AT13148 Is a Novel, Oral Multi-AGC Kinase Inhibitor with Potent Pharmacodynamic and Antitumor Activity

Timothy A. Yap1, Mike I. Walton1, Kyla M. Grimshaw2, Robert H. te Poele1, Paul D. Eve1, Melanie R. Valent1, Alexis K. de Haven Brandon1, Vanessa Martins1, Anna Zetterlund1, Simon P. Heaton1, Kathrin Heinzmann1, Paul S. Jones3, Ruth E. Feltel2, Matthias Reule2, Steven J. Woodhead2, Thomas G. Davies2, John F. Lyons2, Florence I. Raynaud1, Suzanne A. Eccles1, Paul Workman1, Neil T. Thompson2, and Michelle D. Garrett1

Abstract

**Purpose:** Deregulated phosphatidylinositol 3-kinase pathway signaling through AGC kinases including AKT, p70S6 kinase, PKA, SGK and Rho kinase is a key driver of multiple cancers. The simultaneous inhibition of multiple AGC kinases may increase antitumor activity and minimize clinical resistance compared with a single pathway component.

**Experimental Design:** We investigated the detailed pharmacology and antitumor activity of the novel clinical drug candidate AT13148, an oral ATP-competitive multi-AGC kinase inhibitor. Gene expression microarray studies were undertaken to characterize the molecular mechanisms of action of AT13148.

**Results:** AT13148 caused substantial blockade of AKT, p70S6K, PKA, ROCK, and SGK substrate phosphorylation and induced apoptosis in a concentration and time-dependent manner in cancer cells with clinically relevant genetic defects in vitro and in vivo. Antitumor efficacy in HER2-positive, PIK3CA-mutant BT474 breast, PTEN-deficient PC3 human prostate cancer, and PTEN-deficient MES-SA uterine tumor xenografts was shown. We show for the first time that induction of AKT phosphorylation at serine 473 by AT13148, as reported for other ATP-competitive inhibitors of AKT, is not a therapeutically relevant reactivation step. Gene expression studies showed that AT13148 has a predominant effect on apoptosis genes, whereas the selective AKT inhibitor CCT128930 modulates cell-cycle genes. Induction of upstream regulators including IRS2 and PIK3IP1 as a result of compensatory feedback loops was observed.

**Conclusions:** The clinical candidate AT13148 is a novel oral multi-AGC kinase inhibitor with potent pharmacodynamic and antitumor activity, which shows a distinct mechanism of action from other AKT inhibitors. AT13148 will now be assessed in a first-in-human phase I trial.

©2012 AACR.

Introduction

The class I phosphoinositide 3-kinases (PI3K) are key mediators of intracellular signaling between the membrane-bound receptor tyrosine kinases (RTKs) and downstream effector molecules, which control many vital cellular functions, including survival, growth, proliferation, and motility (1, 2). Downstream of these PI3Ks lies a network of serine/threonine kinases, including several members of the AGC kinase family, such as AKT, also known as protein kinase B (PKB), phosphoinositide-dependent kinase 1 (PDK1), p70S6 kinase (p70S6K), p90 ribosomal S6 kinase (RSK), serum- and glucocorticoid-induced kinase (SGK), and Rho kinase (ROCK; refs. 3, 4). The PI3K-AKT axis of this signaling network is hyperactivated in multiple cancers through different mechanisms, including the deregulation of upstream RTKs, for example, insulin-like growth factor-1 receptor (IGF-1R), and genetic alterations of PIK3CA, PTEN, or AKT genes, AKT1, 2, and 3 (1, 2). Thus, pharmacologic inhibition of this pathway is an area of great therapeutic interest (5).

Several drugs targeting the PI3K-AKT pathway are currently in clinical development, including inhibitors of PI3K, AKT, and mTORC1/2 (5, 6). However, inhibiting PI3K-AKT signaling at a single node has shown relatively limited clinical efficacy to date. There are several possible explanations for this. First, AKT inhibition has been shown to relieve feedback suppression of RTK expression and activity, which may attenuate antitumor activity (7). Second, PI3K deregulation may promote cancer through both AKT-
**Translational Relevance**

Deregulated phosphoinositide 3-kinase (PI3K)-AKT pathway signaling through AGC kinases is implicated in many cancers. The simultaneous inhibition of multiple AGC kinases may increase antitumor activity and minimize clinical resistance compared with a single kinase target. The clinical candidate AT13148 is a novel, oral, multi-AGC kinase inhibitor, which has potent pharmacodynamic and antitumor activity in human tumor xenografts with clinically relevant genetic defects in *vivo* and *in vivo*, and shows a distinct mechanism of action from selective AKT inhibitors. AT13148 showed linear pharmacokinetics, achieved therapeutically active drug concentrations, and induced biomarker changes consistent with AGC inhibition in human tumor xenografts. Moreover, we show for the first time that induction of AKT phosphorylation at serine 473 by AT13148, as reported for other ATP-competitive inhibitors of AKT, is not a therapeutically relevant reactivation step for this compound. These detailed preclinical and mechanistic data will facilitate the forthcoming first-in-human phase I trial of AT13148.

dependent and AKT-independent mechanisms, the latter involving the AGC kinases PDK1 and SGK (8). Third, inhibition of a single node such as PI3K or AKT may allow clinical resistance as reported for the selective BRAF inhibitor vemurafenib (PLX4032, Roche; ref. 9). Furthermore, concurrent blockade of multiple components of the PI3K network may have greater therapeutic value than inhibition of any single target (10). Thus, the simultaneous inhibition of several essential nodes of the PI3K signaling network may provide greater overall suppression of key pathways, with the potential for improved therapeutic efficacy across a broader range of cancer types with less opportunity for resistance to develop.

A possible strategy for such a targeted combinatorial blockade is the development of a drug that inhibits AKT together with other key AGC kinases that form part of the PI3K signaling network. In this paper, we present the first detailed pharmacokinetic characterization of the novel, oral clinical drug candidate AT13148, a potent multi-AGC kinase inhibitor. AT13148 was identified using high-throughput X-ray crystallography and fragment-based lead discovery techniques (11–14). We show that this orally bioavailable drug candidate achieves active tumor exposure, induces robust pharmacodynamic biomarker modulation and apoptosis in cancer cells with clinically relevant genetic defects both *in vitro* and *in vivo*, and exhibits anti-tumor efficacy in multiple human tumor xenograft models. Moreover, we show that AT13148-induced hyperphosphorylation of Ser473 AKT is not a therapeutically relevant reactivation step for this compound, in contrast to previous suggestions for other ATP-competitive inhibitors of AKT (15). Finally, we describe gene expression microarray studies that characterize the underlying molecular mechanisms of action of AT13148, including compensatory feedback loops that reveal differences compared with a more selective AKT inhibitor. On the basis of these studies, AT13148 is now undergoing preclinical development before entry into phase I clinical trials.

**Materials and Methods**

**Cell culture and reagents**

All human cancer cell lines were purchased from the American Type Culture Collection and grown in their recommended culture medium, containing 10% FBS at 37°C in an atmosphere of 5% CO₂ and passaged for less than 6 months. AT13148 (16), CCT128930 (17), and LY294002 (Calbiochem, Merck Biosciences) were made up as 10 mmol/L stocks in dimethyl sulfoxide (DMSO).

**In vitro kinase assays**

AT13148 was assayed against 40 kinases (National Centre for Kinase Profiling, Dundee, UK) and the percentage inhibition at 10 μmol/L of AT13148 was determined. Individual IC₅₀ values were measured for selected kinases using ATP concentrations equivalent to the Km for each enzyme (Invitrogen).

**Protein immunoblotting and immunoassay**

Cells were harvested, lysates prepared, protein estimations conducted, and Western blots undertaken as described (18), using the following antibodies: pSer473 AKT, AKT, pSer9 GSK3β, GSK3β, pSer235/236 S6 ribosomal protein (S6RP), S6RP, pSer330 NDRG, NDRG, pSer157 VASP, VASP, pSer19 MLC2, MLC2, pThr24 FOXO1/pThr32 FOXO3a, FOXO1, PRAS40, cleaved PARP, IRS2, pThr1135Rictor, Rictor, cyclin E2, c-MYC (Cell Signaling Technology). PIK3P1 (Abcam), pThr246 PRAS40 (Upstate), cleaved caspase-3 (Epitomis), cyclin D1, p27, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Neomarkers).

**Cell cycle effects and Annexin V staining**

For studies using the Meso Scale Discovery (MSD) electrochemoluminescence platform, U87MG cell lysates were prepared as described earlier and probed for pSer473 AKT, AKT, pSer9 GSK3β, GSK3β, pSer240/244 S6RP, S6RP, pThr1462 tuberin, tuberin, and cleaved PARP (Cell Signaling Technology). Primary antibodies were labeled with infrared IRDye-labeled secondary antibodies (LI-COR Biosciences) and proteins visualized using the Odyssey Infrared Imager (LI-COR Biosciences).

For studies using the Mesoscale Discovery (MSD) electrochemiluminescence platform, U87MG cell lysates were prepared as described earlier and probed for pSer473 AKT, AKT, pSer9 GSK3β, GSK3β, pThr421/424 p70S6K, pThr381/385 p70S6K, and p70S6K according to the manufacturer’s instructions (19).

**OF2**

Clin Cancer Res; 18(14) July 15, 2012

Clinical Cancer Research

Published OnlineFirst July 10, 2012; DOI: 10.1158/1078-0432.CCR-11-3313
iodide as described and analyzed by flow cytometry (18). The proportion of cells in each phase of the cell cycle was determined using either the Cell Quest Pro Software package (BD Biosciences) or WinMDI 2.8. Annexin V staining was carried out according to the manufacturer’s instructions (BD PharMingen).

**In vivo studies**

**Pharmacokinetic and pharmacodynamic analyses.** All procedures were in accordance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986, approved by The Institute of Cancer Research’s Ethics Committee and in accordance with published guidelines (20). Mice were allowed access to food and water *ad libitum*. For pharmacokinetic analysis, male athymic BALB/c mice were obtained from Harlan. AT13148 was formulated in 10% DMSO, 1% Tween-20, and 89% saline and administered at 5 mg/kg i.v. or p.o. Duplicate samples of heparinized whole blood were collected by cardiac puncture at 1, 2, 4, 6, 8, 16, 24, and 72 hours after dosing. Plasma and tissues (liver, kidney, spleen, and muscle were also taken) were prepared and frozen at −20°C until analysis. AT13148 was extracted from plasma and tissues using acetonitrile containing an internal standard and quantified using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method and appropriate standard curves. Pharmacokinetic parameters were determined using WinNonLin software version 5.2.

To assess pharmacokinetic and pharmacodynamic relationships, a single dose of AT13148 (30, 40, or 50 mg/kg p.o.) was given to groups of 3 female athymic (CrlTac:Ncr-Fox1nu) mice bearing established subcutaneous xenografts of MES-SA, BT474, or male athymic mice bearing PC3 xenografts. AT13148 was extracted from plasma and tissue homogenates and quantified as described earlier. For pharmacodynamic studies, tumors were ground to a powder under liquid nitrogen, lysed and centrifuged to remove debris. Protein content was measured using BCA or Bradford reagent and samples evaluated by Western blots or by MSD analysis as described earlier.

**Efficacy studies**

Human MES-SA, BT474, and PC3 tumor cells were injected s.c. into the right flank of female or male athymic mice. When tumors reached ~100 mm³ mean volume, animals were randomized and treated with vehicle (10% DMSO, 1% Tween-20, and 89% saline) or AT13148 p.o. using the dosage schedules detailed in the figures. Body weight and tumor size were determined as previously described (17) 3 times weekly. For the MES-SA study, %T/C represents mean tumor volume of treated animals divided by mean control tumor volume, expressed as a percentage on any particular day. For the BT474 and PC3 studies, %T/C represents mean tumor weight of treated animals divided by mean control tumor weight at the end of the experiment, expressed as a percentage.

**Statistical analyses**

Statistical significance was determined using 1- or 2-tailed *t* tests as appropriate with GraphPad Prism 5.0. Other methods including those for crystallography and microarrays studies are detailed in the Supplementary Material.

**Results**

**Identification of a potent ATP-competitive inhibitor of key AGC kinases**

AT13148 was discovered using fragment-based screening combined with structure-based design as previously described (refs. 13 and 16; Fig. 1A). The structure of AT13148 bound in the ATP pocket of the PKA-AKT chimera was solved by X-ray crystallography (Fig. 1B). The use of this PKA-based surrogate provides a robust and validated structural system.
for understanding interactions between inhibitors and AKT (14). As expected, the structure shows that AT13148 fulfills the requirements of the canonical 3-point pharmacophore needed for potent binding to the ATP site of AKT, forming hydrogen-bonding interactions with the kinase hinge, electrostatic interactions with the ribose site, and hydrophobic contacts with a lipophilic pocket in the glycine-rich loop.

Screening of AT13148 against a panel of kinases at 10 μmol/L revealed >80% inhibition of the structurally related AGC kinases AKT, PKA, ROCK2, p70S6K, MSK, RSK1/2, and SGK (Supplementary Table S1). Further studies showed that IC₅₀ values for p70S6K, PKA, ROCK1, and ROCK2 were all less than 10 nmol/L and those for AKT1, 2, and 3 were 38, 402, and 50 nmol/L, respectively (Supplementary Table S2). For the related AGC kinases RSK1 and SGK3, the IC₅₀ values were 85 and 63 nmol/L, respectively. In contrast, IC₅₀ values for the non-AGC kinases CHK2 and Aurora B were both greater than 800 nmol/L. Therefore, AT13148 is a potent inhibitor of the AGC kinases p70S6K, PKA, ROCKI, and ROCKII that also potently inhibits the related family members AKT1, AKT2, AKT3, RSK1, and SGK3 (Supplementary Table S2).

**AT13148 inhibits the proliferation and AGC kinase activity of cancer cells**

AT13148 potently inhibited proliferation with GI₅₀ values of 1.5 to 3.8 μmol/L across a selected panel of cancer cell lines (Supplementary Table S3) representing common human malignancies with deregulation of PI3K-AKT-mTOR or RAS-RAF pathways. The effect of 1-hour exposure to AT13148 on AKT and p70S6K signaling was initially explored in PTEN-deficient U87MG glioblastoma cells (Fig. 2A). Marked induction of pSer473 AKT occurred at all concentrations. Nevertheless, phosphorylation of the 2 AKT substrates GSK3β and PRAS40 was inhibited at AT13148 concentrations >1 and 5 μmol/L AT13148, respectively.

---

**Figure 2.** The effect of AT13148 exposure on AGC kinase biomarker expression. PTEN-deficient U87MG human glioblastoma cells were incubated with AT13148 for 1 hour (A and B) at the concentrations indicated or (C) with 10 μmol/L AT13148 for the times indicated. D, PTEN-deficient MES-SA human uterine sarcoma cells were incubated for 1 hour with AT13148 at the concentrations indicated. Immunoblotting was carried out for the proteins indicated. GAPDH was used as a loading control. C, no treatment control; D, DMSO vehicle control; LY, positive control (PI3K inhibitor LY294002, 30 μmol/L).
Phosphorylation of the p70S6K substrate, Ser235/236 S6RP, was also inhibited at concentrations >5 μmol/L. Total protein levels remained generally constant throughout this study, apart from PRAS40, which increased with AT13148 treatment (Fig. 2A).

Immunoblotting after 1-hour treatment with AT13148 in U87MG cells also showed clear inhibition of phosphorylation on direct substrates of the AGC kinases PKA, ROCK, and SGK, namely Ser157 VASP, Ser19 MLC2, and Ser330 NDRG1, respectively (Fig. 2B). Inhibition of pSer157 VASP and pSer19 MLC2 was observed from 0.5 μmol/L AT13148, whereas pSer330 NDRG1 was inhibited from 5 μmol/L AT13148. Expression of total protein for all 3 AGC kinase substrates remained constant up to 20 μmol/L AT13148.

We next determined the effect of 10 μmol/L AT13148 on AKT and p70S6K signaling output over time in U87MG cells (Fig. 2C). Induction of pSer473 AKT was detected at the earliest time point assayed (0.5 hours) and sustained throughout the compound exposure time. Decreases in both pSer9 GSK3β and pThr246 PRAS40 were also observed from 0.5 to 24 hours, while a decrease in pSer235/236 S6RP was first detected at 1 hour. Total protein levels remained generally constant throughout the time course.

Inhibition of AKT and p70S6K signaling was also observed in PTEN-deficient MES-SA cells after treatment with AT13148 (Fig. 2D). A 1-hour drug exposure markedly inhibited phosphorylation of the AKT substrates GSK3β and tuberin, and the downstream p70S6K substrate S6RP, at AT13148 concentrations of 3 μmol/L or above, with minimal effects on total protein levels. Taken together, these biomarker data clearly show that AT13148 can inhibit a number of AGC kinase substrates, including AKT, in human tumor cell lines in vitro.

**Phosphorylation of AKT substrates remains suppressed, despite pSer473 AKT induction by AT13148**

ATP competitive inhibitors of AKT induce hyperactivation of AKT in vitro when dissociated from the inhibitor, thus potentially leading to activation of AKT targets in cells, and in turn promoting oncogenesis. To address this, we monitored both pSer473 AKT and phosphorylation of targets downstream of AKT in U87MG cells in vitro after exposure to AT13148 at 1 and 10 μmol/L for 1 and 24 hours, followed by the removal of compound for 0, 4, 8, or 24 hours (Supplementary Fig. S1A and S1B). Analysis of total and phospho-protein signals using the quantitative MSD electro-luminescence immunoassay revealed that the pSer473 AKT signal was strongly induced in U87MG cells at both concentrations and all time points, and was sustained even after 24 hours of compound removal. In contrast, both the pSer9 GSK3β and pThr421/424 S6K signals were inhibited under the same conditions and only showed partial recovery up to 24 hours after compound removal.

From these studies it can be concluded that although AT13148 induces phosphorylation on Ser473 AKT, compound removal does not cause downstream phosphorylation signals to recover rapidly or to rebound to greater than pretreatment levels.

**Pharmacokinetics and pharmacodynamics of AT13148**

To determine if therapeutically active concentrations of AT13148 can be achieved in vivo, the pharmacokinetic profile of this drug candidate was investigated in BALB/c mice. After administration of 5 mg/kg i.v., AT13148 showed a low plasma clearance of 1.68 L/h/kg, which is less than half liver blood flow, and a large volume of distribution of 9.05 L/kg with a terminal half-life of 2.83 hours (Fig. 3A; Supplementary Table S4). Oral drug administration of 5 mg/kg of AT13148 resulted in complete bioavailability. Increasing oral doses from 5 to 50 mg/kg showed linear pharmacokinetics, with plasma AUC₀₋∞ increasing in proportion with dose (Fig. 3B).

Figure 3C shows the concentrations of AT13148 achieved in athymic nude mouse plasma and HER2-positive PIK3CA-mutant BT474 human breast cancer xenografts after the administration of 2 daily doses of 40 mg/kg p.o. Consistent with the large volume of distribution described, tumor AT13148 concentrations greatly exceeded plasma concentrations at 2, 6, and 24 hours with tumor:plasma ratios of 8.5, 7.0, and 13.6, respectively. Moreover, tumor AT13148 concentrations were at least 9 times greater than the in vitro GI₅₀ value of 1.8 μmol/L for this cell line, maintained over 24 hours.

Pharmacodynamic biomarker changes measured in the same BT474 xenografts by MSD immunoassay are shown in Fig. 3D. There was an increase in pS473 AKT at 2, 6, and 24 hours after AT13148 treatment, consistent with in vitro observations (Fig. 2C). Importantly, phosphorylation of the AKT substrate GSK3β and the downstream target p70S6K was significantly decreased, consistent with sustained inhibition of AKT activity by AT13148 in vivo at this dose. Comparable studies in PTEN-deficient PC3 human prostate tumor xenografts also showed high AT13148 tumor:plasma ratios and significant decreases in the phosphorylation of AKT biomarkers (Supplementary Fig. S2A and S2B). Clear inhibition of phosphorylation of the AKT substrate GSK3β, tuberin, and the p70S6K target S6RP were also observed in PTEN-deficient MES-SA human uterine tumor xenografts after treatment with 40 and 50 mg/kg p.o. of AT13148 over 24 hours (Fig. 4A; Supplementary Fig. S3). Importantly, induction of cleaved PARP was observed at these doses of AT13148 over 24 hours, indicating that AT13148 induces apoptosis in solid tumors.

Taken together, these data suggest that AT13148 markedly inhibits the activity of both AKT and p70S6K AGC kinases in human tumor xenografts with differentially activated PI3K pathways after oral administration.

**In vivo antitumor activity of AT13148**

Following the demonstration of clear in vitro activity and promising in vivo pharmacokinetic and pharmacodynamic

www.aacrjournals.org Clin Cancer Res; 18(14) July 15, 2012 OF5

Published OnlineFirst July 10, 2012; DOI: 10.1158/1078-0432.CCR-11-3313

Downloaded from clincancerres.aacrjournals.org on July 14, 2017. © 2012 American Association for Cancer Research.
properties indicating target modulation, the antitumor activity of AT13148 was assessed in multiple human tumor xenograft models. Figure 4B shows that AT13148 markedly inhibited the growth of established MES-SA human uterine sarcoma tumor xenografts at the same doses of 40 and 50 mg/kg that also resulted in pharmacodynamic changes indicative of target engagement (Fig. 4A; Supplementary Fig. S3); the percentage treated/control (%T/C) was 41% and 54% at 40 and 50 mg/kg p.o. of AT13148, respectively, when measured on day 11 of treatment.

Further antitumor studies were undertaken in established BT474 (Fig. 4C) and PC3 human tumor xenograft models (Supplementary Fig. S2C). AT13148 inhibited the growth of both these models giving T/C values of 35.8% on day 26 and 65.4% on day 27, for BT474 and PC3 respectively. These results are consistent with both the pharmacokinetic and pharmacodynamic data (Figs. 3A–D and Fig. 4A; Supplementary Figs. S2A, S2B, and S3), which indicate that for this oral route of administration, plasma drug concentrations were at or just above the in vitro GI50 values, whereas tumor drug concentrations greatly exceeded GI50 values for at least 24 hours for these tumor cells. AT13148 also showed growth inhibition in the mutant KRAS A549 lung adenocarcinoma xenograft model (Supplementary Fig. S4). Tumor growth was significantly inhibited in the MES-SA, BT474, and PC3 tumor xenografts (P < 0.01), with marked growth inhibition in A549 (P = 0.0788) and minor animal weight loss in all studies (Supplementary Figs. S2D, S4B, S5A, and S5B). Our results therefore clearly show that orally administered AT13148 induces sustained inhibition of the AGC kinases AKT and p70S6K and exhibits marked antitumor effects in 4 genetically relevant human tumor xenograft models.
Gene expression microarray analysis

The molecular and cellular responses of the multi-AGC kinase inhibitor AT13148 drug candidate were explored by gene expression microarray and compared with those of the previously described AKT-selective inhibitor CCT128930 (17) after 6-hour treatment of U87MG cells at 0.1 μmol/L, 1 × GI50, and 3 × GI50 concentrations (Figs. 5A–D and 6A–C). The expression levels of 563 genes were significantly altered after AT13148 treatment, whereas the expression levels of 898 genes were significantly changed with CCT128930 (Fig. 5A and B; Supplementary Table S5A and S5B). Importantly, there was an overlap of 147 genes that exhibited significantly altered expression in response to both AT13148 and CCT128930 (Fig. 5B).

The effect of each compound on AKT pathway signaling was also simultaneously confirmed by Western blot analysis (Fig. 5C). Effects on AKT signaling were reflected by perturbations in the gene expression network (Fig. 5C). Expression of the FOXO1 gene is itself transcriptionally regulated by FOXO3a (23, 24), and FOXO1 expression was increased upon treatment with both compounds by gene expression microarray and confirmed at the protein level by Western blot analysis (Figs. 5C and 6B; Supplementary Table S5A and S5B).

Interestingly, the gene expression network analysis also revealed evidence of the induction of both negative and positive feedback mechanisms (Fig. 6C). Both PIK3CA and IRS2 can be transcriptionally regulated by FOXO3a and induction of both has been reported after treatment of cells with PI3K inhibitors (25–27). This could potentially lead to reactivation of the PI3K/AKT pathway. We observed increased expression of both PIK3CA and IRS2 genes after treatment with AT13148 and CCT128930 (Fig. 6C and Supplementary Table S5A and S5B). We also noted a concentration-dependent band shift downward of IRS2 on the Western blots for CCT128930, which was less marked for AT13148 (Fig. 5C). We confirmed using lambda phosphatase that this band shift is because of hypophosphorylation of IRS2 (Fig. 5D). AKT RNA interference (RNAi) studies confirmed that this band shift was AKT-dependent (Fig. 5D). The more specific AKT inhibitor CCT128930 induced a greater mobility shift and marked accumulation of IRS2 protein, compared with that observed for AT13148 or AKT RNAi, suggesting a greater blockade on targets that impact on IRS2. However,
this loss of phosphorylation on IRS2 does not lead to downstream reactivation of the PI3K-AKT pathway, as can be seen by the continued suppression of phosphorylation of AKT substrates (Fig. 5C). Expression of the tumor suppressor PIK3IP1, a potential transcriptional target of FOXO1 and CREB1 (28), was also induced at the gene transcription level following treatment with both compounds (Fig. 6C; Supplementary Table S5A and S5B). This induction was confirmed at the protein level (Fig. 5C). PIK3IP1 has been shown to decrease PI3K p110α activity both in vitro and in vivo (29, 30).

Gene ontology (GO) analysis (31, 32) for the biologic processes of the 147 common genes that showed significantly altered expression in response to both AT13148 and CCT128930 revealed that most genes affected were involved in regulation of the cell cycle, and apoptosis (Figs. 5B and 6A and B; Supplementary Table S6A–S6C). GO analysis for the genes showing altered expression with the specific AKT inhibitor CCT128930 revealed that “cell cycle” was the most common term in the top 20 most statistically significant (by P value) GO categories, which is consistent with gene expression changes previously observed with PI3K inhibitors (refs. 33, 34; Supplementary Table S6B). The effects of CCT128930 on the expression of cell-cycle genes were generally greater than with AT13148 (Fig. 6A). For example, the downregulation of positive cell-cycle regulators such as CDC25A, CDC6, and CCNE1 was more pronounced with CCT128930 than AT13148. However, in contrast to the downregulation of CCND1 in response to CCT128930, a modest but significant and reproducible increase was observed with AT13148, which was confirmed by TaqMan (data not shown). The downregulation of cyclin D1 by CCT128930 was also confirmed at the protein level, whereas AT13148 had no effect on the level of cyclin D1 protein (Fig. 5C; Supplementary Fig. S6A and S6B). CCT128930 also caused a decrease in the expression of the proapoptotic marker c-MYC, both at the gene and protein level, whereas AT13148 did not (Fig. 5B; Supplementary Fig. S6A and S6B).

In contrast to CCT128930, the GO analysis for AT13148 identified “cell death,” “programmed cell death,” and
Figure 6. Gene ontology analysis for the biologic processes of the 147 common genes that showed significantly altered expression in response to 6-hour treatment of both AT13148 and CCT128930 in U87MG human glioblastoma cells. A, cell-cycle genes; B, genes involved in apoptosis; C, PI3K pathway genes.
"apoptosis" as the most common terms in the 20 GO categories with the most statistically significant P values. (Supplementary Table S6C). These data are consistent with the potent induction of apoptosis seen after treatment with AT13148 both in vitro and in vivo. Furthermore, a direct comparison of the cellular effects of AT13148 (GI50 = 3.3 μmol/L; Supplementary Table S3) versus CCT128930 (GI50 = 6.3 μmol/L; ref. 17) identified a marked increased in PARP and caspase-3 cleavage with AT13148 versus CCT128930 at approximately equipotent doses of 10 and 20 μmol/L, respectively (Supplementary Fig. S6A and S6B). This was further emphasized by the fact that AT13148 showed a significantly greater degree of Annexin V staining than CCT128930 at equipotent concentrations, again indicative of a higher level of apoptosis with AT13148 (Supplementary Fig. S6C). Moreover, AT13148 inhibited the phosphorylation of the AKT substrates GSK3β, PRAS40, FOXO1, FOXO3a at similar concentrations to CCT128930 (Fig. 5C; Supplementary Fig. S6A and S6B), but was 10-fold more potent against the ROCK substrate MCL2 (Supplementary Fig. S6A and S6B), suggesting that inhibition of this AGC kinase may contribute to the increased apoptotic cell death. Furthermore, there was a clear difference in the cell-cycle effects of the 2 compounds, with CCT128930 causing a predominant G1/S arrest with loss of S-phase at increasing concentrations, in contrast to a predominant G2/M arrest seen with AT13148 (Supplementary Figs. S6D, S6E, and S7).

Summarizing this gene network analysis, both the AGC kinase inhibitor AT13148 and the more AKT-selective CCT128930 show molecular effects in cancer cells consistent with blockade of AKT signaling, leading to changes in gene expression that include induction of upstream regulators. However, the 2 agents clearly also have distinct effects in cancer cells. Although CCT128930 primarily modulates genes in the network regulating cell cycle and causes a G1 phase arrest, AT13148 has a predominant effect on apoptosis genes and causes a greater apoptotic phenotype, with a secondary effect on cell cycle at the G2–M phase.

Discussion

PI3K signaling is commonly deregulated in cancer and the oncogenic signal is transmitted predominantly through AGC kinases, such as AKT, p70S6K, PKD1, SGK, and ROCK (3). We report for the first time the detailed biologic activity of a novel, potent, oral clinical drug candidate AT13148, which is a multi-AGC kinase inhibitor discovered using fragment-based screening combined with structure-based design. We have showed that AT13148 is a potent inhibitor of selected AGC kinases including AKT, p70S6K, PKA, SGK, and ROCK (Supplementary Table S2).

In this study, we have used the pharmacologic audit trail that we originally conceptualized and subsequently advocated (35, 36) to guide the biomarker-driven drug discovery and development of AT13148. Thus, we incorporated detailed pharmacokinetic and pharmacodynamic studies to confirm adequate drug exposure with concomitant target and pathway blockade. We have showed that AT13148 has antiproliferative activity in a range of in vitro models harboring different genetic abnormalities, including pathogenic PTEN, KRAS, PIK3CA, and HER2 aberrations (Supplementary Table S3). Interestingly, the GI50 values obtained from these tumor cell lines were widely similar despite the different oncogenic alterations and therefore we intend to expand these observations to a much larger panel of human cancer cell lines. These results may be in part because of the simultaneous blockade of different AGC kinases, reducing opportunities for the disruption of negative feedback loops and cross-talk with other key signaling pathways, and thus attenuating the potential for intrinsic resistance. Furthermore, in our in vitro studies with PTEN-deficient U87MG human glioblastoma and similarly PTEN-deficient MES-SA uterine sarcoma tumor cells, we have shown that AT13148 causes substantial blockade of AKT, p70S6K, PKA, ROCK, and SGK substrate phosphorylation in both a concentration- and time-dependent manner, confirming that AT13148 can simultaneously inhibit multiple AGC kinases in these cancer cells (Fig. 2A–D).

Having showed promising pathway modulation and antiproliferative effects on cancer cells in vitro, our subsequent pharmacokinetic studies showed that drug exposure is related linearly to the administered oral dose of AT13148 (Fig. 3A and B). Furthermore, oral administration of AT13148 gave high tumor:plasma concentrations for at least 24 hours in mice bearing HER2-positive, PIK3CA-mutant BT474 breast or PTEN-deficient PC3 human prostate cancer xenografts (Fig. 3C; Supplementary Fig. S2A). These exposures greatly exceeded the in vitro antiproliferative GI50 values that would be predicted to produce pharmacodynamic biomarker modulation, pathway blockade, and antitumor efficacy. Indeed, inhibition of signaling output was confirmed by the marked inhibition of phosphorylation on both AKT and p70S6K substrates for up to 24 hours in both of these cancer models, as well as in PTEN-deficient MES-SA uterine tumor xenografts (Figs. 3D and 4A; Supplementary Figs. S2B and S3). Subsequently, oral efficacy studies with AT13148 showed antitumor effects in all 3 clinically relevant human tumor xenografts (Fig. 4B and C; Supplementary Fig. S2C). These data indicate that AT13148 exhibits promising single-agent antitumor activity after oral administration and support its clinical evaluation. Apart from a monotherapy drug development strategy, AT13148 may also be considered for rational combination regimens, especially with other targeted therapies that induce the activation of compensatory pathways, for example AKT phosphorylation after mTORC1 inhibition observed with everolimus (37). In keeping with other AKT inhibitors currently in the clinic, toxicologic studies with AT13148 revealed some early hyperglycemia, but the effects were largely equivocal (data not shown). As might be expected from its target kinase profile, AT13148 also showed vascular smooth muscle contraction, hypotension, and tachycardia (data not shown), but these perturbations returned to normal after repeat dosing, suggesting an adaptive response. Gene expression microarray analysis of
normal tissues might help to identify those genes associated with drug toxicity.

We have shown that robust inhibition of AGC kinase activity occurs in cancer cells both in vitro and in vivo, despite the observed induction of phosphorylation on Ser473 of AKT by AT13148, which binds into the ATP pocket of this kinase. This type of induction has been seen with other ATP-competitive inhibitors of AKT and shown to be because of direct inhibitor binding, rather than a regulatory pathway feedback mechanism (15). Furthermore, it has been shown that this phosphorylated form of AKT is hyperactive suggesting that in vivo treatment with an ATP competitive inhibitor of AKT may promote tumor cell growth (15). However, our studies have shown that despite the induction of pSer473 AKT, the removal of AT13148 from cancer cells in vitro does not lead to increased phosphorylation of AKT substrates (Supplementary Fig. S1A and S1B). Furthermore, our in vivo pharmacodynamic and efficacy studies with AT13148 (Figs. 3D and 4A–C; Supplementary Figs. S2–S4), using the doses and schedules presented in this paper, indicate that AKT is not hyperactivated and does not promote tumor cell growth but rather, signaling output and tumor growth are inhibited. It is not possible to conclude at this point whether the observed inhibition of AKT signaling output is because of the fact that AT13148 inhibits multiple AGC kinases, or is associated with the pharmacologic properties of this inhibitor. However, our data provide evidence both in vitro and in vivo that the AKT pathway is inhibited rather than activated with the AGC kinase inhibitor AT13148 and that such an inhibitor approach is a viable therapeutic anticancer strategy.

Our gene expression microarray studies in PTEN-deficient U87MG human glioblastoma cells identified an overlap of 147 genes that exhibited significantly altered expression in response to both AT13148 and CCT128930 (Fig. 5B). This suggests a component of shared mechanism of action on the gene network that correlated with inhibition of the IGFIPI3K- AKT-mTOR pathway (Figs. 5C and 6A–C). Of interest, increased expression of upstream positive regulators was observed especially of IRS2, which also seems to be at least partly regulated by FOXO1 and FOXO3a. The increased expression of genes encoding IRS2 and PI3K p110α could potentially lead to reactivation of the pathway, as has been shown previously for IRS2 with the pan-class I PI3K inhibitor, GDC-0941 (34). However, although we observed increased Ser473 phosphorylation on AKT with both CCT128930 and AT13148 (Fig. 5C), downstream targets were still dephosphorylated and the pathway remained inactive.

Both compounds altered the expression of genes involved in cell-cycle regulation and apoptosis (Figs. 5C and 6A and B; Supplementary Fig. S6). The enrichment for cell-cycle genes is very similar to that seen with the dual pan-class I PI3K/mTOR inhibitor PI-103 (33), suggesting that these gene expression changes are pathway related. However, effects were greater for CCT128930 than AT13148, consistent with the former being a more AKT-selective compound. Downregulation of positive cell-cycle regulators, such as cyclin E and CDC6, and upregulation of negative cell-cycle regulators including p27kip1 in response to treatment with both AT13148 and CCT128930, correlated with a substantial decrease in S-phase cells. However, although CCT128930, like other inhibitors of PI3K/AKT signaling, gave a predominant G1 arrest, AT13148 did not. This may be explained by the fact that in contrast to CCT128930, AT13148 did not cause decreased expression of the major G1 regulator, cyclin D1 at either the gene expression or protein level (Fig. 5C; Supplementary Fig. S6A and S6B). Consequently, there was an equal distribution of cells either side of S phase, expressed as an increase in the G2/M phase (Supplementary Figs. S6D, S6E, and S7). Conversely, the most enriched population of genes showing altered expression with AT13148 but not CCT128930 are those involved in the control of apoptosis. This molecular phenotype is recapitulated at the cellular level where a much greater induction of apoptosis is observed with AT13148 compared with CCT128930 at the same concentrations (Supplementary Fig. S6A–S6C). We hypothesize that these differences are a result of the targeting of several key AGC kinases by AT13148, in contrast to the more AKT-specific effects of CCT128930.

In conclusion, we have disclosed here for the first time the detailed mechanism of action and therapeutic potential of the novel, potent, multi-AGC kinase inhibitor, and oral drug candidate AT13148. We report the preclinical pharmacologic audit trail for AT13148 that supports its clinical development, including the pharmacokinetic—pharmacodynamic—antitumor activity relationship in clinically relevant human tumor xenografts. In addition, our detailed gene expression microarray analysis has revealed that AT13148 shows a distinct gene expression profile that correlates with a marked apoptotic rather than cytostatic phenotype, emphasizing the functional differences between its properties as a multi-AGC kinase inhibitor in contrast to a more AKT-selective inhibitor. In view of the potential mechanistic advantages detailed above, and the potent antitumor activity observed at well-tolerated doses against established human tumor xenografts with clinically relevant genetic drivers, the clinical use of such an AGC kinase inhibitor strategy will now be assessed in a first-in-human phase I trial of AT13148.

Disclosure of Potential Conflicts of Interest
T.A. Yap, M.I. Walton, R.H. te Poele, P.D. Eve, M.R. Valenti, A.K. de Haven Brandon, V. Martins, A. Zetterlund, S.P. Heath, R. Heinemann, F.J. Rynes, S.A. Eccles, P. Workman, and M.D. Garrett are current or former employees of The Institute of Cancer Research, which has a commercial interest in the development of AKT inhibitors, including AT13148, and operates a rewards for inventors scheme. K.M. Grimshaw, R. Feltell, M. Reule, S.J. Woodhead, T.G. Davies, J.F. Lyons, and N.T. Thompson are current or former employees of Astex Therapeutics, which also has a commercial interest in the development of AKT inhibitors including AT13148. Both Astex Therapeutics and The Institute of Cancer Research have been involved in a commercial collaboration with Cancer Research Technology Limited (CRT) to discover and develop inhibitors of AKT and intellectual property arising from this program has been licensed to AstraZeneca. P. Workman has a commercial research grant from Yamanouchi (now Astellas), Piramed Pharma, and Astex Pharmaceuticals; ownership interest (including patents) from Piramed Pharma (acquired by Roche) and Chroma Therapeutics; and is a consultant/advisory board member for Piramed Pharma, Chroma Therapeutics, Novartis, Wilks, and Nextech Ventures.
Grants Support was provided to T.A. Yap, M.I. Walton, P.D. Eve, M.R. Valenti, A.K. de Haven Brandon, V. Martins, A. Zetterlund, F.I. Raynaud, S.A. Eccles, P. Workman, and M.D. Garrett by Cancer Research UK [CR-UK] grant C309/A8274, to S.P. Heaton by CR-UK grant C51/A6883, to R.H. te Poele by CR-UK grant C51/A7401, to K. Heinzeimann by Marie Curie Early Stage Funding, and to P. Workman by CR-UK grant number C309/A8992. P. Workman is a Cancer Research Life Fellow. Additional support was provided to S.A. Eccles and M.D. Garrett by The Institute of Cancer Research. This work was carried out as part of a funded research collaboration with Astex Therapeutics. The authors acknowledge NHS funding to the NIHR Biomedical Research Centre.

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.

Received December 23, 2011; revised May 1, 2012; accepted May 15, 2012; published OnlineFirst July 10, 2012.

References

AT13148 Is a Novel, Oral Multi-AGC Kinase Inhibitor with Potent Pharmacodynamic and Antitumor Activity

Timothy A. Yap, Mike I. Walton, Kyla M. Grimshaw, et al.

Clin Cancer Res  Published OnlineFirst July 10, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-3313

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/07/10/1078-0432.CCR-11-3313.DC1

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, contact the AACR Publications Department at permissions@aacr.org.