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AT7867 is a potent and oral inhibitor of AKT and p70 S6 kinase that induces pharmacodynamic changes and inhibits human tumor xenograft growth

Kyla M Grimshaw¹, Lisa-Jane K Hunter², Timothy A Yap², Simon Heaton², Mike I Walton², Steve Woodhead¹, Lynsey Fazal¹, Matthias Reule¹, Thomas G. Davies¹, Lisa C. Seavers¹, Victoria Lock¹, John F. Lyons¹, Neil T. Thompson¹, Paul Workman², and Michelle D. Garrett^{2,*}

¹ Astex Therapeutics, 436 Cambridge Science Park, Milton Road, Cambridge, CB4 0QA, UK.

² Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, 15 Cotswold Road, Sutton, SM2 5NG, UK.

Abstract

The serine/threonine kinase AKT plays a pivotal role in signal transduction events involved in malignant transformation and chemoresistance, and is an attractive target for the development of cancer therapeutics. Fragment-based lead discovery combined with structure-based drug design have recently identified AT7867 as a novel and potent inhibitor of both AKT and the downstream kinase p70S6K and also of PKA. This ATP-competitive small molecule potently inhibits both AKT and p70S6K activity at the cellular level, as measured by inhibition of GSK3 β and S6RP phosphorylation, and also causes growth inhibition in a range of human cancer cell lines as a single agent. Induction of apoptosis was detected by multiple methods in tumor cells following AT7867 treatment. Oral or intraperitoneal administration of AT7867 (90mg/kg p.o. or 20mg/kg i.p.) to athymic mice implanted with the PTEN-deficient U87MG human glioblastoma xenograft model caused inhibition of phosphorylation of downstream substrates of both AKT and p70S6K, as well as induction of apoptosis, confirming the observations made *in vitro*. These doses of AT7867 also resulted in inhibition of human tumor growth in PTEN-deficient xenograft models. These data suggest that the novel strategy of AKT and p70S6K blockade may have therapeutic value and supports further evaluation of AT7867 as a single agent anticancer strategy.

* **Corresponding Author:** Michelle D. Garrett, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom. Tel: +44-20-8722-4352 Fax: +44-20-8722-4126 michelle.garrett@icr.ac.uk.

Potential conflicts of Interest: Kyla M. Grimshaw, Steve Woodhead, Thomas G. Davies, Lynsey Fazal, Matthias Reule, Lisa C. Seavers, Victoria Lock, John F. Lyons and Neil T. Thompson are or were employees of Astex Therapeutics, which has a commercial interest in the development of AKT inhibitors including AT7867. Lisa-Jane K. Hunter, Timothy A. Yap, Simon Heaton, Mike I. Walton, Paul Workman and Michelle D. Garrett are, or were employees of The Institute of Cancer Research, which also has a commercial interest in the development of AKT inhibitors, including AT7867 and operates a rewards for inventors scheme. 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.

Keywords

AT7867; AKT; p70 S6 kinase; cancer; therapeutic

Introduction

AKT (also known as protein kinase B; PKB) is a serine/threonine kinase that lies downstream of phosphatidylinositol 3-kinase (PI3K) and plays a key role in a range of cellular functions, including cell growth, proliferation, metabolism and survival (1, 2). Three closely related isoforms of AKT with overlapping cellular functions have been identified, termed AKT1, 2 and 3 (3).

AKT activation requires the association of its N-terminal pleckstrin homology (PH) domain with cell membrane phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) produced by PI3K (4). This facilitates phosphorylation of AKT at threonine 308 and serine 473, both of which are necessary for full activation of the protein. Phosphorylation of threonine 308 requires phosphoinositide-dependent kinase 1 (PDK1) (5, 6). A number of kinases have been reported to phosphorylate serine 473, the most prominent being the mammalian target of rapamycin complex 2 (mTORC2) (7-10). The activation of AKT is antagonized by the tumor suppressor PTEN (phosphatase and tensin homolog on chromosome 10) through the dephosphorylation of PIP₃ (11).

There are numerous proteins downstream of AKT which, when phosphorylated, participate in the regulation of critical cellular processes including growth, proliferation, metabolism and survival. Those involved in cell growth include p70 S6 kinase (p70S6K), S6 ribosomal protein (S6RP), and the mammalian target of rapamycin complex 1 (mTORC1) (12), while cell proliferation and metabolism are regulated through glycogen synthase kinase-3 β (GSK3 β) phosphorylation (13). A number of pro-apoptotic proteins including BAD (14), caspase 9 (15) and the forkhead family of transcription factors (16), enable AKT to regulate cell survival. Importantly, negative feedback loops have been described, which link proteins downstream of AKT with those upstream, or with AKT itself (17).

Aberrations along the PI3K/AKT pathway have been shown to drive a range of malignancies through mechanisms including activation of upstream receptor tyrosine kinases, *PIK3CA* mutations, *PTEN* mutations, *AKT* amplifications and mutations, as well as over-expression and hyperactivation of AKT proteins themselves (2, 18-21). Thus, the pharmacological ablation of AKT activity represents a rational approach to anticancer therapy. Moreover, PI3K/AKT pathway activation is a frequent hallmark of tumors resistant to treatment with chemotherapy or targeted therapies such as growth factor inhibitors (22-24). Therefore, AKT inhibition in these tumor types may also have therapeutic value either as monotherapy or in rational combinations with other antitumor agents (25).

Small molecules have been described which target various vital components of the PI3K/AKT pathway by blocking activation of AKT or its downstream targets (2). These include the PI3K inhibitors LY294002 and wortmannin, and more recently, isoform-specific PI3K inhibitors with differing biological profiles (2, 26, 27). These agents, and also drugs

such as rapamycin and its analogs that inhibit mTOR, are currently progressing through clinical trials in a number of cancer types (28). The latter compounds provide proof of principle that the PI3K–AKT pathway can be successfully targeted for clinical benefit in cancer (2).

A number of compounds which block the activation of AKT through a range of different mechanisms have recently been described, emphasizing the validity and current interest in AKT as an antitumor drug target (2). The inhibition of both AKT and p70S6K with a single agent has not been previously described. Targeting these two key components of the PI3K–AKT pathway through specific vertical inhibition may have therapeutic value.

We have utilized high-throughput X-ray crystallography and fragment-based lead discovery technologies to identify fragment hits against AKT. These fragments were validated by structural studies, and rapidly transformed into potent lead compounds using structure-based design to increase the efficiency of the medicinal chemistry. This research was recently described in detail and has identified potent, low molecular weight inhibitors of AKT that exhibit drug-like properties (29–32).

In this paper, we describe the detailed pharmacological profile of one of these compounds, AT7867, and also identify this agent as a potent inhibitor of p70S6K in cells. We demonstrate that this orally bioavailable small molecule causes appropriate biomarker modulation and apoptosis both *in vitro* and *in vivo*, and exhibits antitumor efficacy in human tumor xenograft models.

Methods

Cell Culture and Reagents

All cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in their recommended culture medium, which was supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂ and passaged for less than six months. All reagents were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. AT7867 (31) and LY294002 (Calbiochem, Merck Biosciences, Nottingham, UK) were dissolved to a 10mM stock in dimethyl sulphoxide (DMSO) while okadaic acid (Calbiochem, Merck Biosciences, Nottingham, UK) was dissolved to a 50(M stock in DMSO. The recombinant purified AKT2 enzyme used was the PH-truncated AKT2-Piftide as previously described (29), which was kindly provided by Professor David Barford (The Institute of Cancer Research, London, UK). GSK3 β , Histone H1, protein kinase A (PKA) and p70S6K enzymes, phospho-glycogen synthase peptide-2, PKA and p70S6K substrates were purchased from Upstate Biotechnology (Dundee, UK), while protein kinase C (PKC) and glycogen synthase derived peptide GS1-8 were from Calbiochem. CDK2/cyclinA was prepared in-house at Astex Therapeutics.

In Vitro Kinase Assays

Kinase assays for AKT2, PKA, p70S6K and CDK2/cyclinA were all carried out in a radiometric filter binding format. Assay reactions were set up in the presence of compound. For AKT2, the AKT2 enzyme and 25 μ M AKTide-2T peptide (HARKRERTYSFGHHA)

were incubated in 20mM MOPS, pH 7.2, 25mM β -glycerophosphate, 5mM EDTA, 15mM $MgCl_2$, 1mM sodium orthovanadate, 1mM DTT, 10 μ g/ml BSA and 30 μ M ATP (1.16Ci/mmol) for 4 hours. For PKA, the PKA enzyme and 50 μ M peptide (GRTGRRNSI) were incubated in 2 mM MOPS, pH 7.2, 25mM β -glycerophosphate, 5mM EDTA, 15mM $MgCl_2$, 1mM orthovanadate, 1mM DTT and 40 μ M ATP (0.88Ci/mmol) for 20 minutes. For p70S6K, the p70S6K enzyme and 25 μ M peptide substrate (AKRRRLSSLRA) were incubated in 10mM MOPS, pH 7, 0.2mM EDTA, 1mM $MgCl_2$, 0.01% β -mercaptoethanol, 0.1mg/ml BSA, 0.001% Brij-35, 0.5% glycerol and 15 μ M ATP (2.3Ci/mmol) for 60 minutes. For CDK2, the CDK2/cyclinA enzyme and 0.12 μ g/ml Histone H1 were incubated in 20mM MOPS, pH 7.2, 25mM β -glycerophosphate, 5mM EDTA, 15mM $MgCl_2$, 1mM sodium orthovanadate, 1mM DTT, 0.1mg/ml BSA and 45 μ M ATP (0.78Ci/mmol) for 4 hours. Assay reactions were stopped by adding an excess of orthophosphoric acid and the stopped reaction mixture was then transferred to Millipore MAPH filter plates and filtered. The plates were then washed, scintillant added and radioactivity measured by scintillation counting on a Packard TopCount. IC₅₀ values were calculated from replicate curves using GraphPad Prism software. AKT1 and 3 enzyme assays were carried out at Invitrogen Ltd (Paisley, UK), while all other enzyme assays were performed at Upstate Biotechnology (Dundee, UK).

Alamar Blue Cell Proliferation Assay

Cells were plated in 96-well microplates at 5000 cells per well in medium supplemented with 10% FBS, and grown for 24 hours before treatment with AT7867. Inhibitor or vehicle control was added to the cells for 72 hours. Following this, Alamar Blue solution (BioSource, Nivelles Belgium) was added as stated in the manufacturer's instructions. The IC₅₀ value for each inhibitor was calculated in GraphPad Prism (San Diego, USA) using non-linear regression analysis and a sigmoidal dose-response (variable slope) equation.

Phospho-GSK3 β (Serine 9) Cellular ELISA Assay

This assay was based on a previously described protocol (33). Cells were plated in 96-well microplates at 16,000 cells per well in medium supplemented with 10% FBS, and grown for 24 hours before treatment with AT7867. AT7867 or vehicle control were added to the cells for 1 hour. Following this, cells were fixed with 3% paraformaldehyde, 0.25% glutaraldehyde, 0.25% Triton-X100, washed and blocked with 5% milk in tris-buffered saline with 0.1% Tween-20 (TBST) prior to overnight incubation with a phospho-GSK3 β (serine 9) antibody (Cell Signaling Technology, Boston, MA, USA). The plates were then washed, secondary antibody added, and enhancement of the signal performed using DELFIA reagents (Perkin Elmer, Beaconsfield, Bucks, UK) as stated in the manufacturer's instructions. Europium counts were normalized to the protein concentration, and the IC₅₀ value for each inhibitor was calculated in GraphPad Prism using non-linear regression analysis and a sigmoidal dose-response (variable slope) equation.

Protein Immunoblotting and Immunoassay

Floating and adherent cells were collected and washed with ice-cold phosphate buffered saline (PBS), harvested, lysates prepared and protein estimations performed as described previously (34). Samples were separated by SDS-PAGE and transferred to 0.45 μ m

Immobilon-P transfer membrane (Millipore, Watford, Herts, UK). The membranes were blocked for 1 hour in blocking solution (5% milk in TBST) before incubating at 4°C, shaking overnight in the following antibodies diluted in blocking solution: AKT, phospho-AKT (Ser473), phospho-GSK3β (Ser9), phospho-S6RP (Ser240/244), FKHR, cleaved PARP (Asp214) all at 1:1000, S6 ribosomal protein at 1:2000, phospho-S6RP (Ser235/236) at 1:10,000, phospho-FKHR (Thr24)/FKHRL1 (Thr32) at 1:500 (all purchased from Cell Signaling Technology), GSK3β at 1:1000 (BD Biosciences, Oxford, UK), cyclin D1 Ab-1 (DCS-6) at 1:1000 (NeoMarkers, Lab Vision, Fremont, CA, USA) and GAPDH at 1:2×10⁶ (Chemicon International, Millipore, Watford, Herts, UK). The membranes were washed before incubation in goat anti-rabbit (1:1000 or 1:5000) or anti-mouse (1:10,000) HRP-conjugated secondary antibody for 1 hour at room temperature. Bands were visualized with ECL Western Blotting Detection Reagents and developed using Hyperfilm (GE Healthcare, Bucks, UK).

For the electrochemiluminescent immunoassay, the Meso Scale Discovery (MSD[®]) platform (Gaithersburg, Maryland, USA) was utilized. Cell lysates were prepared as described and probed for phosphorylation of Ser473 AKT, Ser9 GSK3β, Ser235/236 S6RP, and total forms of AKT, GSK3β, and S6RP according to the manufacturer's instructions. Analysis was completed as recommended by the manufacturer (33, 35, 36).

Annexin V Staining

U87MG cells were plated in 6-well plates at 2×10⁵ cells per well in 2ml growth media and grown for 24 hours before treatment. Inhibitor or DMSO was added at the specified concentrations. After the respective treatment times, adherent and floating cells were collected and washed in PBS by centrifugation. Cells were then washed in annexin-binding buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl₂), resuspended in 10μg/ml annexin V solution and incubated at room temperature for 15 minutes. Immediately prior to reading on a FACS Calibur flow cytometer, 10μg/ml propidium iodide was added to the cell mix.

Pharmacokinetic Studies

Male athymic BALB/c mice (nu/nu) were obtained from Harlan (Harlan UK, Bicester, UK) and allowed access to food and water *ad libitum*. The care and treatment of experimental animals were in accordance with the United Kingdom Coordinating Committee for Cancer Research (UKCCCR) guidelines (37).

A single dose of AT7867 was administered to BALB/c mice at 5mg/kg intravenously (i.v.) and 20mg/kg per os (p.o.) Compound was formulated in a vehicle containing 10% DMSO, 20% water and 70% hydroxypropyl-β-cyclodextrin (25% aqueous, w/v). Plasma samples were collected from duplicate animals at each of the following time points; 0.083, 0.167, 0.33, 0.67, 1, 2, 4, 6, 16 and 24 hours following i.v. dosing and at 0.25, 0.5, 1, 2, 4, 6 and 24 hours following p.o. dosing. Mice were bled by cardiac puncture and all blood samples were centrifuged to obtain plasma, which was then frozen at -20°C until analysis. For bioanalysis, all plasma samples were prepared by protein precipitation with acetonitrile containing internal standard. Quantification of sample extracts was by comparison with a standard calibration line constructed with AT7867 and using an inhibitor specific liquid

chromatography tandem mass spectrometry (LC-MS/MS) method. Pharmacokinetic parameters were determined using WinNonLin[®] software.

***In vivo* Tumor Studies**

Human MES-SA uterine sarcoma cells or U87MG human glioblastoma cells were injected subcutaneous (s.c.) in the right flank of each animal. Animals were randomized and treatment was started with vehicle or AT7867 when established tumors were ~100mm³ in mean volume. Control mice received vehicle only (10% DMSO, 90% saline) and treated mice received 20mg/kg AT7867 intraperitoneally (i.p.) or 90mg/kg AT7867 p.o. once every 3 days. Tumor size and body weight were monitored three times a week. Tumor size was evaluated by measurement with digital calipers and changes in tumour volume in treated mice (T) versus control mice (C) are given as T divided by C (T/C ratios).

To assess the pharmacokinetic (PK) and pharmacodynamic (PD) profile, a single dose of AT7867 (90mg/kg p.o. or 20mg/kg i.p.) was given to mice bearing MES-SA tumor xenografts. Compound was formulated in vehicle containing 0.1% Tween-20. Plasma and tumor samples were harvested at 2, 6 and 24 hours following dosing. Mice were bled by cardiac puncture and plasma samples were collected and frozen at -20°C until analysis. Tumors were dissected and divided into two approximately equal pieces. One piece was frozen at -20°C until PK analysis whilst the other was snap frozen in liquid nitrogen until PD analysis. For PK bioanalysis, all tissue samples were first homogenized in 5 volumes (w/v) of acetonitrile/water (50/50). AT7867 was extracted from plasma and tissue homogenates and quantified as described above. For PD studies, tumors were ground to a powder under liquid nitrogen, lysed and centrifuged to remove debris. Protein content was analyzed using BCA reagent and samples run on western blots as described above.

Results

Identification of a potent ATP-competitive inhibitor of AKT

AT7867 was discovered using fragment-based screening combined with structure-based design, and was previously referred to as compound 8a by Saxty *et al* (31). The molecule is a pyrazole, linked via the 4-position to a geminally substituted 4,4-biaryl piperidine, with the terminal aromatic group incorporating a *para*-chloro substituent (Figure 1A). The compound is further distinguished by its relatively low molecular weight of 337 Da. For the first time, we report here the detailed biological activity of AT7867. This compound exhibited nanomolar potency towards all 3 AKT isoforms using isolated enzyme assays (Figure 1B). AT7867 also displayed potent activity against the structurally related AGC kinases p70S6K and PKA, but showed a clear window of selectivity against kinases from other kinase sub-families (Figure 1B).

The inhibition of AKT2 by AT7867 was shown to be ATP-competitive with a K_i of 18nM. Binding at the ATP site was confirmed by determining the three-dimensional structure of the AT7867-AKT2 complex using X-ray crystallography (Figure 1C). The structure revealed that AT7867 fulfills a 3-point pharmacophore required for potent binding to AKT2, forming hydrogen bonding interactions with the kinase hinge region, electrostatic interactions with

the ribose site, as well as hydrophobic contacts with a lipophilic pocket in the glycine-rich loop. Specifically, the pyrazole makes a bi-dentate interaction with the hinge, forming hydrogen bonds with the backbone carbonyl of Glu 230, and the amide nitrogen of Ala 232. The central phenyl ring is slightly twisted with respect to the pyrazole, and is sandwiched between the hydrophobic side-chains of Val 166 and Met 282, which form the top and bottom of the ATP-cleft. The ribose binding site is occupied by the piperidine ring, where the basic nitrogen forms electrostatic and hydrogen bonding interactions with the side-chain of Glu 236, and the backbone carbonyl of Glu 279. The final element of the AKT-pharmacophore is fulfilled by the chlorophenyl, which is directed towards a lipophilic pocket formed by the side-chains of Lys 181, Leu 183, and the face of the glycine-rich loop in the region of Gly 161 and Gly 164.

AT7867 inhibits AKT activity in cells

In vitro growth inhibition studies showed that AT7867 blocked proliferation in a number of human cancer cell lines (Figures 2A and 2B). These cell lines represent common cancers that have been reported to exhibit deregulation of the PI3K/AKT pathway by mechanisms such as *PTEN* or *PIK3CA* mutations. AT7867 appeared to be most potent at inhibiting proliferation in MES-SA uterine, MDA-MB-468 and MCF-7 breast, and HCT116 and HT29 colon lines (IC_{50} values range from 0.9-3 μ M), and least effective in the two prostate lines tested (IC_{50} values range from 10-12 μ M), as indicated in the figure.

The U87MG glioblastoma cell line is *PTEN*-deficient and exhibits a high level of phosphorylation of serine 473 on AKT (38), suggesting an overactive AKT pathway. This cell line has previously been used to investigate the cellular properties of several PI3K pathway inhibitors (35, 36, 39-41). For these reasons, further detailed investigations were performed using U87MG cells.

The ability of AT7867 to inhibit AKT activity in human tumor cells was measured by investigating the phosphorylation state of the direct downstream substrate GSK3 β following a 1 hour incubation with compound (Figure 2C). For accurate quantification of AKT inhibition activity in cells, a 96-well plate-based ELISA was established, which allowed quantification of the level of phosphorylation of GSK3 β at serine 9 in intact cells. Figure 2C shows western blot data and the corresponding IC_{50} data generated using the cell-based ELISA. The assay was transferable between cell lines, thus allowing the quantification of AT7867 activity in multiple lines. These experiments showed that AT7867 was equipotent at inhibiting phosphorylation of GSK3 β across all cancer cell lines tested with IC_{50} values in the range 2 – 4 μ M (Figure 2D).

Investigation of additional AKT pathway components demonstrated that a 1 hour treatment of the U87MG cell line with AT7867 induced effects on the levels of a number of cellular proteins in a concentration-dependent fashion (Figure 3A). This included the phosphorylation of the following AKT direct substrates: GSK3 β , pro-apoptotic transcription factors FKHR (FoxO1a) and FKHL1 (FoxO3a) and the downstream target S6RP. Expression of the cell cycle protein cyclin D1 was also measured. In addition to the decrease in phosphorylation of downstream substrates and a decrease in expression of cyclin D1,

there was an induction of AKT phosphorylation at serine 473. However, at higher concentrations of compound, phosphorylation on AKT itself was also inhibited.

An additional study investigating the cellular consequences over time of AKT inhibition was also carried out. This confirmed the loss of phosphorylation on the direct AKT substrates GSK3 β , FKHR and FKHL, as well as on the downstream target S6RP (Figure 3B). Between 1-8 hours, there was a substantial loss of all phosphorylation signals under investigation. This inhibition was sustained together with loss of total proteins at 24 hours in the presence of AT7867. Loss of cyclin D1 expression was seen at one hour post-treatment and was maintained over the 24-hour period of the experiment, at which time the amount of AKT pathway proteins was reduced to below control levels. Since loss of phosphorylation on the pro-apoptotic proteins FKHR and FKHL allows their entry into the nucleus to facilitate gene transcription leading to activation of apoptosis, the effect of AT7867 on apoptosis was studied (16). Cleavage of the protein poly(ADP-ribose) polymerase (PARP) is an event that occurs early in the apoptotic process and as such, cleaved PARP serves as a marker of cells undergoing apoptosis (42). The appearance of cleaved PARP was observed in cells exposed to AT7867 from one hour onwards for the duration of the study and correlated with the reduction in protein levels described above for the pathway components (Figure 3B). Apoptosis was further investigated by Annexin V staining, which detects phosphatidylserine on the external surface of intact cells, another hallmark of apoptosis (43). A concentration- and time-dependent increase in the amount of Annexin V staining was observed (Figure 3C).

In view of the effects of AT7867 on AKT and p70S6K, the MSD[®] electrochemiluminescent immunoassay platform was used to quantify the inhibitory effects of this compound on the phosphorylation of GSK3 β , p70S6K and S6RP following AT7867 treatment in U87MG glioblastoma cells (Figure 3D). At one hour post-treatment, the IC₅₀ for pSer9 GSK3 β was 7.1 μ M. An IC₅₀ value could not, however, be obtained for pThr421/424 of p70S6K, as it was greater than the maximal concentration of AT7867 used in the assay (20 μ M). In contrast, IC₅₀ values of 10.7 μ M and 12.3 μ M were obtained for pSer235/236 and pSer240/244 of S6RP.

***In vivo* activity of AT7867**

Having shown concentration-dependent and time-dependent effects of AT7867 *in vitro*, the properties of the compound were evaluated *in vivo* in mice. The plasma clearance of AT7867 following 5mg/kg i.v. administration was moderate and the compound was still detectable in plasma at 24 hours post administration (Figure 4A). Following oral administration at 20mg/kg, the elimination of AT7867 from plasma appeared to be similar to that observed after i.v. administration (Figure 4A). Plasma levels of AT7867 remained above 0.5 μ M for at least 6 hours following an oral dose of 20mg/kg. Assuming linear pharmacokinetics following i.v. administration, the bioavailability by the oral route was calculated to be 44% (Figure 4B).

From *in vitro* cell growth studies, the MES-SA uterine cell line appeared to be the most sensitive to growth inhibition by AT7867. This cell line does not express PTEN and contains high levels of phosphorylated AKT, indicating an overactive pathway (Figure 4C). *In vivo*

PD biomarker studies were therefore performed with this model. Following pharmacokinetic (see above) and tolerability studies, doses of AT7867 (90mg/kg p.o. or 20mg/kg i.p.) were administered to athymic mice bearing MES-SA tumors and the phosphorylation status of GSK3 β and S6RP in tumors was monitored over time. Clear inhibition of phosphorylation of the two markers of pathway activity was seen at 2 and 6 hours following treatment with AT7867 (Figures 5A and 5B). By 24 hours, total levels of both GSK3 β and S6RP were greatly reduced, as also seen in the *in vitro* studies. For both routes of administration and at each time point investigated, the level of compound in the plasma and tumor exceeded the cellular IC₅₀, which was consistent with the prolonged mechanistic biomarker effect (Figures 5C and 5D). As seen in studies *in vitro*, sustained compound exposure induced an increase in cleaved PARP, indicating that AT7867 also induces apoptosis in the xenograft tumor cells (Figures 5A and B).

Next we determined the effect of AT7867 on the growth of MES-SA tumor xenografts. Marked inhibition of tumor growth, as assessed by changes in volume, were seen with this model when dosed at the same levels and routes utilized for the PD studies described above. Specifically, a T/C of 0.37 was observed at 20mg/kg i.p. (measured at the termination of the experiment on day 12; Figure 6A) and of 0.38 at 90mg/kg p.o. (measured on day 10; Figure 6B). Additional *in vivo* xenograft studies were carried out using the U87MG cell line which was sensitive to the effects of AT7867 *in vitro*, although less so than the MES-SA cell line. Figure 6C shows a T/C of 0.51 (measured on day 13), when 20mg/kg AT7867 was dosed on this schedule via the i.p. route of administration.

Discussion

AKT activity has been implicated in the regulation of tumor cell growth, proliferation, metastasis and apoptosis (1). Recently the PI3K/AKT signal transduction pathway has become the focus of intense interest as a critical regulator of tumor cell survival, and a number of AKT pathway inhibitors have been disclosed with a wide variety of potencies and specificities (2, 44, 45). In this report, we describe the *in vitro* and *in vivo* effects of AT7867, a recently developed inhibitor of AKT and p70S6K, which has been identified using fragment-based lead discovery and structure-based design technologies (29-32).

AT7867 is a potent inhibitor of AKT and p70S6K that blocks the cellular phosphorylation of the AKT substrate GSK3 β and the p70S6K substrate S6RP and inhibits the proliferation of a range of human tumor cell lines. It is interesting to note that while the IC₅₀ for growth inhibition varied 12-fold (1-12 μ M), the IC₅₀ for GSK3 β phosphorylation was much more consistent (2-4.5 μ M) across the same cell line panel. This discrepancy is perhaps not surprising since the response of different cell lines to the same level of pathway inhibition may be dependent on factors other than genetic alterations on the PI3K/AKT pathway. Further detailed studies in the PTEN-deficient human glioblastoma U87MG cell line demonstrated that AT7867 caused both a concentration-dependent and time-dependent reduction in the phosphorylation of proteins downstream of AKT, including the AKT substrate GSK3 β and p70S6K target S6RP. Cyclin D1 expression and thus cell cycle progression is regulated at the post-translational level by GSK3 β and also by protein translation through p70S6K via mTOR (12). The demonstration that AT7867 suppresses the

expression of cyclin D1 is consistent with growth inhibition being mediated via these kinases. Induction of phosphorylation on AKT itself at serine 473 was also noted, consistent with the compensatory but futile feedback regulation of AKT previously described with other inhibitors of this pathway (46-48).

Growing evidence has challenged the view of a linear PI3K/AKT/mTOR pathway, with the identification of crosstalk and feedback regulation within the pathway (8, 18). Despite induction of pSer473 AKT, the phosphorylation of downstream AKT targets was markedly decreased in the presence of AT7867 both in cancer cell lines *in vitro* and human tumor xenografts in immunosuppressed mice. Recent papers have suggested that dual inhibition of PI3K and mTOR could overcome these feedback mechanisms through vertical blockade of key components of the pathway (35, 41, 49). AT7867 has little activity outside the AGC kinase family members but is a potent inhibitor of the structurally related AGC kinases PKA, and p70S6K, as well as AKT. In order to dissect the respective contributions of AKT and p70S6K inhibition along this pathway to AT7867-mediated effects, the relative modulation in substrate phosphorylation signals were quantified in U87MG cells. Whilst the IC₅₀ for pSer9 GSK3β was 7.1 μM, an IC₅₀ value could not be generated for p70S6K as it was greater than the highest concentration of 20 μM used in this assay. This suggests a dilution of the inhibition of the pathway signal, as p70S6K is more distal than GSK3β from the AKT target. In contrast, IC₅₀ values were obtained for both pSer235/236 and pSer241/244 of S6RP of 10.7 μM and 12.3 μM respectively confirming the direct inhibitory effects of AT7867 against p70S6K. It is an intriguing possibility that the combined effects of targeting p70S6K and AKT with this compound may serve to improve its therapeutic potential by delivering a double vertical blockade to the pathway.

It should be noted that, based on kinase profiling in *in vitro* biochemical assays, it is possible that inhibition of PKA may contribute to the cellular effects of AT7867. However, as there are no suitable methods or reagents that can accurately dissect the specific effects of PKA on its target substrates in the cell lines used, this possibility could not be pursued at this time. Further studies are also needed to profile the potential effects of AT7867 on other signaling pathways and gene expression profiling studies using cDNA microarray are now underway to address this in an unbiased fashion.

A major function of AKT in cancer cells is to prevent apoptosis by the phosphorylation and inactivation of pro-apoptotic targets, such as the forkhead transcription factors and BAD (50). We have shown that AT7867 prevents phosphorylation of FKHR and FKHL1 and inhibits tumor cell growth, an effect that may in part be attributed to the induction of apoptosis caused by the compound. The pro-apoptotic effect of AT7867 is supported by the induction of cleaved PARP and annexin V staining in treated cells, which correlated with the reduction in protein levels for PI3K/AKT pathway components at later time points (Figure 3B).

Although the PI3K/AKT pathway has often been associated with survival signaling and apoptosis (1), there has been equivocal evidence in support of this hypothesis from studies involving the pharmacological ablation of key components of this key signaling cascade. We have previously shown that the potent and selective class I PI3K and mTOR inhibitor PI-103

does not cause significant apoptosis in a wide range of tumor cell lines tested (35, 41). In contrast, we have shown here that AT7867 results in extensive apoptosis, both *in vitro* and *in vivo*, suggesting a difference in the simultaneous effects of inhibiting both AKT and p70S6K versus PI3K and mTOR. These data suggest that there may be unknown mechanisms resulting in this vital difference in survival signaling, with the possibility of crosstalk or other undiscovered pathway proteins playing a key role. Also, the results suggest that combined inhibitors of AKT and p70S6K may potentially be efficacious as monotherapy.

Pharmacokinetic studies showed that potentially active concentrations of AT7867 could be achieved in plasma and tumor after 20mg/kg i.p. or 90 mg/kg p.o. reflecting the relatively high oral bioavailability of this compound. The *in vitro* PD biomarker effects of AT7867 described above were also observed in tumor models *in vivo*. Administration of AT7867 p.o. or i.p. to mice bearing human tumor xenografts caused suppression of phosphorylation of the AKT and p70S6K molecular biomarkers GSK3 β and S6RP and a concomitant increase in the levels of cleaved PARP. These PD effects were achieved at plasma and tumor drug concentrations similar to those required to suppress the same markers *in vitro* and at doses that cause inhibition of human tumor xenograft growth (Figures 6A-C). Therefore, these data are consistent with inhibition of AKT and p70S6K as the mechanism of tumor growth suppression for AT7867 *in vivo*.

In conclusion, we have shown that AT7867 suppresses tumor cell proliferation, induces apoptosis and exhibits oral antitumor activity. Importantly, we show that AT7867 suppresses the phosphorylation of downstream markers of AKT and p70S6K in tumor tissue at doses that also inhibit human xenograft tumor growth. The therapeutic response to AKT and p70S6K pathway inhibition observed in the current study makes this approach attractive for clinical application, where such a novel inhibitor of both AKT and p70S6K may have the potential to cause antitumor activity as a single agent or in combination with other agents.

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Abbreviations

GSK3β	glycogen synthase kinase-3 β
mTORC	mammalian target of rapamycin complex
PKD1	phosphoinositide-dependent kinase 1

PI3K	phosphatidylinositol 3-kinase
PIP₃	phosphatidylinositol (3,4,5)-trisphosphate
PTEN	phosphatase and tensin homolog on chromosome 10
p70S6K	p70 S6 kinase
S6RP	S6 ribosomal protein
TBST	tris-buffered saline with 0.1% Tween-20

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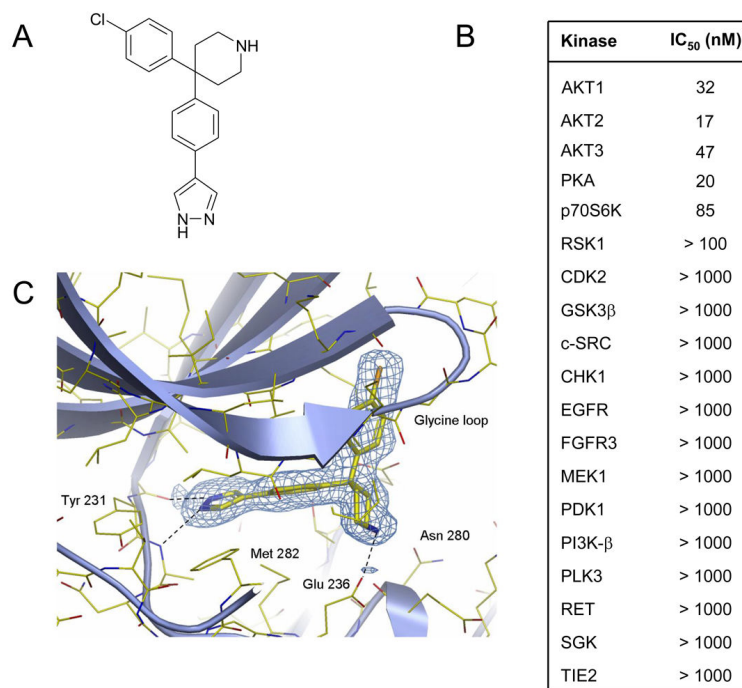
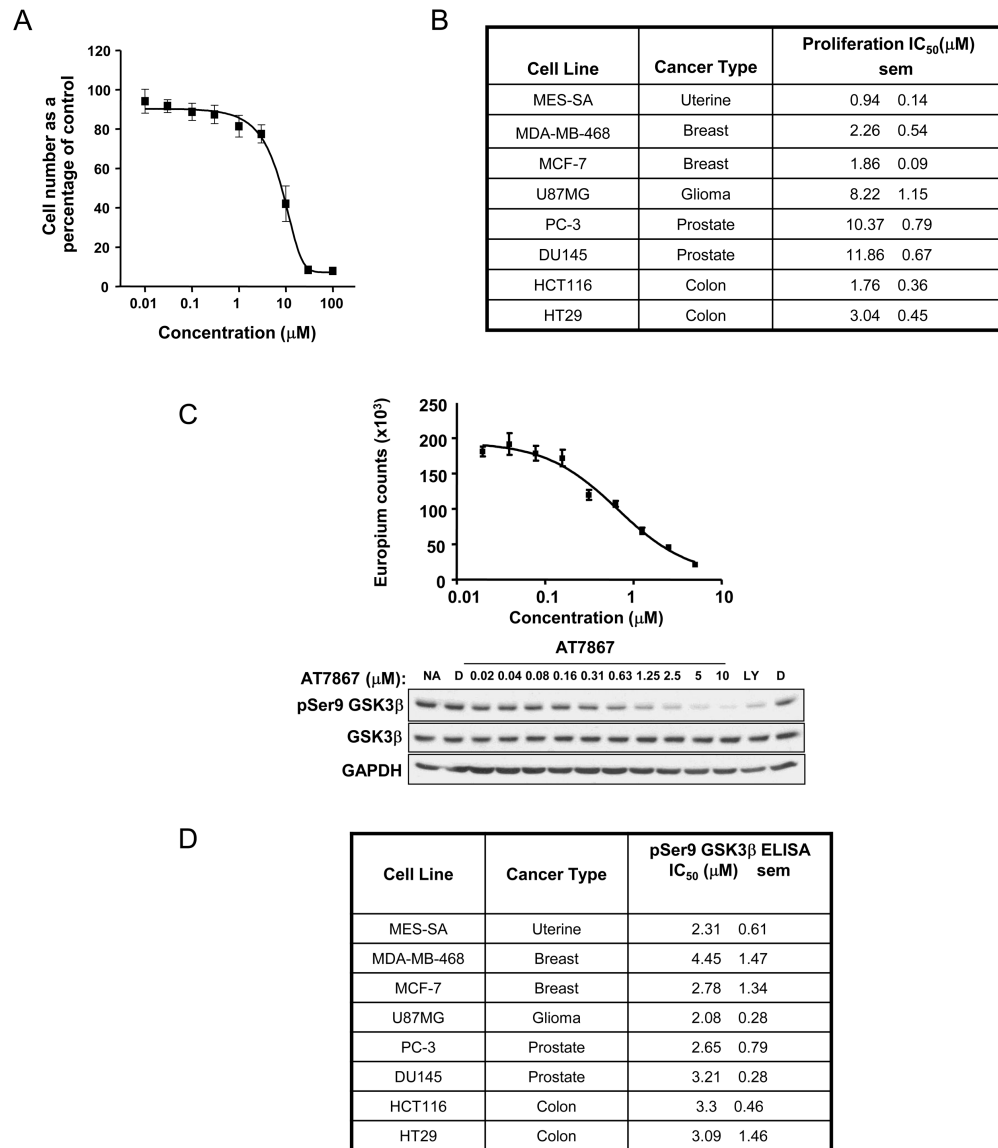
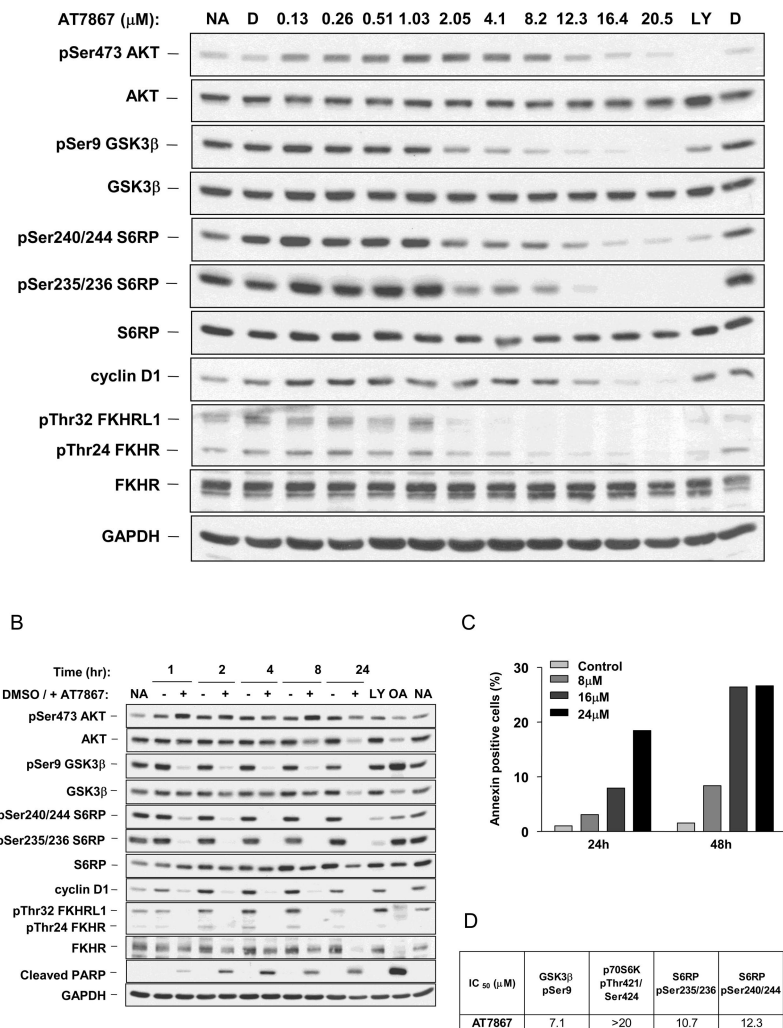


Figure 1. Structure and kinase inhibitory activity of AT7867. *A*, chemical structure of AT7867. *B*, activity of AT7867 against selected kinases as determined by *in vitro* kinase assays. *C*, X-ray structure of AT7867 bound to the ATP binding site of AKT2.

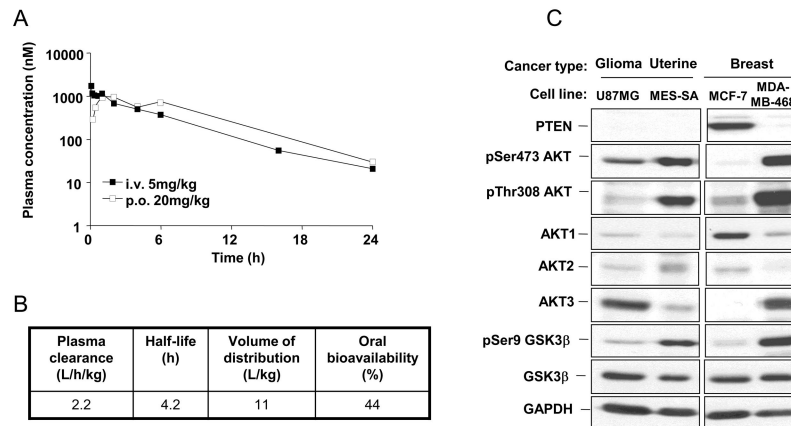
**Figure 2.**

AT7867 inhibits proliferation of human cancer cell lines and reduces phosphorylation of GSK3β. *A*, PTEN-negative U87MG human glioblastoma cells were treated with a range of concentrations of AT7867 for 72 hours. Alamar blue cell viability assays were performed to measure cell growth inhibition. *B*, inhibition of cell growth across a panel of human cell lines from various tumor types. IC₅₀ values are shown as the mean (n=3) with the standard error of the mean (sem). *C*, Cells were incubated with a range of concentrations of AT7867 for 1 hour and the in-cell phospho-GSK3β ELISA was performed in parallel with western blot of cell lysates in U87MG cells. No addition (NA) and DMSO (D) vehicle were used as negative controls, and 20μM LY294002 (LY) was used as a positive control. *D*, a panel of human tumor cell lines were exposed to AT7867, the in-cell GSK3β ELISA performed and the IC₅₀ for phospho-Ser9 GSK3β signal determined.

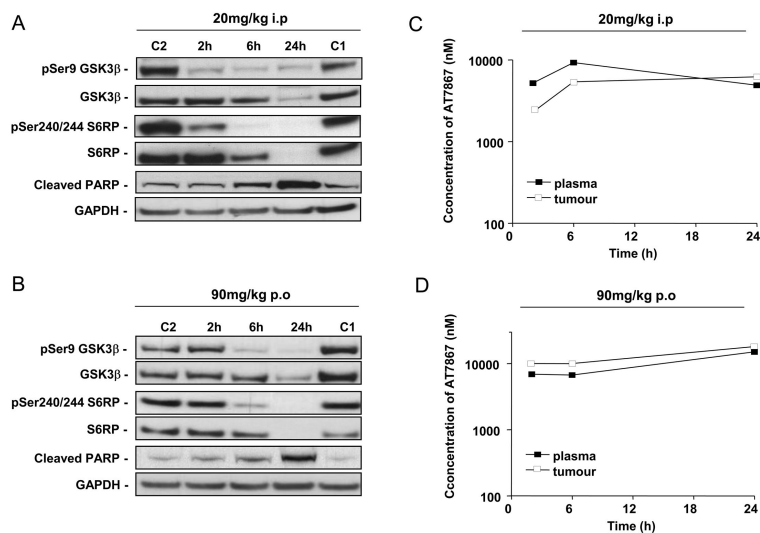
**Figure 3.**

AT7867 suppresses AKT signalling in U87MG human glioblastoma cells in a concentration-dependent and time-dependent manner and induces apoptosis. *A*, PTEN-negative U87MG human glioblastoma cells were incubated with AT7867 for 1 hour and western blot analyses performed to assess the phosphorylation and expression of AKT pathway proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. No addition (NA) and DMSO (D) vehicle were used as negative controls, and 20 μM LY294002 (LY) was used as a positive control. *B*, U87MG cells were treated with 16 μM AT7867 for, 1, 2, 4, 8 and 24 hours, and western blot analyses performed for AKT pathway proteins as indicated in the figure and the appearance of cleaved PARP. GAPDH was used as a loading control. No addition (NA) and DMSO (D) vehicle were used as negative controls, 20 μM LY294002 (LY) was used as a positive control. 100 nM Okadaic acid for 24 hours (OA) was used as a positive control for apoptosis. *C* annexin V staining of U87MG cells following treatment with 8, 16 or 24 μM AT7867 or DMSO vehicle (Control) for 24 or 48 hours. *D*, U87MG cells were treated with 0.1-20 μM AT7867 for 1 hour. Lysates were prepared and

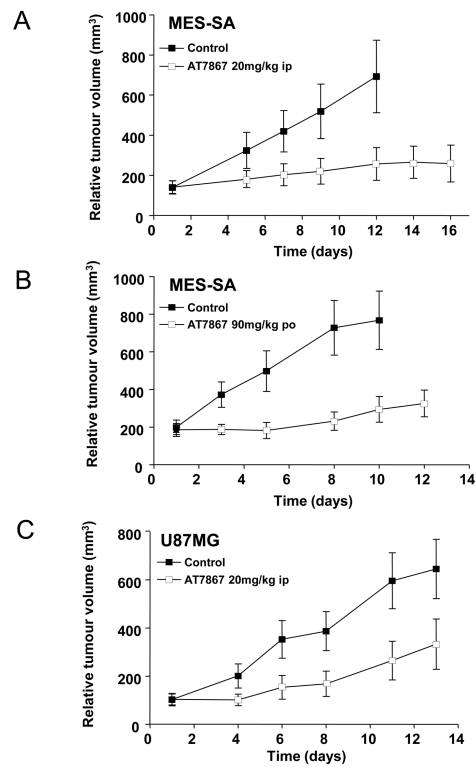
analysis of AKT pathway proteins was performed by electrochemiluminescent immunoassay (MSD[®]) as indicated in the figure.

**Figure 4.**

The plasma pharmacokinetic (PK) profile of AT7867 *in vivo* and comparison of expression of AKT pathway proteins in the MES-SA human uterine versus U87MG glioblastoma and MCF-7 and MDA-MB-468 breast cell lines. *A* and *B*, plasma PK in mice following administration of AT7867 at 20mg/kg p.o. and 5mg/kg i.v. *C*, western blot analysis of AKT pathway proteins in lysates prepared from MES-SA, U87MG, MCF7 and the MDA-MB-468 cell lines. GAPDH was used as a loading control.

**Figure 5.**

The pharmacodynamic (PD) and pharmacokinetic (PK) profile of AT7867. Athymic BALB/c mice bearing MES-SA human uterine xenograft tumors were treated with a single dose of AT7867 at 20mg/kg i.p. and 90mg/kg p.o.. Plasma and tumors were harvested at 2, 6 or 24 hours after dosing. *A* and *B*, phospho and total GSK3 β , S6RP, cleaved PARP and GAPDH were assessed from tumor protein lysates by western blotting at each dose as indicated in the figure. C1 (control 1), C2 (control 2). *C* and *D*, plasma and tumor concentrations of AT7867 were assessed by LC-MS/MS at each dose as indicated in the figure.

**Figure 6.**

AT7867 suppresses MES-SA human uterine and U87MG human glioblastoma xenograft tumor growth. *A*, athymic BALB/c mice bearing MES-SA xenograft tumors were administered AT7867 20mg/kg i.p. once every 3 days for 16 days. *B*, athymic BALB/c mice bearing MES-SA xenograft tumors were administered AT7867 at 90mg/kg p.o. once every 3 days for 12 days. *C*, athymic BALB/c mice bearing U87MG xenograft tumors were administered AT7867 at 20mg/kg i.p. once every 3 days for 13 days. Tumor size was monitored three times a week and relative tumour volumes calculated.