A Novel Small-Molecule Inhibitor of Protein Kinase C\textsuperscript{\textalpha} Blocks Transformed Growth of Non–Small-Cell Lung Cancer Cells

Melody Stallings-Mann, Lee Jamieson, Roderick P. Regala, Capella Weems, Nicole R. Murray, and Alan P. Fields

Department of Cancer Biology, Mayo Clinic College of Medicine, Jacksonville, Florida

Abstract

We recently showed that atypical protein kinase C\textsuperscript{\textalpha} (PKC\textsuperscript{\textalpha}) is required for transformed growth of human non–small-cell lung cancer (NSCLC) cells by activating Rac1. Genetic disruption of PKC\textsuperscript{\textalpha} signaling blocks Rac1 activity and transformed growth, indicating that PKC\textsuperscript{\textalpha} is a viable target for development of novel therapeutics for NSCLC. Here, we designed and implemented a novel fluorescence resonance energy transfer–based assay to identify inhibitors of oncogenic PKC\textsuperscript{\textalpha} signaling. This assay was used to identify compounds that disrupt the interaction between PKC\textsuperscript{\textalpha} and its downstream effector Par6, which links PKC\textsuperscript{\textalpha} to Rac1. We identified aurothioglucose (ATG), a gold compound used clinically to treat rheumatoid arthritis, and the related compound, aurothiomalate (ATM), as potent inhibitors of PKC\textsuperscript{\textalpha}–Par6 interactions in vitro (IC\textsubscript{50} = 1 \textmu mol/L). ATG blocks PKC\textsuperscript{\textalpha}–Par6–related signaling to Rac1 and inhibits transformed growth of NSCLC cells. ATG-mediated inhibition of transformation is relieved by expression of constitutively active Rac1, consistent with a mechanism at the level of the interaction between PKC\textsuperscript{\textalpha} and Par6. ATG inhibits A549 cell tumor growth in nude mice, showing efficacy against NSCLC in a relevant preclinical model. Our data show the utility of targeting protein-protein interactions involving PKC\textsuperscript{\textalpha} for antitumor drug development and provide proof of concept that chemical disruption of PKC\textsuperscript{\textalpha} signaling can be an effective treatment for NSCLC. ATG and ATM will be useful reagents for studying PKC\textsuperscript{\textalpha} function in transformation and represent promising new agents for the clinical treatment of NSCLC. (Cancer Res 2006; 66(3): 1767-74)

Introduction

Lung cancer is the leading cause of cancer death in the United States, accounting for an estimated 160,440 deaths in 2004 (1). Most lung cancers (~80%) are classified as non–small-cell lung cancer (NSCLC), with most remaining cases (~18%) being small-cell lung cancer (2). Early-stage NSCLC tumors are often treated with surgery and radiotherapy whereas advanced-stage metastatic disease often receives combination chemotherapy (2). Unfortunately, despite surgical removal and adjuvant therapy, many early-stage NSCLCs relapse and become fatal. The 5-year survival rate for NSCLC is only 14%, underscoring the need for more effective modalities for prognosis, prevention, and treatment.

Recently, we showed that the atypical protein kinase C (PKC) isoform, PKC\textsuperscript{\textalpha}, plays a requisite role in NSCLC cell growth (3). Expression of PKC\textsuperscript{\textalpha} is elevated in established human NSCLC cell lines and genetic disruption of PKC\textsuperscript{\textalpha} signaling by expression of a dominant-negative, kinase-deficient PKC\textsuperscript{\textalpha} allele blocks anchorage-independent transformed growth and tumorigenicity of NSCLC cells in nude mice (3). PKC\textsuperscript{\textalpha} expression is also elevated in the vast majority of primary NSCLC tumors and PKC\textsuperscript{\textalpha} expression correlates with poor survival in patients with NSCLC (4). PKC\textsuperscript{\textalpha} expression predicts poor survival independent of tumor stage, indicating that PKC\textsuperscript{\textalpha} expression may be useful in identifying NSCLC patients with early-stage disease who are at elevated risk for relapse. In addition, the PKC\textsuperscript{\textalpha} gene is a frequent target for tumor-specific gene amplification in a significant subset of NSCLC, particularly in squamous cell carcinoma of the lung (4). PKC\textsuperscript{\textalpha} gene amplification drives PKC\textsuperscript{\textalpha} expression and disruption of PKC\textsuperscript{\textalpha} signaling in NSCLC cells that harbor PKC\textsuperscript{\textalpha} gene amplification blocks transformed growth, showing the importance of PKC\textsuperscript{\textalpha} gene amplification in NSCLC biology (4). Taken together, our data identified PKC\textsuperscript{\textalpha} as a critical oncogene in NSCLC.

A major molecular mechanism by which PKC\textsuperscript{\textalpha} drives transformed growth was recently elucidated by our laboratory. Specifically, we showed that PKC\textsuperscript{\textalpha} is required for NSCLC transformation by activating a PKC\textsuperscript{\textalpha}–Rac1–Pak–Mek–Erk signaling axis that is required for transformed growth in vitro (3). Furthermore, we showed that expression of the PB1 domain within the NH\textsubscript{2}-terminal regulatory region of PKC\textsuperscript{\textalpha} blocks transformed growth by uncoupling PKC\textsuperscript{\textalpha} from Rac1 activation (3). Taken together, our data suggest that PKC\textsuperscript{\textalpha} is an attractive target for the development of mechanism-based therapeutics for the treatment of NSCLC. Here, we report the design and use of a novel fluorescence resonance energy transfer (FRET)–based high-throughput assay to identify chemical inhibitors of the interaction between PKC\textsuperscript{\textalpha} and its effector Par6. Using this assay, several high-affinity compounds have been identified that disrupt the PB1 domain–mediated interactions between PKC\textsuperscript{\textalpha} and Par6. These compounds effectively inhibit PKC\textsuperscript{\textalpha} signaling to Rac1, the downstream target of the PKC\textsuperscript{\textalpha}–Par6 interaction, and exhibit potent antitumor effects on NSCLC cell growth in vitro and in vivo. Our data provide important proof of principle that chemical disruption of protein/protein interactions involving the PB1 domain of PKC\textsuperscript{\textalpha} is an effective route to the development of novel therapeutics for the treatment of patients with NSCLC.

Materials and Methods

Reagents. The Rac1 antibody was from Transduction Labs (Palo Alto, CA) and was used at a concentration of 1:5,000. Aurothioglucose (ATG) was from Research Diagnostics (Concord, MA). Aurothiomalate (ATM) was from Taylor Pharmaceuticals (Decatur, IL) (Myochrysine). Rac/cdc42 assay reagents were...
from Upstate (Charlottesville, VA). The GenPlus Collection of compounds was from Microsource Discovery Systems, Inc. (Gaylordsville, CT).

**Plasmids, cloning, and recombinant proteins.** The PB1 domain of human PKC\(\alpha\) (amino acids 1-113) was generated by PCR amplification of a plasmid containing the full-length human PKC\(\alpha\) cDNA (5). The PB1 domain of human Par6 (amino acids 1-125) was generated by PCR amplification from a human K562 cell cDNA library (Clontech, Palo Alto, CA). Isolated clones were transformed into a human K562 cell cDNA library (Clontech, Palo Alto, CA). The PB1 domain of human Par6 (amino acids 1-125) was generated by PCR amplification from Microsource Discovery Systems, Inc. (Gaylordsville, CT).

The GenPlus Collection of compounds was from Upstate (Charlottesville, VA). The GenPlus Collection of compounds was screened in triplicate. Specific FRET was determined by monitoring emission at 475 nm for CFP and Par6/CFP and at 529 nm for YFP and PKC/CFP. Emission profiles were obtained using an excitation wavelength of 434 nm for CFP and Par6/CFP and 495 nm for YFP and PKC/CFP. Results were expressed as normalized fluorescence for each protein.

**Expression, purification, and characterization of recombinant proteins.** His-tagged PKC\(\alpha\)/YFP, CFP, and YFP recombinant proteins were isolated from the soluble fraction of bacterial lysates by affinity purification using the B-PER 6×His Purification Kit (Pierce, Rockford, IL) and purified proteins were dialyzed against Tris buffer [50 mmol/L Tris (pH 8.0), 135 mmol/L NaCl, 10% glycerol, 0.002% EDTA] containing 2 mol/L urea. Isolated PKC\(\alpha\)/YFP recombinant protein was isolated from inclusion bodies using the B-PER reagent (Pierce). Briefly, inclusion bodies were solubilized in Tris buffer containing 8 mol/L urea and dialyzed against the same buffer containing 4 mol/L urea for 4 hours, and then overnight against Tris buffer containing 2 mol/L urea. Yields were quantitated by protein assay and measurement of fluorescence in a SpectraMax Gemini microplate reader (Molecular Devices, Union City, CA). Purified recombinant PKC\(\alpha\)/YFP, Par6/CFP, YFP, and CFP were analyzed for their spectral properties using a SpectraMax Gemini fluorescence plate reader. Excitation profiles were determined by monitoring emission at 475 nm for CFP and Par6/CFP and at 529 nm for YFP and PKC/CFP. Emission profiles were obtained using an excitation wavelength of 434 nm for CFP and Par6/CFP and 495 nm for YFP and PKC/CFP. Results were expressed as normalized fluorescence for each protein.

**A FRET-based Par6-PKC\(\alpha\) interaction assay and compound library screen.** For compound library screening, 50 μL of PKC\(\alpha\)/YFP (containing 4,000 fluorescence units) and 50 μL of Par6/CFP (containing 400 fluorescence units) were added to each well of 96-well, clear-bottom black plates (Costar 3631), followed by 10 μL of each of 960 compounds from the Microsource GenPlus library to a final concentration of 1 mmol/L. Plates were incubated at 4°C for 4 hours and fluorescence emission was measured at 475 nm (cutoff, 455 nm) and 529 nm (cutoff, 515 nm) on a SpectraMax Gemini plate reader using an excitation wavelength of 395 nm. The entire library of compounds was screened in triplicate. Specific FRET was determined by taking the fluorescence ratio (529/475 nm) after subtracting the background ratio in samples using Imaging software (Amersham). Appropriate primers were used to amplify PKC\(\alpha\)/YFP, Par6/CFP, YFP, and CFP and directionally clone them into the pRSET vectors for high-level expression of NH\(_2\)-terminally His-tagged fusion proteins in bacteria. The PB1 domain of PKC\(\alpha\) L from a human K562 cell cDNA library (Clontech) was cloned into the PinPoint expression vectors (BD Biosciences, Palo Alto, CA) to generate pPKC\(\alpha\)/YFP plasmid containing the full-length human PKC\(\alpha\) L gene. Human PKC\(\alpha\) L was from Microsource Discovery Systems, Inc. (Gaylordsville, CT).

**Rac1 activity assay.** Rac1 activity was assayed in parental A549 cells in the presence and absence of ATG and in A549/pBabe and A549/kdPKC\(\alpha\) cell transfectants as previously described (3, 9). Briefly, cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 20 mmol/L MgCl\(_2\), 5 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% NP40, 25 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin] at 4°C for 5 minutes. Cellular debris was removed by centrifugation at 20,000 × g for 5 minutes and supernatants were transferred to new tubes An aliquot of each supernatant was reserved to determine total Rac1 expression by immunoblot analysis before addition of 20 μL of GST-p21-binding domain of PAK1 coupled to agarose beads (Upstate). Following a 30-minute incubation at 4°C, the agarose beads were collected by centrifugation and washed thrice with lysis buffer. Bound proteins were solubilized by the addition of 30 μL of SDS sample buffer, resolved by SDS-PAGE, and subjected to immunoblot analysis for Rac1 as previously described (3, 9). An equivalent amount of cell lysate was subjected to immunoblot analysis to assess total Rac1 expression. Signal intensity was quantitated using ImageQuant software.

**Soft agar growth and tumorigenicity assays.** Anchorage-independent growth of A549/LZRS and A549/RacV12 cells was assessed by their ability to grow as colonies in soft agar in the presence or absence of 10 mmol/L ATG as previously described (3, 4). The ability of A549 cells to grow as established s.c. tumors and in the presence and absence of ATG was studied in athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN) as previously described (3). Four- to six-week-old female nude mice were injected s.c. with 5 × 10\(^3\) A549 cells in 100 μL of growth medium. When tumors had reached an average volume of 50 mm\(^3\) (day 21 postinjection), the mice were randomly divided into two groups. The treatment group received daily injections of ATG at 200 mg/kg and the other group received an equal volume of diluent (0.9% sterile saline) daily for a 2-week period. Tumor dimensions were measured using calipers and tumor volume (mm\(^3\)) was calculated using the following formula: 0.5236L × W \(\times\) H, where L is tumor length, W is width, and H is height. At the conclusion of the study, mice were sacrificed by CO\(_2\) asphyxiation.

**Results**

**A FRET-based assay to detect PB1-PB1 domain interactions between PKC\(\alpha\) and Par6.** Our previous studies have shown that PKC\(\alpha\) is an oncogene in NSCLC (3, 4). Specifically, we showed that PKC\(\alpha\) expression is elevated in the majority of NSCLC cell lines and primary tumors, that PKC\(\alpha\) expression is predictive of poor outcome in NSCLC patients, and that the PKC\(\alpha\) gene is a target for frequent tumor-specific gene amplification in squamous cell carcinoma of the lung (4). Mechanistically, we showed that PKC\(\alpha\) activates a PKC\(\alpha\)-Rac1–Par6–Mek–Erk pathway that is required for transformed growth in vitro and in vivo (3). Based on our
studies, we proposed that PKC\(_\text{L}\) is an attractive target for development of novel mechanism-based therapeutics for NSCLC.

To assess the therapeutic potential of PKC\(_\text{L}\) as a drug target, we developed a novel assay using FRET technology to identify potential inhibitors of PKC\(_\text{L}\) signaling. We chose not to screen for compounds that directly inhibit PKC\(_\text{L}\) catalytic activity because the catalytic domain of the enzyme is highly conserved among PKC isozymes and the larger superfamily of protein kinases, making identification of highly specific compounds problematic. Rather, we targeted the PB1 domain of PKC\(_\text{L}\) because it is uniquely present in atypical PKCs and not other PKC isozymes and it is required for the oncogenic function of PKC\(_\text{L}\) (3). The PB1 domain of PKC\(_\text{L}\) represents a highly specific protein-protein interaction domain that mediates binding of PKC\(_\text{L}\) to several adaptor molecules, particularly Par\(_6\) and p62, which link PKC\(_\text{L}\) to downstream effector pathways (10). We have shown that expression of the PB1 domain of PKC\(_\text{L}\) blocks PKC\(_\text{L}\)-mediated activation of Rac1 and the transformed growth of A549 lung adenocarcinoma cells (3).

The FRET-based assay was developed to measure binding between the PB1 domains of PKC\(_\text{L}\) and Par\(_6\). We chose to use two variants of green fluorescent protein, enhanced CFP and enhanced YFP, because it has been previously shown that these two fluorophores are highly suited for FRET (6, 11, 12). Specifically, the emission spectrum of CFP partially overlaps the excitation spectrum of YFP; both CFP and YFP are relatively photostable; and CFP is sufficiently bright to permit accurate ratiometric measurement of both donor (CFP) and acceptor (YFP) by a variety of fluorescence detectors, including fluorescence plate readers (12).

Therefore, we constructed plasmids that allowed us to express and purify the PB1 domain of Par\(_6\) as a His-tagged fusion protein with CFP (Par\(_6\)/CFP) and the PB1 domain of PKC\(_\text{L}\) as a His-tagged fusion protein with YFP (PKC\(_\text{L}/\text{YFP}\); Fig. 1A). To determine whether the Par\(_6\)/CFP and PKC\(_\text{L}/\text{YFP}\) fusion proteins retained spectral properties similar to native CFP and YFP proteins, we analyzed the excitation and emission spectra of these proteins compared with His-tagged CFP and His-tagged YFP expressed in the same bacterial system. Both the excitation (Fig. 1B and C) and emission (Fig. 1D and E) spectra of Par\(_6\)/CFP (Fig. 1B and D) and PKC\(_\text{L}/\text{YFP}\) (Fig. 1C and E) are virtually identical to those of CFP and YFP, respectively.

We next determined whether these proteins are suitable for FRET-based detection of interactions between the PB1 domains of Par\(_6\) and PKC\(_\text{L}\). The basis for the binding assay is illustrated in Fig. 2A. Based on the excitation spectral data in Fig. 1, we chose an excitation wavelength of 395 nm, which is within the excitation range of Par\(_6\)/CFP, but not of PKC\(_\text{L}/\text{YFP}\). Thus, at this wavelength, Par\(_6\)/CFP should emit blue light that can be detected at 475 nm whereas PKC\(_\text{L}/\text{YFP}\) should emit little or no light. When purified Par\(_6\)/CFP is exposed to 395-nm light, the characteristic emission spectrum of CFP with a peak emission at 475 nm is observed (Fig. 2B). In contrast, when purified PKC\(_\text{L}/\text{YFP}\) is exposed to 395-nm light, only a small peak is observed at 529 nm, which is not

Figure 1. Characterization of Par\(_6\)/CFP and PKC\(_\text{L}/\text{YFP}\) fusion proteins. A, Coomassie blue–stained polyacrylamide gel of bacterially expressed and purified PKC\(_\text{L}/\text{YFP}\) and Par\(_6\)/CFP fusion proteins. B and C, excitation spectra of CFP (B) and YFP (C; solid lines) compared with Par\(_6\)/CFP (B) and PKC\(_\text{L}/\text{YFP}\) (C; dashed lines) fusion proteins, respectively. Emission was measured at 475 nm for CFP and Par\(_6\)/CFP and at 529 nm for YFP and PKC\(_\text{L}/\text{YFP}\). D and E, emission spectra of CFP (D) and YFP (E; solid lines) compared with Par\(_6\)/CFP (D) and PKC\(_\text{L}/\text{YFP}\) (E; dashed lines) fusion proteins, respectively. Excitation wavelength was 434 nm for CFP and Par\(_6\)/CFP and 495 nm for YFP and PKC\(_\text{L}/\text{YFP}\).
appreciably above the background emission associated with buffer alone (Fig. 2B). However, when Par6/CFP and PKC\textsubscript{L}/YFP are incubated together, we observe a decrease in the fluorescence peak at 529 nm associated with Par6/CFP emission and a second emission peak with a maximum at 529 nm characteristic of FRET (Fig. 2C). As expected, no emission peak at 529 nm is observed when Par6/CFP is tested alone or when the nonbinding control protein YFP does not emit appreciably above buffer alone. C, emission spectra of the Par6/CFP in the absence (solid line) or presence (long dashed line) of its binding partner PKC\textsubscript{L}/YFP. The binding reaction was also carried out in the presence of SDS (short dashed line) and a nonfluorescent competitive inhibitor consisting of the PB1 domain of PKC\textsubscript{L} fused to GST (dotted line). E, FRET measurements were obtained for Par6/CFP in the presence of increasing amounts of the nonbinding control protein YFP (●) or its binding partner PKC\textsubscript{L}/YFP (▲). Results are expressed as the fluorescence ratio of emission 529/475 nm. The nonbinding control values were subtracted from the Par6/CFP:PKC\textsubscript{L}/YFP values to obtain the specific FRET associated with the saturable binding between Par6/CFP and PKC\textsubscript{L}/YFP (dotted line).

Specific FRET between CFP- and YFP-containing binding protein pairs is often expressed as the ratio of the fluorescence emission at 529 and 475 nm (fluorescence ratio 529/475) of a solution containing the CFP/YFP binding pair minus the fluorescence ratio 529/475 of a solution containing equivalent fluorescence units of a nonbinding CFP/YFP pair. To optimize the binding reaction to obtain the highest FRET value with the least interference from direct excitation of unbound PKC\textsubscript{L}/YFP, we incubated increasing amounts of YFP or PKC\textsubscript{L}/YFP with a set amount of Par6/CFP and PKC\textsubscript{L}/YFP fusion proteins alone in solution after exposure to 395-nm light. Par6/CFP emits a spectrum characteristic of CFP whereas PKC\textsubscript{L}/YFP does not emit appreciably above buffer alone (Fig. 2B). However, when Par6/CFP and PKC\textsubscript{L}/YFP are incubated together, we observe a decrease in the fluorescence peak at 529 nm associated with Par6/CFP emission and a second emission peak at 475 nm that is characteristic of the FRET-mediated emission of YFP when it is excited by the donor molecule. When Par6/CFP and PKC\textsubscript{L}/YFP are incubated together, we observe a decrease in the fluorescence peak at 475 nm associated with Par6/CFP emission and a second emission peak with a maximum at 529 nm characteristic of FRET (Fig. 2C). As expected, no emission peak at 529 nm is observed when Par6/CFP is tested alone or when the nonbinding control protein YFP does not emit appreciably above buffer alone. C, emission spectra of the Par6/CFP in the absence (solid line) or presence (long dashed line) of its binding partner PKC\textsubscript{L}/YFP. The binding reaction was also carried out in the presence of SDS (short dashed line) and a nonfluorescent competitive inhibitor consisting of the PB1 domain of PKC\textsubscript{L} fused to GST (dotted line). E, FRET measurements were obtained for Par6/CFP in the presence of increasing amounts of the nonbinding control protein YFP (●) or its binding partner PKC\textsubscript{L}/YFP (▲). Results are expressed as the fluorescence ratio of emission 529/475 nm. The nonbinding control values were subtracted from the Par6/CFP:PKC\textsubscript{L}/YFP values to obtain the specific FRET associated with the saturable binding between Par6/CFP and PKC\textsubscript{L}/YFP (dotted line).
Par6/CFP by FRET. Thus, these optimized FRET conditions were used to screen our compound library.

**Screening of a compound library for inhibitors of PKC-PAR6 interactions.** Having developed and optimized a FRET-based assay capable of detecting binding between the PBI domains of PKC\(\alpha\) and Par6, we next screened the GenPlus chemical compound library from Microsource Discovery Systems for compounds that inhibit this binding. The GenPlus library is a unique collection of ~960 known bioactive compounds that permit the simultaneous evaluation of hundreds of marketed drugs and biochemical standards. All of the compounds in the library have well-characterized pharmacokinetic and bioavailability profiles, making them excellent first hit candidates. A representative collection of nine compounds from the library screen are shown in Fig. 3A. As can be seen, one of these compounds, ATG, showed a marked decrease in the fluorescence ratio approaching that observed with the control nonbinding protein pair Par6/CFP + YFP (which gave a fluorescence ratio value of ~0.2). Subsequent analysis showed that ATG causes a dose-dependent loss of FRET with maximum inhibition in the 100 \(\mu\)mol/L range with an apparent IC\(_{50}\) in the low micromolar range (Fig. 3B). Incubation of purified YFP-PKC\(\alpha\) or CFP-Par6 alone with ATG at concentrations up to 1 mmol/L had no effect on the intrinsic fluorescence of these proteins, indicating that ATG does not inhibit FRET by quenching CFP or YFP fluorescence (data not shown).

We next wished to confirm that ATG inhibits binding between Par6 and PKC\(\alpha\). For this purpose, we devised a complimentary binding assay. In this assay, full-length biotinylated Par6 was bound to streptavidin-coated 96-well plates and binding of PKC\(\alpha\)/YFP was determined by quantifying the amount of PKC\(\alpha\)/YFP fluorescence bound to the plates in the presence of increasing concentrations of ATG (Fig. 4A). As can be seen, addition of increasing concentrations of ATG to the binding mixture led to a dose-dependent loss of bound PKC\(\alpha\)/YFP. In this assay, maximal inhibition of binding was achieved at 10 \(\mu\)mol/L ATG with an apparent IC\(_{50}\) of ~1 \(\mu\)mol/L, in line with the values obtained in our initial FRET-based screen. In addition to ATG, we also tested ATM, a structurally related gold-containing compound that is also used in the treatment of rheumatoid arthritis. Like ATG, ATM caused a dose-dependent loss of PKC\(\alpha\)/YFP binding with potency similar to ATG (Fig. 4B).

Taken together, these results provide confirmation of our initial drug screen and show that both ATG and ATM are potent inhibitors of PKC-PAR6 interactions in vitro with apparent IC\(_{50}\)s in the low micromolar range. Examination of the chemical structures of ATG and ATM, respectively.
and ATM reveals that a similar thio gold moiety is likely to be involved in their inhibitory activity (Fig. 4C and D).

**ATG inhibits PKC*-mediated cellular signaling.** Having identified ATG as an inhibitor of binding between Par6 and PKCs, we next assessed whether ATG was an effective inhibitor of PKC signaling in cells. We recently showed that PKC* plays a requisite role in the transformed growth of human lung adenocarcinoma A549 cells through activation of the small molecular weight GTPase Rac1 (3). Expression of a dominant-negative kdPKC* leads to inhibition of Rac1 activation and transformed growth of A549 cells (3). The PKC*-dependent activation of Rac1 involves the PB1 domain of PKC* because expression of the PB1 domain of PKC* inhibits coupling of PKC* to Rac1 activation. Others have shown that Par6 couples PKC* to Rac1 by binding PKC* via its PB1 domain while also binding Rac1 through a distinct CRIB domain (Fig. 5A; for a review, see ref. 13). Thus, we hypothesized that if ATG disrupts binding between PKC* and Par6, it should block PKC*-mediated activation of Rac1. Indeed, incubation of A549 cells with increasing concentrations of ATG leads to a dose-dependent decrease in Rac1 activity (Fig. 5B). At 10 and 100 μmol/L ATG, inhibition of Rac1 activity was comparable to that observed in A549 cells expressing kdPKC*, which we previously showed disrupts PKC*-dependent Rac1 activation (3, 9). Quantitative analysis of multiple independent experiments showed that 10 μmol/L ATG is equipotent with kdPKC* at inhibiting Rac1 activity (Fig. 5C). ATG at 100 μmol/L had no further inhibitory effect on Rac1 (data not shown). It should be noted that not all Rac1 activity is PKC*-dependent, a finding that is not surprising, considering the many potential upstream effectors of Rac1 activity. These data are consistent with the apparent IC₅₀ of ATG for inhibition of the Par6-PKC*

**Figure 5.** ATG inhibits PKC*-dependent activation of Rac1. A, schematic diagram illustrating how Par6 mediates coupling between PKC* and Rac1 by binding these proteins via distinct binding domains on Par6. B, A549 cells were treated with ATG at the concentrations indicated and assayed for active, GTP-bound Rac1 as previously described (3). Expression of kdPKC* in A549 cells also leads to Rac1 inhibition. C, active and total Rac1 was assessed in A549 cells treated with 10 μmol/L ATG and in cells transfected with kdPKC*. Rac1 activity is expressed as active, GTP-bound Rac1/total Rac1. Columns, mean (n = 3); bars, SE; *, P < 0.05, compared with control cells.

**Figure 6.** ATG inhibits the transformed growth of A549 cells in vitro and in vivo. A, anchorage-dependent growth of A549 cells in the presence or absence of 10 μmol/L ATG. Columns, mean (n = 3); bars, SE. B, anchorage-independent growth of A549/LZRS and A549/RacV12 cell transfectants in soft agar in the presence or absence of 10 μmol/L ATG. Columns, mean (n = 4); bars, SE. *, P = 0.0001, compared with cells treated with diluent (0.1% DMSO). **, P < 0.05. C, tumorigenic growth of A549 cells as s.c. xenografts in nude mice. Mice were injected daily with ATG (200 mg/kg) or vehicle for 14 days and tumor volume was monitored as previously described (3). Points, mean (n = 10); bars, SE.

interaction in vitro because on a pharmacologic basis, one would expect a concentration of drug 10-fold higher than the IC₅₀ to cause near complete inhibition. Similar results were obtained with ATM, confirming the ability of a structurally related compound to inhibit PKC* signaling (data not shown).

**ATG blocks PKC*-dependent transformation and tumorigenicity.** We recently showed that expression of kdPKC* has no effect on A549 cell survival or growth in adherent culture but has a dramatic inhibitory effect on transformed growth in vitro and in vivo (3). Therefore, we assessed whether the effects of ATG on adherent versus transformed growth of A549 cells were similar to kdPKC*. Addition of 10 μmol/L ATG to adherent cultures of A549 cells had no appreciable effect on cell growth (Fig. 6A) or survival (data not shown), consistent with the effect of kdPKC* under adherent growth conditions (3). However, 10 μmol/L ATG resulted
in a significant reduction in the ability of A549 cells to grow as colonies in soft agar (Fig. 6B). These results are entirely consistent with our results using kdPKC\(_{i}\) (3).

We previously showed that expression of a constitutively active Rac1 mutant, RacV12, in cells expressing kdPKC\(_{i}\) restores their transformed growth in soft agar (3, 9). If, as our data indicate, ATG disrupts the PKC\(_{i}\)-Par6 complex, thereby inhibiting PKC\(_{i}\)-dependent activation of Rac1, then expression of RacV12 should cause cells to be resistant to the inhibitory effects of ATG. Indeed, A549/ RacV12 cells grow as soft agar colonies equally well in the absence and presence of 10 \(\mu\)mol/L ATG (Fig. 6B). These results provide genetic evidence for a mechanism of action of ATG as an inhibitor of PKC\(_{i}\) signaling upstream of Rac1.

We next assessed whether ATG is an effective therapeutic agent against A549 cell tumor growth in vivo. Athymic nude mice were injected with A549 cells to establish s.c. tumors. Once tumors were established, the mice were randomly divided into two groups that received daily injections of either ATG (200 mg/kg) or diluent (0.9% saline), and tumor volume was monitored as previously described (3). In the presence of ATG, A549 cell tumors exhibited a dramatic reduction in growth kinetics compared with control tumors (Fig. 6C). Our results indicate that ATG has a cytostatic rather than cytotoxic effect on A549 tumors. A similar cytostatic effect was observed in A549 cell tumors expressing kdPKC\(_{i}\) (3), consistent with a mechanism of action for ATG at the level of inhibition of PKC\(_{i}\) signaling.

**Discussion**

We recently showed that the atypical PKC isozyme PKC\(_{i}\) is an oncogene in NSCLC (4). Specifically, we showed that PKC\(_{i}\) expression is elevated in the vast majority of NSCLC cases and that PKC\(_{i}\) expression is predictive of poor clinical outcome (4). We also showed that the PKC\(_{i}\) gene undergoes frequent tumor-specific genetic alteration by gene amplification in NSCLC. PKC\(_{i}\) gene amplification drives PKC\(_{i}\) expression, showing the functional importance of gene amplification in NSCLC tumorigenesis (4). Mechanistically, we showed that PKC\(_{i}\) plays a requisite role in transformed growth by activating a PKC\(_{i}\)-Rac1-Pak-Mek-Erk signaling axis that drives transformed growth (3). Our data indicated that PKC\(_{i}\) signaling is an attractive target for development of novel mechanism-based therapeutics for the treatment of lung cancer.

In an effort to develop therapeutics targeting PKC\(_{i}\), we developed a novel high-throughput assay to screen for compounds that inhibit PKC\(_{i}\) signaling. Our FRET assay is based on our previous observation that PKC\(_{i}\)-dependent transformation requires the PB1 domain of PKC\(_{i}\) and coupling of PKC\(_{i}\) to the downstream effectors Rac1 (3). There is ample evidence that PB1-PB1 domain interactions between PKC\(_{i}\) and the adapter molecule Par6 are responsible for coupling PKC\(_{i}\) to Rac1 during the establishment of epithelial cell polarity (14). Based on these observations, we reasoned that PB1-PB1 domain interactions between Par6 and PKC\(_{i}\) represent a viable drug target for the development of antitumor agents. Through a high-throughput screen of a compound library consisting of 960 drugs that have been used in humans, we identified a promising antitumor agent, ATG. ATG and the related gold compound ATM have been used for many years in the treatment of rheumatoid arthritis (15). However, their use has become limited recently due to the development of effective nonsteroidal anti-inflammatory drugs with efficacy against rheumatoid arthritis and lower toxicity. Despite their extensive use in clinical practice, the precise mechanism of action of gold compounds in rheumatoid arthritis is largely unknown. These compounds exhibit potent anti-inflammatory properties that have been postulated to be mediated through inhibition of nuclear factor \(\kappa\)B (NF-\(\kappa\)B) signaling (16, 17). However, the molecular mechanism underlying the ability of these compounds to inhibit NF-\(\kappa\)B signaling has not been definitively elucidated although a recent report indicated that gold compounds may inhibit NF-\(\kappa\)B signaling through inhibition of I\(\kappa\)B kinase activation (18).

In the present study, we identified ATG (and ATM) as potent inhibitors of PB1 domain-mediated interactions between Par6 and PKC\(_{i}\). Both ATG and ATM inhibit this interaction in a dose-dependent manner with an apparent IC\(_{50}\) in the low micromolar range. Biochemical evidence for this mechanism of action in intact cells is provided by our observation that ATG blocks PKC\(_{i}\)-mediated activation of the downstream effector of the Par6-PKCBi signalosome, Rac1. Indeed, 10 \(\mu\)mol/L ATG causes complete inhibition of PKC\(_{i}\)-dependent Rac1 activation, consistent with the fact that this concentration of ATG also elicits maximal inhibition of Par6-PKCBi interactions in vitro. Furthermore, expression of a constitutively active Rac1 mutant renders A549 cells resistant to the inhibitory effects of ATG on transformed growth, consistent with a mechanism at the level of PKC\(_{i}\) upstream of Rac1.

Interestingly, PB1 domain interactions involving PKC\(_{i}\) have also been implicated in the coupling of PKC\(_{i}\) to NF-\(\kappa\)B signaling in some cell systems (19, 20). In fibroblasts, PKC\(_{i}\) has been reported to couple to the NF-\(\kappa\)B signaling cascade through PB1-PB1 domain interactions between PKC\(_{i}\) and the adaptor molecule p62 (19, 20). Crystallographic and solution structural analysis of PB1 domain interactions between PKC\(_{i}\) and Par6 on the one hand, and between PKC\(_{i}\) and p62 on the other hand, indicate that the same binding surface of PKC\(_{i}\) mediates binding to both of these two proteins (21–24). These results suggest that ATG is likely to also be a potent inhibitor of PKC\(_{i}\)-p62 interactions. If this were the case, one would predict that ATG might inhibit PKC\(_{i}\)-dependent activation of NF-\(\kappa\)B in cell types in which PKC\(_{i}\) couples to NF-\(\kappa\)B through p62 interactions. This hypothesis would provide a unifying molecular mechanism underlying the actions of ATG as an anti-inflammatory agent in rheumatoid arthritis and as an antitumor agent in cancer cells. In this regard, we have shown that in A549 cells, PKC\(_{i}\) does not effectively couple to NF-\(\kappa\)B and that NF-\(\kappa\)B is not a critical mediator of oncogenic PKC\(_{i}\) signaling (3). We have observed that ATG does not affect NF-\(\kappa\)B signaling in A549 cells, \(^1\) consistent with the fact that NF-\(\kappa\)B signaling in A549 cells is largely independent of PKC\(_{i}\) (3). In future studies, it will be of interest to determine whether ATG inhibits NF-\(\kappa\)B in cell systems in which PKC\(_{i}\) is tightly coupled to this signaling pathway.

Our present data show that ATG and ATM possess potent antitumor activities against NSCLC in vitro and in vivo. Our data are consistent with the interpretation that the antitumor effects of ATG and ATM are due to their inhibitory effects on PKC\(_{i}\)-Par6 interactions and subsequent coupling to Rac1. Furthermore, the inhibitory actions of ATG are strikingly similar to those obtained when PKC\(_{i}\) signaling is genetically disrupted using a dominant-negative PKC\(_{i}\) mutant allele, kdPKC\(_{i}\). The potency of ATG as an antitumor agent and as an inhibitor of PKC\(_{i}\)-mediated activation of

---

\(^1\) Unpublished observation.
Rac1 are virtually identical, and reconstitution of Rac1 activity by expression of a constitutively active Rac1 allele, RacV12, provides further genetic evidence for the molecular mechanism of action of ATG revealed here. Our results show that ATG and ATM will be useful in the molecular dissection of PKC-mediated cellular signaling by providing a pharmacologic mechanism for inhibiting PKCs that is complementary to existing genetic approaches. ATG and ATM also hold promise as potential novel therapeutic agents for the treatment of NSCLC patients. With regard to the potential clinical use of ATG and/or ATM as antitumor agents, it is important to note that in our studies, we achieved maximal inhibition of PKCα signaling in NSCLC at concentrations of 10 μmol/L. Pharmacokinetic studies on rheumatoid arthritis patients receiving a standard regimen of gold therapy with ATG or ATM show that steady-state blood levels in the 10 μmol/L range are routinely achievable with limited adverse toxicity (25). Thus, it is likely that therapeutically relevant levels of ATG can be achieved in cancer patients using well-established dosing regimens already approved for rheumatoid arthritis. Further preclinical studies will be required to evaluate the efficacy of ATG and ATM as single agents in multiple preclinical NSCLC tumor models and to assess whether ATG and/or ATM might be effective in combination with existing standards of therapy for NSCLC. It will also be of interest to assess whether these agents also show efficacy against other tumor types.

Acknowledgments


Grant support: Mayo Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. D. Zacharias for expert advice on the design and implementation of FRET-based assays and Dr. Chris Eckman (Department of Neuroscience, Mayo Clinic, Jacksonville, FL) for providing the Microsource GenPlus chemical library and for advice on high-throughput drug screening.

References


A Novel Small-Molecule Inhibitor of Protein Kinase C\(\text{\textbeta}\) Blocks Transformed Growth of Non–Small-Cell Lung Cancer Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/3/1767

Cited articles
This article cites 24 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/3/1767.full#ref-list-1

Citing articles
This article has been cited by 19 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/3/1767.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/66/3/1767.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.