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RESEARCH PAPER

Preclinical Pharmacokinetic and Pharmacodynamic Evaluation of Novel Anticancer Agents, ON01910.Na (Rigosertib, Estybon™) and ON013105, for Brain Tumor Chemotherapy

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ABSTRACT

Purpose To evaluate a mitotic inhibitor, ON01910.Na, as a potential chemotherapeutic agent for brain tumors using a series of PK/PD studies, which led to the evaluation of its structural analog, ON013105, a prodrug of the more lipophilic product, ON013100.

Methods Systemic PK characterization of ON01910 and ON013105 was completed in healthy mice. Using an orthotopic U87 glioma mouse model, brain and brain tumor distribution under steady-state conditions were evaluated for ON01910.Na and ON013105/ON013100; anticancer potential following a multiple-dose schedule of 250 mg/kg/day IP for 7 days was evaluated for ON01910.Na.

Results ON01910 exhibited low brain and brain tumor distribution with quasi-steady-state brain/plasma (Css_{brain}/Css_{plasma}) and brain tumor/plasma ($Css_{brain\ tumor}/Css_{plasma}$) concentration ratios of 0.03 $\pm$0.02 and 0.14$\pm$0.08, respectively. Significant antiangiogenic potential and antiproliferative capacity of ON01910 in the intracerebral model was absent. ON013100 showed high brain and brain tumor penetration with Css_{brain}/Css_{plasma} and $Css_{brain\ tumor}/Css_{plasma}$ ratios of 0.92$\pm$0.26 and 1.35$\pm$0.40, respectively; its prodrug ON013105 showed negligible brain and brain tumor penetration.

Conclusions ON013105, not ON01910.Na, was identified as a potential anticancer drug candidate for further investigation in brain tumor chemotherapy based on the properties of ON013100.

KEY WORDS brain tumor disposition · glioma · intracerebral · ON013105 · ON01910.Na

ABBREVIATIONS

AUC	area under the curve
BBB	blood-brain barrier
CDK4	cyclin-dependent kinase 4
CNS	central nervous system
Css_{brain}	quasi-steady-state normal brain concentration
$Css_{brain\ tumor}$	quasi-steady-state brain tumor concentration
Css_{plasma}	steady-state plasma concentration
IP	intraperitoneal
IV	intravenous
LLOQ	lower limit of quantitation
MCF-7/DOX	doxorubicin resistant breast cancer cell line
MCL	mantle cell lymphoma
MDS	myelodysplasia
MOLT ALL	methotrexate resistant acute lymphoid leukemia
MRP1	multidrug resistance protein 1
MRPs	multidrug resistance proteins

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mTOR	mammalian target of Rapamycin
MVD	microvessel density
PARP	Poly (ADP-ribose) polymerase-1
PB	physiologically based
PB-PK	physiologically based-pharmacokinetic
P-gp	P-glycoprotein
PI3K	phosphatidylinositol-3-kinase
PPB	plasma protein binding
TCA	trichloroacetic acid
VEGF	vascular endothelial growth factor
VNI	vascular normalization index
α-SMA	α-smooth muscle actin

INTRODUCTION

ON01910.Na (also referred to as, Rigosertib, Estybon™) is a synthetic low molecular-weight drug (473.47 Da) belonging to the class of benzyl styryl sulfones (Fig. 1a) whose pharmacological activity is attributed to the free acid, ON01910. It is a non-ATP competitive compound (1) and is under evaluation as an anticancer agent. Having completed a Phase I trial (2), ON01910.Na is currently in Phase II clinical trials as a single agent and in combination with conventional chemotherapy in advanced and metastatic tumors. It is also being investigated for the treatment of myelodysplasia (MDS) due to its inhibitory effect on cyclin D1 accumulation and selective toxicity towards trisomy 8 cells and is currently in Phase I/II/III trial in MDS patients with refractory anemia (3). ON01910 inhibits mitotic progression by arresting cells at the G2/M phase of the cell cycle and induces abnormalities in mitotic spindle formation eventually leading to selective apoptosis in tumor cells, but not in normal cells (1). In mantle cell lymphoma (MCL) cell lines, ON01910 was reported to interfere with cell signaling, wherein it was shown to downregulate cyclin D1 by suppressing cyclin D1 mRNA translation and induced apoptosis via the mitochondrial pathway by inhibition of the PI3K/Akt/mTOR pathway (4) while in HeLa cells, it inhibited polo like kinase 1 (plk1, a serine-threonine kinase) activity (1).

ON01910 has exhibited potent antitumor activity with a low toxicity profile in various preclinical tumor xenograft models as a single agent while showing complete tumor regression in combination with chemotherapeutic agents such as oxaliplatin and doxorubicin in human liver and breast cancer xenografts, respectively (1). It inhibited angiogenesis, migration and invasion in an *in vitro* glioma (SNB19 glioma cell line) model (5). Complete regression of tumor growth and angiogenesis was also observed in mice bearing intracranial SNB19 human glioma xenografts; however in this case ON01910.Na was administered by direct intratumoral injection bypassing the blood–brain barrier (BBB) (5). Even though direct injection into the brain tumor suggested a problem with BBB penetration following systemic administration, it also provided the initial motivation to further evaluate ON01910.Na in brain tumor models.

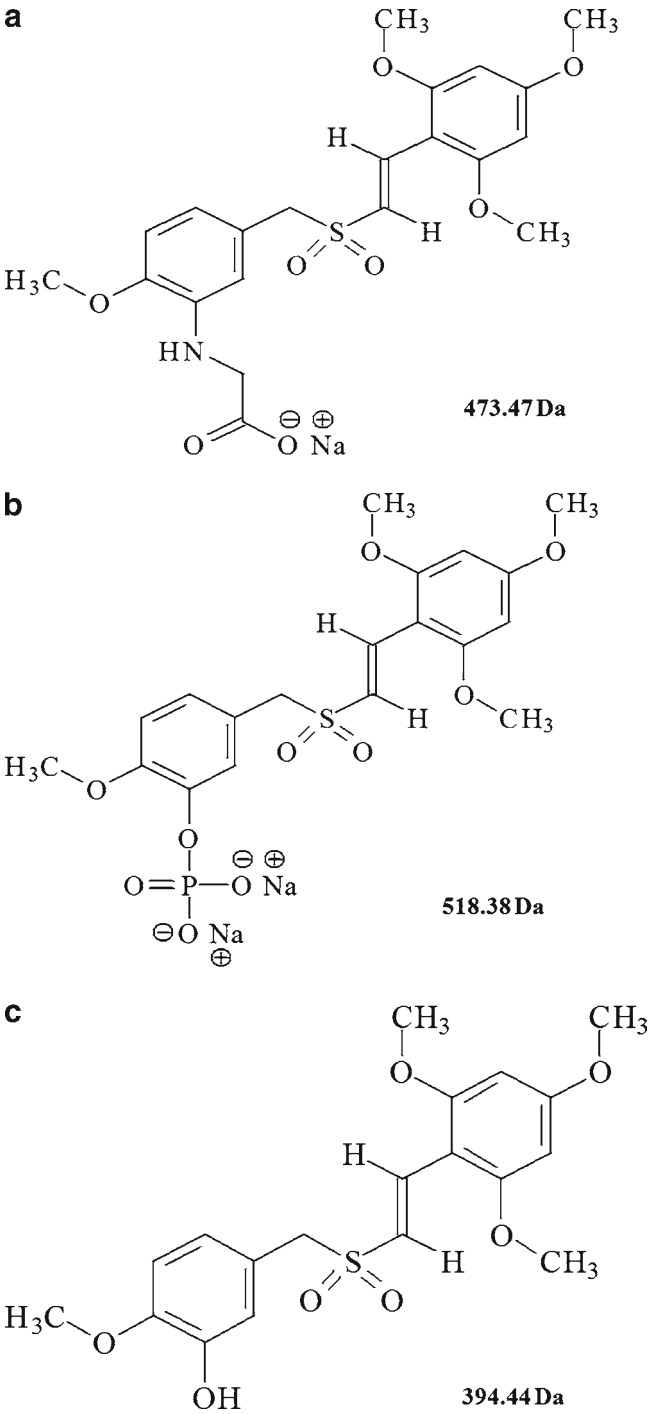

Fig. 1 Chemical structures of ON01910.Na (**a**), ON013105 (**b**) and ON013100 (**c**).

ON013105 (Fig. 1b) and ON013100 (Fig. 1c) are structural analogs of ON01910.Na, belonging to the class of styryl benzyl sulfones constituting non-ATP competitive

novel antineoplastic agents (6). ON013105, being a water soluble phosphate prodrug, is converted to an active dephosphorylated form, ON013100, which exhibited potent cytotoxicity against various human tumor cell lines including drug resistant tumor cell lines at nanomolar concentrations, typically with IC50 values of less than 10 nM (6) that surpass the activity of ON01910 whose IC50 values ranged from 50 to 170 nM in various cancer cell lines (1). ON013100 caused G2/M arrest in mantle cell lymphoma (MCL) and prostate cancer cells (6,7), inhibited the expression of various proteins involved in cell cycle regulation such as cyclin D1, cyclin dependent kinase 4 (CDK4), and cdc2 p34 (7) and similar to ON01910, induced apoptosis in various human tumor cell lines while exhibiting relatively low toxicity towards normal cells (6). The cytotoxic potency of ON013100 was increased by one log scale in combination with cytotoxic agents such as doxorubicin and vincristine in MCL cell lines (7). ON013105, a soluble form of ON013100, when administered to breast cancer xenografts, readily inhibited the tumor growth (6). ON013105 is currently in phase I clinical development in patients with relapsed/refractory lymphoma and acute lymphocytic leukemia.

Malignant gliomas are the most lethal cancers that originate in the central nervous system (CNS) (8). Despite standard medical interventions of surgery and intensive use of radiation and chemotherapy, the median survival is 12–15 months for patients with WHO classification IV glioma, glioblastoma multiforme (8,9). ON01910.Na has yet to be evaluated in patients with primary brain tumors, and given its reported favorable activity when applied locally to intracerebral tumors in mice (5), an evaluation of its potential as a targeted drug for brain tumors following systemic administration was warranted. In addition, cyclin D1, a target of ON01910 (4), has been shown to play a role in promoting angiogenesis (10,11), and was reported to be upregulated in glioma with its expression level correlated with the degree of malignancy (12). In summary, given the favorable antitumor activity of ON01910, its potential antiangiogenic activity, and the absence of data on its brain tumor penetration, we pursued a preclinical pharmacokinetic/pharmacodynamic evaluation of it as a new treatment for brain tumors. Subsequent to these analyses, a structural analog, ON013100, with appreciable glioma cell toxicity was also evaluated for brain and brain tumor distribution.

MATERIALS AND METHODS

Materials

ON01910.Na, ON012380, ON013100, ON013105 and other structural analogs of ON01910.Na were provided by Dr. M.V. Ramana Reddy. Centrifree® centrifugal filter devices were purchased from Millipore Corporation (Bedford, MA). Mouse plasma was purchased from Lampire Biological Laboratories (Pipersville, PA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Cellgro (Manassas, VA), fetal bovine serum from Invitrogen (Carlsbad, CA) and sulforhodamine B from Sigma-Aldrich (St. Louis, MO). Rat anti-CD31 antibody was purchased from BD PharMingen (San Jose, CA), rabbit anti-collagen IV from Millipore Chemicon (Temecula, CA) and mouse anti-α-smooth muscle actin (clone 1A4) from Sigma-Aldrich (St. Louis, MO). Antirat, antirabbit and antimouse biotinylated IgG, and Vectastain Elite ABC and Vectastain Alkaline Phosphatase kits were bought from Vector Laboratories (Burlingame, CA). All other chemicals and reagents were purchased from commercial suppliers.

Human glioblastoma cell line, U87MG, was purchased from American Type Culture Collection. U87MG cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Cells were grown under standard cell culture condition in a humidified atmosphere with 5% CO_2 at 37°C.

Male NIH Swiss nude mice were supplied by Taconic Co. and maintained in the American Association for the Accreditation of Laboratory Animal Care–accredited University Laboratory Animal Resources of Temple University. All animal studies were approved by the Institutional Animal Care and Use Committee. Male nude mice at the age of 6–9 weeks and weighing 23 to 29 g were used for all studies.

Cytotoxicity Assay

The cytotoxicity of ON01910, ON013105 and ON013100 to U87 human glioma cells was determined using a colorimetric sulforhodamine B-based assay (13). U87 cells were allowed to grow in standard culture medium. About 2000 cells/100 μl of culture medium/well were seeded in a 96-well plate and allowed to attach to the bottom of the wells by overnight incubation. A series of concentrations of ON01910.Na (0.1 nM to 50 μM), ON013105 (0.05 nM to 10 μM) or ON013100 (0.05 nM to 10 μM) were added to the wells and incubated for 72 h. At the end of the treatment, cells were fixed with 100 μl of 10% trichloroacetic acid (TCA), washed and then stained with 0.4% sulforhodamine B. The absorbance of the dye was measured with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm. The 50% inhibitory concentration or IC50 value was defined as the drug concentration that reduced the viable cell population by 50% compared with control treatment (vehicle alone). A sigmoidal dose–response model was used to determine IC50 values by nonlinear regression. Each mean IC50 value was based on 3 independent experiments.

In Vitro Plasma and Brain Binding Assay

Binding of ON01910 to plasma proteins and brain tissue was determined *in vitro* using an ultrafiltration method. Centrifree®

centrifugal filter devices consisting of low-adsorptive hydrophilic membranes of 30 kDa molecular weight cut-off were used. The following concentrations of ON01910.Na were prepared—0.2 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, 100 μg/ml and 500 μg/ml in blank mouse plasma and 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, 100 μg/ml and 200 μg/ml in blank brain homogenate (25% homogenate in isotonic 10 mM PBS). Spiked plasma or brain homogenate samples and filter devices were preincubated at 37°C for 15 min before filtration. An aliquot of each sample was kept aside for the analysis of total drug, and 400 μl of plasma or brain homogenate was transferred to the filter device and centrifuged at 1200xg for 5 min at room temperature. Ultrafiltration was performed in triplicates at each concentration level. Protein leakage through the filters was tested using a Bio-Rad protein assay. Nonspecific binding of ON01910 to the filter apparatus was determined by adding the same concentrations of ON01910.Na to 10 mM PBS (pH 7.4) and performing ultrafiltration using the same volume of sample as plasma/brain homogenate. ON01910 was analyzed in plasma before and after filtration using HPLC with UV detection while that in brain tissue homogenate was analyzed before and after filtration using LC/MS/MS. The percentage of plasma protein binding (PPB) was calculated as [1 - (unbound ultrafiltrate drug concentration/total plasma drug concentration)] × 100 while the percentage of brain tissue binding was calculated as $[1-(f_{u\text{-}hom}/(D_f-(D_f-1)\times f_{u\text{-}hom}))]\times 100$ where $f_{u\text{-}hom}$ represents the unbound ultrafiltrate drug concentration in the diluted homogenate and D_f represents the dilution factor (14).

Binding of ON013100 to plasma proteins and brain tissue was determined *in vitro* using an equilibrium dialysis method carried out using a 96-well Equilibrium Dialysis Block system (Model HTB96b, HTDialysis LLC, Gales Ferry, CT). Various concentrations of ON013100 were prepared in plasma and brain tissue homogenate (25% homogenate in isotonic 0.067 M phosphate buffer) (0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, 30 μg/ml, 100 μg/ml and 200 μg/ml). Aliquots (150 μl) of spiked plasma/brain homogenate samples were dialyzed against an equal volume of isotonic 0.067 M phosphate buffer (pH 7.4) with the plasma/brain homogenate and buffer units separated by a dialysis membrane (MWCO 12-14 K, HTDialysis LLC). The units were maintained at 37°C and rotated for 8 h that was previously determined to represent an equilibration time. Post-dialysis plasma and buffer volumes were measured and analyzed for drug concentrations by LC/MS/MS. The percentage of bound drug in plasma/brain homogenate was calculated as follows-

$$\%\ \text{bound} = \frac{(D_{Te} - D_F)\cdot V_e/V_i}{[(D_{Te} - D_F)\cdot V_e/V_i] + D_F} \times 100$$

where D_{Te} and D_F represent the total plasma/brain homogenate concentration at equilibrium (sample side) and the free concentration (dialysate side), respectively, and V_i and V_e represent the initial and equilibrium plasma/brain homogenate volumes, respectively (15). The percent bound in diluted brain homogenate so obtained from the above formula was then converted to percent bound in brain tissue using the same formula as that used for ON01910 brain tissue binding (14). ON013105 binding to plasma proteins and brain tissue could not be determined as it was not stable under the conditions of the *in vitro* assay.

Single-Dose Pharmacokinetic Studies of ON01910.Na

The pharmacokinetics of ON01910.Na was determined in healthy athymic mice at three dose levels—5 mg/kg, 50 mg/kg and 250 mg/kg (n=7–10/dose level) following a short-term 10 min constant rate IV infusion via a tail vein of the sodium salt of ON01910 dissolved in saline. One day before drug administration, a vascular cannula (vinyl tubing of I.D 0.28 mm × O.D 0.61 mm, Scientific Commodities Inc., Lake Havasu City, AZ) was surgically implanted in the right common carotid artery to allow for serial collection of blood (20 μl of blood per time point). On the next day when mice were fully recovered, they were placed in individual metabolic cages for the duration of the pharmacokinetic studies to allow for the collection of urine and feces. Blood samples were collected at a maximum of 11 different time points over a 24 h interval at 5 mg/kg (pre-dose, end of infusion (10 min), 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h and 24 h) and 50 mg/kg (pre-dose, end of infusion (10 min), 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h) dose levels, whereas sampling was extended to 48 h (pre-dose, end of infusion (10 min), 15 min, 30 min, 1 h, 2 h, 4 h, 7 h, 12 h, 24 h, 36 h and 48 h) for the 250 mg/kg dose. Plasma immediately harvested from blood by centrifugation at 3500 rpm for 5 min, and urine and feces, collected over the same time intervals as blood, were stored at −80°C until analyzed by LC/MS/MS for ON01910. Total volume of the urine collected was recorded before storing at −80°C.

Single-Dose Pharmacokinetic Studies of ON013105 and its Active Form, ON013100

The systemic pharmacokinetics of the phosphate prodrug, ON013105 and its active form, ON013100 in healthy athymic mice was determined at a dose level of 37.5 mg/kg following an IV bolus dose of ON013105 dissolved in saline via a tail vein. Blood sampling was carried out at pre-dose, 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h post drug administration and plasma harvested from blood, was stored at −80°C until analyzed by LC/MS/MS for ON013105 and ON013100.

Brain and Brain Tumor Disposition Study

Mice were anesthetized with 0.1 ml/20 g body weight of a 3:2:1 (v/v/v) mixture of ketamine hydrochloride (20 mg/ml), acepromazine maleate (2 mg/ml), and xylazine hydrochloride (4 mg/ml) given intraperitoneally, and then secured in a stereotactic apparatus. A suspension of U87 glioma cells (1×10^6 cells in 8 μl of phosphate buffered saline), prepared fresh from culture, was then injected into the caudate putamen at a position 2.2 mm lateral, 0.7 mm anterior to bregma and at a depth of 2.5 mm using a 10 μl Hamilton syringe (Hamilton Co., Reno, NV). Following recovery from anesthesia, animals were returned to the animal care facility and received regular mouse diet and water *ad libitum*. Mice were monitored at least once a day for body weight and CNS symptoms that included an arched back, unsteady gait or unkempt fur, and were entered into the brain and brain tumor pharmacokinetic study at the earliest signs of CNS symptoms or a 2-day total body weight loss of 10%.

ON01910.Na (dissolved in saline) was given intravascularly (n=7) to achieve steady-state plasma concentrations of 10 μg/ml by using a bolus injection of 77.4 mg/kg followed by an intra-arterial infusion through the carotid artery cannula for 3 h at a constant rate of 31 mg/hr/kg using a syringe pump (Harvard Apparatus, Holliston, MA). The steady-state infusion rate (R_o) and loading dose were calculated as follows: R_o=CL×Css (R_o=rate of infusion (mg/hr/kg), CL=Clearance L/hr/kg) and Css=targeted steady-state plasma concentration (mg/L)); loading dose=Vss×Css (Vss=volume of distribution at steady-state (L/kg), Css=targeted steady-state plasma concentration (mg/L)). At the end of infusion, mice were anesthetized using inhaled isoflurane and exsanguinated via the posterior vena cava. Normal brain and brain tumor samples were rapidly excised and flash frozen on ice, and stored along with plasma samples at −80°C until processed for measurement of ON01910 by LC/MS/MS.

To determine the brain and brain tumor disposition of ON013105 and ON013100, the same glioma model and surgery procedures as for ON01910.Na study were used. Mice bearing intracerebral U87 tumors (n=8) received a bolus of 3.95 mg/kg ON013105 followed by infusion through the right carotid artery for 3 h at a constant rate of 57.63 mg/hr/kg to attain a steady-state plasma concentration of 4.5 μg/ml. Plasma, brain and brain tumor samples were collected at the end of infusion and analyzed for ON013105 and ON013100 using LC/MS/MS.

Antiangiogenic Evaluation of ON01910.Na Following Multiple-Dose Administration

Three weeks post intracerebral tumor implantation, mice were randomized into control and treatment groups (n=6/group) and received IP administration of either saline or 250 mg/kg of ON01910.Na for 7 days. On day 7, animals were sacrificed by exsanguination under inhalation anesthesia at 2 h following the last dose of saline or ON01910.Na. Three mice (one from control group and two from treatment group) were sacrificed on day 6 following drug administration based on their neurological symptoms and were included in the analysis. Brain and brain tumors were collected as before and processed for immunohistochemical staining of angiogenesis markers and Ki-67 (tumor cell proliferation).

The antiangiogenic potential of ON01910 was based on immunohistochemical analysis of microvessels (CD31), pericytes (α-SMA) and the thickness of the basement membrane (collagen IV) that permitted the calculation of the vascular normalization index (VNI) as –

$$\mathrm{VNI} = \mathrm{MVD} \times \frac{\mathrm{Density}_{\alpha-\mathrm{SMA}}}{\mathrm{Density}_{\mathrm{Collagen\ IV}}}$$

This serves as a measure of the quality of the vasculature, and has been previously used to assess the pharmacodynamic effects of sunitinib, an angiogenesis inhibitor, on the tumor vasculature (16). In addition to the angiogenesis markers, the proliferation index of the tumor was also assessed using Ki-67 staining (17).

For immunohistochemistry, a similar method as previously published from our lab was used (16). Briefly, 10 μm cryostat tumor sections were fixed, washed with PBS (pH 7.5) and incubated with 3% hydrogen peroxide in methanol at room temperature to quench endogenous peroxidase. Sections were then incubated overnight at 4°C with appropriate dilutions of rat anti-CD31, rabbit anti-collagen IV and mouse anti-α-smooth muscle actin (clone 1A4) primary antibodies followed by incubation with the appropriate biotinylated IgGs for 1 h at room temperature. The remaining steps were carried out using the Vectastain Elite ABC kit for CD31 and α-SMA, or the Vectastain Alkaline Phosphatase kit for collagen IV and Ki-67, according to the manufacturer's protocols. All sections were then counterstained with 0.5% methyl green. Semiquantitation of digital images (Leica DC500 camera and DM4000B microscope) for each marker (CD31, collagen IV, and α-SMA) in each tumor sample was done by measuring the pixel area of the positive staining of individual markers in at least six random areas at 200x magnification using Image-Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD). The percentage of the positive area stained by the marker was calculated as (marker-positive area/area of optical field) x 100 (16). The Ki-67 proliferation index was calculated as the number of positive cells per total number of counted cells, expressed as a percentage. Extensive necrotic areas were avoided. The differences in means of density of microvessels, α-SMA and collagen IV and difference in the means of proliferation index between control and treatment groups were evaluated using the equal-

variance *t*-test. A P-value of less than 0.05 was considered statistically significant.

Quantitative Analysis of ON01910

An analytical method for the quantitation of ON01910 concentrations in mouse plasma, urine, feces, brain and brain tumor using liquid chromatography with tandem spectrometry (LC/MS/MS) has been previously described (18). Briefly, aliquots of plasma (10 µl), urine (50 µl), feces (30 µl of a 5% homogenate), brain (20 µl of a 10% homogenate (pharmacokinetic study)/25% homogenate (binding assay)), brain ultrafiltrate (20 µl) and brain tumor (50 µl of a 10% homogenate) samples were processed in a single protein precipitation step by adding three times the volume of methanol containing 500 ng/ml of the internal standard (ON012380, provided by Reddy lab), and vortexing for 1 min followed by centrifugation for 5 min (plasma) or 15 min (urine, feces, brain and brain tumor) at 15,000 rpm. Aliquots of the resultant supernatant were injected into the LC/MS/MS. The LC system consisted of a C18 guard cartridge and a Luna C18 analytical column with an isocratic mobile phase consisting of 30:70 v/v of acetonitrile:10 mM ammonium formate. The flow rate was 0.3 ml/min and the injection volume was 5 µl. The instrument was operated in positive ion scan mode with ESI-SRM ion transitions of m/z 469.00→284.00 for ON01910 and 466.00→208.00 for the internal standard.

ON01910 concentration in plasma and plasma ultrafiltrate samples from the *in vitro* plasma protein binding study was measured by HPLC with UV detection at 309 nm. The mobile phase, analogous to that used for the LC/MS/MS procedure, was operated at a flow rate of 200 µl/min with a sample injection volume of 10 µl. The total run time was 5 min and ON01910 produced a chromatographic peak at 2.8 min. The sample preparation method for plasma was the same as described for plasma analysis by LC/MS/MS while ultrafiltrate samples were directly injected into the HPLC without any prior sample preparation. The inter-day accuracy and precision determined for ultrafiltrate samples were within 8% while for plasma, intra- and inter-day assay accuracy and precision were within 20%.

Quantitative Analysis of ON013105 and ON013100

ON013105 and ON013100 concentrations in plasma, normal brain and brain tumor were quantitated using an API 4000 triple quadrupole liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) system. Aliquots of plasma (10 µl), brain tumor (30 µl of a 10% homogenate), normal brain (30 µl of a 10% homogenate (pharmacokinetic study)/20 µl of a 25% homogenate (binding study)) and phosphate buffer (20 µl) were processed using a single protein precipitation step that consisted of adding equal volumes of a stock mixture of ON013100 and ON013105 dissolved in 50% acetonitrile (standards) or blank 50% acetonitrile (samples) and vortexing for 30 s, followed by addition of internal standard (ON01910.Na) dissolved in 50% acetonitrile (1.99 µg/ml) equivalent to sample volume and vortexing for 30 s. Finally five times the sample volume of 100% acetonitrile was added and vortexed for 1 min followed by centrifugation for 10 min (plasma) or 15 min (brain and brain tumor) at 15,000 rpm. Aliquots of the resultant supernatant were injected into the LC/MS/MS. The LC system consisted of a C18 guard cartridge and a Luna C18 analytical column. Gradient elution was used with mobile phase A that consisted of 10 mM ammonium acetate with 0.1% of ammonium hydroxide (29%) while mobile phase B consisted of methanol containing 0.1% of ammonium hydroxide (29%). The flow rate was 0.3 ml/min and an injection volume of 5 µl was used. The instrument was operated in negative ion scan mode with ESI-SRM ion transitions of m/z 472.90→79.00 for ON013105, 392.90→256.80 for ON013100 and 450.00→418.00 for the internal standard. The lower limits of quantitation of ON013105 and ON013100 were 2.20 ng/ml and 7.32 ng/ml, respectively in plasma, brain and tumor homogenates. The intra- and inter-day accuracy and precision determined for plasma, brain and tumor samples were within 15% for both ON013105 and ON013100.

Pharmacokinetic Analysis

Analyses of ON01910, ON013105 and ON013100 plasma concentrations from individual mice following single dose administration were performed using non-compartmental method with Phoenix® WinNonlin® version 6.2 (Pharsight, St. Louis, MO). Pharmacokinetic parameters calculated included systemic clearance (CL), volume of distribution (V_d), terminal elimination rate constant (λ_n), terminal elimination half life ($t_{1/2}$) and the total area under the plasma concentration-time curve ($AUC_{0-\infty}$). The peak plasma concentrations (C_{max}) were obtained by visual inspection of the plasma concentration-time curves. Renal and fecal excretion of ON01910 were expressed as the fraction of the dose excreted unchanged in urine and feces, respectively, and renal and biliary clearances were calculated as the fraction of dose excreted unchanged in urine or feces multiplied by total systemic clearance.

Statistical Analysis

Differences in pharmacokinetic parameters between the three dose groups were evaluated using one-way ANOVA (Tukey-Kramer *post hoc* test) while differences in the pharmacokinetic

parameters between two dose groups were evaluated using equal variance 2-sided *t*-test. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Pharmacokinetic and Pharmacodynamic Characterization of ON01910.Na

Systemic Pharmacokinetics of ON01910.Na

A comprehensive analysis of ON01910, the free acid of ON01910.Na, systemic disposition was completed based on data obtained over a 50-fold dose range; 5 mg/kg, 50 mg/kg and 250 mg/kg of ON01910.Na given IV. Plasma concentration-time profiles (see Fig. 2) of ON01910 exhibited a rapid distribution phase with a relatively slow terminal elimination phase. The plasma concentrations at 5 mg/kg were below the lowest limit of quantitation beyond 1.5–3 h, which prevented the terminal elimination phase from being captured completely, and thus, accurate estimation of pharmacokinetic parameters was not possible (C_{max}=5.72±1.25 μg/ml). Application of non-compartmental analysis to plasma ON01910 concentrations obtained at the two higher dose levels indicated dose-dependent pharmacokinetics (see Table I). The total clearance changed 3–4 fold over the dose range from 50 mg/kg to 250 mg/kg consistent with nonlinear elimination, as evidenced by a more than proportional increase in the area under the plasma concentration-time curve. The apparent volume of distribution decreased 2.8 fold at the 250 mg/kg dose when compared to 50 mg/kg dose group.

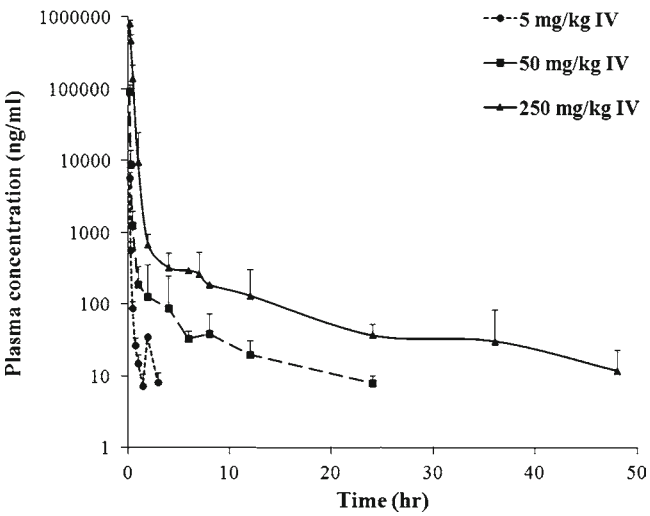

Fig. 2 Time courses of mean plasma concentrations of ON01910 following short term IV infusions of 5 mg/kg, 50 mg/kg and 250 mg/kg ON01910.Na to healthy athymic mice (*n*=7–10). *Bars*, SD. No error bars for concentrations with $n < 3$.

The percent of ON01910 excreted unchanged in urine was 10–16% at all dose levels indicating that renal clearance is not a primary route of drug elimination (Table II). The major route for ON01910 elimination was biliary as indicated by the percent of the drug excreted unchanged in feces, being greater than 50% at all the dose levels (Table II). Based on the amount of ON01910 eliminated in feces, biliary clearance was significantly reduced at the highest dose level (250 mg/kg) when compared to the 50 mg/kg dose level. Thus, biliary excretion being the major elimination pathway, partly contributes to the saturable total clearance. A recent report by Chun *et al.* (19) on the preclinical pharmacokinetics of ON01910.Na in mice, rat and dog found results not inconsistent to ours with nonlinear clearance and plasma protein binding reported.

In Vitro Plasma and Brain Binding of ON01910

The plasma protein and brain tissue binding of ON01910 was studied over a wide range of concentrations (200 ng/ml to 500 μg/ml ON01910.Na in plasma and 100 ng/ml to 200 μg/ml ON01910.Na in brain homogenate). Selected plasma concentrations corresponded to measured values at the three dose levels. In fact, the maximum plasma concentration (C_{max}=822.89 μg/ml) at 250 mg/kg exceeded the highest concentration (500 μg/ml) used in the protein binding assay. ON01910 exhibited saturable plasma protein and brain tissue binding that was high (93–95%) and was essentially constant in the concentration range from 500 ng/ml to 10 μg/ml in plasma and from 100 ng/ml to 5 μg/ml in

Table I PK Parameters (mean±SD) of ON01910 in Plasma in Healthy Athymic Mice Receiving Short Term IV Infusion of ON01910.Na at Dose Levels—50 mg/kg and 250 mg/kg. Parameters were Obtained from Non-compartmental Analysis of Plasma Concentrations from Individual Mice

Variables	Dose levels	
	50 mg/kg	250 mg/kg
n	8	10
C_{max} (μg/ml)	93.24±21.70	822.89±96.64
Area under the plasma concentration-time curve—$AUC_{0\rightarrow\infty}$ (μg.hr/ml)[b]	14.60±3.69	247.98±55.45
Clearance (L/hr/kg)	3.61±0.85	1.06±0.25[a]
Volume of distribution -V_d (L/kg)	49.54±18.61	17.56±11.67[a]
Terminal elimination rate constant - λ_n (1/hr)	0.08±0.03	0.079±0.03
Terminal elimination half life[c] (hr)	8.66±3.30	8.75±3.67

[a] $p < 0.05$ comparing with 50 mg/kg

[b] % $AUC_{0\rightarrow\infty}$ extrapolated after C_{last} (mean±SD) for 50 mg/kg and 250 mg/kg were 0.91%±0.27 and 0.09%±0.10, respectively

[c] Harmonic mean±pseudo SD

Table II Fraction of ON01910 (mean ± SD) Excreted Unchanged in Urine and Feces in Healthy Athymic Mice Receiving Short Term IV Infusion of ON01910.Na at 3 Dose Levels—5 mg/kg, 50 mg/kg and 250 mg/kg

Dose	Fraction excreted unchanged in urine	Renal CL L/hr/kg	Fraction excreted unchanged in feces	Biliary CL L/hr/kg
5 mg/kg[b]	0.13 ± 0.04	–	0.60 ± 0.08	–
50 mg/kg	0.10 ± 0.03	0.36 ± 0.14	0.70 ± 0.15	2.47 ± 0.59
250 mg/kg	0.16 ± 0.05[a]	0.17 ± 0.07[a]	0.60 ± 0.15	0.64 ± 0.23[a]

[a] $p < 0.05$ comparing with 50 mg/kg

[b] Urine and feces were collected for 24 h

brain (Supplementary Table SI), yet above 10 μg/ml (plasma) and 5 μg/ml (brain), the percentage of bound drug decreased to about 77% and 80% in plasma and brain, respectively. From the systemic pharmacokinetic study, ON01910 exhibited a decrease in the volume of distribution at the highest dose level that may indicate saturation of ON01910 tissue binding. If saturable tissue binding also occurred in other tissues as in brain, then the nonlinear volume of distribution as observed is possible. Typically, saturable binding to plasma proteins could lead to increase in the volume of distribution in the presence of constant tissue binding; however this appears not to be the case for ON01910. Nonlinear tissue binding in conjunction with nonlinear serum binding has been observed for certain drugs such as quinidine (20), which could lead to a decrease in the volume of distribution as we observed here.

Brain and Brain Tumor Disposition of ON01910

Brain and brain tumor penetration of ON01910 was evaluated in an orthotopic U87 glioma xenograft under steady-state conditions (Table III). Both the quasi-steady-state normal brain to plasma concentration (Css_{brain}/Css_{plasma}) and brain tumor to plasma concentration ($Css_{brain\ tumor}/Css_{plasma}$) ratios were low being less than 5% and 15%, respectively. The higher ratio in brain tumor, representing a 5-fold greater exposure than normal brain, is consistent with a compromised BBB in brain tumors (21).

The unbound fraction in brain tissue can also be used to assess the potential to achieve active drug concentrations in target tissues since it is assumed that the unbound drug is pharmacologically active. Based on the fraction of ON01910 unbound in brain tissue (see Supplementary Table SI), unbound brain tumor concentration can be estimated (22). In the steady-state study, the mean unbound brain tumor concentration was 0.13 μg/ml [calculated as total brain tumor concentration (2.17 μg/ml) × fraction unbound in brain (0.062)] compared to the estimated unbound IC50 value of 4.7 ng/ml [calculated as total IC50 concentration (72 ng/ml) × fraction unbound in plasma (0.065)], which assumes the fractional binding of ON01910 is equivalent in mouse plasma and fetal bovine serum. This simple analysis suggests that ON01910 can achieve efficacious concentrations in mice bearing intracerebral tumors at dose levels that are acceptable.

In Vitro Cytotoxicity and In Vivo Antiangiogenic Evaluation of ON01910 in U87 Glioma Model

ON01910 showed potent activity against U87 glioma cells, with an IC50 value of 136 ± 16.1 nM (mean ± SD). The U87 cell line has null PTEN, a partial regulator of the PI3K/Akt pathway, and tends to confer drug resistance; so the nanomolar IC50 value supported further analysis of ON01910.Na as an anticancer agent against brain tumors.

The potential effect of ON01910 on tumor angiogenesis was evaluated in an intracerebral U87 glioma xenograft after a 7-day treatment period with 250 mg/kg/day ON01910.Na administered by the IP route. Gliomas are highly angiogenic and invasive in nature which is a key component of their metastatic potential (23), and thus, drugs able to target angiogenesis are considered valuable as a part of emerging combination regimens for brain tumors. There was no significant difference in the microvessel density, pericyte coverage and basement membrane thickness, as measured by the density of CD31, α-SMA and collagen IV positive areas, respectively between the control and treated groups (2-sided student's *t*-test, $p > 0.05$; Supplementary Figure S1). These endpoints are makers for angiogenesis and have been used to calculate the

Table III Brain and Brain Tumor Disposition of ON01910 in an Orthotopic Brain Tumor Model ($n=7$) Following Steady-state Infusion of ON01910.Na for 3 h (mean ± SD)

Steady-state concentration	μg/ml (mean ± SD)
Plasma (Css_{plasma})	16.97 ± 6.50
Normal brain (Css_{brain})	0.42 ± 0.21
Brain tumor ($Css_{brain\ tumor}$)	2.17 ± 1.07
Steady-state concentration ratios	Mean ± SD
Brain/Plasma (Css_{brain}/Css_{plasma})	0.03 ± 0.02
Brain tumor/Plasma ($Css_{brain\ tumor}/Css_{plasma}$)	0.14 ± 0.08
Brain tumor/Brain ($Css_{brain\ tumor}/Css_{brain}$)	5.37 ± 2.36

vascular normalization index (16). The proliferation potential of tumor cells, as measured by Ki-67 positive staining nuclei, also did not differ significantly between the control and treatment groups (2-sided student's *t*-test, $p>0.05$; Supplementary Figure S1C).

Evaluation of ON013105 and ON013100

In Silico and In Vitro Screening, Selection and In Vitro Cytotoxicity Evaluation of ON013105 and ON013100

Based on the low brain penetration of ON01910 that could be partly attributed to its low lipophilicity (predicted log D=−1.07), 55 analogs of ON01910.Na were screened *in silico* using ADMET predictor™ 3.0 for the prediction of various physicochemical properties of the compounds. Compounds that were considered viable candidates for further evaluation in the preclinical brain tumor model satisfied each of the following criteria; modified Lipinski's rules for CNS penetration of molecular weight ≤400, hydrogen bond donor ≤3 and hydrogen bond acceptor ≤7 (24), user defined rules of a molecular weight <400 and a log D between 1–4, and *in vitro* cytotoxic IC50 value of <<1 μM in U87 glioma cells. Low molecular weight compounds with appreciable lipophilicity values show better BBB permeability owing to their transport across the cell membrane by simple diffusion (24,25) while a log D value of 1–4 was shown to be associated with brain uptake of CNS drugs (26). Three compounds of the 55 compounds met these criteria [ON01500 (molecular weight=393.46, predicted log D=2.24, *in vitro* U87 IC50=5.7±1.0 nM), ON013100 (molecular weight=394.44, predicted log D=2.53, *in vitro* U87 IC50=5.4±0.7 nM) and ON015640 (molecular weight=377.46, predicted log D=3.18, *in vitro* U87 IC50=97.8±18.0 nM)], and due to ongoing pharmacological studies of ON013100 in Dr. E.P. Reddy's lab, we selected ON013100 for further evaluation. Given the lipophilic nature of ON013100, an aqueous soluble form, ON013105, was synthesized by the addition of a phosphate group at 3-hydroxyl position on the benzene ring to facilitate intravascular administration. ON013105 also showed very potent cytotoxic activity against U87 cells with IC50 value of 6.9±0.2 nM that can be attributed to its conversion to ON013100 in the cell culture medium (unpublished data from Dr. Stephen Cosenza, Mount Sinai school of Medicine, NY). Hence ON013105 was chosen for further evaluation of brain and brain tumor disposition in a mouse glioma model.

Systemic Pharmacokinetics of ON013105 and ON013100

Systemic pharmacokinetic characterization of ON013105 and its active form, ON013100 was carried out following 37.5 mg/kg ON013105 administered intravenously. This dose was in the range of previous doses used in efficacy studies (6). ON013105 and its active form, ON013100 yielded plasma concentration-time profiles that showed rapid conversion of ON013105 to ON013100 (see Fig. 3 and Table IV) with a C_{max} of 25.83 μg/ml as early as 2 min after ON013105 administration. ON013100 showed a rapid distribution phase with a prolonged elimination phase where low levels of the drug persisted in the body for up to 8–12 h. The decline in plasma concentrations of ON013100 was relatively slower than that of the ON013105 suggesting that the formation of ON013100 was not a rate-limiting step. The apparent volume of distribution of ON013105 indicated its distribution into a much larger space than plasma volume (Table IV).

In Vitro Plasma and Brain Binding of ON013100

The plasma protein and brain tissue binding of ON013100 was studied over a wide range of concentrations (100 ng/ml to 200 μg/ml in both plasma and brain homogenate). ON013100 exhibited high plasma protein (97-98%) and brain tissue (98–99%) binding and unlike ON01910, binding of ON013100 to plasma proteins and brain tissue was essentially constant in the concentration range from 100 ng/ml to 200 μg/ml (Supplementary Table SII).

Brain and Brain Tumor Disposition of ON013105 and ON013100

The quasi-steady-state normal brain and brain tumor distribution of ON013100 was appreciable with Css_{brain}/Css_{plasma} and $Css_{brain\ tumor}/Css_{plasma}$ ratios of 0.92 and 1.35, respectively (Table V). The prodrug, ON013105 was not detectable in normal brain while its tumor penetration was very low being less than 1% that is attributed to its hydrophilic nature and rapid conversion to ON013100. As observed with ON01910.Na, the

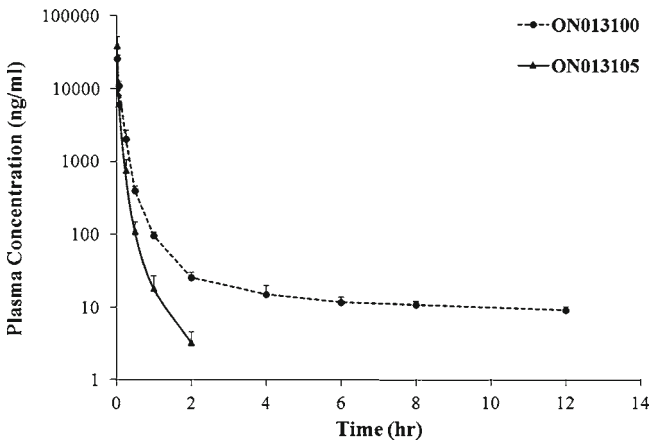

Fig. 3 Time courses of mean plasma concentrations of ON013105 and ON013100 following single IV bolus administration of 37.5 mg/Kg ON013105 to healthy athymic mice (*n*=4). *Bars*, SD.

Table IV PK Parameters (n=4, mean ± SD) of Prodrug, ON013105 and its Active Form, ON013100 in Plasma in Healthy Athymic Mice Receiving an IV Bolus Dose of 37.5 mg/kg ON013105. Parameters Were Obtained from Non-compartmental Analysis of Plasma Concentrations from Individual Mice

Variables	37.5 mg/kg IV ON013105	
	ON013105 (prodrug)	ON013100 (active form)
C_{max} (μg/ml)	39.54 ± 12.19	25.83 ± 3.32
Area under the plasma concentration-time curve – $AUC_{0\rightarrow\infty}$ (μg.hr/ml)[a]	4.83 ± 1.65	3.18 ± 0.30
Clearance – CL (L/hr/kg)	8.47 ± 2.84	–
Volume of distribution - V_d (L/kg)	3.21 ± 1.71	–
Terminal elimination rate constant - λ_n (1/hr)	3.06 ± 1.45	0.13 ± 0.08
Terminal elimination half life[b] – $t_{1/2}$ (hr)	0.23 ± 0.13	5.24 ± 3.35

[a] % $AUC_{0\rightarrow\infty}$ extrapolated after C_{last} (mean ± SD) for ON013105 and ON013100 at 37.5 mg/kg were 0.09% ± 0.11 and 3.12% ± 2.09, respectively

[b] Harmonic mean ± pseudo SD

exposure in brain tumor was greater than normal brain thus consistent with a compromised BBB in brain tumors (21).

Utilizing the brain tissue binding data of ON013100 (Supplementary Table SII) and as for ON01910.Na, converting total to unbound drug concentrations for ON013100, the unbound IC50 would be about 0.05 ng/ml [calculated as total IC50 concentration (2.14 ng/ml)×fraction unbound in plasma (0.022)] and in the steady-state study, the mean unbound brain tumor concentration was 0.15 μg/ml [calculated as total brain tumor concentration (9.01 μg/ml)×fraction unbound in brain (0.017)], and suggests that cytotoxic brain tumor concentrations can be achieved in mice, yet further studies are needed to establish ON013100 dose-efficacy relationships.

DISCUSSION

ON01910.Na is an anticancer agent under clinical evaluation for various solid tumors and hematologic malignancies. It exhibited potent antitumor activity in various preclinical models such as human liver, breast cancer and pancreatic cancer xenografts (1). It has also been reported to cause complete tumor regression in an intracranial glioma model when administered locally (5). Based on these data and our own *in vitro* cytotoxicity results that showed potent activity, we were motivated to analyze ON01910.Na as an anticancer drug for brain tumors. We considered that one potential hurdle to its use in this regard could be limited BBB penetration. To address this issue, we chose a steady-state tissue distribution study based on the pharmacokinetic properties acquired in the single-dose investigations of ON01910.Na. A steady-state drug administration was implemented as it is parsimonious in terms of animal usage and provides an accurate indication of specific tissue distribution, since systemic exposure is essentially constant. This latter condition is readily met for drugs undergoing linear or first-order kinetics; however, for drugs obeying saturable elimination like ON01910.Na, the condition may be violated depending on the targeted steady-state plasma concentrations. We targeted a steady-state plasma concentration of 10 μg/ml, which was close to the C_{max} of about 6 μg/ml achieved at the low 5 mg/kg single-dose study, and thus, the linear assumption was likely valid. Furthermore, we conducted a pilot study in a small cohort of mice that confirmed that steady-state ON01910 plasma concentrations were maintained over the 3 h infusion period (data not shown). Although direct confirmation of steady-state concentrations in brain was not pursued, steady-state brain and brain tumor concentrations

Table V Brain and Brain Tumor Disposition of Prodrug, ON013105 and its Active Form, ON013100 in an Orthotopic Brain Tumor Model Following Steady-state Infusion of ON013105 for 3 h (n=8, mean ± SD)

Steady-state concentration	ON013105	ON013100
	μg/ml (mean ± SD)	μg/ml (mean ± SD)
Plasma (Css_{plasma})	25.62 ± 8.83	7.22 ± 2.25
Normal brain (Css_{brain})	ND*	6.26 ± 1.43
Brain tumor ($Css_{brain\ tumor}$)	0.04 ± 0.02	9.01 ± 1.25
Steady-state concentration ratios	ON013105 (mean ± SD)	ON013100 (mean ± SD)
Brain/Plasma ratio (Css_{brain}/Css_{plasma})	ND*	0.92 ± 0.26
Brain tumor/Plasma ratio ($Css_{brain\ tumor}/Css_{plasma}$)	0.0015 ± 0.0007	1.35 ± 0.40
Brain tumor/Brain ratio ($Css_{brain\ tumor}/Css_{brain}$)	–	1.48 ± 0.24

*ND**, not detected

may be assumed based on the likely linear relationship between plasma and brain concentrations at the targeted concentration of ON01910. Using the steady-state design and the intracerebral U87 tumor model, we found that the distribution of ON01910 in normal brain was less than 5% of plasma concentration and about 14% in brain tumors. The approximate 5-fold greater distribution of ON01910 in brain tumor relative to normal brain is consistent with a compromised BBB that can contain gaps between the normally tight configurations of adjacent endothelial cells (27,28) that results in enhanced vascular permeability (29). In this case, the low diffusional transport across the BBB can be offset somewhat by transport between cells (30,31). The limited access of ON01910 to the brain can be attributed to its low lipophilicity, with a predicted log D value of −1.07, and high plasma protein binding (93–95%) at the measured steady-state plasma concentrations. Both variables contribute to low transport across the BBB by diffusion due to limited partitioning into the BBB and a minimal unbound drug concentration gradient across the BBB. The contribution of drug efflux pumps at the BBB such as P-glycoprotein (P-gp) or multidrug resistance proteins (MRPs), to the limited brain distribution of ON01910 is unknown and cannot be ruled out at this time. ON01910.Na was found to be active against drug resistant cell lines, such as MOLT ALL (methotrexate resistant acute lymphoid leukemia cell line) and MCF-7/DOX (doxorubicin resistant breast cancer cell line), as well as cell lines overexpressing transporters such as P-gp and multidrug resistance protein 1 (MRP1), which suggested that ON01910.Na is not a P-gp or MRP1 substrate (32). Even though the specific roles of transporters in the active efflux of ON01910 at the BBB remain to be defined, its distribution into brain is limited.

Cyclin D1 is an important regulator of G1/S transition of the cell cycle, and was reported to be overexpressed in gliomas (12) making it a potential drug target. Furthermore, cyclin D1 may play a role in tumor angiogenesis by regulating the production of secreted angiogenic factors such as vascular endothelial growth factor (VEGF) from tumor cells as well as endothelial cells (11). Thus, the inhibitory action of ON01910 on cyclin D1 (4) could yield deleterious effects on both tumor and endothelial cells. Specifically, to address its antiangiogenic action, we chose to monitor microvessel density, as measured by CD31, pericyte maturity, as measured by α-SMA, and basement membrane thickness, as measured by collagen IV, all implicated in angiogenesis and vascular normalization (16) following multiple-dose administration of 250 mg/kg IP ON01910.Na for 7 days. The dose of 250 mg/kg IP was chosen based on earlier efficacy studies published on ON01910.Na where the animals were dosed on alternate days (1). A 7-day treatment regimen should be sufficient to see a pharmacological response as our previous studies with the antiangiogenic agent, sunitinib produced beneficial effects on the tumor vasculature within 7 days (33). The ON01910.Na treated group showed no significant difference in any of these parameters as well as in the tumor cell proliferation index as compared to the control group, and thus it is concluded that ON01910 lacked antiangiogenic and antiproliferative activity.

In the multiple dose study, actual brain tumor concentrations were not assessed during the 7-day ON01910.Na treatment. Based on the analysis presented above on the *in vitro* unbound concentrations of ON01910 in brain tissue that suggested cytotoxic concentrations could be achieved, we conducted a simulation using a physiologically based-pharmacokinetic (PB-PK) membrane-limited model to predict unbound ON01910 brain tumor concentrations following the multiple-dose regimen. The PB-PK model (described in Supplementary Material Figure S2) predicted that effective unbound drug concentrations (>4.7 ng/ml) were not maintained for more than 3 h in a 24 h dosing interval in brain tumor (Supplementary Figure S3), and thus, the lack of ON01910 anticancer activity may be attributed to its rapid decline in tumor.

To circumvent the limited brain distribution of ON01910, a screening strategy was applied to a series of ON01910.Na structural analogs. We identified ON013100 to be a suitable candidate for evaluation in brain tumors based on its physical properties of low molecular weight, predicted log D value of 2.53 and meeting the criteria of the modified Lipinski's rules for CNS penetration (24), all of which were shown to be associated with greater CNS penetration (24–26). In addition, its potent cytotoxic activity (IC50=5.4 nM) against U87 glioma cells supported our investigation of ON013100 disposition into brain and brain tumor. Similar to ON01910.Na study, we undertook a steady-state infusion study design for the determination of brain and brain tumor penetration in an intracerebral U87 glioma model. A steady-state ON013105 plasma concentration of 4.5 μg/ml was targeted which was much greater than the *in vitro* IC50 value of ON013100 and at the same time being less than C_{max} of ON013105 at 37.5 mg/kg IV. It was also a concentration that could be achieved within the constraints of drug solubility and administration volumes. As for ON01910.Na, a pilot study was conducted to confirm that steady-state ON013105/ON013100 concentrations were maintained during the 3 h infusion (data not shown). Although observed steady-state plasma concentrations of ON013105 were greater than the targeted concentration (possibly related to a cachexic condition in the brain tumor-bearing mice that may have decreased clearance, and correspondingly elevated plasma concentrations), steady-state plasma concentrations of both ON013105 and ON013100 were maintained over the 3 h infusion period (data not shown) indicating that the assumption of linear kinetics was valid, and support the attainment of steady-state brain and brain tumor concentrations at 3 h. The distribution of ON013100 was quite high, being 92% in brain and 135% in brain tumor relative to plasma concentration while ON013105 had absent

or negligible penetration into normal brain and brain tumor, consistent with its very low lipophilicity (predicted log D=−2.45).

CONCLUSION

In summary, pharmacokinetic and pharmacodynamic studies of ON01910.Na in an intracerebral U87 brain tumor mouse model indicated limited utility of this compound with once a day dosing. A more lipophilic derivative of ON01910.Na, ON013100 showed appreciable brain tumor penetration and potent *in vitro* cytotoxicity suggesting its potential for further investigation in brain tumor chemotherapy. Future studies will analyze the pharmacodynamic activity of ON013105 and its active form ON013100 in preclinical brain tumor models.

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Dr. E.P. Reddy is the scientific founder, stock holder, board member and paid consultant of Onconova Therapeutics Inc. He is also one of the inventors of the patents that describe the compounds described here.

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