In Vitro and In Vivo Antitumor and Anti-Inflammatory Capabilities of the Novel GSK3 and CDK9 Inhibitor ABC1183[§]

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Received October 18, 2017; accepted January 19, 2018

ABSTRACT

Glycogen synthase kinase-3s (GSK3 α and GSK3 β) are constitutively active protein kinases that target over 100 substrates, incorporate into numerous protein complexes, and regulate such vital cellular functions as proliferation, apoptosis, and inflammation. Cyclin-dependent kinase 9 (CDK9) regulates RNA production as a component of positive transcription elongation factor b and promotes expression of oncogenic and inflammatory genes. Simultaneous inhibition of these signaling nodes is a promising approach for drug discovery, although previous compounds exhibit limited selectivity and clinical efficacy. The novel diaminothiazole ABC1183 is a selective GSK3 α/β and

CDK9 inhibitor and is growth-inhibitory against a broad panel of cancer cell lines. ABC1183 treatment decreases cell survival through G2/M arrest and modulates oncogenic signaling through changes in GSK3, glycogen synthase, and β -catenin phosphorylation and MCL1 expression. Oral administration, which demonstrates no organ or hematologic toxicity, suppresses tumor growth and inflammation-driven gastrointestinal disease symptoms, owing in part to downregulation of tumor necrosis factor α and interleukin-6 proinflammatory cytokines. Therefore, ABC1183 is strategically poised to effectively mitigate multiple clinically relevant diseases.

Introduction

Glycogen synthase kinase (GSK3 α and GSK3 β) protein kinases regulate diverse cellular functions by incorporating into distinct intracellular complexes and targeting over 100 substrates (Beurel et al., 2015). GSK3 regulates proliferation, in part, through β -catenin (Ha et al., 2004) and c-Myc (Schild et al., 2009), and inflammation through nuclear factor (NF)- κ B-induced cytokine expression, such as tumor necrosis factor (TNF) α , interleukin (IL)-1 β , and IL-6 (Beurel et al., 2010). Consequently, excessive GSK3 signaling is implicated in several hyperproliferative and inflammatory diseases, including cancer and chronic intestinal inflammation (Hofmann et al., 2010; Walz et al., 2017). Efforts to inhibit GSK3 initially focused on suppressing neuroinflammation in neurologic diseases such as Alzheimer disease; however, interest in evaluating GSK3 inhibitors as anticancer drugs is growing (Duffy et al., 2014; Morales et al., 2014; Vincent et al., 2014; Beurel et al., 2015).

Post-translational modification of, and by, GSK3 dictates much of the enzymatic activity and downstream signaling output. GSK3 α/β Ser21/9 phosphorylation (pSer21/9) by upstream kinases, such as protein kinase B (Cross et al., 1995) and protein kinase A (Fang et al., 2000), is typically viewed as inhibitory since this results in self-substrate priming and occlusion of other primed substrates from GSK3 binding. In general, GSK3 substrates require priming by prior phosphorylation events that enable interaction with the GSK3 binding pocket (Beurel et al., 2015). Therefore, pSer21/9 is one mechanism to regulate activity and substrate selectivity of GSK3. GSK3 activity can also be monitored through downstream signaling. For example, GSK3 mediates serine phosphorylation of glycogen synthase (GS) (Rayasam et al., 2009) and β -catenin (Stamos and Weis, 2013) that influences activity or stability, respectively. In the case of β -catenin, only a small subset of total β -catenin is associated with the proteasomal destruction complex and does not affect membrane-bound pools. Overall, measuring multiple post-translational events can give a broad picture of GSK3 activity.

Another enzyme attracting interest as a target for anticancer drugs is cyclin-dependent kinase (CDK) 9 (Krystof et al., 2012; Morales and Giordano, 2016). In contrast with most CDKs that regulate the cell cycle, CDK9, in conjunction with cyclin T1, T2a, or T2b, forms the catalytic core of the positive transcription elongation factor b (P-TEFb), and thus regulates RNA production. CDK9 is elevated and provides oncogenic transcription profiles in several cancers owing, at least in part,

This work was supported by a grant from the Commonwealth of Pennsylvania, Department of Health, and by National Institutes of Health National Cancer Institute (Grant 5 P01 CA203628).

Portions of these data were presented at AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics: Discovery, Biology, and Clinical Applications, November 29–December 2, 2016; ICM–International Congress Center Munich, Munich, Germany.

<https://doi.org/10.1124/jpet.117.245738>.

[§] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: CDK, cyclin-dependent kinase; DAI, Disease Activity Index; DSS, dextran sulfate sodium; GS, glycogen synthase; GSK, glycogen synthase kinase; IBD, inflammatory bowel disease; IL, interleukin; LY, LY2090314; MPO, myeloperoxidase; NF, nuclear factor; PBS, phosphate-buffered saline; Pol2, polymerase 2; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

to its requirement for transcriptional activation of MYC-targeted genes, such as MCL1 (Lam et al., 2001; Gregory et al., 2015). CDK9 also promotes the expression of inflammatory cytokine genes (Brasier, 2008; Brasier et al., 2011) and COX-2 (Keum et al., 2013), and the extravasation of leukocytes into sites of inflammation (Berberich et al., 2011). Mechanistically, CDK9 signals through nuclear factor (NF)- κ B, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and extracellular signal-regulated kinases (Takada and Aggarwal, 2004; Haque et al., 2011). Interestingly, pNF- κ B is required for CDK9 binding to P-TEFb (Nowak et al., 2008; Fang et al., 2014), and so crosstalk between the GSK3 and CDK9 pathways is critical for inflammatory responses. Therefore, therapies inhibiting both enzymes could potentiate anti-tumor and anti-inflammation pathways to abrogate multiple diseases.

To date, dual GSK3/CDK9 inhibitors have demonstrated nonspecific CDK inhibition and shown limited clinical efficacy. Diaminotriazoles that inhibit various CDKs or microtubule assembly have been previously described as potent anticancer agents (Thomas et al., 2014; Vasudevan et al., 2015). Here we report the preclinical pharmacological analysis of ABC1183, an orally available novel selective GSK3 and CDK9 inhibitor. The analysis includes kinase inhibition selectivity, in vitro cytotoxicity, target modulation, in vivo antitumor response, and in vivo anti-inflammatory activity across multiple models.

Materials and Methods

Synthesis of ABC1183

4-(4-Amino-2-*p*-tolylamino-thiazole-5-carbonyl)-benzonitrile (ABC1183). At 0°C, a mixture of *p*-tolyl-isothiocyanate (0.15 g, 1.0 mmol) and cyanamide (0.042 g, 1.0 mmol) in solution of tetrahydrofuran/methanol (4:1, 5 ml) was treated with potassium *tert*-butoxide (0.124 g, 1.0 mmol) for 30 minutes. 4-Cyanophenacyl bromide (0.224 g, 1.0 mmol) was added, and the suspension was stirred for 12 hours. The solvent was removed, and the semisolid residue was triturated with water to give the crude product, which was filtered and dried in a vacuum oven at 50°C for 2 hours. The dried crude product was recrystallized from EtOAc/hexane (1:3), which provided 0.170 g (51%) of ABC1183 as a yellow solid powder with a melting point of 252–254°C, in methanol. Synthesis and batch-to-batch uniformity was confirmed through high-performance liquid chromatography, mass spectroscopy, and nuclear magnetic resonance.

In Vitro Kinase Assay

ABC1183 was screened by ThermoFisher Scientific SelectScreen services (Waltham, MA) at a single 10- μ M dose against a panel of 414 human kinases using ATP at Km [app]. All kinases were screened in duplicate and data were confirmed to meet stringent quality control standards for reproducibility. Kinases inhibited by greater than 60% were further confirmed through dose response IC₅₀ assays, carried out by ThermoFisher Scientific.

Cell Culture and Treatments

LNCAp human prostate cancer cells were maintained in improved minimum essential media (IMEM) supplemented with 5% fetal bovine serum (FBS) and supplemented with 2 mmol/l of L-glutamine. FaDu human squamous cell carcinoma, B-16 F10 murine melanoma, Pan02 murine pancreatic, Mia-Paca2 human pancreatic, BxPC3 human pancreatic, and SK-N-MC human neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All media was supplemented with 100 IU/ml penicillin-streptomycin.

ABC1183, LY2090314, and flavopiridol were dissolved in dimethyl sulfoxide (DMSO) and treated at indicated concentrations.

Sulforhodamine B Assay

To determine IC₅₀ values, cells were seeded in 96-well plates and 24 hours later treated with 0–100 μ M ABC1183 for 72 hours. Cell viability was determined by a standard sulforhodamine B assay as described previously (Schrecengost et al., 2015).

Flow Cytometry

Indicated cells were treated and seeded in hormone-proficient or -depleted conditions, as indicated, and labeled with BrdU (Invitrogen/Thermo Fisher Scientific) 2 hours prior to harvest. Cells were fixed in 100% ethanol, stained with FITC-conjugated anti-BrdU antibody (BD Biosciences, San Jose, CA), and processed using FACSCalibur (BD Biosciences).

Western Blot Analysis

Cell lysates were produced using buffer containing 25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS (Sigma Aldrich, St. Louis, MO). Total cell lysates were resolved by 10% or 4%–12% gradient SDS-PAGE, transferred to polyvinylidene fluoride, and immunoblotted with the following antibodies: phospho-GSK-3 α/β (Ser21/9), GSK-3 β , phospho-GS (Ser 641), GS, phospho- β -catenin (Ser33/37, Thr 41), β -catenin, MCL1 (Cell Signaling Technology, Danvers, MA), and HRP-GAPDH (GeneTex Inc., Irvine, CA).

Cellular Thermal Shift Assay

To determine physical engagement of ABC1183 with target proteins, cellular thermal shift assay (CETSA) was employed as previously described (Martinez Molina et al., 2013; Jafari et al., 2014). Preliminary experiments identified 55°C as the lowest thermal point for GSK3 α/β protein degradation. LNCAp cells were treated with ABC1183 or DMSO for the indicated times and trypsinized. One half of the cells were resuspended in PBS with protease inhibitors, then heated for 3 minutes at 55°C and the other half was not heated to serve as a loading control. If proteins are bound by ABC1183 they will be protected from degradation.

Animal Models

All procedures involving mice were performed in accordance with Pennsylvania State University IACUC protocols.

Tumor Models. Seven-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were subcutaneously injected in the flank as indicated in 100 μ l PBS or PBS/Geltrex (Life Technologies/Thermo Fisher Scientific). When tumors reached ~100–150 mm³ mice were administered 5 mg/kg ABC1183 or vehicle (50% PEG₄₀₀, 35% propandiol, 5% Tween 5% EtOH, 5% saline) by oral gavage five times per week. Body weight was monitored throughout and tumor volume was measured with calipers. Supernatants for the homogenates of each tumor were used to determine the levels of TNF α , IL-6, and IL-1 β using Luminex assays performed by the Cytokine Core Laboratory at the University of Maryland, Baltimore. Cytokine values were determined as picogram per milliliter and normalized on the basis of protein concentration to achieve picogram per milligram of tumor tissue.

Trinitrobenzene Sulfonic Acid-Induced Crohn's Disease.

To model inflammation-induced Crohn's disease, trinitrobenzene sulfonic acid (TNBS) was used to provoke disease onset as previously described (Maines et al., 2010). Briefly, male C57BL/6 mice were anesthetized, and a 0.1-ml solution of 50 μ g/g TNBS (Sigma Aldrich) in 50% ethanol/PBS was slowly administered through a stainless steel catheter carefully inserted into the rectum and advanced into the colon until tip was 4 cm proximal to the anus. To ensure retention, mice were inverted for 30 minutes. Colitis was induced by delivery of

TNBS on experimental days 0 and 7. Starting on day 6 and proceeding through day 9, animals were treated daily by oral gavage with either vehicle or 50 mg/kg ABC1183. On day 10, animals were killed by CO₂ asphyxiation and cervical dislocation, per institutional IACUC requirements, and the colons were removed. The colons were measured, weighed, and distal 3 cm was scored for macroscopic inflammation (macro score) as previously described (Fitzpatrick et al., 2000). Supernatants for the homogenates of each colon were used to determine the levels of TNF α , IL-6, and IL-10 using Luminex assays performed by the Cytokine Core Laboratory at the University of Maryland, Baltimore.

Dextran Sulfate Sodium–Induced Ulcerative Colitis. To test anti-inflammatory properties of ABC1183, the acute dextran sulfate sodium (DSS)–induced ulcerative colitis model was employed as previously described (Maines et al., 2008). Briefly, male C57BL/6 mice were divided into groups for vehicle-only treatment, DSS- (40,000 mol. wt.; MP Biomedicals, Santa Ana, CA)–plus-vehicle treatment, or DSS-plus-ABC1183 (50 mg/kg) treatments. DSS was administered as a 2% solution continuously in drinking water and vehicle/ABC1183 was administered once daily by oral gavage in a volume of 0.1 ml per dose. The Disease Activity Index, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was scored for each animal on days 4–6. Mucosal myeloperoxidase (MPO) activity was determined by assaying the middle one-third of the colon for MPO by quantifying the metabolism of tetramethylbenzidine as described by Fitzpatrick et al. (2000), as a measure of granulocyte and monocyte infiltration into the colon.

Statistics

All results were analyzed using the two-tailed Student's *t* test (adjusted for variance) or Mann-Whitney test. For all analyses, *P* < 0.05 was deemed significant.

Results

Specific Inhibition of GSK3 and CDK9 Activity. A novel diaminothiazole, ABC1183, was designed as described (Fig. 1A). To determine if ABC1183 functions as a selective kinase inhibitor, a panel of 414 human kinases was screened at a single 10- μ M dose, as described in *Materials and Methods*. ABC1183 modulated enzyme activities from –17% to 85%; however, only four targets, CDK9 (inactive), GSK3 α , GSK3 β , and CDK9/cyclinT1 were inhibited more than 60% (Fig. 1B). In fact, despite similarities between GSK3 and CDK ATP binding pockets, CDK2 was the next most inhibited CDK at 44% inhibition. IC₅₀ values for ABC1183 against GSK and CDK9 targets were determined to be 657 nM for GSK3 β , 327 nM for GSK3 α , and 321 nM for CDK9/cyclin T1 (Fig. 1C). Additionally, ABC1183 was identified as an ATP-competitive inhibitor for both GSK3 isoforms but a noncompetitive ATP inhibitor for CDK9/cyclinT1 (Supplemental Fig. S1).

To determine if ABC1183 physically engages with GSK3 in a whole-cell setting, CETSA were used. Incubation of cell lysates at 55°C resulted in protein degradation when unbound by ABC1183. However, within 15 minutes of incubation, ABC1183 was able to physically associate with GSK3 α and GSK3 β , which is sufficient to protect from degradation (Supplemental Fig. S2). On the basis of these data, we hypothesized that ABC1183 selectively inhibits GSK3 α , GSK3 β , and CDK9.

ABC1183 Inhibits Cell Growth and Abrogates Signaling. To determine if inhibition of GSK3 and CDK9 activity negatively influences cell proliferation, a panel of murine and human cancer cell lines were treated with increasing

Fig. 1. ABC1183 selectively inhibits GSK3 α/β and CDK9. (A) Structure of ABC1183. (B) Top, in vitro kinase screen with 10 μ M ABC1183 and graphical representation of inhibition. Bottom, four kinases with inhibition >60%. (C) Dose-response assay calculating IC₅₀ values of GSK3 α , GSK3 β , and CDK9.

concentrations of ABC1183, then analyzed for cytotoxicity. IC₅₀ cytotoxicity values, as determined by sulforhodamine B assay, ranged from 63 nM to 2.6 μ M and were similar to in vitro kinase inhibition values for GSK3 and CDK9 (Fig. 2A). GSK3 inhibitors have been reported to arrest cell cycle progression, whereas CDK9 inhibitors promote cell apoptosis

Fig. 2. ABC1183 exhibits cytotoxicity against a panel of murine and human cancer cells. (A) The indicated cell lines were seeded into 96-well plates at determined subconfluent concentrations and 24 hours later were treated with a dose response of ABC1183 for 72 hours. Cell survival was quantified by sulforhodamine B assay. IC₅₀ value for each cell line shown. (B) Pan02 cells were treated with dimethyl sulfoxide (DMSO) or 3 μM ABC1183 for 24 hours and cell cycle was analyzed by propidium iodide staining. Representative histogram plots are shown.

(Sonawane et al., 2016; Walz et al., 2017). Analysis of cell cycle phases following treatment demonstrated that, relative to control, ABC1183-treated cells had a significantly decreased number of cells in replicating G1 and S phases, and contained an increased number of cells in G2/M and sub-G1 cycle phases (Fig. 2B). This suggests that ABC1183 is disrupting cellular proliferation by blocking cell cycle progression and promoting cell death.

GSK3 α/β activity can be monitored through pSer21/9 levels, downstream GS pSer641, and β -catenin pSer33/37/Thr41. Likewise, MCL1 and RNA polymerase 2 (Pol2) are major downstream targets of CDK9 activity. To determine if ABC1183 can modulate GSK3 and CDK9 activity within a whole-cell environment, multiple cell lines were treated for 2–24 hours with ABC1183, or known GSK3 inhibitor LY2090314 (LY), and cellular signaling was queried. As shown in Fig. 3, ABC1183 treatment decreased GSK3 α/β pSer21/9 in a time-dependent fashion, although GSK3 β was more dramatically decreased compared with GSK3 α . Cell-specific differences were also observed, including transiently decreased GSK3 β phosphorylation in LNCaP cells (Fig. 3A, lane 2) and a rapid and persistent downregulation of GSK3 β phosphorylation in FaDu cells (Fig. 3C, lanes 1–4). Consistent with GSK3 inactivation, GS phosphorylation was also decreased in a cell-type-specific fashion. For example, in LNCaP and FaDu cells, ABC1183 most significantly decreased pSer641 GS 24 hours after treatment (Fig. 3, A and C) and to a similar extent as 24-hour LY treatment. Pan02 cells, by

comparison, exhibited a less robust inhibition of GS phosphorylation; however, LY treatment was likewise ineffective (Fig. 3B). β -catenin pSer33/37/Thr41, which targets the protein for proteasomal degradation (Stamos and Weis, 2013), was increased between 6 and 24 hours following ABC1183 treatment in all cell lines (Fig. 3; Supplementary Fig. S3). On the basis of these data, ABC1183 modulates GSK3 activity, which negatively regulates multiple downstream signaling events.

To address inhibition of CDK9, downstream targets were evaluated at protein and mRNA levels. MCL1 total protein levels were measured following ABC1183 treatment and determined to be decreased most discernibly in LNCaP and Pan02 cells after 24 hours (Fig. 3, A and B). There was also a robust decrease in Pol2 activity, measured by pSer5 Pol2, following ABC1183, which was observable to similar levels in cells treated with CDK9 inhibitor flavopiridol (Supplemental Fig. S4A). Furthermore, the CDK9-regulated gene *Hexim1* was detected to be significantly decreased at the mRNA level following 24 hours of ABC1183 treatment (Supplemental Fig. S4B). Overall, these in vitro and cell-based experiments demonstrate that ABC1183 specifically targets GSK3 α/β and CDK9, inhibiting cell proliferation and cell cycle progression and modulating intracellular signaling.

Anti-Tumor Effects of ABC1183. To extend the antiproliferative findings of ABC1183 treatment, several allogenic tumor models were employed to understand in vivo therapeutic response. First, murine melanoma B16 tumors were propagated in C57BL/6 mice. Following initial tumor growth,

Fig. 3. Multiple tumorigenic signaling pathways are inhibited by ABC1183. (A) LNCaP, (B) Pan02, (C) FaDu cells were treated with 3 μ M ABC1183 for 2–24 hours or 20 nM LY2090314, as indicated. Cell lysates were immunoblotted with MCL1, pSer21/9 GSK3 α/β , GSK3 β , pSer641 GS, GS, pSer33/37 Thr41 β -catenin, β -catenin, and GAPDH.

mice were treated with oral ABC1183 or vehicle. As depicted in Fig. 4A, tumor growth was decreased more than 70% within 8 days by ABC1183 treatment, relative to vehicle, which was maintained until termination of study. On the basis of body weight, gross toxicities were not observed (Fig. 4A, right). Next, murine pancreatic Pan02 allograft tumors were propagated and, relative to B16 tumors, exhibited less aggressive growth kinetics that allowed for extended treatment of tumors. Oral treatment significantly suppressed tumor growth, which was visible by day 5 and persisted throughout the 22-day treatment course (Fig. 4B). Again, on the basis of body weight, no toxicities were observed. Finally, prostate TRAMP-C2 tumors were treated with oral ABC1183 or vehicle over the course of 21 days. Throughout the experiment, ABC1183 treatment diminished tumor size, compared with vehicle, and no toxicities were observed (Fig. 4C).

Monitoring pharmaceutical on-target action is critical for understanding response rates in clinical trials. Therefore, to determine if ABC1183 impinges on intratumoral GSK3 signaling, representing a clinically relevant biomarker candidate, previously untreated tumor-bearing mice were treated with oral ABC1183 (5 mg/kg) or intraperitoneal ABC1183 (25 mg/kg, i.p.), for 2 and 12 hours to analyze GSK3 α/β phosphorylation and downstream GS phosphorylation. GSK3 α/β pSer21/9 was decreased in all treatment groups, compared with vehicle, with no remarkable changes to total GSK3 β expression (Fig. 4D, compare lanes 1–3 with 4–15). Interestingly, low-dose oral treatment was as effective as high intraperitoneal dose, demonstrating robust oral bioavailability. GS pSer641 levels were also appreciably diminished by all ABC1183 treatment conditions, compared with vehicle, without altering total GS expression.

Since GSK3 and CDK9 can activate inflammatory signaling pathways, it was also of interest to determine whether inflammatory cytokines within tumor samples were altered in response to ABC1183 administration. Pan02 tumor-bearing mice were treated with vehicle or 25 mg/kg ABC1183, and TNF- α , IL-6, and IL-1 β cytokines were quantified from harvested tissue. Consistent with ABC1183 inhibiting GSK3

and CDK9, 12 hours following compound administration intratumoral TNF- α had been reduced 65%, IL-6 decreased 30%, and IL-1 β diminished by 45%, relative to control (Supplemental Fig. S5). In sum, these tumor models demonstrate that ABC1183 inhibits tumor growth in vivo, in part by impinging on GSK3 signaling and reducing inflammatory cytokines. GSK3 and GS phosphorylation levels can also be used as pharmacodynamic biomarkers to assess target engagement in tumor samples.

Historically, some GSK3 inhibitors manifested deleterious side effects that halted clinical development (Eldar-Finkelman and Martinez, 2011). To begin to identify potential ABC1183 side effects, 100 and 200 mg/kg (20- to 40-fold above antitumor efficacy) were administered orally for 7 days followed by gross organ analysis and serum chemistry profiles. Heart, liver, kidney, and overall body weights did not differ appreciably from vehicle (Fig. 5A). Blood chemistry analysis revealed normal levels of red blood cells, white blood cells, and platelets. Furthermore, alanine aminotransferase (ALT), creatinine, and glucose were also measured and normal, with the exception of elevated ALT at the highest 200 mg/kg dose (Fig. 5A). These high-dose, short-term exposure studies, in concordance with the in vivo tumor studies, provide evidence that ABC1183 is a safe and efficacious treatment option.

ABC1183 Reverses Inflammation-Induced Pathologic Inflammatory Bowel Disease. GSK3 isoforms influence disparate disease progression, in part through proinflammatory signaling. Inflammatory bowel disease (IBD) is the clinical manifestation of chronic colonic inflammation, often with debilitating symptoms. To address the ability of ABC1183 to abrogate progression of IBD, disease symptoms were induced by multiple models. First, TNBS-induced colitis was modeled. Relative to vehicle alone, rectal TNBS administration on days 0 and 7 resulted in significant incidence of disease within the colon, as determined by macroscopic inspection (Fig. 5B, hatched bars). However, addition of oral ABC1183 on days 6–10 significantly decreased macroscopic symptoms of inflammation, such that these colons were indistinguishable from

Fig. 4. Inhibition of tumor growth by ABC1183. C57BL/6 mice were injected subcutaneously with (A) B16 melanoma cells suspended in PBS, (B) PAN02 pancreatic cancer cells suspended in PBS/growth matrix, or (C) TRAMP-C2 murine prostate cancer cells suspended in PBS/growth matrix. After tumor volume of at least 100 mm³, animals were treated five times per week by oral gavage of either 100 μ l vehicle (open squares), 5 mg/kg ABC1183 (A and B), or 2 mg/kg ABC1183 (C) (gray squares). Tumor volume measurement was performed for time course as indicated; $n = 5$ per group in (A) and (C), $n = 10$ per group for (B). * $P < 0.05$; ** $P < 0.01$. (D) Pan02-bearing mice were treated with 5 or 25 mg/kg for 2 or 12 hours. Tumors were excised, snap frozen, processed, and tumor lysates immunoblotted with pSer21/9 GSK3 α/β , GSK3 β , pSer641 GS, and GS.

vehicle. Analysis of cytokines within the colons revealed that TNBS treatment increased the proinflammatory cytokines TNF- α and IL-6 (Fig. 5C). Consistent with reduced

evidence of inflammation seen through macroscopic inspection, TNF α and IL-6 levels in colons were reduced 60% and 85%, respectively, in ABC1183-treated animals.

A

Parameter	Vehicle	100 mg/kg	200 mg/kg
Weight loss (%)	0.29±0.17	1.64±0.32	1.16±0.3
Heart weight (g)	0.165±0.004	0.148±0.009	0.1537±0.005
Liver weight (g)	1.383±0.137	1.464±0.008	1.56±0.046
Kidney weight (g)	0.354±0.018	0.346±0.012	0.378±0.009
Red Blood cells (x10 ³ mm ³)	9.26±0.8	9.34±0.11	8.55±0.42
White blood cells (x10 ³ mm ³)	7.45±1.01	6.75±0.75	5.85±0.29
Platelets (x10 ³ mm ³)	1066±93	1401±192	987±118
ALT (IU/L)	14.75±1.89	17.33±3.18	37.0±20.6
Creatinine (mg/dl)	0.20±0.04	0.20±0	0.13±0.03
Glucose (mg/dl)	180±17.2	199.3±10.2	190.3±20.2

Fig. 5. ABC1183 is safe for oral administration and reduces severity of TNBS-colitis model, at least in part, through inflammatory cytokines. (A) Seven-day toxicity of ABC1183. The indicated dose of ABC1183 was administered by oral gavage daily for 7 days, and the mice were then sacrificed for hematology, blood chemistry, and organ weight analyses. Values are mean $\pm$ S.E.M., $n = 3$ or 4 mice/group. (B) C57BL/6 mice were treated with rectal 50% ethanol and oral vehicle (black bars); rectal TNBS and oral vehicle (cross hatched bars), or rectal TNBS or oral ABC1183 (gray bars 50 mg/kg). Animals were sacrificed on day 10 and macroscopic inflammation within the distal 3 cm of each colon was scored. (C) Mice were treated as in (B) and TNF- α and IL-6 cytokines levels were determined from colon samples. * $P < 0.05$; ** $P < 0.01$.

B

C

Therefore, TNBS-induced colitis represents a scenario in which ABC1183 treatment is sufficient to reverse or diminish the negative physiologic effects of inflammation signaling.

Another clinically relevant model of IBD was reproduced by dextran sulfate sodium (DSS) administration and disease progression was determined by Disease Activity Index (DAI), which accounts for pathologic symptoms including bloody and loose stool. Consistent with previous data and compared with vehicle alone, exposure of mice to DSS/vehicle in drinking water induced IBD, which progressively increased DAI between days 4 and 6 (Fig. 6A, hatched bars). In comparison, treatment of animals with ABC1183 (50 mg/kg, once daily) dramatically decreased overall disease intensity between days 4 and 6 (Fig. 6A, gray bars). Additionally, colon length, which decreases following DSS-induced colitis and is indicative of overall damage and scarring, was measured. Compared with vehicle treatment, colons from DSS-treated mice were significantly shortened, whereas the colon lengths of ABC1183-treated mice were indistinguishable from controls (Fig. 6B). To measure the colonic neutrophil infiltration resulting from elevated inflammation, which contributes to IBD tissue damage, MPO activity was quantified from day-6 colons. As indicated in Fig. 6C, DSS treatment significantly increased MPO activity, compared with vehicle, whereas DSS in combination with ABC1183 attenuated neutrophil influx and resultant MPO activity. The findings from DAI score, colon length, and MPO activity combine to demonstrate that ABC1183 effectively attenuates proinflammatory signaling that drives symptoms of IBD. To further examine the impact of ABC1183 on inflammation signaling, cytokine levels were analyzed from colon samples. DSS-treatment corresponded with increased proinflammatory TNF α and IL-6 cytokines and decreased anti-inflammatory IL-10 (Fig. 6D, hatched bars). In

contrast, ABC1183 reversed IL-6 and IL-10 expression patterns but did not influence TNF- α levels (Fig. 6D, gray bars). Overall, these findings promote the hypothesis that the novel GSK3 α/β and CDK9 inhibitor ABC1183 negatively regulates cell growth and proinflammatory signaling sufficiently to abrogate tumor proliferation and IBD progression.

Discussion

Previous studies exploring the impact of GSK3 demonstrated both tumor suppressor and oncogenic capabilities, whereas dual GSK3/CDK9 inhibitors were often nonselective and targeted multiple CDKs. Here, we present data demonstrating the selective GSK3- and CDK9-inhibitory function of a novel aromatic diaminothiazole, ABC1183. This compound inhibits growth of a broad panel of cancer cell lines, with IC₅₀ values ranging from 63 nM to 2.8 μ M. ABC1183 treatment decreases cell survival through G2/M arrest and modulates GSK3 and CDK9 activity as indicated by changes in GSK3, GS, β -catenin phosphorylation, Pol2 phosphorylation and MCL1 expression. Additionally, oral administration diminishes tumor growth in multiple animal models, reverses inflammation-driven gastrointestinal disease symptoms, owing in part to pro- and anti-inflammatory cytokine modulation, and does not promote any associated gross toxicities. Therefore, ABC1183 is strategically poised to effectively mitigate multiple clinically relevant diseases.

On the basis of the data, it is hypothesized that ABC1183 inhibits cell proliferation and tumor growth of various cancer types by specifically attenuating GSK3 and CDK9 activity. Both molecules were identified from a human kinome screen for selective inhibition by ABC1183. CDK9 specificity was further queried by analyzing Pol2 and MCL1 expression, which were decreased. Interestingly, ABC1183 altered GSK3

Fig. 6. Effect of oral ABC1183 on disease parameters in the mouse DSS-induced ulcerative colitis. C57BL/6 mice were treated with oral vehicle (black bars), 2% DSS-positive control (horizontal hatched bars), or 2% DSS with oral ABC1183 50 mg/kg twice a day (gray bars), and the following parameters were measured: (A) Disease Activity Index (DAI), which monitors disease severity; (B) colon length on day 6 of treatment; (C) myeloperoxidase (MPO) activity measured from the colons of animals on day 6 of treatment. (D) Cytokine values: TNF α , IL-6, and IL-10 cytokine levels were determined from colon samples. * $P < 0.05$; ** $P < 0.01$.

activity as indicated by decreased GSK3 α/β and GS phosphorylation and increased β -catenin phosphorylation. These findings are in conflict with canonical GSK3 signaling, whereby inhibition promotes autoinhibitory pSer21/9 and subsequent decrease of GS phosphorylation. Therefore, ABC1183 treatment causes a mechanistic paradox of signaling output. Treatment with LY2090314, a known GSK3 inhibitor, decreased GSK3 and GS phosphorylation as did ABC1183 (Fig. 3 lane 5). Findings herein have been corroborated by other GSK3 inhibitor studies. Treatment of renal cancer cells with GSK3 inhibitor 9-ING-41 caused G2/M arrest, tumor growth inhibition, and decreased pGS. (Pal et al., 2014). Likewise, SB216763 inhibitor decreased GSK3 β pSer9 (Hilliard et al., 2011; Koo et al., 2014) and decreased pGS (Benakanakere et al., 2010; Wang et al., 2011). In hepatocellular carcinoma, stemness was conferred through increased GSK3 Ser9 phosphorylation and subsequent stabilization of β -catenin expression (Chua et al., 2015). Therefore, despite the current understanding of GSK3 activity, the disparate findings reported here and elsewhere demonstrate the depth of complexity and crosstalk.

GSK3-mediated phosphorylation of β -catenin signals proteasomal degradation and inhibition of progrowth target genes (Shang et al., 2017). However, on the basis of data herein, ABC1183 treatment increases β -catenin serine phosphorylation, despite inhibiting GSK3 activity. We hypothesize that the reported findings of ABC1183 inhibition results from the differential priming requirement for substrates. In general, GSK3 substrates require a priming step for engagement

within the binding pocket (Beurel et al., 2015). β -catenin phosphorylation within the destruction complex is one example, however, whereby GSK3-mediated phosphorylation is independent of pSer21/9 status (Stamos and Weis, 2013). Therefore, although ABC1183 impinges on pSer21/9, it does not negatively affect the ability of GSK3 to signal through a separate pool of β -catenin.

ABC1183 specifically inhibits GSK3 and CDK9 but no other closely related CDK molecules, which is unique since previously described dual inhibitors typically antagonized CDKs broadly, such as flavopiridol, AT7519, and roscovitine. Flavopiridol is a pan-CDK inhibitor that targets CDKs 2, 4/6, 7, and 9, and has been in clinical studies since 1994 for numerous tumor types. Although cell proliferation was abrogated via G2/M cell cycle arrest by ABC1183, this result is not specifically attributed to GSK3 and CDK9. AT7519 is a pan-CDK inhibitor that likewise caused G2/M arrest and decreased GSK3 β ser9 phosphorylation. Multiple phase II clinical trials have been completed but, although treatment is well tolerated, low response rates were observed (Seftel et al., 2017). Therefore, ABC1183 represents a novel molecule for selectively inhibiting CDK9 and GSK3.

ABC1183 treatment of multiple allogenic tumor types significantly reduces growth kinetics, relative to vehicle conditions. Within tumor specimens, GSK3 signaling is inhibited on the basis of reduced GSK3 and GS serine phosphorylation, which is consistent with cellular findings. These data confirm that ABC1183 engages the anticipated targets and sufficiently abrogates tumor progression. In

preclinical and clinical scenarios, post-translation modifications, rather than GSK3 expression, was most frequently associated with a disease state, and numerous studies support the biologic relevance of GSK3 signaling in cancer progression. In cell-based models, GSK3 β Ser9 levels corresponded with cisplatin resistance in ovarian cancer cells (Cai et al., 2007) and antagonized cell apoptosis (Gao et al., 2014). Novel protein kinase B and AGC kinase family inhibitors decreased pSer21/9 GSK3 α/β , which corresponded with decreased cancer cell survival (Yap et al., 2011, 2012). In a retrospective analysis, GSK3 pSer9 was present in 47% of invasive mammary carcinomas and correlated with a worse clinical outcome (Armanious et al., 2010), and also correlated with a poor prognosis in lung carcinomas (Zheng et al., 2007). A phase II trial investigating LY treatment of acute myeloid leukemia patients demonstrated on-target effects on the basis of β -catenin expression; however, limited clinical benefit was observed (Rizzieri et al., 2016). Phase I/II trial with the protein kinase C inhibitor enzastaurin, which decreased GSK3 β phosphorylation in culture (Graff et al., 2005), was shown to decrease pSer9 in peripheral blood monocytes from patients with high-grade glioma (Kreisl et al., 2010). Therefore, these studies corroborate the biologic significance and utility of intratumoral detection of protein phosphorylation states as clinically relevant biomarker options.

Multiple models of inflammation-driven gastrointestinal disease provide evidence that ABC1183 effectively attenuates proinflammatory cytokines TNF- α and IL-6, increases anti-inflammatory cytokine IL-10, modulates immune cell infiltration, and subsequently diminishes symptoms of disease. These results underscore the proinflammatory role of both GSK3 and CDK9 implicated in many disease types. Although GSK3 regulates multiple mechanisms promoting inflammation, the transcriptional activity of NF- κ B is paramount. GSK3 inhibition decreases nuclear accumulation of NF- κ B and regulates cytokine production (Cortes-Vieyra et al., 2012), which is similar to our findings. CDK9 also influences inflammatory immune response by promoting expression of proinflammatory cytokine genes (Brasier, 2008; Brasier et al., 2011) and the extravasation of leukocytes into sites of inflammation (Berberich et al., 2011). NF- κ B is also a dominant player in this signaling as pNF- κ B is required for CDK9 incorporation into P-TEFb (Nowak et al., 2008; Fang et al., 2014). These overlapping mechanisms of inflammation have profound implications, as elevated inflammation is also associated with neurologic disorders, such as Alzheimer and Parkinson diseases, gastrointestinal disorders, cancer, and others (Morales et al., 2014; Kalia and Lang, 2015). Furthermore, given that the tumor studies described herein used immune-competent animals, the antitumor function of ABC1183 could be associated with reduced NF- κ B signaling and suppressed proinflammatory production. Future studies will characterize tumor-associated inflammation and the ability of ABC1183 to resolve symptoms associated with inflammation in neurologic diseases.

ABC1183 is a novel small-molecule dual inhibitor of GSK3 and CDK9 that is effective as a single agent to inhibit clinically relevant models of disease. Mechanistic studies highlight the antiproliferative capability of ABC1183 and underscore the complex GSK3 and CDK9 signaling networks. ABC1183 inhibits preclinical models of tumor growth as an orally available single agent with on-target action. Pharmacodynamic biomarkers from

tumor biopsies are advantageous for monitoring treatment efficacy. We have also demonstrated the potent anti-inflammatory effect of dual GSK3 and CDK9 abrogation and the ability to antagonize cytokine-induced inflammatory disease. Overall, ABC1183 is positioned for further development and progression into clinical investigation.

Authorship Contributions

Participated in research design: Schrecengost, Maines, C. D. Smith.

Conducted experiments: Schrecengost, Green, Keller, R. A. Smith, Maines.

Contributed new reagents or analytic tools: Zhuang.

Performed data analysis: Schrecengost, Keller, R. A. Smith, Maines.

Wrote or contributed to the writing of the manuscript: Schrecengost, Green, C.D. Smith.

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