Preclinical Pharmacological Evaluation of a Novel Multiple Kinase Inhibitor, ON123300, in Brain Tumor Models

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Abstract

ON123300 is a low molecular weight multikinase inhibitor identified through a series of screens that supported further analyses for brain tumor chemotherapy. Biochemical assays indicated that ON123300 was a strong inhibitor of Arks and CDK4, as well as growth factor receptor tyrosine kinases such as B-type platelet-derived growth factor receptor (PDGFRβ). ON123300 inhibited U87 glioma cell proliferation with an IC50 of 3.4 ± 0.1 μmol/L and reduced phosphorylation of Akt, yet it also unexpectedly induced Erk activation, both in a dose- and time-dependent manner that subsequently was attributed to relieving Akt-mediated C-Raf S259 inactivation and activating a p70S6K-initiated PI3K-negative feedback loop. Cotreatment with the EGFR inhibitor gefitinib produced synergistic cytotoxic effects. Pursuant to the in vitro studies, in vivo pharmacokinetic and pharmacodynamic studies of ON123300 were completed in mice bearing intracerebral U87 tumors following intravenous doses of 5 and 25 mg/kg alone, and also at the higher dose concurrently with gefitinib. ON123300 showed high brain and brain tumor accumulation based on brain partition coefficient values of at least 2.5. Consistent with the in vitro studies, single agent ON123300 caused a dose-dependent suppression of phosphorylation of Akt as well as activation of Erk in brain tumors, whereas addition of gefitinib to the ON123300 regimen significantly enhanced p-Akt inhibition and prevented Erk activation. In summary, ON123300 demonstrated favorable pharmacokinetic characteristics, and future development for brain tumor therapy would require use of combinations, such as gefitinib, that mitigate its Erk activation and enhance its activity. Mol Cancer Ther; 13(5); 1–12. ©2014 AACR.

Introduction

Glioblastoma multiforme represents the most common primary brain tumor in adults and is among the most lethal of all cancers. Despite multimodality treatment consisting of surgical resection followed by concurrent or sequential treatment with radiation and chemotherapy, the prognosis for patients with glioblastoma multiforme is poor, with a median survival time of approximately 14 months (1). Standard chemotherapy is based on DNA alkylating agents, most often temozolomide; however, due to modest long-term benefits, there is substantial interest to identify molecularly targeted agents that can be used in combination with temozolomide.

Receptor tyrosine kinase (RTK)/RAS/PI(3)K signaling cascades are among the most frequently altered signaling pathways in glioblastoma multiforme that are likely key requirements for disease progression in a majority of glioblastoma multiforme (2–5). RTK signaling hyperactivation are most commonly caused by EGFR mutation/amplification or PDGFR amplification/overexpression, largely mediated through the PI3K/Akt/mTOR and Ras/mitogen-activated protein kinase (MAPK) downstream signaling pathways. Pathologic fibroblast growth factor receptor 1 (FGFR1) signaling also occurs in glioblastoma multiformes which exhibit FGFR1 kinase domain gain-of-function mutations (6). These pathway aberrations have stimulated an effort to discover novel modulators, albeit not necessarily directed at glioblastoma multiforme, due in part to its orphan disease status and the more restrictive requirements to identify novel compounds with adequate blood brain barrier (BBB) transport (7, 8).

Our own efforts in anticancer drug discovery have led to a number of agents in clinical trials (9–13), and more recently we have employed a pharmacokinetic/pharmacodynamic-driven drug development paradigm to identify agents suitable for brain tumor chemotherapy (14–16). Application of a pharmacokinetic/pharmacodynamic-driven approach to the ON123 series, 154 low molecular weight moieties, produced ON123300 as the lead compound (Fig. 1A) that possessed favorable...
pharmacokinetic properties, including the ability to penetrate the BBB. A biochemical kinase screen indicated ON123300 was a multitargeted kinase inhibitor with primary targets of Ark5 and CDK4, PDGFRβ, FGFR1, proto-oncogene Ret receptor tyrosine kinase, and proto-oncogene Fyn tyrosine-protein kinase. Ark5 is a member of the AMPK family and found to be directly phosphorylated and activated by Akt to prevent cell death. It was reported that Ark5-mediated mTOR phosphorylation induced by IGF-1 plays a key role in tumor malignancy and transient RNAi-mediated ARK5 knockdown caused significant reductions in cell proliferation and brain invasion in a glioma xenograft mouse model (17–20). CDK4 has been shown to be responsible for hyperphosphorylation of tumor suppressor protein Rb releasing its inhibition on G1–S cell-cycle progression through activation of the transcription factor E2F (21). CDK4-silenced cells undergo apoptosis and displayed decreased colony formation capacity (22). Small-molecule inhibitors of CDK4 and CDK6 have shown preclinical efficacy in glioblastoma multiforme models (23, 24). Given the interesting and potential importance of ON123300’s targets and its positive pharmacokinetic profile we undertook a detailed pharmacokinetic/pharmacodynamic analysis in a common preclinical brain tumor model (U87MG).

Materials and Methods

ON123300 (Fig. 1A), ON1231120, and ON1231320 compounds were supplied by Dr. M.V. Reddy (Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai; ref. 16). Gefitinib was purchased from LC Laboratories. Temozolomide and β-actin antibody was purchased from Sigma-Aldrich, and IRDye 800CW-conjugated and Alexa Fluor 680-conjugated secondary antibodies were from Rockland and Invitrogen, respectively.
All other antibodies were purchased from Cell Signaling Technology. Ninety-six-well p-Akt and p-Erk whole-cell lysis kits were purchased from Meso Scale Discovery. Human phospho-MAPK array kit was purchased from R&D Systems. All other chemicals and solvents were obtained from commercial sources.

U87MG (U87) and U251 human glioma cells were purchased from American Type Culture Collection in August 2011. U87/EGFRvIII and U87/PTEN cell lines were a generous gift from Dr. Webster Cavenee (University of California-San Diego, La Jolla, CA) and Dr. Paul Mischel (University of California Los Angeles, Los Angeles, CA) obtained in December 2009, respectively. Cells were authenticated using Western blotting assays in June 2013. GBM10 and GBM39 cells were obtained from Dr. Jann Sarkaria (Mayo Clinic, Rochester, MN) in December 2011. Cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% standard FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and maintained in a humidified atmosphere of 5% CO2 in air at 37°C.

NIH SWISS nude mice were purchased from Taconic and maintained in the American Association for the Accreditation of Laboratory Animal Care accredited Laboratory Animal Resources of Icahn School of Medicine at Mount Sinai (New York, NY). All study procedures were approved by the Institutional Animal Care and Use Committee.

**In vitro cytotoxicity and combination drug studies**

The cytotoxicity of ON123300 was determined using a colorimetric sulforhodamine B (SRB)-based assay (25). Suspensions of glioma cells (100 µL containing 2 x 10^5 cells) were seeded in 96-well plates and allowed to attach to the surface by overnight incubation. The cells were then treated with increasing concentrations of ON123300 for 72 hour. At the end of the treatment, cells were fixed with 10% (v/v) trichloroacetic acid (TCA) and stained with 10% (v/v) Triton X-100 for 30 minutes at room temperature. The cell cycle of intact/attached cells was analyzed on a flow cytometer (FACScalibur, BD Biosciences) and only live cells were gated and quantitated by FlowJo software (Tree Star, Inc.). For Annexin V studies, U87 cells seeded on 12-well plates were cultured overnight and treated with ON123300 and gefitinib alone or concurrently for 24 hours. Both floating and attached cells were harvested, stained with AnnexinV-FITC 633 (10 µg/mL) in binding buffer (10 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 5 mmol/L KCL, 1 mmol/L MgCl2, 1.8 mmol/L CaCl2), and analyzed by flow cytometry (FACScan, BD Biosciences; 5,000 events/sample).

**Live cell microscopy**

U87 cells were seeded in 6-well plates overnight and treated with ON123300, gefitinib or the combination for 24 hours. Treated cells were then directly visualized with an Olympus IX70 fluorescence live cell microscope (Olympus Imaging) controlled by InVivo software (Media Cybernetics).

**Protein sample preparation**

Cell lysates from treated cells or brain tumors were prepared in a cell lysis buffer (27) containing 1 mmol/L phenylmethylsulfonylfluoride, phosphatase inhibitor I, phosphatase inhibitor II, and protease inhibitor cocktail at a ratio of 600 µL of buffer to 80%–90% confluent cells in 10 cm plate or 20 µL of buffer to 1 mg of brain tumor tissue. After adding cell lysis buffer the cells and tissues were briefly sonicated, incubated on ice for 30 minutes, and then centrifuged at 21,000 x g for 10 minutes at 4°C. The supernatants were collected and stored at −80°C. Protein concentrations of both supernatants were measured by a BSA assay according to the manufacturer’s instructions.

**Phospho-MAPK array**

Human phospho-MAPK arrays were processed according to the manufacturer’s protocol. Briefly, U87 cells treated with 6.3 µmol/L ON123300 for 1 hour were rinsed with ice-cold PBS and solubilized with lysis buffer to reach a cell concentration of 1 x 10^7/mL. Cell supernatants were collected after cell lysates were rocked at 4°C for 30 minutes and then centrifuged for 5 minutes. Subsequently, 200 µg of the cell supernatants were adjusted to final volume of 1.5 mL with array buffer 1 followed by the addition of 20 µL of a detection antibody cocktail.
and incubated at room temperature for 1 hour. The prepared sample–antibody mixtures were added to membranes that had been preincubated with array buffer and 5% nonfat milk at room temperature for 1 hour, and subsequently probed with various primary antibodies for 1 hour at room temperature or overnight at 4°C. The blots were washed and then incubated with IRDye 800CW-conjugated or Alexa Fluor 680-conjugated secondary antibodies at room temperature for 1 hour and then quantitated for protein using an Odyssey 2.1 (LI-COR Biotechnology) system and the ImageJ software. All Western blots were repeated at least three times.

**Western blot assay**

Equal amounts of protein samples (20 μg) were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes that were blocked with 5% nonfat milk at room temperature for 1 hour, and subsequently probed with various primary antibodies for 1 hour at room temperature or overnight at 4°C. The blots were washed and then incubated with IRDye 800CW-conjugated or Alexa Fluor 680-conjugated secondary antibodies at room temperature for 1 hour and then quantitated for protein using an Odyssey 2.1 (LI-COR Biotechnology) system and the ImageJ software. All Western blots were repeated at least three times.

**Meso Scale Discovery assay**

Meso Scale Discovery (MSD) 96-well multispot p-Akt/total Akt and p-Erk/total Erk assays were carried out as per the manufacturer’s protocol with minor modifications. Briefly, plates were blocked with the MSD blocking solution for 1 hour at room temperature while shaking and then washed 4 times with Tris wash buffer. For each well, 25 μL of protein (20 μg for Akt and 5 μg for Erk) was added to the plate in duplicate and incubated overnight at 4°C. The plates were washed 4 times with Tris wash buffer and incubated with 25 μL of detection antibody in each well at room temperature for 2 hours with shaking. The plates were analyzed on a SECTOR 6000 instrument (Meso Scale Discovery) after washing with Tris wash buffer and addition of 150 μL of read buffer. Both the phosphor- and total signals were corrected for background (BSA spots) and for any effects of the lysis buffer. The percentage of phosphorylation against total protein was calculated and the expression of phosphorylation in each treated group was expressed as percentage relative to the control group.

**Orthotopic glioma model and dosing studies**

The U87 glioma model used in this study was described previously. Briefly, NIH Swiss nude mice were anesthetized with a cocktail (ketamine:acepromazine:xylazine: saline = 3:2:1:24 volume ratio; ketamine: 100 mg/mL; acepromazine: 10 mg/mL; xylazine: 20 mg/mL) at an intraperitoneal dose of 5 mL/kg. After securing in a stereotactic apparatus, mice were implanted with a suspension of U87 cells (10⁶ cells in 10 μL PBS) into the caudate putamen at a position 0.7 mm anterior and 2.2 mm lateral from the bregma at a depth of 2.5 mm using a 10 μL Hamilton syringe (Hamilton Co.). Once the animals recovered from anesthesia (about 20 minutes) they were returned to the animal care facilities, and provided food and water ad libitum. Mice were monitored daily and then entered into the pharmacokinetic/pharmacodynamic studies upon the appearance of clinical symptoms (i.e., unkempt appearance, arched back, unsteady gait) or a body weight loss of 2 g over 2 consecutive days.

ON123300 was administered to groups of brain tumor-bearing mice as an intravenous bolus at a dose of either 5 or 25 mg/kg via a tail vein. Blood, normal brain, and brain tumor were collected from 3 to 4 mice at each time point (5, 15, 30 minutes and 1, 2, 4, 6, 10 hours postadministration) in which the mice were briefly anesthetized with isoflurane and exsanguinated by cardiac puncture. Plasma was separated by centrifugation (14,000 rpm, 2 minutes, 4°C) and brain tissues obtained by gross dissection. Normal brain and brain tumor samples were rinsed thoroughly with 0.9% saline solution and together with plasma samples stored at –80°C until analyzed by liquid chromatography-tandem mass spectrometry (LC/MS-MS). For the ON123300-GFN treatment study, ON123300 was administered at an intravenous dose of 25 mg/kg via a tail vein 1 hour after an oral dose of 200 mg/kg gefitinib (set time point of gefitinib administration of as of -1), and had blood, normal brain, and brain tumor samples collected at each time point (0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 10, 17 hours post-ON123300 administration) and analyzed as described for ON123300 only treatment.

**LC/MS-MS analysis**

All plasma and tissue samples from the pharmacokinetic studies were analyzed with an electrospray ionization LC/MS-MS system (HPLC; QTrap 5500, Applied Biosystems) described previously (16). To 10 μL samples of plasma, normal brain or brain tumor homogenate (20% w/w tissue/water), 40 μL of cold acetonitrile was used to precipitate proteins followed by centrifugation at 15,000 rpm for 5 minutes. For both plasma and brain samples, 10 μL aliquots of the resultant supernatant were injected into the LC/MS-MS system. Data collection and analysis were performed using Analyst 1.5.1 software (Applied Biosystems MDS Sciex).

**Pharmacokinetic and pharmacodynamic data and statistical analysis**

Noncompartmental analyses (NCA) were performed using WinNonlin Phoenix, version 6.3 software [Pharsight Corporation] to estimate pharmacokinetic and pharmacodynamic parameters that included for each compound the areas between the effect–time curve in brain tumor (ABEC), and the areas under the drug concentration–time curve in plasma, brain, and brain tumor (AUCp, AUCb, and AUCbt, respectively), half-life (t₁/₂), systemic clearance (CL), and the apparent volume of distribution at steady state (Vss). The normal brain or brain tumor partition coefficient (Pb/Po) was calculated as the ratio of either the AUCb or AUCbt over the corresponding AUCp. All AUC calculations were based on the area to the last
operative concentration using the linear-log interpolation method. Data were statistically evaluated by a Student t test or one-way ANOVA with the level of significance chosen as P < 0.05.

Results

In vitro studies

Our previous pharmacokinetic/pharmacodynamic-driven screen of 154 ON123 compounds indicated ON123300 had potential as an anticancer drug for brain tumor chemotherapy based on in vitro cytotoxicity and an integrated panel of in vitro pharmacokinetic assays (i.e., metabolic stability, BBB permeability, and plasma and brain tissue binding; ref. 16). Further delineation of its in vitro cytotoxicity of ON123300 to U87 cells indicated it had an IC50 equal to 3.4 (mean) ± 0.1 (SD) μmol/L. In addition, ON123300 inhibition of cell proliferation in a panel of 11 glioma models, including a patient-derived model (GBM10), was quite comparable with that observed in U87 cells (Supplementary Fig. S1). A previously completed biochemical screen of 288 kinases using recombinant proteins of ON123300 found the following primary targets: Ark5 (IC50 = 5 nmol/L) and CDK4 (IC50 = 3.9 nmol/L), as well as appreciable inhibitory action against, PDGFRB (IC50 = 26 nmol/L), FGFR1 (IC50 = 26 nmol/L), proto-oncogene Ret receptor tyrosine kinase (RET, IC50 = 9.2 nmol/L) and proto-oncogene Fyn tyrosine-protein kinase (FYN, IC50 = 11 nmol/L).

To determine how ON123300 target modulation influenced cell signaling, we measured the activities of several critical molecules in both the PI3K/Akt/mTOR and Ras/MAPK signaling cascades using a phospho-MAPK array containing 26 different capture antibodies (Supplementary Fig. S2). Selected proteins that were significantly different (P < 0.05) in treated and control are shown in Fig. 1B. ON123300 decreased expression of phosphorylated Akt, CREB, and JNK pan decreased, whereas p-Erk and p-p38 increased in cells. On the basis of these disparate findings related to Akt and Erk, we further evaluated these related proteins, including Ark5, a key target, by Western blot analyses. ON123300 inhibited phosphorylation of Akt and its downstream signaling components, P70S6K, 40S ribosomal protein S6 (rpS6), and Rb S780 (see Fig. 1C). In conjunction with p-Ark5 and p-Akt inhibition there was an increase in p-Erk upon exposure to ON123300 (see Fig. 1C). Two additional ON123 series compounds, ON1231120 and ON1231320, had negligible effects on these proteins. As measured by Western blot and MSD assays the opposite effects of ON12300 on both p-Akt and p-Erk were concentration-dependent with an IC50 for p-Akt inhibition equal to 5.2 μmol/L (Fig. 2A), which roughly correlated to that for cytotoxicity, and 100% increase in p-Erk at a concentration of about 7 μmol/L compared with vehicle. Using the same experimental system the time-dependent behavior of p-Akt inhibition and p-Erk activation is shown in Fig. 2B in which the effects of ON123300 on p-Akt can be seen as early as 10 minutes, a nadir at 30 minutes and sustained inhibition over 6 hours at 6.3 μmol/L of ON123300. The p-Erk time profile was oscillatory in nature with a rapid increase at 5 minutes followed by a decline and then a second peak at 2 hours with another modest decline over 6 hours yet still elevated about 2-fold relative to pretreatment. Similar dose- and time-dependent behavior on p-Akt and p-Erk was also observed in two other glioma models; U251 glioma cells and GBM39 cells, a patient-derived brain tumor model (Supplement Fig. S3). Specifically, in the GBM39 model at 4 μmol/L ON123300 (close to that used in U87 cells), nadir p-Akt inhibition was 55% and peak p-Erk activation was 174% relative to the control.

ON123300 did not directly inhibit Akt based on the biochemical kinase screen but did show appreciable inhibitory action against upstream tyrosine kinase receptors (PDGFRB, FGFR1, and VEGFR3) that we inferred was the cause for reduced Akt activity. To elucidate the importance of FGFR1 and PDGFRB signaling in Akt inhibition, we examined their response upon stimulation by their cognate ligands, bFGF (20 ng/mL) and PDGF-BB (50 ng/mL), respectively (28, 29). The activation status of the receptor and Akt and Erk were determined by Western blot analysis (Fig. 2C) in U87 cells that were serum-starved for 18 hours and then treated for 2 hours with 3 μmol/L ON123300 before stimulation with each ligand for 10 minutes. Ligand stimulation with bFGF and PDGF-BB induced high levels of p-Akt and p-Erk in control cells that was abolished completely for p-Akt and partially for p-Erk by ON123300.

Previous studies reported that mTOR inhibition activates Erk by release of a concomitant negative feedback loop in which activation of p70S6K suppresses its upstream signaling component PI3K and causes inactivation of Erk (30, 31). To test whether ON123300-induced Erk activation was mediated through this PI3K/Ras/Raf/Erk negative feedback loop, PI3K activity was inhibited either by pretreating cells with 50 μmol/L LY294002 or 1 μmol/L wortmannin for 1 hour or augmented by using U87/PTEN cells that possess normal PTEN activity. As shown in Fig. 2D, abrogation of the PI3K feedback by pharmacologic inhibition of PI3K or by overexpression of PI3K suppressor PTEN reduced Erk activation in cells treated with either vehicle or ON123300. These data suggested that Erk activation may be partially modulated by release of feedback inhibition via PI3K activity but may also involve other signaling pathways. Under certain conditions, ligand concentration-dependent and time-dependent, Akt directly phosphorylates C-Raf at a highly conserved serine residue S259 and this phosphorylation results in recruiting 14-3-3 protein binding to C-Raf causing inactivation leading to Erk inactivation (32, 33). Thus, inhibition of Akt by ON123300 could prevent phosphorylation of C-Raf S259 via this cross-talk mechanism and promote Raf and then Erk activation. ON123300 treatment alone, in either U87 or U87/PTEN cells, partially decreased p-C-Raf S259 expression (Fig. 2D) and when combined with LY294002 or wortmannin even greater inhibition of p-C-Raf S259 was achieved supporting this pathway.

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connection in Erk activation. In summary, our results demonstrated that ON123300-induced Erk activation is caused by the release of the p70S6K/PI3K/Ras/Raf/Erk negative feedback loop and by suppression of Akt-induced Raf inhibition. Figure 2E illustrates the proposed mechanisms involved in ON123300 signaling.

In vitro combination studies

On the basis of the fact that ON123300 activated Erk through PI3K and Raf mechanisms, we considered that the addition of the EGFR inhibitor gefitinib could prevent this negative effect and enhance ON123300 activity. First, the in vitro cytotoxicity assays and associated combination index values being less than 1 (range 0.25–0.7) for the ON123300-gefitinib combination indicated synergy for all designated degrees of cell toxicity in all glioma cell lines (Fig. 3A; ref. 26). We completed a combination study with ON123300 and temozolomide and found an antagonistic effect based on the combination indices being >1 (Supplementary Table S1). Although initially perplexing this antagonism could be explained by the ability of temozolomide to induce Erk1/2 in U87 cells (34, 35), yet further investigations would be indicated to further characterize this combination. To investigate the molecular mechanisms underlying the synergistic cytotoxic effect of the combination of ON123300 and gefitinib Western blot
analyses of U87 cells were completed that demonstrated gefitinib not only enhanced ON123300 blockade of p-Akt, p-Ark5, and p-mTOR (S2448 and 2481), but also decreased p-Erk levels relative to control (Fig. 3B). The favorable responses to gefitinib are attributed to EGFR inhibition that is upstream of both the PI3K/Ras/Raf/Erk negative feedback loop and Akt-Raf activation that led to Erk activation.

To examine the effects of the gefitinib and ON123300 combination on cell dynamics, live cell microscopy and flow cytometry were completed. Consistent with cytotoxicity assays, the live cell microscopy showed a dramatic decrease of total cell number of U87 cells following single or combination treatments for 24 hours (Fig. 3C). Flow cytometry of live cells demonstrated significant effects on cell-cycle progression following 24 hours drug exposures (Fig. 3D) with ON123300 alone and in combination with gefitinib causing an approximate 2.5-fold increase in the percentage of cells in the G2–M phase [86% ± 3.1% and 102% ± 5.2% when treated with ON123300 alone and reduced to 64% ± 2.8% and 84% ± 4.5% when treated in combination]. C–E, after treatment of U87 cells with 6.3 μmol/L ON123300, 13.3 μmol/L gefitinib, or both for 24 hours, cells were analyzed by live cell imaging for morphology (C), and by flow cytometry for cell cycle (D) and apoptosis quantification (E). Scale bars, 20 μm. Data, percentages of the mean value and mean ± SD of three experiments. **, P < 0.01.

Figure 3. Mechanisms of action of concurrent treatment with ON123300 and gefitinib (GFN). A, combination indices for ON123300 and gefitinib combination therapy. U87 parental, U87 VIII, U87/PTEN cells were treated with different concentration of ON123300, gefitinib, or with a fixed concentration ratio of 0.2 of both for 72 hours. Combination indices were computed using the CompuSyn program for designated degrees of toxicity; ED50, ED75, and ED90. B, immunoblot analysis was performed on Akt, Erk, mTOR, and Ark5 expression after treatment of U87 cells with 6.3 μmol/L ON123300, 13.3 μmol/L gefitinib or both for 1 hour. p-Akt and p-Erk expression levels were quantitated by ImageJ software. The expression of p-mTOR S2448 and p-mTOR S2481 were 86% ± 3.1% and 102% ± 5.2% when treated with ON123300 alone and reduced to 64% ± 2.8% and 84% ± 4.5% when treated in combination. C–E, after treatment of U87 cells with 6.3 μmol/L ON123300, 13.3 μmol/L gefitinib, or both for 24 hours, cells were analyzed by live cell imaging for morphology (C), and by flow cytometry for cell cycle (D) and apoptosis quantification (E). Scale bars, 20 μm. Data, percentages of the mean value and mean ± SD of three experiments. **, P < 0.01.

In vivo pharmacokinetic/pharmacodynamic studies

From our prior pharmacokinetic screening analyses of the ON123 series, ON123300 was the lead compound that possessed favorable pharmacokinetic characteristics suggestive of distribution into brain (16). To determine
whether these findings translated to an in vivo setting, a series of pharmacokinetic/pharmacodynamic studies of ON123300 alone and combined with gefitinib were conducted in mice bearing intracerebral U87 tumors. These efforts were based on the measurement of two biomarkers, p-Akt and p-Erk in brain tumor samples and plasma, normal brain, and brain tumor concentrations of ON123300 and GFN following doses.

**In vivo pharmacodynamic profiles**

In agreement with in vitro pharmacodynamic profiles, in vivo pharmacodynamic results showed decreased p-Akt expression and increased p-Erk activity in brain tumors upon ON123300 administration at both intravenous doses of 5 and 25 mg/kg (Fig. 4A and B). p-Akt rapidly declined and reached nadir values of 73% and 60% of control within 30 minutes after 5 and 25 mg/kg dose levels, respectively. This was followed by a rapid and pronounced rebound within the first 2 hours of dosing and then a more sustained period of p-Akt inhibition with levels at the higher dose reaching near nadir values of 60% at 10 hours. To ascertain the cumulative effect of ON123300 on p-Akt levels the ABEC parameters were 63.2% and 304.9%-h at intravenous doses of 5 and 25 mg/kg, respectively, which is a proportional dose-dependent inhibition (5-fold change, see Table 1). Unlike the p-Akt profiles that shared similar patterns of inhibition at both dose levels, the p-Erk profiles were somewhat dissimilar with the 25 mg/kg dose group exhibiting appreciable activation that was biphasic reaching a maximum of 161% relative to baseline, whereas at the lower dose of ON123300 the biphasic pattern was muted with a singular spike of p-Erk at 5 minutes of 159%. As shown in Table 1, The ABEC values of p-Erk were 50%-h below the baseline and 144%-h above the baseline following administration of ON123300 at 5 and 25 mg/kg i.v., respectively. This suggests that at the lower 5 mg/kg dose of ON123300 the Akt-dependent feedback inhibition mechanisms were not completely blocked.

The addition of gefitinib to the ON123300 25 mg/kg dose regimen produced prominent effects on both the p-Akt and p-Erk profiles. As with single-agent ON123300, the p-Akt profile was biphasic yet the signal was further reduced (ABEC: 765%-h, Table 1) and achieved a nadir of about 30% at 4 hours after the ON123300 dose (Fig. 4C). The p-Erk response, although biphasic, was attenuated to a large degree with only a single peak activation of 120% at 5 minutes that was followed by a more durable decrease (ABEC: 273.9%-h below the baseline, Table 1) that was at 80% of control at 17 hours following the ON123300 dose (Fig. 4C). Overall, the addition of gefitinib to ON123300 produced similar effects on p-Akt and p-Erk responses in brain tumor–bearing mice as that observed in U87 cells in vitro.

**In vivo pharmacokinetic profiles**

The concentration–time profiles of ON123300 in plasma, brain, and brain tumor following single intravenous doses of 5 and 25 mg/kg are shown in Fig. 5A and B and the corresponding noncompartmental analysis results are summarized in Table 1. The pharmacokinetic profiles of plasma ON123300 concentration were multiexponential.

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**Figure 4.** Pharmacodynamic profiles of brain tumor in U87 brain tumor–bearing mouse administered at an intravenous dose of 5 or 25 mg/kg alone or combinational after 1 hour of oral dosing of 200 mg/kg gefitinib. p-Akt (A and C) and p-Erk (B and C) expression levels against total Akt and Erk expression in brain tumor homogenates at various time points quantified by MSD analysis. Data, percentages of the mean value and mean ± SD of at least three mice in each time point.
and overall declined fairly rapidly with terminal elimi-
nation half-lives of 1.5 hours. The AUCp values did not increase in a dose-proportional manner, being 1,206 ng-
h/mL at 5 mg/kg and 4,224 ng-h/mL at 25 mg/kg or a 3.5-
fold increase that translated into an increase in CL at the higher dose that could reflect saturable plasma protein 
binding for a low clearance drug. Previously, we showed that ON123300 is highly bound (99.4%) to plasma proteins 
in mice (16). The apparent volume of distribution of 
ON123300 was elevated at the higher dose also consistent 
with saturable plasma protein binding (Table 1).

ON123300 rapidly penetrated into brain with peak con-
centrations at 5 minutes of 5735 ng/g and 12432 ng/g at 
doses of 5 and 25 mg/kg, respectively. Distribution of 
ON123300 into normal brain as measured by the brain 
partition coefficient (Pb) was equal to 2.6 and 2.8 at the low 
and high doses, respectively. As expected in brain tumors 
where the BBB is compromised Pb was elevated to 5.7 and 15 at the 5 and 25 mg/kg doses levels, respectively. Whether the elevated partition-
ning into brain tumor at the 25 mg/kg dose could partially 
be attributed to saturation of BBB efflux transporters is 
unknown at this time; however, based on previous studies it is unlikely to be a substrate for the ABC transporters (16).

The concentration–time profiles of ON123300 in mice pretreated with a single 200 mg/kg oral dose of gefitinib were similar in nature to those in mice treated with single-agent ON123300 (See Fig. 5C and Table 1). There was a marked reduction (33%) in the total clearance of 
ON123300 in the presence of gefitinib. Normal brain and brain tumor partitioning of ON123300 was analogous to 
that when ON123300 was given alone, and although 
distribution into brain tumor was less in the presence of 
gefitinib it is more likely a reflection of interanimal var-
nability in BBB integrity because normal brain distribution 
was unaltered.

Discussion

Despite the plethora of new anticancer drugs under study, appreciable improvements in patient survival have not yet followed. Specifically, with respect to brain tumors, successful chemotherapy must not only inhibit key pharmacodynamic targets, but overcome the BBB that can greatly limit drug access to the desired target. By application of a pharmacokinetic/pharmacodynamic-
driven drug development strategy ON123300 surfaced as the lead candidate in the ON123 series based on toxicity 
to glioma cells and proficient BBB penetration and accumu-
lation in normal brain (16). The current investigation

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</tr>
<tr>
<td>Cmax</td>
<td>ng/mL</td>
<td>5,753</td>
<td>12,432.5</td>
<td>16,300</td>
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</tr>
<tr>
<td>Tmax</td>
<td>h</td>
<td>0.083</td>
<td>0.083</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Pd</td>
<td></td>
<td>2.6</td>
<td>2.8</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Brain tumor AUCbt</td>
<td>h ng/mL</td>
<td>6,886</td>
<td>63,221</td>
<td>49,440</td>
<td></td>
</tr>
<tr>
<td>Cmax</td>
<td>ng/mL</td>
<td>6,483</td>
<td>27,250</td>
<td>20,900</td>
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<td>Tmax</td>
<td>h</td>
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<td>1</td>
<td>0.5</td>
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<tr>
<td>Pbt</td>
<td></td>
<td>5.7</td>
<td>15</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>ABEC (%)h</td>
<td>p-Akt (%)h</td>
<td>63.2</td>
<td>304.9</td>
<td>765.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-Erk (%)h</td>
<td>50.0</td>
<td>144.1a</td>
<td>273.9</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: NCA were performed using WinNonlin Phoenix, version 6.3 software (Pharsight Corporation) to estimate pharmacokinetic and pharmacodynamic parameters that included for each compound the areas under the drug concentration–time curve in plasma, brain, and brain tumor (AUCp, AUCd, and AUCbt, respectively), the areas between the effect-time curve in brain tumor (ABEC), half-life (t1/2), systemic clearance (CL), and the apparent volume of distribution at steady state (Vss). The normal brain or brain tumor partition coefficient (Pb and Pbt) was calculated as the ratio of either the AUCb or AUCbt over the corresponding AUCp. All AUC calculations were based on the area to the last observable concentration using the linear-log interpolation method. It is assumed that the density of tissues equals 1 g/mL.

aABEC above the baseline.
was designed to characterize the pharmacokinetics/pharmacodynamics of ON123300 and assess its potential in brain tumor chemotherapy by focusing on the components in the Ras/MAPK and PI3K/Akt/mTOR pathways that are often dysregulated in patients with brain tumor (7, 8, 38).

In U87 glioma cells, ON123300 did inhibit p-Akt in a dose- and time-dependent manner, yet unexpectedly also stimulated p-Erk that would be detrimental to its anticancer activity. The observation that inhibitors along the RTK/PI3K/Akt/mTOR pathway produce undesirable anti-complimentary effects has been noted previously (29–33, 39–41). Specifically, the PDGFR inhibitor imatinib elicited Erk activation that was ligand dependent, in which imatinib inhibited signaling mediated by PDGF-BB, but not by PDGF-AA or stem cell factor (29, 40). The mTOR1 inhibitor rapamycin and its analogues can activate Ras and its downstream counterparts by relief of the brake supplied by PI3K and triggered by p70S6K (30, 31). This p70S6K/PI3K/Ras/Raf/MEK feedback loop exists in both cancer patients and in preclinical animal models that appeared drug schedule-dependent as we observed (30, 31). In addition, Akt antagonizes Raf activity by direct phosphorylation of S259, and thus, treatment with a PI3K or Akt inhibitor, such as LY294002, results in Erk hyperactivation by decreasing inhibitory S259 phosphorylation of c-Raf (32, 33). Our data demonstrate that ON123300 treatment appears to cause MAPK activation not only by relief of PI3K feedback inhibition triggered by p70S6K, but also by downregulation of c-Raf S259 phosphorylation. The overall action of ON123300—decreased Akt activity and activated Erk—was observed in three different glioblastoma multiforme models, including a patient-derived model that supported the need to find another drug that could synergize with ON123300 and prevent Erk activation.

Our previous studies of single-agent gefitinib suggested it would be effective in mitigating the rapid Erk activation caused by ON123300 due to its rapid and appreciable penetration of the BBB and its inhibitory action upstream of PI3K and Akt and their points of interaction with the MAPK pathway (42–44). Other studies employing gefitinib or an EGFR inhibitor also suggested tumor growth and Erk suppression when used in combination with either an mTOR inhibitor (rapamycin) or Akt inhibitor (45). In the latter study (45), the Akt inhibitor activated Erk yet when combined with gefitinib (150 mg/kg three times/week) growth of NCI-H292 tumors in mice were significantly retarded compared with the Akt inhibitor alone. These positive preclinical findings of EGFR inhibitors and Akt/mTOR pathway inhibitors have not yet translated to the clinic as noted for the combinations of gefitinib and everolimus or sirolimus (46–48). Pharmacodynamic analyses of intratumoral MAPK, PI3K, and mTOR signaling were not completed leaving the cause of the lack of efficacy unresolved (46–48). Other agents, possibly those directly inhibiting Raf\MEK\Erk signal cascades, such as the MEK inhibitor AZD6244, may also curtail ON123300-induced Erk activation (49, 50); however, with the gefitinib–ON123300 combination not only was Erk activation suppressed, Akt inhibition was also enhanced.
In summary, through a series of in vitro and in vivo pharmacokinetic/pharmacodynamics investigations of ON123300 we confirmed it possessed suitable pharmacokinetic properties for brain tumor chemotherapy accentuated by brain:plasma partitioning of more than 1. Of particular interest and significance was this multikinase inhibitor produced opposing effects on the PI3K/Akt/mTOR and Ras/MAPK pathways that were attributed to release of feedback inhibition mechanisms that were dose-dependent, and would likely curtail future development as a single agent. Because this feature may be characteristic of inhibitors of the PI3K/Akt/mTOR pathway, it is worthwhile to consider confounding pathway activations early in the drug development scheme and whether rational combination therapy can overcome, and in the process, improve disruption of oncogenic pathways.

Disclosure of Potential Conflicts of Interest

M.V. Ramana Reddy is a consultant in Onconova Therapeutics, Inc and has ownership interest in several patents on kinase inhibitors through Temple University. E. Premkumar Reddy is a director, a consultant, an advisory board member of Onconova Therapeutics Inc, and has received commercial research grant from and has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The outcome of this research project could affect the value of these patents and of Onconova Therapeutics.

Authors’ Contributions

Conception and design: X. Zhang, M.V. Ramana Reddy, J.M. Gallo


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Lv, R. Elkholi, J.E. Chipuk, E.P. Reddy

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhang, R. Elkholi, J.E. Chipuk, E.P. Reddy, J.M. Gallo

Writing, review, and/or revision of the manuscript: X. Zhang, J.E. Chipuk, E.P. Reddy, J.M. Gallo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhang

Study supervision: J.M. Gallo

Other: Design and synthesis of all ON123300 and related compounds, M.V. Ramana Reddy.

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References


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