

Research Article

Integrated Pharmacokinetic-Driven Approach to Screen Candidate Anticancer Drugs for Brain Tumor Chemotherapy

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Abstract. The goal of the study was to develop an effective screening strategy to select new agents for brain tumor chemotherapy from a series of low molecular weight anticancer agents [ON123x] by the combined use of *in silico*, *in vitro* cytotoxicity, and *in vitro* ADME profiling studies. The results of these studies were cast into a pipeline of tier 1 and tier 2 procedures that resulted in the identification of ON123300 as the lead compound. Of the 154 ON123xx compounds, 13 met tier 1 screening criteria based on physicochemical properties [*i.e.*, MW < 450 Da, predicted log *P* between 2 and 3.5] and *in vitro* glioma cell cytotoxicity [*i.e.*, IC₅₀ < 10 μM] and were further tested in tier 2 assays. The tier 2 profiling studies consisted of metabolic stability, MDCK-MDR1 cell permeability and plasma and brain protein binding that were combined to globally assess whether favorable pharmacokinetic properties and brain penetration could be achieved *in vivo*. *In vivo* cassette dosing studies were conducted in mice for 12 compounds that permitted examination of *in vitro/in vivo* relationships that confirmed the suitability of the *in vitro* assays. A parameter derived from the *in vitro* assays accurately predicted the extent of drug accumulation in the brain based on the area under the drug concentration–time curve in brain measured in the cassette dosing study ($r^2=0.920$). Overall, the current studies demonstrated the value of an integrated pharmacokinetic-driven approach to identify potentially efficacious agents for brain tumor chemotherapy.

KEY WORDS: brain tumor; CNS; drug development; pharmacokinetics; preclinical.

INTRODUCTION

Nearly 20,000 new primary brain tumors, many classified as grade IV glioblastoma multiforme (GBM), and about 200,000 metastatic brain tumor cases are reported each year in the U.S.A. (1). The overall survival of these patients is dismal and the majority of survivors suffer disabling toxicities from the aggressive treatment regimens of surgery, radiation, and chemotherapy. The discovery and development of new medicines to cure brain tumors poses a number of hurdles not the least of which is to discover compounds that are both efficacious and sufficiently penetrate the blood–brain barrier (BBB). It is well-known that in addition to the anatomic features of the BBB that limit drug access, active transporters located on the BBB act as drug efflux pumps also contribute to limited brain access (2,3). Measurement of the unbound

drug fractions in plasma and target tissues is becoming part of the standard drug discovery procedures since in addition to membrane permeability limitations that may retard drug access to brain tumors, high plasma protein binding and low target tissue binding of drugs can also limit tissue distribution and the concentration of pharmacologically active free drug. All drug discovery programs interested to identify new compounds for brain diseases employ different methods to assess brain delivery including *in silico* QSAR models (4–6), *in vitro* assays and various cell models (7,8), and finally animal models. The wealth of experiences gained especially during the last decade has led to significant advances in our understanding of CNS penetration and distribution, yet there is no single approach that may be considered universal.

The ON123 series was derived from an anticancer drug discovery partnership between academia and Onconova Therapeutics Inc. which is focused on the discovery of low molecular weight inhibitors of protein kinases. These intracellular enzymes are involved in the regulation of a large variety of biochemical pathways that are often overexpressed and mutated in cancers. Rational design and development of such molecularly targeted agents is a burgeoning endeavor based on their improved toxicity profiles compared to traditional chemotherapy, and their ability to dissect oncogenic pathways which facilitate the design of more rational combination regimens. In this study, we sequentially applied a

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combination of *in silico* and *in vitro* absorption, distribution, metabolism, and excretion (ADME) screening assays to the ON123 series of compounds that proved to be an accurate approach to predict *in vivo* brain exposure. One compound, ON123300, selected from the approach exhibited favorable cytotoxicity and brain penetration.

MATERIALS AND METHODS

Materials

All ON123 compounds were synthesized in the lab of Dr. R. Reddy and can be described as 2-arylamino-6-substituted-8-alkyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidine derivatives (Fig. 1). All test compounds had a purity of at least 95%.

U87MG human glioma cells were purchased from the American Type Culture Collection, cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc.) supplemented with 10% standard fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin and maintained in a humidified atmosphere of 5% CO₂ in air at 37°C.

MDCK-MDR1 cells were obtained from NIH (Bethesda, MD) and maintained in DMEM with 10% standard fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 80 ng/mL colchicine. For transport experiments, cells with passage numbers of 24–33 were seeded at a density of 60,000 cells/cm² on Transwell plates (Costar, pore size 0.4 µm; diameter 6.5 mm; insert growth area 0.33 cm², Pittston, PA).

Mouse pooled liver microsomes male (CD-1), 20 mg/mL, and HLM (human liver microsomes) pooled male donors, 20 mg/mL, were purchased from BD Gentest (Woburn, MA).

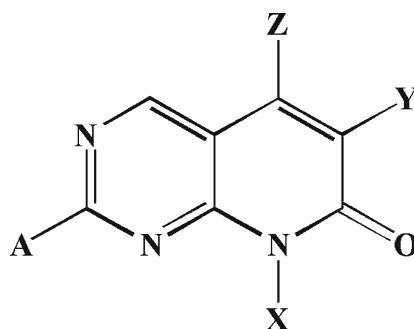
β-Nicotinamide adenine dinucleotide phosphate (β-NADPH) was purchased from Sigma-Aldrich Co., (St. Louis, MO). All other chemicals were reagent grade and obtained from Sigma-Aldrich Co.

In Silico Prediction

The ADMET Predictor™ (V5.0, Simulations Plus, Inc., Lancaster, CA) program was used to predict the log *P* (octanol-water partition coefficient) and ADMET Risk (Table I), derived from a set of predicted physicochemical properties, for 154 compounds in the ON123 series based on the 2D chemical structures. Compounds with a molecular weight less than or equal to 450, log *P* values between 2 and 3.5, and an ADMET risk score of less than 1 were selected for *in vitro* cytotoxicity.

Cytotoxicity Assay

The cytotoxicity of 56 compounds in U87 glioma cells was evaluated by a colorimetric SRB-based assay (9). Cells were seeded into 96-well culture plates at a density of 2×10³ cells/well in 100 µL of culture medium. The cells were then treated with vehicle control, 1- and 10-µM concentrations of the selected ON123 compounds for 72 h. At the end of the treatment, cells were fixed with 10% (*v/v*) TCA and stained with 0.4% SRB. The optical densities were measured with a



Compound	X	Y	Z	A
1231030	CH ₃	4- CH ₃ PhSO ₂	H	NH-6-indolyl
1231060	C ₆ H ₁₁	CN	H	NH-4-indolyl
123300	C ₅ H ₉	CN	H	NH-(N-CH ₃ piperazino)benzene
123650	C ₅ H ₉	CN	H	NH-4-indolyl
123660	C ₅ H ₉	CN	H	NH-5-indolyl
123680	C ₅ H ₉	CN	H	NH-4-OCH ₃ C ₆ H ₄
123770	C ₅ H ₉	CN	H	NH-3,5-(OCH ₃) ₂ C ₆ H ₄
123780	C ₅ H ₉	CN	H	NH-2,4-(OCH ₃) ₂ C ₆ H ₄
123830	CH ₃	4- CH ₃ PhSO ₂	H	NH-5-indolyl
123850	CH ₃	4- CH ₃ PhSO ₂	H	NH-5-pyrrolopyridine
123870	CH ₃	PhSO ₂	H	NH-5-indolyl
123890	CH ₃	PhSO ₂	H	NH-5-pyrrolopyridine
123230	C ₅ H ₉	4-F-PhNHCO	H	CH ₃ S

Fig. 1. Chemical structures of 13 ON123 compounds used in the tier 2 screens

Table I. *In Silico* Tier 1 Screening Parameters Applied to 154 ON123 Compounds

Descriptors	Physicochemical properties	Criteria
MWt	Molecular weight [g/mol]	<450
Log <i>P</i>	Lipophilicity	2< and <3.5
Peff	Permeability	>0.2219
pH	Acidity at saturation point	>3.5803
HBDch	Hydrogen bond donors	<1.4291
T_PSA	Polar surface area	<230.4
ABSQ	Polarity	<5.15
FormalQ	Formal electric charge	=0

Of the 154 compounds 13 compounds met acceptable criteria of *in silico* physicochemical properties combined with IC₅₀ values of <10 μM in U87 glioma cells

SpectraMax M2 microplate reader (Molecular Devices, Downingtown, PA) at a wavelength of 570 nm and converted to the percentage of viable cells.

In Vitro ADME Profiling

Metabolic Stability

The oxidative metabolism of ON123 compounds was evaluated in both mouse and human NADPH-fortified liver microsomes; a common screening assay in drug discovery that represents the most likely metabolic reactions (10,11). An incubation mixture of 3 mL was prepared in a 100 mM sodium phosphate buffer (pH7.4) containing 1-μM test compound, 1 mg/mL liver microsomal protein, and 6.7 mM magnesium chloride. The final incubation solution contained 0.01% DMSO. After a 10-min preincubation at 37°C, the reaction was initiated by adding NADPH followed by the collection of aliquots of the incubation medium taken at 0, 5, 10, 15, 20, and 30 min, and placed in three volumes of ice-cold acetonitrile (containing an internal standard, IS) to terminate the reaction. The samples were vortexed and centrifuged at 14,000×g for 5 min to collect the supernatant for sample analysis by LC/MS/MS as described in the bioanalysis section below. The turnover rate constant (*k*) in liver microsomes was determined by nonlinear regression of the percentage of the compound remaining (*y*) vs. time (*t*) curve using the equation of $y = y_0 \exp(-kt)$, where *y*₀ is percentage of the compound remaining at time zero estimated from the equation. The microsomal stability half-life (*t*_{1/2}) was calculated using the equation $T_{1/2} = 0.693/k$.

MDCK-MDR1 Cell Permeability

For *in vitro* BBB permeability, MDCK-MDR1 cells (passage number 21–30) were seeded onto 0.33-cm² polycarbonate filters at a density of 60,000 cells/cm² and maintained in culture as previously described (7,8). Confluent MDCK-MDR1 monolayers expressing P-glycoprotein (P-gp) were obtained 3–4 days postseeding and their integrity assessed by measurement of the transepithelial electrical resistance (TEER, ohm square centimeter) with a volt-ohm meter (Millicell-ERS, Millipore Corporation, Billerica, MA). After subtraction of the background TEER (*i.e.*, the resistance exhibited by the filter alone), only MDCK-MDR1 cell monolayers that exhibited a TEER > 1,000 Ω·cm² throughout [measured before and after the study] the experiments were used.

Drug transport across the cell monolayers was measured in both apical to basolateral (A–B) and basolateral to apical (B–A) directions. Experiments were performed in HBSS (Hank's balanced salt solution containing millimolar Hepes buffer, pH~7.4) at 37°C using monolayers that were preincubated for 30 min with prewarmed HBSS. At the start of the experiment, fresh HBSS was added to the receiver compartments and the compounds were independently added to the donor compartments at an initial concentration of 10 μM (diluted from 10 mM DMSO stock to a final DMSO concentration of 0.1%), and then incubated at 37°C for 90 min after which samples were collected from both the receiver and donor compartments. Drug concentrations were determined by LC/MS/MS as described in the bioanalysis section below.

The apparent permeability, *P*_{app} (centimeter per second) was calculated as: $P_{app} = dQ/dt \times 1/(A \times C_0)$, where *dQ/dt* is the transport rate of the compound (moles per second), *A* is the area of the cell monolayers (centimeter square) and *C*₀ is the initial donor concentration (moles per liter).

Plasma Protein and Brain Tissue Binding

The binding of compounds to mouse plasma and brain was determined by a previously described equilibrium dialysis method (12–14). Briefly, untreated plasma or brain homogenate (4× diluted with PBS) was fortified with the test compound to yield a final concentration of 10 μM and then placed in a 96-well equilibrium dialysis block system (Model HTB96b, HTDialysis LLC, Gales Ferry, CT) fitted with a dialysis membrane (MWCO 12-14K, HTDialysis LLC) and incubated at 37°C while being rotated for 6 h. Post-dialysis plasma and buffer volumes were measured and then samples analyzed for drug concentrations by LC/MS/MS as described in the bioanalysis section below. The fraction of unbound drug was calculated as follows;

$$f_u = \frac{D_F}{(D_{Te} - D_F) \cdot \frac{V_i}{V_e} + D_F} \times 100\%$$

where *D*_{Te} and *D*_F represent the total and unbound plasma or brain homogenate concentrations at equilibrium, respectively, and *V*_i and *V*_e represent the initial and equilibrium plasma or brain homogenate volumes, respectively.

In Vivo Cassette Dosing Study

Adult male ICR mice (25–30 g) were supplied by Taconic Farms (Germantown, NY, USA) and maintained in the American Association for the Accreditation of Laboratory Animal Care-accredited University Laboratory Animal Resources of Mount Sinai School of Medicine. All animal studies were approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine.

A cassette dosing study was conducted to evaluate the *in vivo* pharmacokinetics of 12 compounds that underwent *in vitro* analyses. Compounds were dissolved in a mixture of NMP/PEG300/water at a volume ratio of 1:4:5, respectively. Each compound was administered at a dose of approximately 2.5 mg/kg as an intravenous (IV) bolus injection through a lateral tail vein in a total injection volume of 5 mL/kg. At each predetermined sampling time (0.083, 0.25, 0.5, 1, 2, 4, 6 h), groups of mice (three mice for each time point) were briefly anesthetized with isoflurane, and terminal blood samples were collected through the inferior vena cava, followed by rapid extraction of normal

brain. Plasma and normal brain samples were stored at -80°C until analyzed by LC/MS/MS.

Bioanalysis of ON123 Compounds

Sample preparation was adapted to each of the different sample media that consisted of donor–receiver compartments for the MDCK-MDR1 assays, bound-free compound compartments for equilibrium dialysis, and plasma and brain homogenate for the mouse PK studies. In each case, to 10 μL samples, 40 μL of cold acetonitrile was used to precipitate proteins followed by centrifugation at 15,000 rpm.

All samples were analyzed by LC/MS/MS using the multiple reaction monitoring mode specific to each analyte and the internal standards. The LC/MS/MS system consisted of an online degasser (DGU-20A₃, Shimadzu, Kyoto, Japan), two pumps (LC-20 AD, Shimadzu), a column oven (CTO-20 AC, Shimadzu), and autosampler (SIL-20AC_{HT}, Shimadzu), a system controller (CBM-20A, Shimadzu) and a hybrid quadrupole linear ion trap mass spectrometer (QTrap 5500, Applied Biosystems, Foster City, CA) with an ESI source. Chromatographic separation was achieved with a guard cartridge (C18, 4.0 \times 2.0 mm, Phenomenex, Torrance, CA, USA) and analytical column (Luna C18, 3- μM particle size, 50 \times 2.0 mm, Phenomenex, Torrance, CA, USA) using a gradient solvent system that consisted of mobile phase A, 0.5 mM ammonium formate (0.1% formic acid, *v/v*), and B, acetonitrile that was specified according to three gradient programs as summarized in Table SI.

Mass spectrometer conditions were optimized for each compound with some detected in positive ionization mode and others in negative ionization mode. The transitions monitored are shown in Table SII. Both the chromatographic system and the mass spectrometer were controlled by Analyst software version 1.5. (QTrap 5500, Applied Biosystems). Quantification was carried out using standard calibration curves and quality control samples prepared with blank matrix spiked with each ON123 compound. The analytical methods were specific and sensitive with a lower limit of quantification of 1 ng/mL. The intraday and interday variability was less than 15% in all matrixes. The average run time was about 5 min for each sample.

Data Analysis

The hepatic intrinsic clearances ($CL_{h,int}$) of ON123 compounds were estimated from human liver microsome data using the method described by Obach *et al.* (15) and Iwatsubo *et al.* (16). Assuming linear kinetics and similar unbound intrinsic clearance *in vitro* and *in vivo*, the $CL_{h,int}$ was calculated based on the disappearance of the parent drug in liver microsome as follows:

$$CL_{h,int} = \left(\frac{k}{C_{\text{protein}}} \right) \times \left(\frac{45 \text{ mg protein}}{1 \text{ g liver weight}} \right) \times \left(\frac{\text{grams of liver weight}}{\text{kilogram of body weight}} \right)$$

where k is the turnover rate constant from the incubation reactions as described above, and C_{protein} is the microsomal protein concentration. The liver weight relative to body weight in human is 21 g/kg. Assuming the well-stirred model of hepatic clearance, similar protein binding in microsomes and blood, and

rapid equilibrium between blood and the liver, the hepatic blood clearance ($CL_{h,blood}$) was estimated as follows:

$$CL_{h,blood} = \frac{Q_h \times CL_{int}}{Q_h + CL_{int}}$$

where Q_h is the hepatic blood flow with a value equal to 90 and 21 mL/min/kg for the mouse and human, respectively.

Noncompartmental analysis was performed using WinNonlinTM Phoenix software to estimate the pharmacokinetic parameters from the cassette dosing study in mice. The calculated parameters were area under the concentration-time curve (AUC), systemic clearance (CL), the apparent volume of distribution at steady-state (V_{dss}) and the terminal elimination half-life ($t_{1/2}$). All AUC calculations were based on the area to the last observable concentration (AUC(0-t)) using the linear trapezoidal method. The normal brain partition coefficient was calculated as the ratio of brain AUC (AUC_b) over the corresponding plasma AUC (AUC_p).

RESULTS

Tier 1 Screening

The goal of tier 1 screening was to select from the 154 compounds in the ON123 series the compounds with the most favorable physicochemical properties and *in vitro* cytotoxicity data that would then undergo *in vitro* tier 2 screening. Physicochemical properties were predicted from QSAR models available in the ADMET program that predicted log P values as well as a hybrid parameter referred to as the “ADMET_Risk” (lipophilicity > -1.1102 , permeability > 0.2219 , acidity at saturation point > 3.5803 , hydrogen bond donors < 1.4291 , polar surface area < 230.4 , polarity < 5.15 and permanent charge = 0) that is similar to the well-known Lipinski “Rule of Five” used to predict the likelihood of poor oral absorption (4–6). The ADMET_Risk rules were employed as an indication of suitable drug absorption by oral administration that would be the likely and preferred route for the ON123 series, and further as a means to indicate BBB penetration (*i.e.*, fewer H-bond donors lower polar surface area). To assess whether the compounds would cross the BBB by passive diffusion, we choose compounds that had $MWt \leq 450$ g/mol, and $2.0 < \log P < 3.5$; criteria that has been previously used (17,18). After applying these criteria and the ADMET_Risk rules (Table I) to the 154 ON123 compounds, 56 compounds complied with these rules and underwent *in vitro* cytotoxicity testing using U87 glioma cells, a well-known human model for GBM (19–21). Thirteen of these 56 compounds were selected for the tier 2 screening based on favorable cytotoxicity; defined as compounds that inhibited more than 50% of cell proliferation at ≤ 10 μM .

Tier 2 Screening

The goal of tier 2 screening was to establish an integrated approach to *in vitro* ADME profiling based on three key attributes; favorable BBB penetration [MDCK-MDR1 assay] and brain distribution [unbound drug fractions in plasma and brain] and system clearance [liver microsomal assay]. The assays employed have been used previously as tools to screen drug candidates (7,8,12–16); however how they were

combined and validated with *in vivo* studies has not explicitly been done previously.

The *in vitro* liver human microsomal half-life values for the 13 compounds ranged 6.7-fold with a high value of 48.9 min for ON123230. The corresponding predicted clearances (Table II) of the 13 compounds ranged between medium (seven compounds) and high (six compounds) clearances based on the following classifications; low-clearance category: $CL < 6.3$ mL/min/kg, medium-clearance category: $6.3 \text{ mL/min/kg} \leq CL \leq 14.7$ mL/min/kg, high-clearance category: $CL > 14.7$ mL/min/kg (10).

In vitro cell-based BBB permeability and the potential influence of P-gp were assessed using the MDCK-MDR1 model (Table II). Based on criteria that compounds with P_{app} (A–B) $> 3 \times 10^{-6}$ cm/s have a high potential to cross the BBB and those with P_{app} (A–B) $< 1 \times 10^{-6}$ cm/s a low potential, three compounds met the criteria of high potential with two of these being particularly high, ON123300 (9.52×10^{-6} cm/s) and ON123850 (10.82×10^{-6} cm/s), five compounds were of intermediate potential, and four compounds had low potential. A P-gp-mediated efflux ratio [ER], calculated as the ratio of P_{app} (B–A)/ P_{app} (A–B) of > 30 is an indication a compound may be a P-gp substrate and may be unable to reach effective brain concentrations. All of the ON123 series compound evaluated possessed efflux ratios under 30, and thus, are not likely candidates for P-gp-mediated transport.

Although the BBB plays a key role in modulating brain penetration, recent reports have highlighted the importance of drug binding to both plasma proteins and brain tissue as an indicator of drug accumulation in brain (22,23). Drug accumulation is clearly distinguished from the rate of BBB penetration measured by cell permeability assays, and can be predicted by the brain/plasma partition coefficient that can be estimated by the ratio of the unbound fraction in plasma to brain under the proviso of diffusional transport (24,25). As shown in Table II, all 13 ON123 series compounds were highly bound to plasma and brain with brain/plasma partition coefficients clustered between 2 and 8 except ON123890 that had a low ratio of 0.62.

By collectively considering all factors (favorable BBB penetration, brain distribution and system clearance) that

potentially impact brain exposure, the results from the three *in vitro* assays were cast into a graphical analysis (see Fig. 2) to select among the 13 compounds those with the most favorable *in vitro* PK characteristics. The human liver microsomal half-life is singularly expressed on the y-axis as it indicates the rate of drug elimination or system clearance. The x-axis is a linear combination of parameters simply constructed from the idea that high membrane permeability (*i.e.*, P_{app}) and brain partitioning (*i.e.*, F_{up}/F_{ub}) contribute positively to brain distribution and appear in the numerator, whereas higher ER values should restrict brain penetration and appears in the denominator. This graphical analysis readily segregates the most favorable compounds based on their x–y coordinates, and although no single compound possessed the maximum x and y values, one compound ON123300 is distinguished with the maximum x-axis value and intermediate y-axis value and would be predicted to possess the most favorable *in vivo* PK characteristics.

Cassette Dosing Study and *In Vitro/in Vivo* Correlations

Cassette dosing PK analysis is used to improve the throughput of *in vivo* PK screens (26,27) and obtain key PK parameters as shown in Table III. In our case, we conducted *in vivo* cassette dosing studies to verify the *in vitro* results and to examine whether positive correlations existed between the *in vitro* measurements and comparative parameters measured in mice. Specifically, we compared drug clearance and brain partition coefficient [$P_b = AUC_b/AUC_p$] values for 12 of the 13 [insufficient supply of ON123230 for mouse PK study] compounds from the tier I screen. To compare the ability of *in vitro* liver microsomal metabolic stability studies to predict *in vivo* clearance we also performed the former using mouse liver microsomes, and found a positive *in vitro–in vivo* correlation ($R^2 = 0.542$, Fig. 3) between the predicted mouse clearance and the total clearance in the cassette dosing studies. It should be noted that the compounds were well distributed among the categories of low-, medium-, and high-clearance classes that provides a measure of confidence on the utility of the *in vitro* metabolic stability assay.

Table II. Tier 2 Screening Parameters for 13 ON123 Compounds That Passed Tier 1 Screening Criteria

Compound ID	Human Liver Microsomal $t_{1/2}$ (min)	Predicted CL (mL/min/kg)	P_{app} ($\times 10^{-6}$ cm/s)			$F_{unbound}$ (%)		
			A–B	B–A	Efflux ratio	Plasma	Brain	F_{up}/F_{ub}
ON1231030	15.5	14.0	0.90	1.81	2.0	0.14	0.04	3.15
ON1231060	7.3	17.0	2.75	6.07	2.2	0.38	0.11	3.45
ON123300	14.3	14.4	9.52	7.47	0.8	3.64	0.55	6.64
ON123650	10.5	15.7	0.89	0.58	0.6	0.62	0.19	3.34
ON123660	8.4	16.5	2.16	7.47	3.5	0.27	0.07	3.92
ON123680	11.1	15.5	1.87	3.33	1.8	0.38	0.07	5.53
ON123770	17.5	13.5	0.26	0.04	0.2	0.25	0.04	6.62
ON123780	20.3	12.7	2.46	1.22	0.5	0.71	0.15	4.86
ON123830	40.7	9.1	0.27	0.99	3.7	0.12	0.05	2.32
ON123850	10.4	15.7	10.82	19.04	1.8	2.69	0.65	4.17
ON123870	9.6	16.1	3.06	11.50	3.8	3.88	1.66	2.34
ON123890	16.3	13.8	2.51	34.12	13.6	1.27	2.06	0.62
ON123230	48.9	8.2	ND	ND	ND	0.20	0.03	8.09

The parameters were obtained from three assays; microsomal stability [$t_{1/2}$, predicted CL], MDCK-MDR1 [P_{app} , efflux ratio], and unbound fractions in plasma and brain [$F_{unbound}$, F_{up}/F_{ub}]. All values represent the mean of 3
ND none detected

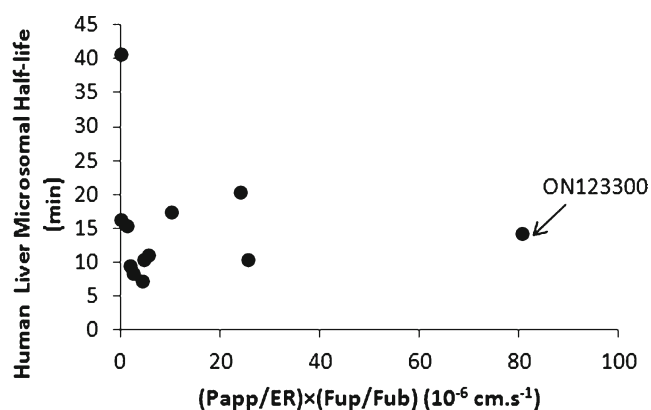


Fig. 2. Graphical analysis of human liver microsomal half-life and a derived parameter obtained from the *in vitro* MDCK-MDR1 [*i.e.*, (P_{app}/ER)] and plasma and brain equilibrium dialysis assays [(F_{up}/F_{ub})]. Each circle corresponds to one of the ON123 compounds analyzed in the tier 2 screening protocols. The compound with the most overall favorable characteristics is ON123300, designated by the arrow

Several recent studies have shown the impact of drug protein and tissue binding on brain distribution (24,25), and based on the 12 compounds in our analyses there was limited agreement between the *in vitro* and *in vivo* brain partition coefficients ($R^2=0.256$ see Fig. 4). For example, ON123770, predicted to have the highest brain partition coefficients (*i.e.*, 6.62) from the *in vitro* binding studies, yielded one of the lowest *in vivo* brain partition coefficients based on the AUC ratio (*i.e.*, 0.11). The lack of correlation could be attributed to the involvement of active transport mechanisms, either influx or efflux, which are not accounted for by the *in vitro* binding assays. To improve the correlation between *in vitro* and *in vivo* brain distribution parameters, parameters obtained from the MDCK-MDR1 cell permeability model were used. The MDCK-MDR1 model is widely used as a means to assess BBB permeability and provide an indication of a compounds potential to be a substrate for P-glycoprotein that serves as a common drug efflux pump on the BBB (7,8). The efflux

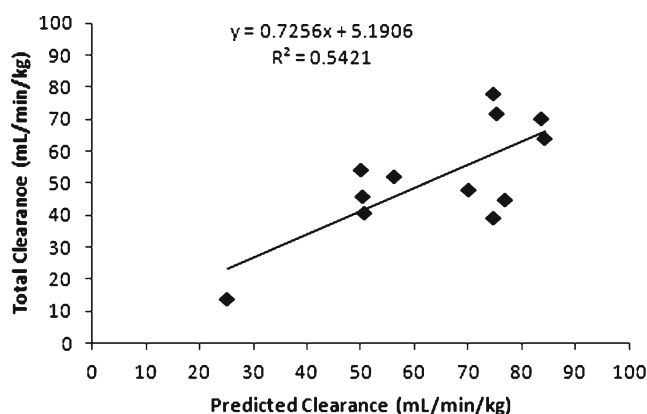


Fig. 3. *In vitro-in vivo* correlation between predicted mouse clearance based on mouse microsomal stability assay and the total clearance from the cassette dosing study for 12 of the ON123 compounds

ratios (*i.e.*, ER, Table I), also generated from the MDCK-MDR1 assays, were relatively low and suggested that P-glycoprotein is not a determinant of brain accumulation for these compounds. However, since the MDCK-MDR1 model does not address the possible involvement of other transporters and due to the likelihood that passive diffusion is not the sole determinant of brain accumulation for these compounds, a role for other transporters seems plausible. By combining both parameters from the MDCK-MDR1 assay (*i.e.*, ER and P_{app}) with the drug binding parameters into a hybrid parameter [$P_{app}/ER \times (F_{up}/F_{ub})$] as described for Fig. 2, the *in vivo* brain partition coefficients [$P_b = AUC_b/AUC_p$] values were predicted reasonably well ($R^2=0.920$, Fig. 5).

DISCUSSION

Development of drugs for brain diseases requires consideration of pharmacokinetic factors beyond what is typical of non-brain diseases due to the challenge of achieving acceptable BBB penetration that often eliminates drug candidates from further development. Moreover, the

Table III. Pharmacokinetic Parameters of 12 ON123 Compounds Obtained from the Cassette Dosing Study

Compound ID	AUC _p (μg/h/L)	AUC _b (μg/h/L)	AUC _b /AUC _p ratio	CL (mL/min/kg)	V _{dss-obs} (mL/kg)	t _{1/2} (h)
ON1231030	111.21	788.03	0.14	52	3,963	2.07
ON1231060	714.28	682.45	0.96	64	1,731	0.85
ON123300	2,933.15	798.92	3.67	54	3,645	1.70
ON123650	934.38	467.19	0.50	45	866	0.33
ON123660	664.41	334.85	0.50	70	2,472	1.90
ON123680	1,113.81	449.73	0.40	39	1,093	0.78
ON123770	107.99	985.29	0.11	41	1,482	1.60
ON123780	873.69	945.84	1.08	46	1,045	0.48
ON123830	210.67	3,327.74	0.06	14	1,368	2.90
ON123850	1,035.51	588.95	1.76	72	2,371	2.18
ON123870	105.57	758.05	0.14	78	1,878	0.35
ON123890	929.48	76.31	0.08	48	1,772	0.75

All compounds were administered as an intravenous bolus dose [2.5 mg/kg each] to mice in a single cassette. Discrete plasma and brain samples were collected over a 6-h period and then were analyzed by LC/MS/MS for drug concentrations. The following parameters were obtained by noncompartmental analyses

AUC_p area under the drug concentration-time curve in plasma, AUC_b area under the drug concentration-time curve in plasma brain, CL total systemic clearance, V_{dss} apparent volume of distribution at steady state, t_{1/2} terminal elimination half-life

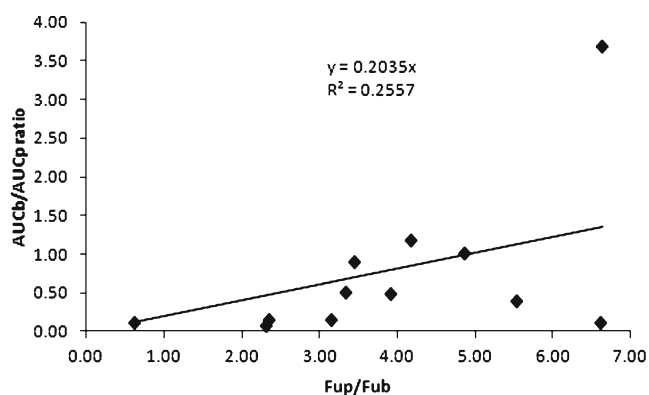


Fig. 4. Relationship between the *in vivo* [(AUCb)/(AUCp)] and *in vitro* [F_{up}/F_{ub}] brain partition coefficients for the 12 of the ON123 compounds evaluated in the cassette dosing studies

complexities of brain tumors, notably heterogeneity and variable BBB function, create additional uncertainties as to which preclinical development paradigms will be most informative on the ability of a candidate drug to achieve therapeutic concentrations. We applied a two-tier screening system to the ON123 series that was relatively parsimonious and efficient to identify compounds suitable for brain tumor chemotherapy.

Tier 1 screening incorporated two types of screens that have rather disparate endpoints, a rapid *in silico* screen to eliminate compounds with physicochemical properties that do not favor brain uptake, and a medium throughput cytotoxicity assay to determine potential efficacy in a standard glioma cell line. The rule-based *in silico* methodology is not uncommon in drug discovery, and here we made use of a general consensus that compounds of molecular weight greater than 450 Da and very low and high octanol/water partition coefficients do not penetrate into the brain (17,18). In addition, we utilized a calculated risk criterion that is similar to the well-known Lipinski's Rule of 5 that is often used as an indicator of oral drug absorption, and further may also indicate brain penetration as CNS active compounds were found to have fewer hydrogen (H)-bond donors and lower polar surface area than compared to non-CNS compounds; criteria used in the ADMET risk parameter

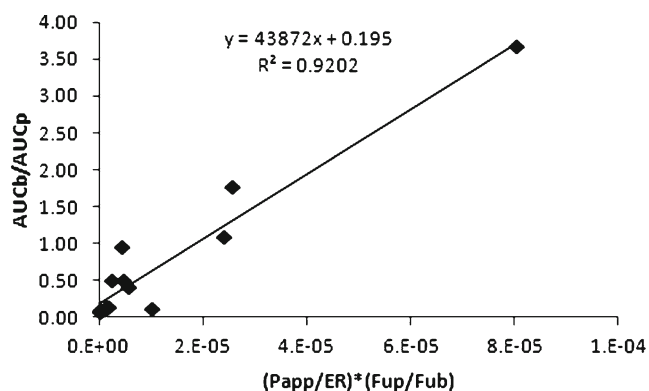


Fig. 5. Relationship between the *in vivo* [(AUCb)/(AUCp)] brain partition coefficient and a derived parameter on the x-axis that is composed of the MDCK-MDR1 membrane permeability [P_{app}] and efflux ratio [ER] values, and the unbound fraction in plasma [F_{up}] and brain tissue [F_{ub}] for 12 of the ON123 compounds

(28). The glioma cell cytotoxicity screen provides the first measure of compound efficacy and could eventually be used to build a pharmacodynamic profile. Some caution is warranted; however, as the results from a single cancer cell line could be misleading in that false negatives are possible in that some compounds may be active in glioma cells with different genetic characteristics. In general, the U87 glioma model is a robust test of drug activity since it lacks the tumor suppressor, PTEN, and is unable to regulate oncogenic PI3K that often leads to drug insensitivity. This seems to be the case with the ON123 series as only 13 of the 56 compounds possessed IC₅₀ values of <10 μ M, and on that basis were chosen for tier 2 screening.

The tier 2 screening procedures used three *in vitro* ADME assays that culminated in the identification of ON123300 as the lead compound in the series. *In vitro* microsomal half-life studies have been used extensively to obtain intrinsic metabolic clearance and elimination half-life data for prediction of *in vivo* hepatic blood clearance (11,29). According to the classification of hepatic blood clearance into low, medium, and high extraction groups, the 13 ON123 compounds were well distributed (see Table II) and a favorable *in vitro*-*in vivo* correlation in mouse clearance was obtained (see Fig. 3).

The two remaining tier 2 assays, MDCK-MDR1 permeability and equilibrium dialysis binding, are used to indicate both the rate [permeability] and extent [binding assays] of drug accumulation in brain. The evaluation criteria for MDCK-MDR1 assay vary with differences due to of expression level of P-gp and cell culture conditions, such as the seeding density and postseeding time. In our study, caffeine, methotrexate, and paclitaxel were used as control compounds for CNS-positive and CNS-negative and P-gp substrates, respectively, and found to agree well with results presented in several recent publications that focused on the use of MDCK-MDR1 cells as screening tool (7,8). Based on such agreement, we chose the same threshold values for BBB permeation (compounds with P_{app} (A-B) > 3×10^{-6} cm/s have high potential to cross the BBB and those with P_{app} (A-B) < 1×10^{-6} cm/s low potential). It should be mentioned that compounds with a P_{app} (A-B) in the MDCK-MDR1 cells of lower than 1×10^{-6} cm/s does not necessarily disqualify a drug candidate as being a potential CNS-acting drug. Other parameters, including efflux ratios and unbound fraction, should be taken into consideration to judge the potential brain distribution. In the present study, estimates of F_{ub} , F_{up} , and F_{up}/F_{ub} ratio varied by 82-, 31-, and 13-fold, respectively, among the 13 compounds examined (Table II) that highlights that even in the presence of highly variable plasma and brain binding the estimated brain partition coefficient varied much less. In fact, without ON123890 included, the range of predicted brain partition coefficients would have been less than 4-fold (*i.e.*, 2.34 to 8.09, Table II).

Implementation of these three *in vitro* ADME screens in the selection of new agents for brain tumor chemotherapy greatly enhanced the throughput and the ability to efficiently triage compounds prior to conducting *in vivo* pharmacokinetic studies. An important challenge to prevent premature elimination of compounds is to adequately balance the properties derived from each *in vitro* assays into a combined selection criteria. As illustrated in Fig. 2, a number of compounds that had

in vitro human liver microsomal half-life value of >14 min, including ON123230 that possessed the most favorable half-life of 48.9 min, were unable to cross the cell membrane in MDCK-MDR1 assay, which can be explained that when compounds become more polar and less lipophilic, they tend to be more stable in the liver microsomal half-life screen (10). Incorporation of the parameters from the MDCK-MDR1 and binding assays into a hybrid parameter (*i.e.*, $(P_{app}/ER) \times (F_{up}/F_{ub})$) proved to be a reliable indicator of *in vivo* brain distribution (see Fig. 5) and showed that the compound, ON123300, with the highest hybrid value also had the highest *in vivo* brain/plasma partition coefficient. Use of either the MDCK-MDR1 assay or equilibrium binding assays (see Fig. 4) alone did not predict brain accumulation to the extent of the hybrid parameter. The hybrid parameter [*i.e.*, $(P_{app}/ER) \times (F_{up}/F_{ub})$] improved the ability to predict the brain exposure and is attributed to it accounting for both the rate and extent of brain distribution. In conclusion, an effective screening strategy was developed to select new agents for brain tumor chemotherapy from a series of low molecular weight anticancer agents [ON123x] by the combined use of *in silico*, *in vitro* cytotoxicity, and *in vitro* ADME profiling studies. One compound, ON123300, selected from the approach exhibited favorable cytotoxicity and brain penetration.

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