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Screening candidate anticancer drugs for brain tumor chemotherapy: Pharmacokinetic-driven approach for a series of (E)-N-(substituted aryl)-3-(substituted phenyl)propenamide analogues

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Summary

A pharmacokinetic [PK]-driven screening process was implemented to select new agents for brain tumor chemotherapy from a series of low molecular weight anticancer agents [ON27x] that consisted of 141 compounds. The screening procedures involved a combination of *in silico*, *in vitro* and *in vivo* mouse studies that were cast into a pipeline of tier 1 and tier 2 failures that resulted in a final investigation of 2 analogues in brain tumor-bearing mice. Tier 1 failures included agents with a molecular weight of > 450 Da, a predicted log P (log P) of either < 2 or > 3.5, and a cytotoxicity IC₅₀ value of > 2 uM. Next, 18 compounds underwent cassette dosing studies in normal mice that identified compounds with high systemic clearance, and low blood-brain barrier [BBB] penetration. These indices along with a derived parameter, referred to as the brain exposure index, comprised tier 2 failures that led to the administration of 2 compounds [ON27570, ON27740] as single agents [discrete dosing] to mice bearing intracerebral tumors. Comparison of ON27570's resultant PK parameters to those obtained in the cassette dosing format suggested a drug-drug interaction most likely at the level of BBB transport, and prompted the use of the *in vitro* MDCK-MDR1 transport model to help assess the nature of the discrepancy. Overall, the approach was able to identify candidate compounds with suitable PK characteristics yet further revisions to the method, such as the use of *in vitro* metabolism and transport assays, may improve the PK-directed approach to identify efficacious agents for brain tumor chemotherapy.

Keywords

Pharmacokinetics; Drug development; Brain tumor; CNS; Preclinical

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Conflict of interest

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.

Introduction

In attempts to improve what are often considered failed drug discovery and development paradigms in the pharmaceutical industry and to expedite translation of basic biomedical sciences to clinical therapeutics, the FDA and NIH have offered the Critical Path Initiative [1] and NIH Roadmap programs [2]. These programs aim to incorporate new technologies into drug discovery strategies that hope to arrive at individualized therapeutics, and thus ample consideration is given to genomics, biomarkers, and clinical trial design. Although pharmacokinetic (PK) and pharmacodynamic (PD) information and model-based drug development is receiving more attention as a supplemental tool in the drug discovery hierarchy, focus on target tissue PK/PD and disease-specific strategies are only beginning to emerge [3,4]. An analysis of standard anticancer drug discovery and development practices reveals that compounds that enter clinical trials are often selected in a semi-empirical manner based on systemic PK properties, toxicology, and tumor size-based efficacy studies completed in mice [2]. The lack of PK/PD data obtained directly from the tumor creates a void that limits how preclinical data can be translated to the clinic and propagates continued use of semi-empirical drug development strategies. Armed with an understanding of a drug's PK/PD properties in tumors enables a quantitative bridge between preclinical animal studies and clinical trials. Therefore, although the notion of PK/PD-driven drug discovery and development has gained traction it has yet to be implemented with tumor-based PK/PD central to the process. Previously, we demonstrated a component of this latter strategy by achieving equivalent PK/PD dosing of gefitinib in EGFR wild-type and mutant tumor models [3,4]; however the broader application of such a PK/PD-driven approach to the complete drug discovery milieu remains to be completed.

The ON27 series was derived from an anticancer drug discovery partnership between academia and Onconova Therapeutics Inc that is focused on the discovery of low molecular-weight inhibitors of oncogenic kinases. The ON27 series is early in development and as shown below a number of candidates possess appreciable cytotoxicity to glioma cells, yet their precise mechanisms of action have yet to be defined. The current investigation describes our first iteration to achieve the goal of an integrated PK/PD-driven drug development scheme and was designed to identify candidate compounds from the ON27 series that possessed the most favorable PK characteristics [moderate to low clearance and superior brain distribution] for brain tumor chemotherapy.

Materials and methods

Materials

Gefitinib was purchased from LC laboratory (Woburn, MA, USA). 1-Methyl-2-pyrrolidinone (NMP), dimethyl sulphoxide (DMSO), trichloroacetic acid (TCA), sulforhodamine B (SRB) were purchased from Sigma-Aldrich. (St. Louis, MI, USA). Polyethylene glycol 300 (PEG300) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Water was prepared from Millipore Synergy UV system (Billerica, MA, USA).

U87MG human glioma cells were purchased from the American Type Culture Collection, cultured in Dulbecco's modified Eagle's medium (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.

Adult male ICR mice (25–30g) and male athymic Swiss mice (nu/nu, 25–30g) 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.

Chemical synthesis

All 141 ON27 compounds were synthesized in the lab of Dr. R Reddy according to the general synthetic method briefly described below. The general structure and those of 18 compounds used in the cassette dosing studies are shown in Figure 1.

To a solution of an aromatic amine (10 mmol) and TEA (10 mmol) in dichloromethane (50 mL) at room temperature was slowly added a solution of an alkyl malonyl chloride (10mmol) in dichloromethane. The reaction mixture was stirred for 1 hour. The reaction material was filtered and the solvent removed under reduced pressure to yield an oily material which was purified by column chromatography to yield an alkyl-2-(N-arylaminoacarbonyl)-acetate.

The alkyl-2-(N-arylaminoacarbonyl)-acetate was refluxed for 2.5 hours in a solution of sodium hydroxide (9.0 g) in water (90 mL) and ethanol (90 mL). The reaction mixture was subsequently cooled and acidified with hydrochloric acid to precipitate the crude product. The crude 3-arylamino-3-oxopropanoic acid was removed by filtration and recrystallized from hot water.

A solution of the arylamino-3-oxopropanoic acid (10 mmol), an aromatic aldehyde (10 mmol) and benzylamine (0.4 mL) was refluxed for 3 hours in glacial acetic acid (10 mL). The solution is then cooled and cold ether (50mL) added. The organic layer was separated and washed with a saturated solution of sodium bicarbonate (30 mL), sodium bisulfite (30 mL) and dilute hydrochloric acid (30 mL). The thin layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield the corresponding N-aryl-3-aryl-2-propenamide.

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise stated. Solvents were dried using standard procedures and reactions requiring anhydrous conditions were performed under N₂ atmosphere. Reactions were monitored by thin layer chromatography (TLC) on preloaded silica gel F254 plates (Sigma-Aldrich) with a UV indicator. Column chromatography was performed with Merck 70– 230 mesh silica gel 60 Å. Yields were of purified product and were not optimized. Melting points were determined using an Electro thermal Mel-Temp 3.0 micro melting point apparatus and are uncorrected. ¹H NMR spectra were obtained with Bruker AM 300 and 400 MHz spectrometers to confirm chemical structures. The purity of the final compounds was determined by HPLC and is 95% or higher.

LC/MS/MS analysis

All samples from the cassette dosing, discrete dosing and plasma protein binding studies were analyzed with an electrospray ionization LC/MS/MS system (HPLC, Shimadzu, Kyoto, Japan; QTrap 5500, Applied Biosystems, Foster City, CA, USA). Prior to animal dosing, compounds were checked for potential isobaric interferences and those that had a potential for interferences with each other or with potential metabolites of other compounds would not be placed in the same cassette.

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 15000 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. The chromatographic separation system consisted of a guard cartridge (C18, 4.0 × 2.0 mm, Phenomenex,

Torrance, CA, USA), an analytical column (Luna C18, 3 μ M particle size, 50 $\times$ 2.0 mm, Phenomenex, Torrance, CA, USA). An HPLC solvent system that consisted of mobile phase A (acetonitrile: water: formic acid, 5/95/0.1, v/v/v) and B (acetonitrile: water: formic acid, 95/5/0.1, v/v/v) was used for all assays, and specified according to three gradient programs corresponding to each of three dosing cassettes as summarized in Table S1. The effluent from the HPLC column was directed into the electrospray ionization interface of the mass spectrometer operated in the positive ion mode with the probe temperature set at 650°C. Nitrogen was used for all gases and set at 45 psi as the curtain gas, and at 45 psi and 65 psi as ion source gases 1 and 2, respectively. The turbo ion spray voltage was 4500V. Tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM) analyses were performed using nitrogen as the collision gas which was set at medium. The mass spectrometer was set to pass protonated molecular ions $[M+H]^+$ through the first quadrupole (Q1) with fragment ions produced via collision induced dissociation (CID) in Q2 isolated and detected using Q3. Data collection and analysis were performed using Analyst 1.5.1 software (Applied Biosystems MDS Sciex, Ontario, Canada). The transitions monitored are shown in Table S2.

The analytical methods were specific and sensitive with a quantification level of 1 ng/ml. The intraday and interday variability was less than 15% in both plasma and brain, see Tables S3 and S4. The average run time was about 7 min for each sample.

***In silico* prediction**

The 2D chemical structures of 141 ON27 compounds were entered into the ADMET Predictor™ (V4.0, Simulations Plus, Inc., Lancaster, CA, USA) computer program to obtain predicted physicochemical properties that were subsequently used to assist in selecting compounds for the cassette dosing studies. From this set of compounds those with log P values between 2 and 3.5 were deemed acceptable for further evaluation.

***In vitro* cytotoxicity assay**

The cytotoxicity of selected ON27 series compounds was determined using a colorimetric SRB-based assay. Suspensions (100 μ L containing 2×10^3 cells) of U87 glioma cells were seeded in 96-well plates and allowed to attach to the surface by overnight incubation in DMEM with 10% standard fetal bovine serum. The cells were then treated with varying concentrations of selected ON27 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 SpectraMax M2 microplate reader (Molecular Devices, Downingtown, PA) at a wavelength of 570 nm. A Sigmoid Emax model (WinNonlin, Pharsight Corporation, Mountain View, CA, USA) was used to calculate IC₅₀ values, which were defined as the drug concentration that was required to reduce the number of viable cells to 50% compared with control treatment (vehicle alone). Each IC₅₀ mean value was obtained from at least three independent experiments.

***In vivo* cassette dosing studies**

Cassette dosing, also referred to as N-in-1 dosing, is an *in vivo* PK screening approach that requires the administration of low doses of multiple compounds to the same animal [5–8]. The method is very efficient in terms of resources but has the potential to facilitate competitive drug-drug interactions that can lead to inaccuracies in the calculated PK parameters. Compounds selected for cassette dosing studies were grouped into three cassettes (6 ON27 compounds per cassette) based on their individual LC/MS/MS characteristics with the intent to minimize drug-drug or drug-metabolite analytical interferences. In each cassette, gefitinib was also included as a reference compound. Gefitinib is a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor

and has been used in brain tumors [9–11]. It undergo extensive metabolism primarily by cytochrome P450 3A4 [12]. Recent studies indicate that gefitinib interacts with various multidrug transporters including ABCG2, P-glycoprotein and MRP1 [13, 14]. Compounds were dissolved in a mixture of NMP:PEG300:water at a volume ratio of 1:4:5, respectively. Each compound, except one, was administered at a dose of approximately 5 mg/kg [15, 16] as an intravenous (IV) bolus injection through a lateral tail vein in a total injection volume of 10 ml/kg for each cassette. Compound ON271320 was given at a dose of 1.67 mg/kg due to a shortage of material. Adult male ICR mice were used for the cassette dosing studies.

At each predetermined sampling time (0.25, 0.5, 2, 4, 6 hr), groups of normal mice [3 mice/time point for each cassette] 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.

Orthotopic glioma model and discrete dosing studies

The U87MG glioma model used in this study was described previously [17]. Briefly, 30 adult male nude mice were anesthetized and secured in a stereotactic apparatus and had implanted a suspension of U87MG cells (10^6 cells in 10 μL phosphate-buffered saline) 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., Reno, NV, USA). Once the animals recovered from anesthesia they were returned to the animal care facilities, and provided food and water *ad libitum*. Mice were monitored daily and entered into the discrete dosing pharmacokinetic 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.

The PK studies for the individual compounds [*i.e.* ON27570 and ON27740] were conducted in an analogous manner as for the cassette dosing studies. Each compound was administered at an IV dose of 5 mg/kg via a tail vein and had blood normal brain and brain tumor samples collected at 0.25, 0.5, 2, 4, 6 hr upon sacrifice. Plasma and tissue samples were stored at -80°C until analyzed by LC/MS/MS.

Plasma protein binding

Methanolic solutions of ON27570 and ON27740 were added to mouse plasma to give concentrations in the range of 0.01–2 $\mu\text{g/mL}$. The methanol did not exceed 5% of the final volume. Aliquots (150 μL) of each of the plasma solutions were dialysed against an equal volume of isotonic 0.067 M phosphate buffer, pH 7.4, using a 96-Well Equilibrium Dialysis Block system (Model HTB96b, HTDialysis LLC, Gales Ferry, CT, USA), with the plasma and buffer being separated by a dialysis membrane (MW cut-off 12–14 kDa HTDialysis LLC). The units were maintained at 37°C whilst being rotated for 8 hr that was previously determined to represent an equilibration time. Post-dialysis plasma and buffer volumes were measured and then were analyzed for drug concentrations by LC/MS/MS. The fraction of bound drug expressed as a percentage (fb) was calculated as follows;

$$\text{fb} = \frac{(D_{\text{Te}} - D_{\text{F}}) \cdot V_{\text{pe}} / V_{\text{pi}}}{[(D_{\text{Te}} - D_{\text{F}}) \cdot V_{\text{pe}} / V_{\text{pi}}] + D_{\text{F}}} \times 100\%$$

where D_{Te} and D_{F} represent the total plasma concentration at equilibrium (sample side) and the free concentration (dialysate side), respectively, and V_{pi} and V_{pe} represent the initial and equilibrium plasma volumes, respectively [18].

MDCK-MDR1 Cell Permeability

Following the completion of the *in vivo* PK studies the *in vitro* MDCK-MDR1 cell model [19–21] for BBB permeability was used to further assess the transport characteristics of the two lead compounds; ON27570 and ON27740. MDCK-MDR1 cells (passage number 21–30) were seeded onto 0.33 cm² polycarbonate filter Transwells at a density of 60,000 cells/cm² and maintained in culture as previously described [21]. Confluent MDCK-MDR1 monolayers expressing P-gp were obtained 3–4 days postseeding and their integrity determined by measuring the transepithelial electrical resistance (TEER, $\Omega \cdot \text{cm}^2$) using an epithelial 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 > 1000 $\Omega \cdot \text{cm}^2$ throughout the experiments were used [measured prior to and at the end of the experiment].

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 mM Hepes buffer, pH ~ 7.4) at 37°C using monolayers that were preincubated for 30 min with pre-warmed HBSS. At the start of the experiment, fresh HBSS was added to the receiver compartments and ON27570 and ON27740 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 1%), and then incubated at 37°C for 90 min. After incubations, samples were collected from both the receiver and donor compartments, and drug concentrations were determined by LC/MS/MS as described in the Sample Analysis Section.

The apparent permeability, P_{app} (cm/s) was calculated as: $P_{\text{app}} = dQ/dt \times 1/(A \times C_0)$, where dQ/dt is the transport rate of the compound (mol/s), A is the area of the cell monolayers (cm²) and C_0 is the initial donor concentration (mol/l).

Pharmacokinetic data analysis

Noncompartmental analysis (NCA) was performed using WinNonlin™ Phoenix software to estimate the pharmacokinetic parameters for each compound in the cassette and discrete dosing studies. The parameters estimated were; the areas under the drug concentration-time curve in plasma and brain (AUC_p and AUC_b), systemic clearance (CL), and the apparent volume of distribution (V_d) and K_e , the terminal elimination rate constant in plasma. In addition, the area under the drug concentration-time curve in brain tumor (AUC_{bt}) was calculated for each compound undergoing discrete dosing studies. In all cases, the individual concentration values obtained from replicates were used in the analyses. All AUC calculations were based on the area from time zero to the last observable concentration using the linear-log interpolation method, and not extrapolated to time infinity. Although for a number of compounds the AUC to infinity would have been appropriate [*i.e.* terminal phase well-characterized] there were some compounds, particularly for the AUC_b, in which this extrapolation would not have been due to uncertainty in the terminal elimination phase, and thus, for the sake of consistency we choose to use the AUC to the last observed concentration. The V_d was calculated as CL/K_e , where K_e was obtained from log-linear regression of the terminal phase of the plasma concentration-time curve. The terminal half-life values, calculated as $0.693/K_e$, ranged from 0.4 to 1.1 hr and agreed with the observed data [K_e r^2 values ranged from 0.84 and 0.99 and were based on a minimum of 3 points]. The normal brain or brain tumor partition coefficient was calculated as the ratio of AUC_b or AUC_{bt} to the corresponding AUC_p and is a measure of the cumulative drug distribution to tissue. A derived parameter the brain exposure index [BEI] was calculated as the ratio of the AUC_b/IC50, where the AUC_b value was that determined in the cassette dosing study and

the IC₅₀ was the *in vitro* cytotoxicity value. The BEI was used to indicate the likelihood that effective brain tumor concentrations could be achieved *in vivo*.

Results

Tier 1 screening

The goal of tier 1 screening was to employ a combination of physicochemical properties and *in vitro* cytotoxicity data to select approximately 20 compounds for cassette dosing studies. Since the BBB is a major obstacle to drug penetration indicators of BBB permeability, namely, molecular weight and log P, which had previously been used as screening criteria [22, 23] were utilized as initial screening criteria. There is an inverse relationship with MW and BBB permeability for compounds with a molecular weight of about 400 Da or less. Of the 141 ON27 compounds, 14 possessed MW greater than 450 Da [max MW = 586 Da] leaving 127 compounds for further evaluation.

The *in silico* program provides a wide range of predicted parameters that can be used to indicate a compounds pharmaceutical and pharmacological properties that pertain to their suitability for use in humans. The range of parameters includes estimates of membrane permeability in different cell types, intrinsic clearances for drug-metabolizing enzymes, and physicochemical properties. Given our interest to identify compounds that could penetrate the BBB, compounds that possessed octanol-to-water partition coefficients or log P values between 2 and 3.5 were deemed acceptable for further consideration. Log P values have been shown to be correlated to BBB permeability [24]; however values > 3.5 or highly lipophilic compounds may remain in the BBB and not actually cross it. Of the 127 ON27 compounds that had MW < 450 Da, 40 compounds had log P values between 2 and 3.5. The selection of compounds for cassette dosing from these 40 compounds was based on favorable cytotoxicity to U87 glioma cells (9 compounds, Table 1); defined as those with IC₅₀ values of < 2 uM, as well as inclusion of outliers for each criterion; MW > 450 Da (3 compounds), log P either < 2 or > 3.5 (4 compounds) or IC₅₀ values > 2 uM (9 compounds). Of the 18 (Figure 1) compounds selected for cassette dosing, 6 compounds met all three criteria, 8 compounds failed 1 criterion, and 4 compounds failed two criteria (Table 1).

Tier 2 screening

The goal of tier 2 screening was to select two compounds from the 18 compounds included in the cassette dosing studies for individual or discrete PK investigations in brain tumor bearing mice. The criteria used to make this selection were based on total systemic clearance [CL] and AUC_b/AUC_p values measured in the cassette dosing studies, and the BEI (see Table 2). Higher BEI values are desirable and indicate the potential that effective drug concentrations can be achieved in brain. The range of total clearance values was very high, being > 2,400-fold, and 5 compounds with very high CL values of > 20 L/hr/kg were eliminated since maintenance of effective drug concentrations would be difficult. The range of brain partition coefficients [P_b = AUC_b/AUC_p] values was greater than 300-fold with a high of 3.2 and using a value of 0.3 as a division between low and moderate to high values five additional compounds with values of < 0.3 were eliminated due to their low brain distribution. Compound ON271370 possessed both a high CL and low brain partition coefficient. Of the remaining 9 compounds the BEI indicated a wide range of values (Table 2) from a low of 0.01 hr [ON27490] to a high of 23.5 hr [ON27740] and using a value of < 1 as a criterion an additional 5 compounds were eliminated from further consideration. Four compounds failed both the P_b and BEI criteria (Table 2). Four compounds [ON27010, ON27040, ON27570, ON27740] possessed acceptable PK properties and did not fail any tier 2 criteria. The 2 compounds [ON27740, ON27570] selected for discrete PK studies in brain tumor bearing mice had the highest BEI values, both > 20, and lower CL values than

both ON27010 and ON27040, which supported the possibility that ON27740 and ON27570 could more readily achieve and maintain effective brain concentrations than either ON27010 or ON27040. The PK parameters of the reference compound gefitinib from the three cassette dosing studies are also listed in Table 2 in which a 2-fold range of systemic PK parameters and brain distribution was found. These differences between cassettes are attributed to drug-drug interactions that most likely involve competition for saturable hepatobiliary elimination and BBB transport that have been associated with the disposition of gefitinib [25, 26]. In hindsight, gefitinib may not have been the optimal choice as a reference compound; however at the dose of 5 mg/kg, plasma C_{max} values were about 50-fold less than those (*i.e.* 100 μ M) reported to inhibit Pgp and Mrp1 [27]. Another group reported that gefitinib doses of 100 mg/kg or 200 mg/kg were needed to inhibit Pgp and Abcg2 *in vivo* [28, 29], and thus, the contribution of gefitinib to transport-based drug interactions is anticipated to be low.

Discrete pharmacokinetic studies

Based on the tier 1 and 2 screening procedures two lead compounds, ON27570 and ON27740 (Figure 1), underwent discrete PK studies in brain tumor-bearing mice using the same dose and sampling schedule as in the cassette dosing studies, and the resultant concentration-time profiles from the two dosing format are shown in Figure 2. A summary of the PK parameters including the brain and brain tumor partition coefficients are provided in Table 3. Comparison of the PK indices between the cassette and discrete dosing investigations reveal some differences that for ON27570 suggested a drug-drug interaction with respect to brain distribution. Firstly, for ON27740 there was agreement in P_b yet differences of 1.5-fold for total clearance [CL] and 2.5-fold for the volume of distribution [Vd] when compared between the discrete and cassette dosing formats. These differences, particularly for ON27740 clearance values, could reflect experimental variability as recently suggested [30] when comparing cassette to discrete dosing PK parameters, but could also reflect competitive inhibition, either at the level of active membrane transporter or metabolism, following cassette dosing. A more likely drug-drug interaction was for the brain distribution of ON27570 in which the normal brain partition coefficient [P_b] was increased 5-fold in the cassette dosing format relative to the discrete format. Systemic PK parameters for ON27570 agreed between the cassette and discrete dosing studies so the alteration in the brain partition coefficient could be attributed to a transport interaction at the BBB or a plasma protein binding displacement interaction. If the latter mechanism occurred in the cassette format a higher unbound fraction of ON27570 in plasma would make more compound available to distribute into brain and via a greater concentration gradient from plasma to brain lead to a higher brain partition coefficient. As can be seen in Table 4 ON27570 is highly bound ($99.5 \pm 0.2\%$), and thus, a viable candidate for protein displacement. To explore the possibility that ON27570 higher partition coefficient in the cassette dosing format was due to a competitive interaction involving a BBB efflux pump we used the Madin-Darby canine kidney (MDCK)-MDR1 *in vitro* cell model for the BBB that overexpressed P-glycoprotein, a well-known drug efflux pump [31]. By measurement of the bi-directional transport of both ON27570 and ON27740 we found that neither compound were likely substrates for Pgp based on the permeability parameters [see Table 5]; Papp (A-B) $> 1 \times 10^{-6}$ cm/s and efflux ratio (Papp (B-A)/Papp (A-B)) < 30 . Other BBB efflux pumps of the Mrp family, such as Abcg2, could be involved in ON27570 transport. As noted above gefitinib, the reference compound for each cassette, is a Abcg2 substrate and 95% bound to plasma proteins in mice [31,32] so the changes observed in its PK parameters could similarly be attributed to plasma protein binding and transport interactions as for ON27570. The primary inference from the comparison of PK parameters in the cassette and discrete dosing formats is that drug-drug interactions are possible, even when the number of the compounds in a cassette is low, and greater use of *in vitro* screens including metabolic stability, protein binding and membrane transport is suggested.

Discussion

Given the advances of new technologies including combinatorial chemistry, bioinformatics and genomic sciences, an optimized strategy to screen a large number of compounds in the discovery phase of drug development would be beneficial. Many new drug discovery strategies have been proposed that incorporate various physicochemical, genetic, and pharmacological elements [5, 6, 33, 34, 35]; however, we are unaware of a comprehensive screening strategy specifically designed for drugs potentially effective against brain tumors. In designing an expeditious approach for anticancer drugs intended for brain tumor chemotherapy our initial attention was placed on properties that have been indicative of BBB permeability, namely, MW and log P, and then proceed to *in vivo* cassette dosing methods. Other BBB permeability assessment methods are available, such as the use of *in vitro* cell models, as we implemented later in the course of this investigation and see that this is a valuable addition to the screening paradigm. The tier 1 screening criteria, in addition to MW and log P, also included *in vitro* cytotoxicity data in glioma cells. The cytotoxicity assays can be completed fairly rapidly and yield a global measure of pharmacodynamic activity since pharmacological details on target inhibition or specificity are not ascertained. Further, since we limited our cytotoxicity screen to a single glioma cell type additional studies will be required to assess potential molecular determinants of drug activity [4]. To summarize the tier 1 screening efforts, an expeditious approach weighted on *in silico* and *in vitro* cytotoxicity methods was employed that identified a relatively small number of compounds for *in vivo* investigations.

The tier 2 screening procedures revolved around the cassette dosing studies that included a number of compounds that did not meet tier 1 criteria. This was done as a means to assess the validity of MW and log P as predictors of brain distribution and to begin to collect a database that may be used to devise models of BBB penetration for ON27 and related chemotypes. Cassette dosing studies have been used to rapidly ascertain oral bioavailability, systemic clearance, and in some cases BBB penetration [7, 8]. The concerns of false negatives and positives obtained with cassette or N-in-1 dosing has led to divergent schools of thought as to their usefulness. As discussed, these concerns were supported as the PK properties differed between the cassette and discrete dosing formats, particularly for ON27570. In the small series of 18 ON27 compounds there is partial agreement between log P and the brain partition coefficient values (Tables 1 and 2) as compounds with the lowest 6 log P values [$\log P < 2.55$] also possessed the 6 lowest brain partition coefficients [$P_b < 0.6$]. Interestingly compound ON271360 with the highest log P of 4.69 did not have the highest brain partition coefficient, rather an intermediate value of 1.3, and provides support for the criteria that compounds with log P values greater than 3.5 may not actually show preferential brain distribution being retained in the BBB. Further, all compounds identified as tier 1 log P failures were also tier 2 failures [$P_b > 0.3$] except ON27550 whose P_b was quite low, being 0.04, yet the log P, again a predicted value, was 2.54. Nonetheless, strict reliance on log P values as a measure of brain distribution is not recommended.

The brain exposure index [BEI = AUC_b/IC_{50}] was used as a tier 2 criterion and using a cut-off of 1 hr identified 11 compound failures, and for 5 compounds it was the sole criterion for failure. By combining an *in vitro* and *in vivo* parameter a direct meaning of the BEI is not obvious, yet the combination of a pharmacokinetic and pharmacodynamic endpoint is appealing and suggests that compounds meeting the criterion are more likely to maintain tumoricidal drug concentrations. Overall, the tier 2 screening procedures were effective in minimizing the set of potential ON27 compounds to a manageable number of compounds two of which were studied independently in mice bearing brain tumors.

The discrete dosing studies revealed some concerns attributed to the likelihood of drug interactions in the cassette dosing format. The most troublesome is that for compound ON27570, in which the brain partition coefficient in the cassette was 0.5 that was appreciably greater than the value of 0.1 when given alone, and in fact, this latter value would not have met the tier 2 criteria of $P_b > 0.3$. The nature of the likely drug-drug interaction for ON27570 was discussed above and attributed to plasma protein displacement and/or BBB transport interactions. The ancillary MDCK-MDR1 transport studies ruled out Pgp as the likely BBB transporter but members of the Mrp family, such as Abcg2, may have played a role [36, 37]. Further studies would be required to delineate the exact mechanism(s) for the proposed drug-drug interaction for ON27570; however more importantly, the discrepancy in the two dosing formats supports the use of additional *in vitro* screens prior to conducting *in vivo* PK investigations. In this regard, use of other *in vitro* procedures to indicate brain permeability, partitioning, and the unbound fraction in brain could set new screening criteria and possibly replace cassette dosing protocols, and further facilitate greater focus on discrete dosing studies in mice bearing intracerebral tumors [38, 39]. As expected both ON27570 and ON27740 showed greater partitioning into brain tumor than in normal brain consistent with the compromised BBB associated with brain tumors. Although the use of brain tumor bearing mice in a cassette dosing protocol was considered it requires more resources and could not be supported as a routine screening tool.

In conclusion, a PK-driven screening procedure was implemented for a series of potential low molecular weight anticancer drugs in consideration of their utility in brain tumors. This stepwise protocol relied on *in silico*, *in vitro* cytotoxicity and both cassette and discrete dosing *in vivo* studies to identify suitable compounds characterized by high cytotoxicity, adequate brain distribution and moderate to low clearance. The procedure was partially successful in that one compound (ON27740) was confirmed to possess these properties in brain tumor bearing mice. The limitations of the current PK-driven approach can be attributed to drug-drug interactions, both plasma protein binding and BBB transport, that were revealed when PK properties were compared from the cassette and discrete dosing formats. The plasma protein binding interactions can be identified by implementing a wider *in vitro* plasma protein binding screen. Similarly, an *in vitro* screen of BBB permeability, such as the MDCK-MDR1 model [40, 41], may prove to be advantageous, and since drug-drug interactions are eliminated. These types of revisions will contribute to a next generation PK-driven screening protocol and ultimately to an optimized strategy for developing drugs for brain tumor chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Challenges and Opportunities Report (CDER). Innovation and stagnation: Challenge and opportunity on the critical path to new medicinal products. US Food and Drug Administration; 2004.
2. Zerhouni E. Medicine. The NIH Roadmap. Science. 2003; 302:63–72. [PubMed: 14526066]
3. Wang S, Guo P, Wang X, Zhou Q, Gallo JM. Preclinical pharmacokinetic/pharmacodynamic models of gefitinib and the design of equivalent dosing regimens in EGFR wild-type and mutant tumor models. Mol Cancer Ther. 2008; 7:407–417. [PubMed: 18281523]

4. Wang S, Zhou Q, Gallo JM. Demonstration of the equivalent pharmacokinetic/pharmacodynamic dosing strategy in a multiple-dose study of gefitinib. *Mol Cancer Ther.* 2009; 8:1438–1447. [PubMed: 19509243]
5. He K, Qian M, Wong H, Bai SA, He B, Brogdon B, et al. N-in-1 dosing pharmacokinetics in drug discovery: experience, theoretical and practical considerations. *J Pharm Sci.* 2008; 97:2568–2580. [PubMed: 17914718]
6. Smith NF, Raynaud FI, Workman P. The application of cassette dosing for pharmacokinetic screening in small-molecule cancer drug discovery. *Mol Cancer Ther.* 2007; 6:428–440. [PubMed: 17308044]
7. Tamvakopoulos CS, Colwell LF, Barakat K, Fenyk-Melody J, Griffin PR, Nargund R, et al. Determination of brain and plasma drug concentrations by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom.* 2000; 14:1729–1735. [PubMed: 11006579]
8. Zhang MY, Kerns E, McConnell O, Sonnenberg-Reines J, Zaleska MM, Steven Jacobsen J, et al. Brain and plasma exposure profiling in early drug discovery using cassette administration and fast liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal.* 2004; 34:359–368. [PubMed: 15013150]
9. Hegi ME, Diserens AC, Bady P, Kamoshima Y, Kouwenhoven MC, Delorenzi M, et al. Pathway analysis of glioblastoma tissue after preoperative treatment with the EGFR tyrosine kinase inhibitor gefitinib—a phase II trial. *Mol Cancer Ther.* 2011; 10:1102–1112. [PubMed: 21471286]
10. Heon S, Yeap BY, Britt GJ, Costa DB, Rabin MS, Jackman DM, et al. Development of central nervous system metastases in patients with advanced non-small cell lung cancer and somatic EGFR mutations treated with gefitinib or erlotinib. *Clin Cancer Res.* 2010; 16:5873–5882. [PubMed: 21030498]
11. Geyer JR, Stewart CF, Kocak M, Broniscer A, Phillips P, Douglas JG. A phase I and biology study of gefitinib and radiation in children with newly diagnosed brain stem gliomas or supratentorial malignant gliomas. *Eur J Cancer.* 2010; 46:3287–3293. [PubMed: 20708924]
12. McKillop D, McCormick AD, Millar A, Miles GS, Phillips PJ, Hutchison M. Cytochrome P450-dependent metabolism of gefitinib. *Xenobiotica.* 2005; 35:39–50. [PubMed: 15788367]
13. Lemos C, Kathmann I, Giovannetti E, Calhau C, Jansen G, Peters GJ. Impact of cellular folate status and epidermal growth factor receptor expression on BCRP/ABCG2-mediated resistance to gefitinib and erlotinib. *Br J Cancer.* 2009; 100:1120–1127. [PubMed: 19277036]
14. Kawamura K, Yamasaki T, Yui J, Hatori A, Konno F, Kumata K, et al. In vivo evaluation of P-glycoprotein and breast cancer resistance protein modulation in the brain using [(11)C]gefitinib. *Nucl Med Biol.* 2009; 36:239–246. [PubMed: 19324269]
15. Watanabe T, Schulz D, Morisseau C, Hammock BD. High-throughput pharmacokinetic method: cassette dosing in mice associated with minuscule serial bleedings and LC/MS/MS analysis. *Anal Chim Acta.* 2006; 559:37–44. [PubMed: 16636700]
16. Smith NF, Hayes A, Nutley BP, Raynaud FI, Workman P. Evaluation of the cassette dosing approach for assessing the pharmacokinetics of geldanamycin analogues in mice. *Cancer Chemother Pharmacol.* 2006; 54:475–486. [PubMed: 15526201]
17. Zhou Q, Gallo JM. Differential effect of sunitinib on the distribution of temozolomide in an orthotopic glioma model. *Neuro Oncol.* 2009; 11:301–310. [PubMed: 18971416]
18. Boudinot FD, Jusko WJ. Fluid shifts and other factors affecting plasma protein binding of prednisolone by equilibrium dialysis. *J Pharm Sci.* 1984; 73:774–780. [PubMed: 6204037]
19. Luo S, Kansara VS, Zhu X, Mandava NK, Pal D, Mitra AK. Functional characterization of sodium-dependent multivitamin transporter in MDCK-MDR1 cells and its utilization as a target for drug delivery. *Mol Pharm.* 2006; 3:329–339. [PubMed: 16749865]
20. Soldner A, Benet LZ, Mutschler E, Christians U. Active transport of the angiotensin-II antagonist losartan and its main metabolite EXP 3174 across MDCK-MDR1 and caco-2 cell monolayers. *Br J Pharmacol.* 2000; 129:1235–1243. [PubMed: 10725273]
21. Wang Q, Rager JD, Weinstein K, Kardos PS, Dobson GL, Li J, et al. Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. *Int J Pharm.* 2005; 288:349–359. [PubMed: 15620875]

22. van de Waterbeemd H, Gifford E. ADMET in silico modelling: towards prediction paradise? *Nat Rev Drug Discov.* 2003; 2:192–204. Review. [PubMed: 12612645]
23. Waterhouse RN. Determination of lipophilicity and its use as a predictor of blood-brain barrier penetration of molecular imaging agents. *Mol Imaging Biol.* 2003; 5:376–389. [PubMed: 14667492]
24. Levin VA. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. *J Med Chem.* 1980; 23:682–684. [PubMed: 7392035]
25. Agarwal S, Sane R, Gallardo JL, Ohlfest JR, Elmquist WF. Distribution of gefitinib to the brain is limited by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2)-mediated active efflux. *J Pharmacol Exp Ther.* 2010; 334:147–155. [PubMed: 20421331]
26. Hofer S, Frei K, Rutz HP. Gefitinib accumulation in glioblastoma tissue. *Cancer Biol Ther.* 2006; 5:483–484. [PubMed: 16582594]
27. Ozvegy-Laczka C, Hegedus T, Várady G, Ujhelly O, Schuetz JD, Váradi A, et al. High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. *Mol Pharmacol.* 2004; 65:1485–1495. [PubMed: 15155841]
28. Carcaboso AM, Elmeliegy MA, Shen J, Juel SJ, Zhang ZM, Calabrese C, et al. Tyrosine kinase inhibitor gefitinib enhances topotecan penetration of gliomas. *Cancer Res.* 2010; 70:4499–4508. [PubMed: 20460504]
29. Kawamura K, Yamasaki T, Yui J, Hatori A, Konno F, Kumata K, et al. In vivo evaluation of P-glycoprotein and breast cancer resistance protein modulation in the brain using [¹¹C] gefitinib. *Nucl Med Biol.* 2009; 36:239–246. [PubMed: 19324269]
30. Nagilla R, Nord M, McAtee JJ, Jolivet LJ. Cassette dosing for pharmacokinetic screening in drug discovery: comparison of clearance, volume of distribution, half-life, mean residence time, and oral bioavailability obtained by cassette and discrete dosing in rats. *J Pharm Sci.* 2011; 100:3862–3874. [PubMed: 21360708]
31. Li J, Brahmer J, Messersmith W, Hidalgo M, Baker SD. Binding of gefitinib, an inhibitor of epidermal growth factor receptor-tyrosine kinase, to plasma proteins and blood cells: in vitro and in cancer patients. *Invest New Drugs.* 2006; 24:291–297. [PubMed: 16502356]
32. McKillop D, Hutchison M, Partridge EA, Bushby N, Cooper CM, Clarkson-Jones JA, et al. Metabolic disposition of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, in rat, dog and man. *Xenobiotica.* 2004; 34:917–934. [PubMed: 15764411]
33. Kerns EH. High throughput physicochemical profiling for drug discovery. *J Pharm Sci.* 2001; 90:1838–1858. [PubMed: 11745742]
34. Hop CE, Cole MJ, Davidson RE, Duignan DB, Federico J, Janiszewski JS, et al. High throughput ADME screening: practical considerations, impact on the portfolio and enabler of in silico ADME models. *Curr Drug Metab.* 2008; 9:847–853. [PubMed: 18991580]
35. Carlson TJ, Fisher MB. Recent advances in high throughput screening for ADME properties. *Comb Chem High Throughput Screen.* 2008; 11:258–264. [PubMed: 18336218]
36. Ebinger M, Uhr M. ABC drug transporter at the blood-brain barrier: effects on drug metabolism and drug response. *Eur Arch Psychiatry Clin Neurosci.* 2006; 256:294–298. [PubMed: 16783492]
37. Tamai I, Tsuji A. Transporter-mediated permeation of drugs across the blood-brain barrier. *J Pharm Sci.* 2000; 89:1371–1388. [PubMed: 11015683]
38. Wan H, Rehngrén M, Giordanetto F, Bergström F, Tunek A. High-throughput screening of drug-brain tissue binding and in silico prediction for assessment of central nervous system drug delivery. *J Med Chem.* 2007; 50:4606–4615. [PubMed: 17725338]
39. Hammarlund-Udenaes M, Fridén M, Syvänen S, Gupta A. On the rate and extent of drug delivery to the brain. *Pharm Res.* 2008; 25:1737–1750. [PubMed: 18058202]
40. Wang Q, Rager JD, Weinstein K, Kardos PS, Dobson GL, Li J, et al. Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. *Int J Pharm.* 2005; 288:349–359. [PubMed: 15620875]
41. Taub ME, Podila L, Ely D, Almeida I. Functional assessment of multiple P-glycoprotein (P-gp) probe substrates: influence of cell line and modulator concentration on P-gp activity. *Drug Metab Dispos.* 2005; 33:1679–1687. [PubMed: 16093365]

Compound	Ar	R
ON 27010	4-OCH ₃ -C ₆ H ₄	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27040	3-NH ₂ , 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27090	3-NH ₂ , 4-OCH ₃ -C ₆ H ₃	3-F, 4-NH ₂ -C ₆ H ₃
ON 27110	3-OH, 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27390	3-NH ₂ , 4-Cl-C ₆ H ₃	3-OCH ₃ , 4-F-C ₆ H ₃
ON 27470	3-OH, 4-OCH ₃ -C ₆ H ₃	2,4-F ₂ -C ₆ H ₃
ON 27490	3-NH ₂ , 4-OCH ₃ -C ₆ H ₃	2,6-(OCH ₃) ₂ , 4-O(CH ₂) ₃ COOH-C ₆ H ₂
ON 27550	3-NH(CH ₃) ₂ COOH, 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27570	3-NHCH ₂ COOH, 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27720	6-NH-quinolinyl	2-pyrrolyl
ON 27730	6-NH-quinolinyl	2-thiophenyl
ON 27740	6-NH-quinolinyl	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27750	5-NH-quinolinyl	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 27830	2-OCH ₃ , 6-NH-quinolinyl	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 271320	4-(3-pyridyl)-phenyl	2-pyrrolyl
ON 271340	3- α -t-BocNHC ₃ H ₅ COO, 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 271360	3-[CH ₃ -N-(CH ₂) ₄ -N-CH ₂ -C ₆ H ₄ -COO], 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂
ON 271370	3-O-Biotin, 4-OCH ₃ -C ₆ H ₃	2,4,6-(OCH ₃) ₃ -C ₆ H ₂

Fig. 1.
Chemical structures of 18 compounds from the ON27 series used in the cassette dosing studies.

A **B**

Fig. 2. Time profiles of ON27740 (A) and ON27570 (B) concentrations in plasma (p) and brain (b) in cassette (C) and discrete (D) dosing. Compounds were administered to mouse at an IV dose of 5 mg/kg via a tail vein and had blood and normal brain samples collected at various time points. Plasma and tissue samples were analyzed by LC/MS/MS. The data shown are the mean $\pm$ SD of at least three mice at each time point.

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Table 1

Molecular weight, predicted log P(S+logP) and mean IC50 values in U87 glioma cells of eighteen compounds selected for three cassette dosing studies. The last column indicates the category [molecular weight = M, log P = P, cytotoxicity = C] of tier 1 failures based on MW > 450 Da, log P < 2 or > 3.5 and IC50 values > 2 uM.

Cassette	Compound ID	MW	S+logP	IC50 [uM]	Failure
A	ON27010	343.38	3.04	0.48	-
	ON27040	358.4	2.22	0.157	-
	ON27090	301.32	1.98	16	P, C
	ON271320	289.34	2.89	50	C
	ON27490	430.43	1.56	12	P,C
	ON27740	364.4	3.16	1.66	-
B	ON27110	359.38	2.6	0.101	-
	ON27550	444.49	2.54	0.205	-
	ON27720	263.3	2.4	18	C
	ON271340	542.59	3.81	0.037	M, P
	ON271360	575.67	4.69	0.176	M, P
	ON271370	585.68	3.25	0.134	M
C	ON27390	320.75	3.47	50	C
	ON27470	305.28	3.49	50	C
	ON27570	416.43	2.01	0.296	-
	ON27730	280.35	3	50	C
	ON27750	364.4	3.09	8.5	C
	ON27830	394.43	3.41	22	C

Table 2

Pharmacokinetic and associated parameters of compounds from three cassette dosing studies. The last column indicates the category [CL, P_b, or BEI] of tier 2 failures defined as either CL > 20 L/h/kg, P_b < 0.3 or BEI < 1.

Cassette	Compound	AUC _p (ng* ^h /mL)	AUC _b (ng* ^h /g)	P _b	CL (L/h/kg)	Vd (L/kg)	BEI (hr)	Failure
A	Gefitinib	2277.3	1968.6	0.9	2.3	8.3	-	-
	ON27010	2810.5	2120.9	0.8	1.8	1.4	15.6	*
	ON27040	1558.3	645.8	0.4	3.2	2.5	14.1	*
	ON27090	2642.1	248.1	0.1	1.9	2.5	0.09	P _b , BEI
	ON271320	521.0	585.7	1.1	3.2	3.3	0.05	BEI
	ON27490	4969.5	44.1	0.009	1.0	1.2	0.01	P _b , BEI
	ON27740	26682.7	14242.0	0.5	0.2	0.2	23.5	*
B	Gefitinib	1073.7	2685.2	2.5	4.3	9.9	-	-
	ON27110	174.0	343.2	2.0	25.0	40.4	9.5	CL
	ON27550	3342.5	140.8	0.04	1.5	1.6	1.6	P _b
	ON27720	14704.3	283.0	0.02	0.4	0.3	0.06	P _b , BEI
	ON271340	7.8	24.8	3.2	577.0	457.3	1.23	CL
	ON271360	16.9	21.3	1.3	269.3	255.2	0.21	CL, BEI
	ON271370	30.5	8.3	0.3	159.2	169.3	0.11	CL, P _b , BEI
C	Gefitinib	1826.3	3491.2	1.9	2.6	8.9	-	-
	ON27390	3659.7	2438.7	0.7	1.4	1.5	0.15	BEI
	ON27470	228.6	439.4	1.9	23.4	32.1	0.03	CL, BEI
	ON27570	5077.6	2729.3	0.5	1.0	1.5	22.1	*
	ON27730	2725.7	2749.0	1.0	1.9	1.8	0.19	BEI
	ON27750	1732.5	824.6	0.5	2.9	4.5	0.27	BEI
	ON27830	5732.9	7442.9	1.3	1.0	1.3	0.84	BEI

Abbreviations are area under the drug concentration-time curve in plasma, AUC_p; total systemic clearance, CL; apparent volume of distribution, Vd; brain partition coefficient, P_b; brain exposure index, BEI, defined as AUC_b/IC₅₀.

Compounds designated with a * in the failure column possessed acceptable properties yet the 2 compounds with the highest BEI values, ON27570 and ON27740, were selected for discrete dosing studies.

Table 3

Mean pharmacokinetic parameters for ON27570 and ON27740 following administration of single IV doses of 5 mg/kg to mice bearing intracerebral tumors.

Compound	AUCp [ng*h/ml]	CL [L/h/kg]	Vd [L/kg]	AUCb [ng*h/g]	AUCbt [ng*h/g]	P _b	Pbt
ON27570	5369.5	0.9	1.1	679.9	1433.5	0.1	0.3
ON27740	15064.0	0.3	0.5	6852.0	9376.1	0.5	0.6

Abbreviations are area under the drug concentration-time curve in plasma, brain and brain tumor, AUCp, AUCb and AUCbt, respectively; total systemic clearance, CL; apparent volume of distribution, Vd; brain partition coefficient, P_b, defined as AUCb/AUCp, and brain tumor partition coefficient, Pbt, defined as AUCbt/AUCp.

Table 4

The bound fraction (f_b) of ON27570 and ON27740 in mouse plasma as a function of concentration.

conc. (ng/mL)	f_b (%)	
	ON27570	ON27740
10	ND	ND
20	ND	91.05±0.69
50	ND	92.88±1.03
100	ND	92.54±0.80
200	ND	90.63±0.61
500	99.81±0.02 [*]	91.82±0.98
1000	99.68±0.04	91.20±0.39
2000	99.47±0.05	91.18±0.42

^{*} Measurements were performed in triplicate (n=3), ND: None Detected.

Table 5

Transport parameters of ON27570 and ON27740 in the MDCK-MDR1 cell model.

Compound	Papp* (10 ⁻⁶ cm/s)		Efflux ratio (Papp (B-A)/Papp (A-B))
	A-B	B-A	
ON27570	2.52±0.32	7.25±0.48	2.93
ON27740	2.59±0.76	3.46±0.39	1.44

* Papp values are expressed as mean ± S.D.; n=3 for each determination.