Novel Inhibitors of Rad6 Ubiquitin Conjugating Enzyme: Design, Synthesis, Identification, and Functional Characterization

Matthew A. Sanders1,2, Ghali Brahemi4, Pratima Nangia-Makker1,3, Vitaly Balan1,2, Matteo Morelli4, Hend Kothayer4,5, Andrew D. Westwell6, and Malathy P.V. Shekhar1,2,3

Abstract

Protein ubiquitination is important for cell signaling, DNA repair, and proteasomal degradation, and it is not surprising that alterations in ubiquitination occur frequently in cancer. Ubiquitin-conjugating enzymes (E2) mediate ubiquitination by selective interactions with ubiquitin-activating (E1) and ubiquitin ligase (E3) enzymes, and thus selective E2 small molecule inhibitor (SMI) will provide specificity unattainable with proteasome inhibitors. Here we describe synthesis and functional characterization of the first SMIs of human E2 Rad6b, a fundamental component of translesion synthesis DNA repair. A pharmacophore model for consensus E2 ubiquitin-binding sites was generated for virtual screening to identify E2 inhibitor candidates. Twelve triazine (TZ) analogs screened in silico by molecular docking to the Rad6B X-ray structure were verified by their effect on Rad6B ubiquitination of histone H2A. TZs #8 and 9 docked to the Rad6B catalytic site with highest complementarity. TZs #1, 2, 8, and 9 inhibited Rad6B-ubiquitin thioester formation and subsequent ubiquitin transfer to histone H2A. SMIs #9 inhibition of Rad6 was selective as BCA2 ubiquitination by E2 UbcH5 was unaffected by SMI #9. SMIs #9 more potently inhibited proliferation, colony formation, and migration than SMIs ##8, and induced MDA-MB-231 breast cancer cell G2–M arrest and apoptosis. Ubiquitination assays using Rad6 immunoprecipitated from SMIs #8–9-treated cells confirmed inhibition of endogenous Rad6 activity. Consistent with our previous data showing Rad6-mediated polyubiquitination stabilizes β-catenin, MDA-MB-231 treatment with SMIs #8 or 9 decreased β-catenin protein levels. Together these results describe identification of the first Rad6 SMIs. Mol Cancer Ther; 12(4); 373–83. ©2013 AACR.

Introduction

Protein ubiquitination involves the activities of an ubiquitin-activating enzyme (E1) that initiates ubiquitination by forming an ATP-dependent thioester bond between its active site cysteine and the ubiquitin carboxyl terminus. The activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2) with resultant formation of a thioester-linked E2-ubiquitin complex, and subsequently transferred to substrate directly or via interaction with an ubiquitin-protein ligase (E3), leading to substrate mono- or polyubiquitination. E2s play a central role in ubiquitin transfer, as they are responsible both for E3 selection and substrate modification. Recent work shows E2s are important for dictating the final ubiquitinated product, that is, a mono- or polyubiquitinated chain of a specific lysine linkage (1–3), and ultimately the fate of the substrate: proteasomal degradation or signaling.

Rad6, the first cloned E2 (4), is essential for postreplication DNA repair. Yeast rad6 mutants lacking the active site cysteine exhibit DNA-damaging agent sensitivity, UV-induced mutagenesis defects (5), impaired proteolysis by the N-end rule pathway (6, 7), and cell-cycle arrest (8), implicating Rad6 ubiquitin-conjugating activity as necessary for its many functions. The yeast Rad6 human homologues, HHR6A and HHR6B (or Rad6A and Rad6B), encode ubiquitin-conjugating enzymes and complement DNA repair and UV mutagenesis defects of Saccharomyces cerevisiae mutant rad6 (9, 10). The requirement for at least one functional Rad6A or Rad6B allele in all somatic cell types is confirmed by the nonviability of mice lacking both Rad6A and Rad6B homologues (11). By differential display gene expression analysis we identified Rad6B and...
overexpression in mouse and human breast cancer lines and tumors. Constitutive Rad6B overexpression in non-transformed human breast cells induces multinucleated cell formation, centrosome amplification, abnormal mitosis, aneuploidy, and transformation (12). Rad6 ubiquitinates histones in the absence of E3 proteins (4, 13). Similarly, Rad6B ubiquitinates β-catenin in vitro in the absence of E3 ligases, and the K63-linked ubiquitinated β-catenin conjugates generated by Rad6B are insensitive to 26S proteasome (14), indicating Rad6B is important for β-catenin stabilization/activation in breast cancer (14, 15). Rad6 and its E3 ligase partner Rad18 mediate proliferating cell nuclear antigen (PCNA) monoubiquitination and K63-linked polyubiquitination (16, 17). These data suggest Rad6 is important for genomic integrity maintenance via its ubiquitin-conjugating activity, and that imbalances in its levels/activity could contribute to genomic instability via error-prone DNA repair and/or ubiquitination of substrates with resultant altered signaling or proteasomal processing.

We report here synthesis, identification, and functional characterization of the first known Rad6B small molecule inhibitors (SMI). These SMIs dock to the Rad6B catalytic site, inhibit Rad6B-induced histone H2A ubiquitination, downregulate intracellular β-catenin, induce G2–M arrest and apoptosis, and inhibit proliferation and migration of metastatic human breast cancer cells.

Materials and Methods

Pharmacophore model for virtual screening

Identification of new potential lead compounds with E2 inhibitory activity was based on computational modeling using MOE 2010.10 (Molecular Operating Environment, Chemical Computing Group Inc.), described in detail elsewhere (18). Briefly, an E2-ubiquitin consensus-binding site was built based on the nuclear magnetic resonance (NMR) structure of ubiquitin-conjugating enzyme Ubc1 ubiquitin complex (19). Identification of the key residues stabilizing the E2-ubiquitin thioester intermediate and conserved across the human E2 family allowed generation of a 4-point pharmacophore model describing key H-donor and H-acceptor points, and was refined by including excluded volumes around these points. Virtual screening of the pharmacophore model against the ZINC database (pre-prepared using drug-like filters) identified the substituted diamino-triazine (TZ) core structure that formed the starting point for analog synthesis.

Experimental chemistry

Melting points were measured on a Griffin apparatus and are uncorrected. Mass spectra were recorded on a Bruker MicroTOF instrument or at the EPSRC National Mass Spectrometry Centre. NMR spectra were recorded on a Bruker AVANCE 500 MHz instrument. Merck silica gel 60 was used for column chromatography. Following purification, all TZ compounds were determined to possess 95% or more purity as determined by spectroscopic and combustion analyses (±0.4% carbon, hydrogen, nitrogen tested in duplicate).

TZ compound synthesis

The TZ compounds synthesis scheme (Fig. 1) involved 3 steps using commercially available starting materials. Step 1 involved arylbiguanide synthesis (Fig. 1, 3a–f; ref. 20)). Step 2 involved (4-amino-6-(phenylamino)-

![Figure 1. Schematic illustration of synthesis of substituted (4-amino-6-(phenylamino)-1,3,5-triazin-2-yl) methyl benzoates.](image-url)
[1,3,5]triazin-2-ylmethanol synthesis (Fig. 1, 5a–f; ref. 21)). Step 3 involved synthesis of (4-amino-6-(phenylamino)-[1,3,5]triazin-2-ylmethyl benzoate, which yielded Tzs #1–11. TZ #13 (4-amino-6-(2-ethylphenylamino)-[1,3,5]triazin-2-ylmethyl 4-hydroxybenzoate) was synthesized from TZ #6 (Fig. 1). Details of chemical synthesis, purification, and NMR and MassSpec data of TZ compounds are shown in Supplementary Data.

Rad6 nomenclature
We previously showed that the Rad6B gene is overexpressed in breast cancer by transcript sequencing and Rad6B-specific short hairpin RNA transfections (12, 14). However, because the peptide we used for Rad6B antibody generation is 91% conserved in human Rad6A protein, our antibody will not distinguish Rad6A and Rad6B proteins. Hence in data from Western blot analysis and immunofluorescence analysis, we refer to it as Rad6 rather than Rad6A or Rad6B.

Cell lines and culture
MDA-MB-231 breast cancer and nontransformed MCF10A cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) or DMEM/F12 as previously described (12). Both cell lines were tested and authenticated by ATCC by short tandem repeat DNA profiling. Cells were initially grown and multiple aliquots were cryopreserved. After resuscitation, cells were used within 10–15 passages or 3 months.

Cell survival assay
MDA-MB-231 cells were seeded at 5–10 × 10^3 cells/well in 96-well dishes, and treated with TZ analogs (or dimethyl sulfoxide vehicle, 0.01% v/v) at 0.5–100 μmol/L in triplicate. Cell viability was assessed at 72 hours by MTT assay.

Cell-cycle and aneuploidy analysis
MDA-MB-231 cells were seeded on 8-well chamber slides and treated 48 hours with vehicle, 10 μmol/L SMI #8 or TZ #15, or with 5 μmol/L SMI #9 for 8–48 hours. Slides were stained with acridine orange/ethidium bromide and at least 6 fields were scored for dye uptake by fluorescence microscopy (22).

Clonogenic assay
MDA-MB-231 cells treated 24 hours with vehicle or 10 μmol/L SMI #8 or 9 were trypsinized, and 100–200 viable cells replated/well in 12-well plates in triplicate. Cultures were fixed with buffered formalin, stained with crystal violet, and colonies containing more than 50 cells scored to compare survival of control versus SMI-treated cells.

Ubiquitin-conjugating activity assay
Histone H2A ubiquitination assays were conducted at room temperature for 1 hour with histone H2A (2.5 μg; Roche Biotech), ubiquitin-activating enzyme E1 (50 μg/mL, BioMol), recombinant human Rad6B (85 μg/mL), ubiquitin (1.25 mg/mL; Roche), 2 mmol/L MgCl2, 4 mmol/L ATP, and energy regeneration system (Boston Biochem) in reaction buffer (50 mmol/L Tris-HCl, pH 7.5; ref. 14). To assess TZ compound effects on Rad6B-mediated ubiquitination, reactions containing Rad6B were preincubated with the compounds (25 mmol/L) or vehicle for 1 hour before adding ubiquitin and histone H2A. Reaction products were separated by SDS-PAGE and analyzed by immunoblotting with anti-ubiquitin antibody.

Immunoblot analysis
MDA-MB-231 cells were treated with 0.5–10 μmol/L of SMIs #8 or 9 for 24 hours, and lysates (25 μg protein) subjected to immunoblot analysis of Rad6, PCNA (Dako), β-catenin (Santa Cruz), α-tubulin (Sigma), and β-actin (Sigma).

Immunofluorescence staining
MDA-MB-231 cells treated with vehicle, SMIs #8 or 9 for 24 hours were fixed with methanol/aceton and incubated with Rad6 and β-catenin antibodies followed by fluorescein isothiocyanate (FITC)- and Texas Red-conjugated rabbit and mouse secondary antibodies, respectively. Cyclin B1 and α-tubulin staining were conducted on synchronized MDA-MB-231 cells. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Slides were stained without primary antibody or with isotype-matched nonimmune immunoglobulin G to assess nonspecific reactions. Images were collected on an Olympus BX60 microscope equipped with a Sony high-resolution/sensitivity camera.

Identification of Functional Rad6 Inhibitors

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Chemotaxis assay

Chemotaxis assays were conducted in Boyden chambers containing 8 μm pore-size polycarbonate membrane. MDA-MB-231 cells were incubated with vehicle, 25 μmol/L of TZs, or 1–100 μmol/L of SMIs #8 or 9 for 1 hour at 37°C and 5% CO₂. Cells were rinsed with serum-free media and 50 × 10⁵ cells were placed in the upper chamber, and 100 μL of serum-free medium containing 100 μg/mL Matrigel (BD Biosciences) was placed in the lower chamber. Cells were incubated for 6 hours at 37°C and 5% CO₂, and migrated cells were fixed, stained with Protocol Hema 3 stain set (Fisher Scientific), and quantitated with NIH Image Version 1.62. Assays were conducted in triplicate.

Molecular docking

To assess interactions between TZs and Rad6B, molecular docking was carried out using PyMol software. The human Rad6B protein X-ray structure (2YB6) was used to estimate binding affinities and TZ compound orientation to the Rad6B active site (24). The average relative mean square deviation (RMSD) between X-ray (2YB6; ref. 24) and NMR (2Y4W; ref. 25) structures is 2.054, and the RMSD at the active site is 1.918. Analogs showing minimum docking score and binding energy were identified as they are predicted to bind favorably to Rad6B.

Results

TZ compound synthesis

Target TZ compounds were synthesized in 3 steps (Fig. 1). Reaction of the appropriate (substituted) aniline (Fig. 1, 1a–f) with dicyandiamide (2) under acidic conditions produced the corresponding biguanide (Fig. 1, 3a–f) as the hydrochloride salt (46%–84% yield) that was used in the next step without further purification. Condensation of the biguanide with ethyl glycolate (4) in a solution of sodium ethoxide in ethanol gave rise to a mixture of products from which the hydrochloride (4) in a solution of sodium ethoxide in ethanol was purified by column chromatography (45%–85% yield). Compound 5a (TZ #15; R = H) was retained for anticancer evaluation. Esterification of the (4-amino-6-(phenylamino)-[1,3,5]triazin-2-yl) methanol compounds using substituted benzoyl chlorides gave the final TZs #1–11 following column chromatography (45%–85% yield). Product TZ #6, containing the benzoxoxy protecting group, was deprotected by hydrogenation to yield TZ #13 (33% yield). TZs #4, 12, and 14 are missing because of failed synthesis or very low quantities/impure products.

Identification of SMI analogs with Rad6B inhibitory activity

TZs with Rad6B inhibitory activity were identified by in vitro ubiquitination assays with recombinant human Rad6B and histone H2A in the presence of TZ compounds. Because histones H2A and H2B are Rad6A and Rad6B ubiquitination targets (26), they are ideal substrates for screening compounds with Rad6 inhibitory activity. Inhibition of Rad6 ubiquitin-conjugating activity was evaluated by effects on Rad6B-ubiquitin thioester formation (Rad6B–Ub) and ubiquitin transfer to histone H2A (Ub-H2A). In control reactions, strong ubiquitination of histone H2A was observed. Rad6B-ubiquitin thioester levels, however, were negligible, suggesting robust ubiquitin transfer to substrate. Reactions including TZs #1, 2, 8, or 9 (Fig. 2A) showed reduced Rad6B-ubiquitin thioester formation and approximately 35% to 50% decrease in histone H2A ubiquitination compared with control (Fig. 2A). These data suggest inhibition of substrate ubiquitination potentially resulted from these compounds interfering with Rad6B-ubiquitin thioester formation. TZs #3 and 5 caused modest inhibition (~30%), although TZs #6, 7, 10, 11, 13, and 15 may be considered as Rad6B activators as strong bands corresponding to both Rad6B-ubiquitin thioester and ubiquitinated histone H2A were detectable (Fig. 2A). Specificity of SMI #9 for Rad6B was tested in ubiquitination assays using recombinant human UbcH5 (an E2 sharing 37% identity with Rad6B), SMI #9, and UbcH5 ubiquitination substrate BCA2 E3 ligase (23). The robust ubiquitination of BCA2 by UbcH5 was unaffected by Rad6B SMI #9 (Fig. 2B). Furthermore, Rad6E E2 activity does not substitute for UbcH5 in BCA2 ubiquitination, verifying the selectivity of Rad6B and SMI #9 (Fig. 2B).

Molecular docking to Rad6B

In silico molecular docking analysis of intermolecular interactions between TZs and Rad6B indicated SMIs #8 and 9 have the highest geometric shape complementarity scores. On the basis of the Rad6B X-ray structure, the Rad6B active site topology suggests SMIs #8 and 9 form noncovalent interactions with Thr69 (in β4 region of Rad6B), Asp90 and Glu93 (adjacent to the Rad6B catalytic site Cys), and Asn119 and Ala122 (in the connecting region between helices α2 and α3; Fig. 2C–E). The validity of these predictions is consistent with our experimental data showing TZs #8 and 9 possess Rad6B ubiquitin conjugation inhibitory activity (Fig. 2A). Consistent with noncomplementary activities of Rad6B and UbcH5 in BCA2 ubiquitination (Fig. 2B), UbcH5 shares only one (Asp90) of 5 noncovalent interaction sites proposed for SMIs #8 and 9 interaction with Rad6B.

Rad6B SMI #9 inhibits MDA-MB-231 cell proliferation and migration

Effects of Rad6B inhibitors on nontransformed MCF10A and metastatic MDA-MB-231 cell proliferation were examined. MCF10A cells express low Rad6B levels unless exposed to DNA-damaging agents whereas MDA-MB-231 cells overexpress Rad6B (12). Among the TZs tested (#1, 2, 8, 9, and 15), only SMIs #8 and 9 inhibited MDA-MB-231 cell proliferation (Fig. 3A and data not shown). SMI #9 inhibited MDA-MB-231 cell
proliferation more robustly (IC₅₀ ~6 μmol/L) than its structurally related SMI #8 analog (IC₅₀ ~25 μmol/L; Fig. 3A). The reduced inhibitory activity of SMI #8 compared with SMI #9 appears to result from poor solubility of SMI #8 as traces of precipitation began appearing at 10 μmol/L concentration. The bulk of MDA-MB-231 cells treated with 10 μmol/L or more SMI #9 displayed a round morphology compared with controls and less than 5 μmol/L doses of SMI #9, whereas treatment with up to 50 μmol/L SMI #8 did not produce pronounced morphologic changes of surviving cells compared with control (Fig. 3B and data not shown). MCF10A cell proliferation was unaffected by SMIs #8 and 9 (Fig. 3C).

We next tested whether TZ compounds affect migration of MDA-MB-231 cells as they migrate robustly. Compared with cells treated with vehicle or other TZs, only SMI #9 potently inhibited MDA-MB-231 cell migration (P < 0.001; Fig. 3D top panel). Simultaneous comparison of SMIs #8 and 9 confirmed SMI #9 inhibits Matrigel-induced migration of MDA-MB-231 cells in a dose-dependent manner compared with SMI #8 (Fig. 3D bottom panel).

**Effect of Rad6 inhibitors on cell survival**

Because MTT assays (Fig. 3A) showed growth inhibitory effects with SMIs #8 and 9, we tested whether inhibition resulted from a cytostatic or cytotoxic response. Cells undergoing apoptosis/necrosis were detected by differential uptake of the fluorescent DNA-binding dyes acridine orange and ethidium bromide. Consistent with MTT data (Fig. 3A), 5 μmol/L SMI #9 treatment triggered morphologic changes consistent with apoptosis in a time-dependent manner. Early apoptosis marked by intercalated acridine orange within fragmented DNA (22) was observed at 8 hours of SMI #9 treatment. Late stages of apoptosis such as apoptotic body separation and presence of reddish-orange color because of binding of acridine orange to fragmented DNA (23) were observed at
24 and 48 hours of SMI #9 treatment (Fig. 4A). Vehicle or 10 μmol/L TZ #15-treated cells were unaffected as more than 98% showed a green intact nuclear structure. A small proportion of SMI #8-treated cells exhibited nuclear ethidium bromide staining (~8.5%), whereas most showed extranuclear ethidium bromide staining (Fig. 4A).

Clonogenic assays were conducted to determine whether SMIs #8 and 9 compromised cell reproductive capacity. Colony-forming efficiencies of MDA-MB-231 cells treated with SMIs #8 or 9 were 44.4% and 3.7%, respectively, of vehicle controls (Fig. 4C). Colonies formed from SMI #9-treated cells appeared to also be defective in migration as dividing cells were long-drawnout and not completely physically separated (Fig. 4B, compare magnified images of colonies from vehicle and SMI #9).

SMI #9 delays cell-cycle progression
To examine if SMI #9 growth inhibitory effects result from cell-cycle arrest, synchronized MDA-MB-231 cells were treated with vehicle or 0.1–5 μmol/L SMIs #8 or
9, and analyzed at 24–72 hours posttreatment. Compared with vehicle, similar proportions of cells treated with 0.1–5 μmol/L of SMI #8 and 0.1–1 μmol/L of SMI #9 were in G0–G1, G2–M, and S-phases. However, 5 μmol/L SMI #9 treatment of MDA-MB-231 cells for 24 hours increased the proportion of G2–M-arrested cells by 2-fold and was accompanied by a proportional decrease in S-phase cells (Fig. 5A, left panel). SMI #9 treatment for 24 hours significantly increased the ratio of cells in G2–M to S-phase (0.665/0.005) compared with vehicle (0.295/0.06; P = 0.0282), whereas differences in ratios of G2–M to G1 between vehicle (0.255/0.05) and SMI #9 (0.46/0.07) were not significant (P = 0.147; Fig. 5A, right panel). At later time points, as cells became unsynchronized, differences between SMI #9 and control cells diminished (G2–M to S ratio of control vs. SMI #9: 0.39 vs. 0.66 at 48 hours; G2–M to S ratio of control vs. SMI #9: 0.52 vs. 0.54 at 72 hours). These data are consistent with MTT assays (Fig. 3A), and agree with our previous data showing maximal Rad6 expression in late G2 to S-phase, which decreases as cells reenter G1 (27).

To analyze SMI #9 effects in G2–M arrest, synchronized MDA-MB-231 cells treated 24 hours with vehicle or SMI #9 were immunostained with cyclin B1 or α-tubulin antibodies. Persistence of cytoplasmic/nuclear cyclin B1 is indicative of G2–M arrest (28–30). Compared with vehicle, SMI #9 significantly increased the percentage of cells with cytoplasmic/nuclear cyclin B1 staining (18.68 ± 1.19, vehicle vs. 31.48 ± 2.52, SMI #9; P = 0.0101). The persistence of cyclin B1 in SMI #9-treated cells suggests that these cells are arrested in G2–M (confirming data in Fig. 5A), and are unable to exit mitosis as they contain pleiomorphic/multiple nuclei (Fig. 5B, compare panels b, d with a, c). α-Tubulin staining confirmed these results. In control cells, α-tubulin staining is localized toward the poles of dividing cells, whereas in SMI #9-treated cells, it is intensely present at the dividing plane of late mitotic cells (Fig. 5B, compare panels b, d with a, c). α-Tubulin staining confirmed these results. In control cells, α-tubulin staining is localized toward the poles of dividing cells, whereas in SMI #9-treated cells, it is intensely present at the dividing plane of late mitotic cells (Fig. 5B, compare panels b, d with a, c). α-Tubulin polymers in late mitotic SMI #9-treated cells is consistent with their inability to normally exit mitosis. Consistent with these observations, flow cytometry analysis revealed increases in aneuploid populations in SMI #9-treated (12.93% and 19.49% at 24 hours and 48 hours, respectively) cells compared with vehicle (2.02% and 9.13% at 24 hours and 48 hours, respectively).
Next we verified whether SMIs #8 or 9 inhibited endogenous Rad6 enzymatic activity. Aliquots of lysates of MDA-MB-231 cells treated with vehicle, SMI #8 or 9 were immunoprecipitated with Rad6 antibody and used as a source of ubiquitin-conjugating enzyme for in vitro histone H2A ubiquitination. Compared with strong histone H2A ubiquitination in vehicle-treated cells, H2A ubiquitination was inhibited in SMI #8- and 9-treated cells but more potently by SMI #9 (Fig. 5C, top panel). Immunoblot analysis indicated that Rad6 protein levels in MDA-MB-231 cells were unaffected by these compounds, suggesting SMIs #8 and 9 possess Rad6 inhibitory activity but have little effect on Rad6 protein steady-state levels (Fig. 5C, bottom panel). Consistent with SMI #9 inhibition of MDA-MB-231 cell proliferation (Fig. 3A), PCNA protein levels were decreased in SMI #9-treated cells compared with vehicle or SMI #8-treated cells (Fig. 5C, bottom panel).

SMI #8 and 9 downregulate β-catenin

We previously showed Rad6B stabilizes β-catenin by K63-linked polyubiquitination that protects it from 26S proteasomal degradation (14). To assess SMIs #8 and 9 effects on β-catenin protein levels, immunoblot analysis was conducted on MDA-MB-231 cells treated with various SMIs #8 or 9 doses. Both compounds decreased high molecular weight polyubiquitinated (14) and nascent β-catenin protein levels in a dose-dependent manner compared with vehicle (Fig. 5D, right panel). The β-catenin protein level decrease was verified by immunofluorescence staining of Rad6 and β-catenin. Whereas control MDA-MB-231 cells showed strong Rad6 and β-catenin staining colocalizing to perinuclear compartments, SMIs #8 or 9 treatments dramatically reduced β-catenin staining as visualized by reduced merged Rad6/β-catenin yellow fluorescence (Fig. 5D, left panel). Consistent with
immunoblotting data in Fig. 5C, SMIs #8 and 9 minimally affected Rad6 staining. These data show that decreased β-catenin levels caused by SMIs #8 and 9 are consistent with their Rad6 inhibitory activities (Fig. 5C, top panel), and provide further support for their Rad6 inhibitory function.

Discussion

The data presented here describe novel SMIs of the postreplication repair or translesion synthesis (TLS) DNA repair E2 ubiquitin-conjugating enzyme Rad6B. Only one E2 enzyme SMI has been described (31), and the SMIs described here are the first that interfere with an E2 enzyme catalytic site. SMIs #8 and 9 both inhibit Rad6B-ubiquitin thioester formation and ubiquitination of its substrate histone H2A in vitro and in reactions using extracts of MDA-MB-231 breast cancer cells treated with the SMIs. SMI #9 treatment of MDA-MB-231 cells delayed cell-cycle progression, inhibited cell survival, and compromised the reproductive capacity of MDA-MB-231 cells as indicated by reduced frequency and size of colonies formed in clonogenic assays. Consistent with Rad6 function in postreplication DNA repair, Rad6 protein is maximally expressed during late G2 to S (27). In this context it is interesting to note that Rad6B SMI #9 treatment induces cell-cycle arrest in the G2–M phase. Our data from cyclin B1 staining showed that SMI #9 treatment induces G2–M arrest; these cells enter into aberrant M-phase as the aneuploid population proportion increases upon SMI #9 treatment compared with controls. We have previously shown that Rad6 is associated with centrosomes at all cell cycle phases, and constitutive Rad6B overexpression induces centrosome amplification, abnormal mitosis, and aneuploidy (12). Staining with α-tubulin antibody showed tubulin polymer retention in late mitotic SMI #9-treated cells, leading us to speculate that SMI #9-induced cell death is caused by G2 arrest and aberrant but incomplete mitosis. It remains to be determined if the impaired microtubule dynamics induced by SMI #9 are caused by loss of Rad6B function at the centrosomes.

In addition to its essential function in TLS, Rad6B also regulates β-catenin levels by inducing K63-linked proteasome-insensitive ubiquitination (14). Rad6B is also a β-catenin transcriptional target setting off a positive feedback loop between Rad6B expression and β-catenin levels in breast cancer cells (32). SMI #8 or 9 inhibition of Rad6B ubiquitin-conjugating activity results in decreased β-catenin protein levels, further demonstrating that SMIs #8 and 9 target the Rad6 ubiquitin-conjugating catalytic site. The decrease in β-catenin protein levels by SMIs #8 and 9 confirms our previous observations of inhibition of β-catenin ubiquitination by Rad6B silencing (14). PCNA steady-state levels are decreased in SMI #9-treated cells. Although this decrease may reflect a general drop in proliferation index of SMI #9-treated cells, it could also be attributed to SMI #9 inhibition of Rad6B-mediated K63-linked PCNA polyubiquitination (16, 17). The mechanism by which Rad6 SMIs inhibit cell migration is unclear, as Rad6 is not known to directly regulate cell migration. The Wnt/β-catenin pathway regulates transcription of several proteins involved in migration, including β1-integrin (33) and Snail (34). Our previous study showed that Rad6B-mediated stabilization of β-catenin requires intact Wnt signaling as β-catenin ubiquitination was impaired in Rad6B overexpressing MDA-MB-231 subpopulations in which Wnt signaling was disrupted with a dominant negative LRP6 mutant (15). It is possible that SMI #9 inhibition of cell migration results from inhibition of β-catenin transcriptional activity and expression of Wnt/β-catenin transcriptional targets.

Molecular modeling indicates that SMIs #8 and 9 make multiple noncovalent interactions with amino acids near the ubiquitin acceptor Cys88 (Asp90 and Gln93) and other amino acids in the pocket around the catalytic site (Thr69, Asn119, Ala122). Although the Rad6A and Rad6B paralogs share all of these amino acids, no other E2 family member has more than 2 identical or conservative charge-preserving substitutions at these 5 amino acids (Fig. 6). This suggests that SMIs #8 and 9 would be selective for Rad6A and Rad6B. Consistent with this observation, SMI #9 did not inhibit ubiquitination of BCA2 by UbcH5B (also known as UBE2D2; Fig. 2B), which only shows Asp90 with Rad6A and Rad6B and has differently charged amino acids in place of Thr69 (Lys) and Asn119 (Asp). The involvement of amino acid residues predicted to interact with SMIs #8 and 9 awaits confirmation by 3-D QASAR and site-directed mutagenesis studies.

As noted above, only one other SMI of an ubiquitin E2 (CDC34a also known as UBE2R1) has been described (31). This SMI acts by an allosteric mechanism different from that described for the Rad6 SMIs in this article. The CDC34 inhibitor does not interfere with CDC34 interaction with its E1 and E3, and only weakly inhibits CDC34-ubiquitin thioester formation. Rather a subtle conformational change initiated by SMI binding appears to inhibit ubiquitin transfer from CDC34 to the substrate (31). Molecular modeling predicts that the Rad6 SMIs may block Rad6-ubiquitin thioester formation by occupying the pocket surrounding the Rad6 catalytic cysteine residue. This prediction is consistent with our experimental data showing that SMIs #8 and 9 inhibit Rad6B activity by inhibiting Rad6B-ubiquitin thioester formation and that the decrease in ubiquitinated-histone H2A levels results from this inhibition. For both the Rad6B SMIs described here and the CDC34 inhibitors described by Ceccarelli and colleagues (31), it may be possible to use these SMI structures as a starting point for designing other E2-specific SMIs.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M.A. Sanders, G. Brahemi, A.D. Westwell, M.P. Shekhar
Development of methodology: M.A. Sanders, G. Brahemi, M. Morelli, H. Kothayer, A.D. Westwell, M.P. Shekhar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.A. Sanders, G. Brahemi, P. Nangia-Makker, H. Kothayer, M.P. Shekhar
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.A. Sanders, G. Brahemi, V. Balan, H. Kothayer, A.D. Westwell, M.P. Shekhar
Writing, review, and/or revision of the manuscript: M.A. Sanders, P. Nangia-Makker, V. Balan, A.D. Westwell, M.P. Shekhar
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.P. Shekhar
Study supervision: A.D. Westwell, M.P. Shekhar

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Figure 6. Sequence alignment of Rad6B with representative E2 family members. Asterisks indicate Rad6B noncovalent interaction sites with SMIs 8 and 9, and those conserved in other E2s. Conserved amino acids in E2s are indicated by solid squares.
Molecular Cancer Therapeutics

Novel Inhibitors of Rad6 Ubiquitin Conjugating Enzyme: Design, Synthesis, Identification, and Functional Characterization

Matthew A. Sanders, Ghali Brahemi, Pratima Nangia-Makker, et al.


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