact with RX-5902 in cancer cells. DARTS method indicated that a band with mobility around 60 kDa was protected from the protease cleavage

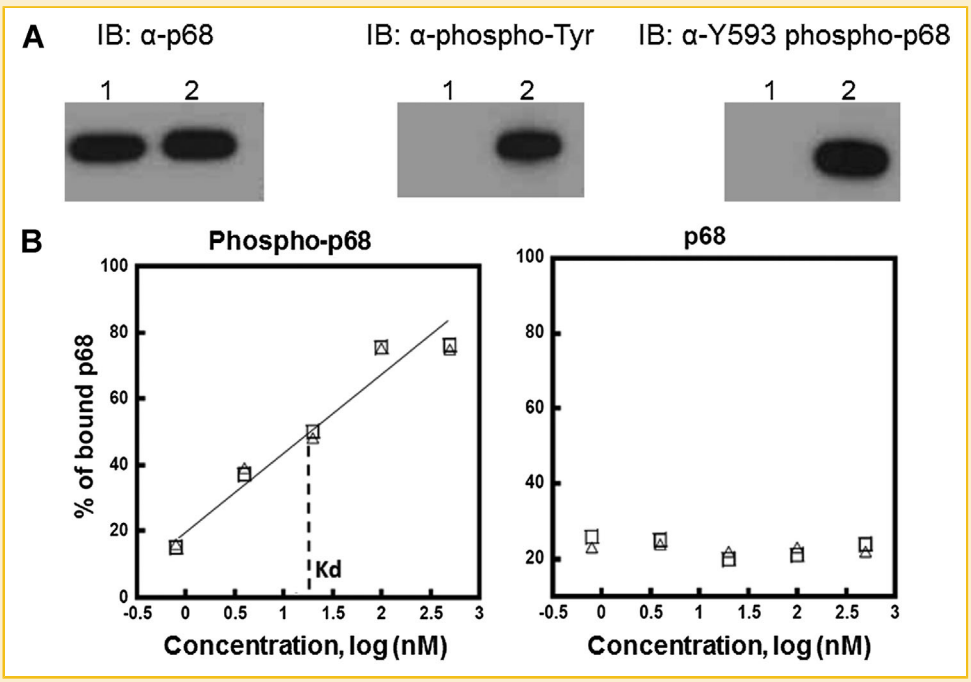

Fig. 2. RX-5902 interacts with tyrosine phosphorylated p68 RNA helicase. Recombinant p68 protein was tyrosyl phosphoylated with c-Abl at 30°C for 30 min. Before (1) and after (2) kinase reaction mixtures were analyzed by western blot analysis against anti-p68, anti-tyrosine and anti-Y593 phospho-p68 antibody (A). The filter-binding curve of ^{3}H-labeled RX-5902 with p68 with (Left, Phospho-p68) and without (Right, p68) the tyrosine phosphorylation. Y-axis is the percentage of p68 bound to the compound calculated based on the ^{3}H scintillation counting. The X-axis is the corresponding p68 concentration in log scale. The Kd is estimated by 50% of p68 bound to RX-5902 (B).

upon interaction with RX-5902 (Fig. 1A). To identify this protected protein, the bands along with control were sliced out, and analyzed with LC-MS/MS by ProtTech (Phoenixville, PA). We carried out western blot analysis using antibodies against several potential candidates from LC-MS/MS analysis that have a similar mobility in SDS-PAGE to confirm the protected protein by RX-5902 treatment. Clearly, this protected band was recognized by the antibody against p68 RNA helicase (Fig. 1B), indicating that RX-5902 may interact with p68 RNA helicase in cells and protect p68 from degradation by thermolysin.

To verify the interaction of RX-5902 with p68, we used ^{3}H-labeled RX-5902. The interaction of ^{3}H-labeled RX-5902 with recombinant p68 protein and the in vitro tyrosyl phosphorylated recombinant p68 protein was probed by filter binding assays [Coombs and Pearson, 1978]. Through western blot analysis we confirmed that p68 was phosphorylated on a tyrosine residue (Fig. 2A). A filter-binding assay clearly showed RX-5902 interacted with the Y593 phospho-p68 with an estimated Kd around 19 nM, but RX-5902 did not interact with unphosphorylated p68 in our filter binding studies (Fig. 2B). Since p68 has been shown to be an RNA-dependent ATPase and an ATP-dependent helicase capable of unwinding RNA [Ford et al., 1988; Yang et al., 2007a], we examined whether RX-5902 would affect ATPase activity of p68. To do this, we measured ATPase activity of recombinant p68 in the presence of RX-5902 and total RNA extracted from yeast. RX-5902 did not affect RNA-dependent ATPase activity of p68 RNA helicase at 0.2 μM and even at high concentrations such as 20 μM, RNA-dependent ATPase activity was inhibited less than 30% (Fig. 3A), indicating RX-5902 had very little effect on RNA-dependent ATPase activity of p68. These data could confirm the result that RX-5902 did not interact with unphosphorylated p68 in filter binding assay (Fig. 2B).

It was demonstrated that interaction with β-catenin and microtubules also stimulates ATPase activity of phospho-p68 RNA helicase [Yang et al., 2006; Wang et al., 2013]. We therefore questioned whether interaction with RX-5902 would affect the β-catenin dependent ATPase activity of phospho-p68. It was evident that the β-catenin dependent ATPase activity of phospho-p68 was largely diminished in the presence of RX-5902 at 0.2 μM (Fig. 3B). Next, we repeated the experiment at lower concentrations of RX-5902 to calculate IC_{50} (Fig. 3C). We determined the IC_{50} of RX-5902 for the inhibition of β-catenin dependent ATPase activity as 61 nM, indicating that RX-5902 potentially disrupts the phospho-p68/β-catenin interaction.

Next, since RX-5902 directly binds to Y593 phospho-p68, we hypothesized treatment of cells with RX-5902 would interfere with the phospho-p68/β-catenin interaction and consequently affect the expression of several growth associated genes, including p-c-Jun, c-Myc and cyclin D, which are regulated by phospho-p68/β-catenin interaction. It has been reported that the interaction between phospho-p68 and β-catenin plays a role in expression of cyclin D1 and c-myc, and also affects activation of c-jun MAP kinase [Yang et al., 2007a]. Thus, we analyzed cyclin D1 and c-myc expression, as well as phosphorylation of c-jun in cells that were treated with RX-5902. We used SK-MEL-28, MDA-MB-231 and normal fetal lung fibroblasts (WI-38) as we found that RX-5902 was effective in apoptosis induction and cell growth inhibition in MDA-MB-231 and

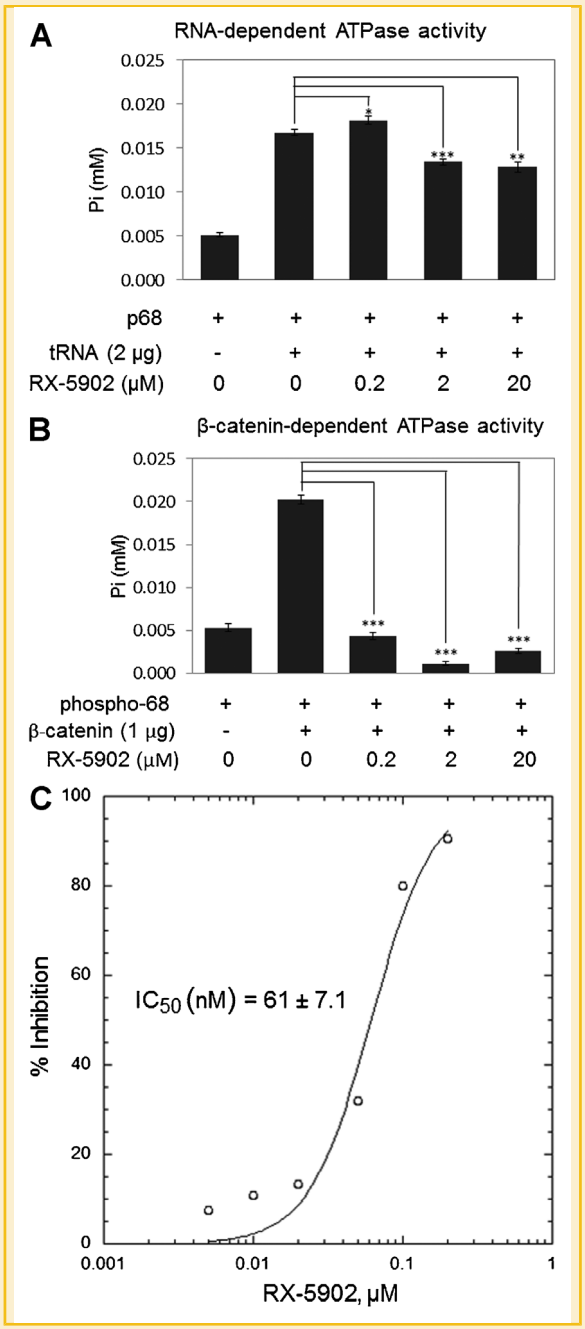

Fig. 3. Effects of RX-5902 on ATPase activity of p68 RNA helicase. ATPase activity of p68 was measured in the presence of 2 μg yeast total RNA, ATP, and recombinant p68 RNA helicase, in the presence or absence of RX-5902 at the indicated concentrations (0–20 μM). The ATPase activity is presented as μmol of released inorganic phosphate from ATP hydrolysis. Data shown are average number ± SEM (n = 4) (A). ATPase activity was measured in the presence of 1 μg β-catenin, 2 mM ATP, and 1 μg of recombinant phospho-p68 RNA helicase, in the presence or absence of indicated concentrations of RX-5902 (0–20 μM). Data shown are average number ± SEM (n = 4) (B). Since RX-5902 had an effect on β-catenin-dependent ATPase activity, the IC_{50} of RX-5902 was measured using the same conditions as previously used except RX-5902 concentrations were between 0 and 0.2 μM. The percentage of inhibition by defining the ATPase activity of phospho-p68 without RX-5902 as zero percent inhibition was calculated and 50% inhibition by RX-5902 was presented as IC_{50} ± SD (C). Statistical analysis (*t*-test): $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

SK-MEL-28 with IC_{50} of 12 and 20 nM, respectively [Lee et al., 2010]. We could not detect phospho-p68 in WI-38 cells and IC_{50} of RX-5902 in this cell line was higher than 10 μM (unpublished data). Although 20 nM of RX-5902 did not change protein levels, it was evident that 70 nM of RX-5902 treatment led to a decrease in expression of both cyclin D1 and c-myc and a decrease in c-jun phosphorylation in SK-MEL-28 without changing the level of total p68 protein, while the similar changes in protein levels were detected at both 20 and 70 nM of RX-5902 in case of MDA-MB-231. But RX-5902 did not result in any significant change in cyclin D1/c-myc expression or c-jun phosphorylation in WI-38 cells (Fig. 4) even at 70 nM. These results clearly demonstrate that the effects of the phospho-p68 and β-catenin interaction on expression of several proliferation and survival related genes are suppressed by RX-5902.

DISCUSSION

p68 RNA helicase is a prototypical member of the DEAD box family of RNA helicases. It is well documented that p68 RNA helicase expression and post-translational modifications play an important role in cancer progression and metastasis [Causevic et al., 2001; Yang et al., 2005a, 2005b]. Particularly, phosphorylation of p68 has been correlated with cancer progression [Yang et al., 2005a]. Phosphorylation of p68 at Y593 facilitates cancer metastasis via promotion of EMT. Furthermore, phosphorylation of p68 at Tyr residues has been shown to confer a survival advantage and block apoptosis via interaction with several important apoptotic and proliferation regulators [Dey and Liu, 2012; Yang et al., 2006, 2007a, 2007b]. Using DARTS method, we identified p68 RNA helicase as one of the binding proteins of RX-5902 and confirmed further with a filter binding assay using radio-labeled RX-5902 that phospho-p68 RNA helicase, not un-phosphorylated p68, is the binding protein of RX-5902. Thus, the data suggest that RX-5902 may target phospho-p68 to exert its activity in inhibiting proliferation and/or inducing apoptosis.

Originally, p68 was shown to be an RNA-dependent ATPase and an ATP-dependent helicase capable of unwinding RNA [Ford et al., 1988; Hirling et al., 1989]. As a helicase, p68 binds to both double- and single- stranded RNA, with greater affinity to double-stranded RNA. Binding to RNA stimulates ATPase activity of p68 and results in unwinding of RNA in both the 5′ to 3′ and 3′ to 5′ direction. Along with its helicase activity, p68 possesses an RNA annealing ability and can catalyze the rearrangement of secondary structures in RNA. With its RNA dependent ATPase activity, ATP-dependent RNA helicase and RNA annealing abilities, p68 RNA helicase plays a very important role in various cellular processes, including ribosome biogenesis, pre-mRNA splicing, and microRNA processing [Janknecht, 2010]. Likewise, dysregulated p68 expression likely plays a role in tumor progression. Indeed, p68 overexpression has been correlated with colon cancinogenesis through coactivation of β-catenin–mediated transcription [Shin et al., 2007]. Additionally, it has been reported that ectopically expressed, Y593 phosphorylated p68 binds to β-catenin and can induce its nuclear translocation through disruption of its interaction with axin [Yang et al., 2006]. Here, we demonstrate that RX-5902 did not have much of an effect on the RNA-dependent ATPase activity of p68 helicase, most likely because of the limited interaction of RX-5902 and the unphosphorylated form of p68. Several studies have shown phosphorylation of p68 at Y593 facilitates cancer cell proliferation and survival, and it was demonstrated that these important functions of phospho-p68 were mediated by interacting with β-catenin in cancer cells [Yang et al.,

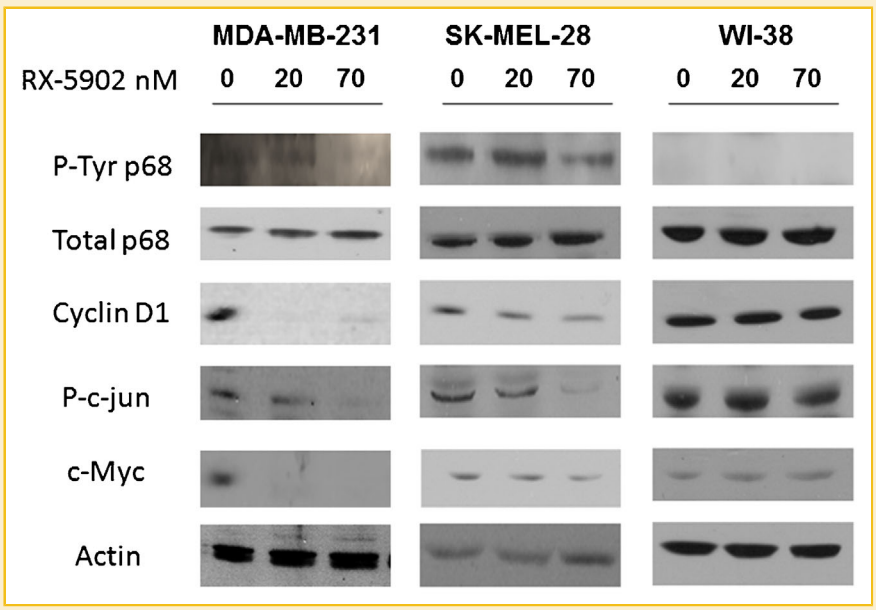

Fig. 4. Effect of RX-5902 on expression of downstream proteins of p68–β-catenin. The cell lysates were prepared after treatment with indicated concentrations of RX-5902. Phospho-p68 RNA helicase, total p68 and β-actin were analyzed by western blot. Expression and activation of multiple genes, including cyclin D1, c-myc, and p-c-Jun that are regulated by p68–β-catenin interaction were also analyzed by western blot.

2006; Carter et al., 2010]. Previous studies have shown that tyrosine-phosphorylated p68 binds β-catenin and facilitates its signaling to the nucleus by inhibiting GSK3β phosphorylation and by displacing axin from β-catenin. Importantly, the p68/β-catenin interaction is essential in PDGF-induced nuclear accumulation of β-catenin and EMT stimulation of HT-29 cells [Yang et al., 2006]. Thus, we reasoned that our compound RX-5902 confers its anti-cancer activity by interfering with the ATPase activity of phospho-p68. By testing this hypothesis by measuring ATPase activity of phospho-p68 in the presence of β-catenin and downstream gene expression of phospho-p68/β-catenin pathway, we determined RX-5902 inhibits the interaction of Y593 phospho-p68 helicase and β-catenin, resulting in decreased expression of several proliferation and survival-related genes such as p-c-Jun, c-Myc and cyclin D.

Due to important biological functions of many protein-protein interactions (PPI) in almost all physiological and pathological processes, development of molecules that would disrupt these disease causative PPIs has emerged as an important approach to develop therapeutics for many diseases, such as Creutzfeldt-Jakob syndrome, Alzheimer's disease and cancer [Gao et al., 2013]. One potential advantage of targeting specific disease related PPIs is that targeting can efficiently achieve disease specific effects while limiting side effects seen with other agents. Phosphorylation of p68 at tyrosine residues has been shown to be closely associated with cancer progression and metastasis. Previous studies have shown interaction between phosphorylated p68 and β-catenin plays a role in cancer cell proliferation and survival [Dey and Liu, 2012; Yang et al., 2005a, 2006, 2007b]. This interaction does not exist in normal cells and in normal physiological processes. Thus, this PPI should be an excellent target for cancer therapies. Certainly, our study sets an example for further development of molecules that would be effective in disrupting the phospho-p68/β-catenin interaction. How RX-5902 interacts with phospho-p68 is an open question. Our results show that the compound did not affect the RNA dependent ATPase activity, but affected the β-catenin dependent ATPase activity of phospho-p68, indicating that RX-5902 likely interacts with phospho-p68 at the C-terminal. This is also consistent with previous observations that post-translational modifications at the C-terminal of p68 mediate pro-growth and anti-apoptosis functions of this RNA helicase. Thus, it would be of interest to test whether RX-5902 also interferes with the functional role(s) of other C-terminal modifications of p68 RNA helicase.

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