Novel inhibitors of Rad6 Ubiquitin Conjugating Enzyme: Design, synthesis, identification and functional characterization

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Abbreviations: TZ, triazine; SMI, small molecule inhibitor; E2, ubiquitin conjugating enzyme

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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 (E2s) mediate ubiquitination by selective interactions with ubiquitin activating (E1) and ubiquitin ligase (E3) enzymes, and thus selective E2 small molecule inhibitor (SMIs) 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. 12 triazine (TZ) analogs screened \textit{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. SMI\#9 inhibition of Rad6 was selective as BCA2 ubiquitination by E2 UbcH5 was unaffected by SMI\#9. SMI\#9 more potently inhibited proliferation, colony formation and migration than SMI\#8, and induced MDA–MB-231 breast cancer cell G2/M arrest and apoptosis. Ubiquitination assays using Rad6 immunoprecipitated from SMI\#8 or 9 treated cells confirmed inhibition of endogenous Rad6 activity. Consistent with our previous data showing Rad6B-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.
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 demonstrates E2s are important for dictating the final ubiquitinated product, i.e., 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 S. 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 overexpression in mouse and human breast cancer lines and tumors. Constitutive Rad6B overexpression in nontransformed human breast cells induces multinucleated cell formation, centrosome amplification, abnormal mitosis, aneuploidy, and transformation (12). Rad6
ubiquititates histones in the absence of E3 proteins (4,13). Similarly, Rad6B ubiquititates 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 PCNA mono- 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 proteosomal processing.

We report here synthesis, identification and functional characterization of the first known Rad6B small molecule inhibitors (SMIs). 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., Montreal), described in detail elsewhere (18). Briefly, an E2-ubiquitin consensus-binding site was built based on the 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 four 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 diaminotriazine (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 (Swansea, U.K.). 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% purity as determined by spectroscopic and combustion analyses (±0.4% CHN tested in duplicate).

**TZ compound synthesis.** The TZ compounds synthesis scheme (Fig. 1) involved three steps using commercially available starting materials. Step 1 involved arylbiguanide synthesis (3a-f; (20)). Step 2 involved (4-amino-6-(phenylamino)-[1,3,5]triazin-2-ylmethanol synthesis (5a-f; (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-yl)methyl 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 demonstrated that the Rad6B gene is overexpressed in breast cancer by transcript sequencing and Rad6B-specific shRNA transfections (12,14). However, since 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 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 ATCC and maintained in DMEM or DMEM/F12 as previously described (12). Both cell lines were tested and authenticated by ATCC by STR DNA profiling. Cells were initially grown and multiple aliquots were cryopreserved. After resuscitation, cells were used within 10-15 passages or three months.

**Cell survival assay.** MDA-MB-231 or MCF10A cells were seeded at 5-10x10^3 cells/well in 96-well dishes, and treated with TZ analogs (or DMSO vehicle, 0.01% v/v) at 0.5–100 μM in triplicate. Cell viability was assessed at 72 h by MTT assay.

**Acridine orange/ethidium bromide staining.** MDA-MB-231 cells (10x10^3) were seeded on 8-well chamber slides and treated 48 h with vehicle, 10 μM SMI#8 or TZ#15, or with 5 μM SMI#9 for 8-48 h. Slides were stained with acridine orange/ethidium bromide and at least six fields were scored for dye uptake by fluorescence microscopy (22).

**Clonogenic assay.** MDA-MB-231 cells treated 24 h with vehicle or 10 μM SMIs#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 >50 cells scored to compare survival of control vs. SMI-treated cells.

**Cell cycle and aneuploidy analysis.** MDA-MB-231 cells synchronized by culturing in serum-free medium for 48–72 h were replated in complete medium. 8 h later, cells were treated with
SMIs#8 or 9, or vehicle. Cells were harvested at 24-72 h post-treatment, and analyzed by flow cytometry (Becton Dickinson).

**Immunoblot analysis.** MDA-MB-231 cells were treated with 0.5-10 μM of SMIs#8 or 9 or vehicle for 24 h, 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 h were fixed with methanol/acetone and incubated with Rad6 and β-catenin antibodies followed by FITC- and Texas Red-conjugated rabbit and mouse secondary antibodies, respectively. Cyclin B1 and α-tubulin staining were performed on synchronized MDA-MB-231 cells. Nuclei were counterstained with DAPI. Slides were stained without primary antibody or with isotype-matched nonimmune IgG to assess nonspecific reactions. Images were collected on an Olympus BX60 microscope equipped with a Sony high resolution/sensitivity camera.

**Ubiquitin conjugating activity assay.** Histone H2A ubiquitination assays were performed at room temperature for 1 h 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 mM MgCl₂, 4 mM ATP and energy regeneration system (Boston Biochem) in reaction buffer (50 mM Tris-HCl, pH 7.5) (14). To assess TZ compound effects on Rad6B-mediated ubiquitination, reactions containing Rad6B were preincubated with the compounds (25 nM) or vehicle for 1 h prior to adding ubiquitin and histone H2A. Reaction products were separated by SDS-PAGE and analyzed by immunoblotting with anti-ubiquitin antibody. To assess the functional impact of SMIs#8 or 9 on endogenous Rad6 activity, lysates (50 μg protein) of vehicle, SMIs#8 or 9-treated MDA-MB-231 cells were immunoprecipitated with Rad6 antibody.
immobilized on Protein A/G Sepharose. Pellets were washed and used as Rad6 source for
histone H2A ubiquitination. To assess Rad6 SMI effects on UbcH5-mediated BCA2
ubiquitination, ubiquitination assays were performed with extracts of COS7 cells transiently
transfected with GST-tagged BCA2 (23) or control vector (generous gifts from Dr. Arun Seth,
Sunnybrook Research Institute and University of Toronto) in the presence or absence of 25 nM
Rad6 SMI#9. To determine if Rad6B can substitute for UbcH5, similar assays were performed
with recombinant human Rad6B.

**Chemotaxis assay.** Chemotaxis assays were performed in Boyden chambers containing 8 μm
pore-size polycarbonate membrane. MDA-MB-231 cells were incubated with vehicle, 25 μM of
TZs, or 1-100 μM of SMIs#8 or 9 for 1 h at 37°C and 5% CO₂. Cells were rinsed with serum-
free media and 50×10³ 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 h 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
performed 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 RMSD between X-ray (2YB6) (24) and NMR (2Y4W) (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 three steps (Fig. 1). Reaction of the appropriate (substituted) aniline (1a-f) with dicyandiamide (2) under acidic conditions produced the corresponding biguanide (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 required (4-amino-6-(phenylamino)-[1,3,5]triazin-2-yl)methanol (5a-f) was purified by column chromatography (14-37% 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 benzyloxy protecting group, was deprotected by hydrogenation to yield TZ#13 (33% yield). TZs#4, 12, and 14 are missing due to 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. Since 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 ~35-50% decrease in histone H2A ubiquitination compared to 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%), while 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). Further, Rad6B 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. Based on 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 five 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 μM) than its structurally related SMI#8 analog (IC50 ~25 μM; Fig. 3A). The reduced inhibitory activity of SMI#8 compared to SMI#9 appears to result from poor solubility of SMI#8 as traces of precipitation began appearing at 10 μM concentration. The bulk of MDA-MB-231 cells treated with ≥10 μM SMI#9 displayed a round morphology compared to controls and <5 μM doses of SMI#9, whereas treatment with up to 50 μM SMI#8 did not produce pronounced morphological changes of surviving cells compared to control (Figure 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 to 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 to SMI#8 (Fig. 3D bottom panel).

**Effect of Rad6 inhibitors on cell survival.** Since 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 μM SMI#9 treatment triggered morphological changes consistent with apoptosis in a time-dependent manner. Early apoptosis marked by intercalated acridine orange within fragmented DNA (22) was observed at 8 h of SMI#9 treatment. Late stages of apoptosis such as apoptotic body separation and presence of reddish-orange color due to binding of acridine orange to fragmented DNA (23) were observed at 24 and 48 h of SMI#9 treatment (Fig.
4A). Vehicle or 10 μM TZ#15 treated cells were unaffected as >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 performed 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-drawn-out 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 μM SMIs#8 or 9, and analyzed at 24 to 72 h post-treatment. Compared to vehicle, similar proportions of cells treated with 0.1–5 µM of SMI#8 and 0.1-1 µM of SMI#9 were in G0/G1, S and G2/M phases. However, 5 µM SMI#9 treatment of MDA-MB-231 cells for 24 h 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 h significantly increased the ratio of cells in G2/M to S phase (0.665 ± 0.005) compared to 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 h; G2/M to S ratio of control vs. SMI #9: 0.52 vs. 0.54 at
72 h). These data are consistent with MTT assays (Fig. 3A), and agree with our previous data showing maximal Rad6 expression in late S/G2 phase which decreases as cells reenter G1 (27).

To analyze SMI#9 effects in G2/M arrest, synchronized MDA-MB-231 cells treated 24 h 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 to 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 towards the poles of dividing cells, whereas in SMI#9-treated cells it is intensely present at the dividing plane of late mitotic cells (Fig. 5C, compare cells indicated by arrows in e with those in f and g). Persistence of 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 and 48 h, respectively) cells compared to vehicle (2.02 and 9.13% at 24 and 48 h, 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 to 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, upper 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 to vehicle or SMI#8 treated cells (Fig. 5C, bottom panel).

**SMI#8 and 9 downregulate β-catenin.** We previously demonstrated 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 performed 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 to 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, upper 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 utilizing 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 S/G2 (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 to 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. While 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). While the Rad6A and Rad6B paralogs share all of these amino acids, no other E2 family member has more than two identical or conservative charge-preserving substitutions at these five 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 (a.k.a. UBE2D2; Fig. 2B), which only shares Asp90 with Rad6A and Rad6B and has differently charged amino acids in place of Thr 69 (Lys) and Asn 119 (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 a.k.a. UBE2R1) has been described (31). This SMI acts by an allosteric mechanism different from that described for the Rad6 SMIs in this paper. 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 et al (31), it may be possible to use these SMI structures as a starting point for designing other E2 specific SMIs.

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References


Figure Legends

Figure 1. Schematic illustration of synthesis of substituted (4-amino-6-(phenylamino)-1,3,5-triazin-2-yl)methyl benzoates.

Figure 2. A. Identification of TZs with Rad6 inhibitory activity. In vitro histone H2A ubiquitination assays were performed as described in Methods. Rad6B-ubiquitin thioester (Rad6B~Ub) and monoubiquitinated histone H2A (Ub-H2A) were detected by ubiquitin antibody. B. In vitro BCA2 ubiquitination assays were performed with UbcH5 or Rad6B, with and without SMI#9. BCA2 and ubiquitinated BCA2 (Ub-BCA2) were detected by BCA2 antibody. Control vector- and BCA2-transfected COS7 extracts are included for identification of expressed BCA2. C and D. Docking model of SMIs#8 and 9, respectively, with human Rad6B showing interactions with the indicated amino acids surrounding the catalytic site Cys88. Predefined color codes of PyMol were used. E. Rad6 binding sites of SMIs#8 and 9.

Figure 3. Rad6 SMIs inhibit MDA-MB-231 cell proliferation and migration. A and C. MTT assays with MDA-MB-231 (A) or nontransformed MCF10A (C) cells. B. Phase contrast micrographs of MDA-MB-231 cells. Magnification X10. D. Migration of MDA-MB-231 cells measured in presence of the indicated TZs (top panel) or doses of SMIs#8 or 9 (bottom panel).

Figure 4. SMI#9 treatment induces MDA-MB-231 cell death. A. Acridine orange/ethidium bromide staining of cells treated with vehicle, 15 μM cisplatin (CDDP), 10 μM SMI#8 or TZ#15, or 5 μM SMI#9. Magnification X40 (SMI#9), X20 (all others). B. Colony survival assays. Representative wells and magnified images of colonies are shown. C. Graphic representation of colony forming frequencies.
Figure 5. Rad6 SMI#9 induces MDA-MB-231 G2/M-arrest and decreases β-catenin protein levels. A. Cell cycle measurements at the indicated SMI#8 or 9 concentrations (left panel) or 10 μM SMI#8 or 9 (right panel). Results are mean ± S.E. from two independent experiments. B. Immunofluorescence (red) staining of cyclin B1 (a-d) and α-tubulin (e-g). Arrows in d indicate cells with pleiomorphic or multiple nuclei; arrows in f,g show retention of α-tubulin polymers at the dividing plane of SMI#9 treated cells compared to their absence (arrows in e) in vehicle controls. Magnification X20 (a,b,f), X40 (c,d), X100 (e), X200 (g). C (upper panel), analysis of endogenous Rad6 activity in MDA-MB-231 cells (see Methods); ubiquitinated (Ub)-histone H2A (arrow). Bottom panel, immunoblot analysis of Rad6 and PCNA. D. Immunoblot analysis of β-catenin (right panel), and immunofluorescence staining (left panel) of Rad6 (FITC) and β-catenin (Texas Red) in MDA-MB-231 cells. Magnification X40.

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 in upper case. E2 catalytic site cysteines are indicated by solid squares.
Reagents and conditions: (i) 3M HCl (aq), 90 °C, 5h; (ii) NaOEt, EtOH, reflux, 3h; (iii) NEt3, CH2Cl2, reflux, 12h; (iv) H2 (g), Pd/C (10%), EtOH, 12h.

Fig. 1
Figure 2
Figure 3
Figure 4

A

B

C

Figure 4
Figure 6
Molecular Cancer Therapeutics

Novel inhibitors of Rad6 Ubiquitin Conjugating Enzyme: Design, synthesis, identification and functional characterization

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