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ORIGINAL ARTICLE

Preclinical pharmacokinetics and in vitro activity of ON 01910.Na, a novel anti-cancer agent

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Abstract
Purpose ON 01910.Na is a novel targeted anti-cancer agent under clinical investigation in Phase I and II trials. The purpose of this research was to evaluate the pharmacokinetic profile of ON 01910.Na across several species, and to evaluate the effects of protein binding and duration of exposure on its in vitro cytotoxic activity.
Methods Data were collated from several preclinical investigations, where the plasma disposition and tissue distribution of ON 01910.Na were assessed after administration (10–150 mg/kg, IP or IV) to several species (mouse, rat, and dog). Plasma protein binding was assessed using ultrafiltration. Cytotoxic activity of ON 01910.Na was determined in DU145 cells, and activity was correlated to unbound drug concentration and the duration of exposure.
Results ON 01910.Na exhibits extensive plasma protein binding and the compound displays rapid elimination from the circulation in all three animal species ($t_{1/2}$ range 0.404–0.870 h). Tissue distribution studies in mice revealed highest drug accumulation in the liver, followed by the kidneys. ON 01910.Na is not extensively metabolized in vivo and urinary excretion is predominant at higher doses. ON 01910.Na cytotoxicity in DU145 cells was adversely affected by protein binding in the incubation medium. Drug cytotoxicity was greatly enhanced upon extending the duration of exposure at reduced drug concentrations.
Conclusions Due to the short half-life and rapid clearance of the drug, administration of ON 01910.Na by continuous IV infusion is a likely treatment option for cancer patients.

Keywords ON 01910.Na · Pharmacokinetics · Plasma binding · Cytotoxicity

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Introduction

ON 01910.Na (Fig. 1) is a novel compound under clinical development by Onconova Therapeutics, Inc. (Newtown, PA). ON 01910.Na is designed around a novel benzyl styryl sulfone chemotype demonstrating ability to inhibit the cell cycle progression. It is a multikinase inhibitor that selectively induces a mitotic arrest leading to apoptosis in cancer cells, while being non-toxic to normal cells [1–3]. In addition, ON 01910.Na cytotoxicity studies exhibit IC_{50} values in the nanomolar range, and the compound has shown efficacy with low toxicity in preclinical animal models [1, 4]. When used in combination with other anti-cancer agents including oxaliplatin, doxorubicin, and gemcitabine, ON 01910.Na displays a strong synergistic effect, in some cases leading to total regression of the tumor [1, 5]. An active analog, designated as ON 01500, has also been identified as a potential metabolite of ON 01910.Na.

A systematic drug development program was initiated to characterize the disposition of ON 01910.Na in various animal species (mouse, rat, and dog) and to determine the influence of protein binding on the free fraction of the

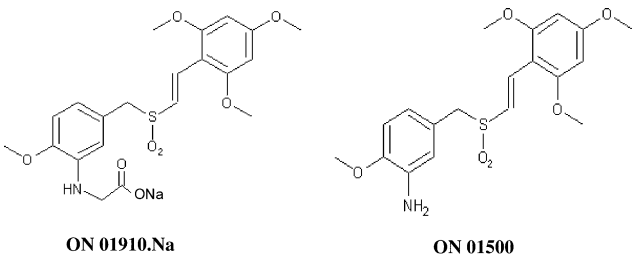

Fig. 1 Structure of ON 01910.Na, $C_{21}H_{24}NNaO_8S$ and main identified metabolite ON 01500, $C_{19}H_{23}NO_6S$

circulating drug, which in turn would be expected to impact its in vivo activity. Based on the results of pharmacokinetic and protein binding studies, in vitro experiments were conducted to assess the cytotoxic activity of ON 01910.Na as a function of unbound drug concentration and the duration of exposure. The collective information obtained from drug disposition, protein binding, and the non-traditional cytotoxicity studies helped to design appropriate clinical regimens for investigating the drug in Phase I clinical trials.

Materials and methods

Chemicals and reagents

ON 01910.Na was provided by Onconova Therapeutics, Inc. (Newtown, PA). Sodium chloride and potassium phosphate monobasic and all HPLC grade solvents used for extraction and chromatography were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used for the preparation of aqueous solutions was filtered and deionized with a Nanopure, ultrapure water system (Barnstead, Dubuque, IA, USA). Drug-free, blank mouse, rat, dog, and human plasma were obtained from Valley Biomedical (Winchester, VA, USA).

Animals

In vivo mouse studies were performed at Fels Institute. Twelve-week-old female CD-1 mice weighing approximately 30 g were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed five per cage. Animals were on an alternating 12-h light/dark cycle and allowed access to food and water ad libitum throughout the experiment. Mouse tissue distribution studies were conducted at Absorption Systems (West Chester, PA, USA). Tissue and systemic exposure of ON 01910.Na was evaluated in 12-week-old SW1 mice weighing 30–35 g. Mice were purchased from Hilltop Lab Animals (Scottsdale, PA). The animals were fasted for at least 16 h prior to dosing.

Rat studies were performed at Ricerca Biosciences, LLC (Concord, OH, USA). Seven to eight-week-old Albino rats (Crl:CD® (SD)(IGS)BR®) with an average weight of 250 g were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed individually with unlimited access to food and water. The rats were maintained on an alternating 12-h light/dark cycle at 18–29°C and 30–70% relative humidity.

Dog studies were performed at Ricerca Biosciences, LLC. Seven-month-old beagle dogs weighing approximately 7 kg were purchased from Marshall Farms (North Rose, NY, USA) and housed individually with $\sim$300 g food daily and unlimited access to water. The dogs were maintained on an alternating 12-h light/dark cycle at 18–29°C and 30–70% relative humidity.

All animal studies were conducted with approval from the Institutional Animal Care and Use Committee (IACUC) at each study site. "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed in all experiments.

In vivo pharmacokinetic studies

Stock solutions were prepared by dissolving ON 01910.Na in a preparation of 1:1 (V:V) polyethylene glycol 400 and 0.016 M phosphate buffer yielding final nominal concentrations of 77 mg/mL. Slight heating and stirring aided in the dissolution of ON 01910.Na. Stock solutions were adjusted to pH 10.0. Intravenous dosing solutions (11 mg/mL) were prepared by mixing one part stock solution with six parts 0.01 M phosphate buffer (pH 7.2). The dosing solution (final pH 7.5) was then passed through a 0.2 μ filter. The intravenous preparations were stored in refrigeration up to 10 days, and stability has been demonstrated over this storage period.

Pharmacokinetic studies in mice

ON 01910.Na was administered by IP injection at a dose of 100 mg/kg in mice. Blood samples were collected following decapitation at 0.083, 0.025, 0.50, 1.0, 4.0, 6.0, and 8.0 h. Blood was collected into heparinized tubes and centrifuged immediately to obtain plasma. All the plasma samples were kept frozen at <-20°C until further analysis.

Tissue distribution of ON 01910.Na was determined following intravenous administration (12.5 mg/kg). Animals were euthanized at 0.25, 2, and 8 h and approximately 0.5 mL of whole blood was collected from the dorsal aorta of each mouse. Tissue samples were extracted from the mice immediately following the blood collection and were homogenized prior to compound extraction.

Pharmacokinetic studies in rats

Jugular vein cannulation was performed on the rats through which a bolus dose of 30, 75, or 150 mg/kg was administered. Blood samples were collected at 0.25, 1, 4, 8, and 12 h through the cannula. Plasma was extracted and frozen at <−20°C until analysis was performed.

For biliary excretion studies, the bile duct of each test animal ($n = 5$/group) was cannulated. Following a 5-day acclimation period, ON 01910.Na was administered intravenously via femoral vein injection at 150, 225, or 300 mg/kg. The animals were placed in metabolism cages following dosing and attached to a tether/swivel system for continuous bile and urine collection. Bile was collected in a container exterior to the cage and surrounded by ice. Urine and feces were collected continuously from each animal until termination. Sample containers were placed over dry ice during collection.

Pharmacokinetic studies in dogs

Beagle dogs received an intravenous injection of ON 01910.Na. Three doses were tested: 10, 25, and 50 mg/kg. Blood was obtained by venipuncture and collected into EDTA-containing tubes at 0.25, 1, 4, 8, and 24 h post-dose. The samples were immediately centrifuged and plasma was retained and stored frozen prior to analysis.

In a separate set of experiments, ON 01910.Na was administered via continuous intravenous infusion for a period of 3 days (108.4 and 325 mg/kg/day). Blood samples were collected by venipuncture at 0.25, 1, 2, 6, 24, 48, and 71.5 h after infusion initiation, and then 0.25, 1, 2, and 4 h after the infusion was terminated. Plasma samples were collected and stored as described previously.

Protein binding

Protein binding of ON 01910.Na was assessed by ultrafiltration. A stock solution of ON 01910.Na was spiked into plasma samples (2 mL) to achieve final ON 01910.Na concentrations of 12.5, 25, 50, and 100 μg/mL. Samples were allowed to equilibrate in a 37° C shaker bath for 1 h. An aliquot (1 ml) was collected for measurement of total drug concentration. A second aliquot (1 mL) of plasma was added to an Amicon Centrifree Micropartition System® (MW cutoff 30,000). The system was centrifuged for 15 min at 1500*g*. Preliminary studies determined that non-specific binding to the device and membrane was <2%. Free fraction of drug was estimated as the ratio of drug concentrations in the ultrafiltrate and plasma. Studies were conducted in plasma from four species: mouse, rat, dog, and human. In order to estimate binding parameters, affinity constant (K_a) and maximum binding capacity (nM), additional experiments were performed in human plasma over a range of ON 01910.Na concentrations between 12.5 and 5000 μg/mL.

Analytical methods

In vitro and in vivo plasma and homogenized tissue samples were prepared for analysis by protein precipitation with the addition of 400 μL acetonitrile to 100 μL sample volume. Samples were vortexed then centrifuged (10,000 rpm) for 10 min prior to evaporation of supernatant under N_2. The samples were reconstituted with 100 μL acetonitrile:water (1:1), vortexed and centrifuged again and the supernatant removed for analysis.

ON 01910.Na concentrations from in vivo plasma, tissues, urine, and bile samples were measured using a validated LC-MS/MS method. A PESCIEX API 2000 system (Concord, ON, Canada) was utilized under gradient LC conditions in positive ionization mode with separation achieved through a reversed phase C18 column. The mobile phase used for separation consisted of 0.05% formic acid in water (A) and 0.05% formic acid in acetonitrile (B). The initial gradient conditions set mobile phase B at 20%, increasing to 65% at 0.5 min and returning to 20% after 2.6 min. The entire program was set to run for 5.5 min per sample with the flow rate set at 0.3 mL/min and an injection volume of 5 μL. The following parameters were set for analysis using MS: nebulizing gas, 35 psig; drying gas (N_2) temperature: 400°C; N_2 flow rate: 7 L/min; ion spray voltage: 5000 V; Q0: −10 V; IQ1: −11 V; IQ2: −30 V; IQ3: −50 V. The precursor $[MH]^+$ ion was monitored at 452.2 amu for ON 01910.Na and 394.0 amu for ON 01500. The product ion was observed at 194.2 amu for ON 01910.Na and 136.2 amu for the metabolite.

Plasma samples from in vitro protein binding studies were analyzed by HPLC with a Shimadzu (Columbia, MD, USA) SPD-10AV UV detector. Separation of the analyte was achieved at 40°C using Alltima HP column (250 mm × 4.6 mm) packed with a 5 μ, C_{18} stationary phase (Alltech Associates, Inc., Deerfield, IL, USA). The mobile phase was composed of 0.01 M potassium phosphate buffer (pH 8.0) and acetonitrile (65% v/v), delivered isocratically at 1.0 mL/min and degassed using a Waters in-line degasser. The detection wavelength was set at 215 nm.

Method validation

Validation was completed prior to analysis in all matrices for both LC-MS/MS and HPLC methods. No interfering peaks were observed in blank plasma, tissues, bile, or urine chromatograms at ON 01910.Na or ON 01500 retention times indicating selectivity of both analytical methods.

For the LC-MS/MS, linearity was determined in the following ranges: 3–1000 ng/mL in mouse tissues and mouse plasma, 10–2500 ng/mL in rat plasma, 0.1–50 μg/mL (drug and metabolite) in rat bile and urine, and 15–5000 ng/mL in dog plasma. The correlation coefficients were >0.995 for calibration curves. For each matrix, quality control (QC) samples were prepared at low, medium, and high concentrations to assess accuracy, precision, and recovery. Intra-day precision from a measurement of a minimum of six replicates was <7.9% in all matrices. The inter-day precision was determined from analysis of standard samples on three consecutive days and the relative standard deviation (RSD) was found to be <8.9%. The overall accuracy of QC samples was in the range of 85–115%. Percentage recovery was calculated as a ratio of the peak areas of drug in matrix and the corresponding peak area of drug in buffer. In plasma samples, greater than 85% drug recovery was accomplished. Recovery was lower in tissues ($\sim$30%). The lower limit of quantification (LOQ) was determined based on intra-run accuracy of LOQ sample replicates for which RSD was <20%. The tested LOQ values were 3, 10, and 5 ng/mL in mouse (plasma and tissues), rat (plasma, bile, and urine), and dog plasma, respectively.

The HPLC method employed for analysis of protein binding samples established linearity in the range of 10–100 μg/mL in mouse, rat, dog, and human plasma and 1–10 μg/mL in their corresponding ultrafiltrates with correlation coefficients exceeding 0.991. Both intra- and inter-day precision were <5.6% and overall accuracy >88%. Across all species, ON 01910.Na recovery from plasma was >86%. The LOQ at 1.0 and 10 μg/mL for ultrafiltrate and plasma, respectively, across species was tested and deemed acceptable based on RSD <15% between replicates.

The stability of ON 01910.Na in plasmas, tissues, bile, and urine and ON 01500 in bile and urine was assessed under different storage and handling conditions. Short-term stability at room temperature was confirmed for 4 h with accuracy of >95%. Samples stored at −70°C exhibited stability for up to one month with >93% accuracy. In addition, all samples were subjected to three freeze-thaw cycles and demonstrated >91% accuracy when compared to original drug concentrations.

Pharmacokinetic analysis

ON 01910.Na plasma concentration versus time data were analyzed by non-compartmental analysis. Pharmacokinetic parameters were generated through WinNonlin® (version 5.0.1, Pharsight Corporation, Mountain View, CA). Area under the concentration versus time curve $\left(AUC_0^{\infty}\right)$ from time 0 to the last experimental time period (C_{last}) was determined using the linear trapezoidal rule (with extrapolation to infinity using the formula C_{last}/k). Clearance and volume of distribution were calculated using standard pharmacokinetic equations.

In vitro anti-tumor activity studies

Anti-tumor activity of ON 01910.Na was evaluated in a human prostate cancer cell line, DU145. Cells were purchased from the American Type Culture Collection (Manassas, VA), and maintained in DMEM cell culture medium supplemented with 10% FBS, 1 mM Na pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Cells were treated with increasing concentrations (0, 1, 2, 10, 25, and 50 μM) of ON 01910.Na for various time periods: 1, 2, 4, 8, and 24 h. After the end of the treatment period, cells were washed 3 times with fresh medium and incubated for 96 h. The total number of viable cells was determined by trypan blue exclusion followed by counting with a hemacytometer, and the average was determined for each duplicate set. The cell survival data was plotted as a percentage of vehicle control.

The effect of albumin binding on the ON 01910.Na activity was also assessed in a DU145 cell line. In these studies, FBS was replaced in the cell culture medium with buffer (serum free medium) or supplemented with 2.5 g/dl human serum albumin. The cells were exposed to drug for 1 h. The cells were then washed 3 times and normal growth medium was placed back into each well. The total number of viable cells was analyzed as described above. ON 01910.Na binding to each matrix was estimated by ultrafiltration at three nominal concentrations (1, 2.5, and 5 μg/ml).

Results

Protein binding

In vitro plasma protein binding experiments were performed by ultrafiltration. The results are summarized in Table 1. Over a concentration range of 12.5–100 μg/ml, ON 01910.Na binding was 86% in mouse plasma, and 98% in rat, dog, and human plasma. In mouse, the binding was non-linear, increasing with decreasing concentration. The binding was nearly linear in the other species among concentrations tested.

Figure 2 presents the binding isotherm (plot of bound versus unbound plasma concentrations) for ON 01910.Na in human plasma. For this experiment, binding was assessed at plasma concentrations up to 5000 μg/mL. Overall, the data were best described by a two-site binding model:

Table 1 Cross-species plasma binding comparison of ON 01910.Na

ON 01910.Na plasma concentration (μg/mL)	% Bound[a]			
	Mouse	Rat	Dog	Human
100	44.8 ± 1.03	88.2 ± 0.290	97.3 ± 0.310	97.7 ± 0.100
50	69.3 ± 0.820	97.0 ± 0.010	98.4 ± 0.910	97.9 ± 0.120
25	84.4 ± 0.400	98.8 ± 0.510	99.2 ± 0.0200	98.1 ± 0.130
12.5	88.5 ± 1.04	99.5 ± 0.0400	99.1 ± 0.320	98.2 ± 0.180

$n = 3$ for each species

[a] Data presented as mean ± standard deviation

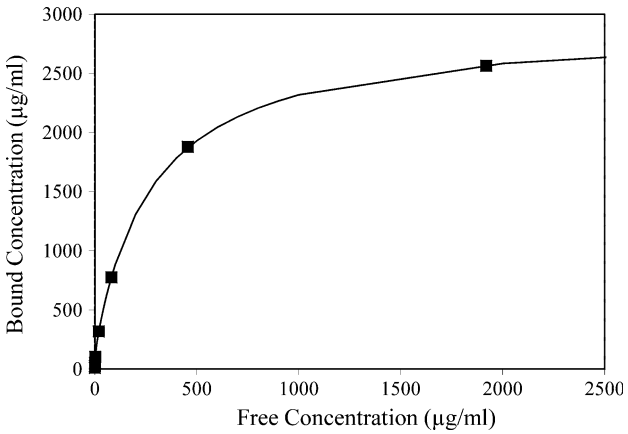

Fig. 2 Binding isotherm for ON 01910.Na in human plasma. The *solid line* represents model-predicted data using two-site binding model (a high affinity, low-capacity site and a low affinity, high-capacity site)

$$C_{\text{Bound}} = \frac{K_{a1} \times nM_1 \times C_{\text{Unbound}}}{1 + K_{a1} \times C_{\text{Ubound}}} + \frac{K_{a2} \times nM_2 \times C_{\text{Unbound}}}{1 + K_{a2} \times C_{\text{Unbound}}}$$

This equation was fitted to the data using WinNonlin®, and the following parameter estimates were obtained: $K_{a1} = 0.44 \pm 0.051$ mL/μg, $nM_1 = 162 \pm 12.3$ μg/mL, $K_{a2} = 0.0036 \pm 0.00012$ mL/μg, and $nM_2 = 2760 \pm 24.8$ μg/mL.

In vivo pharmacokinetic studies

A comparison of ON 01910.Na pharmacokinetic parameters in mouse, rat, and dog is provided in Table 2. The results for each individual species are summarized below.

Pharmacokinetic studies in mice

As presented in Table 2, ON 01910.Na is rapidly eliminated from plasma after IV dosing. This is reflected in the parameter estimates for half-life (0.496 ± 0.0579 h) and clearance (0.718 ± 0.127 L/h/kg).

Plasma and tissue distribution data for ON 01910.Na following intravenous administration (12.5 mg/kg) is presented in Table 3. Concentrations are expressed in terms of nanograms of test compound per gram of matrix (plasma, brain, heart, kidney, or liver). The data illustrates that the highest accumulation is seen in the liver at all sampling times. The distribution of ON 01910.Na to the liver is over 10-fold higher than that seen in the brain, heart, or kidney. In both the kidney and liver, the tissue to plasma concentration ratio increases over time. Brain and heart tissue to plasma concentration ratios increase up to 2 h followed by a marked decrease at 8 h.

Pharmacokinetic studies in rats

A plot of ON 01910.Na plasma concentrations versus time is provided in Fig. 3. ON 01910.Na is rapidly eliminated from the plasma after intravenous injection, as concentrations declined by 99% over the first 4 h. The half-life of this phase was ~0.450 h (Table 2). As shown in the figure, ON 01910 Na elimination includes a prolonged terminal phase, where low plasma concentrations are sustained. ON 01910.Na displayed non-linear kinetics over the range of doses studied, as both clearance and volume of distribution decreased with increasing dose. Plasma elimination half-life was unaffected by dose.

ON 01910.Na biliary and urinary excretion was also studied (Fig. 4). At the lowest dose tested (150 mg/kg), 79.4% of the administered dose was recovered in the urine and bile 8 h post dose. Urinary excretion was the predominant route of ON 01910.Na excretion at higher doses. The administered dose recovered as ON 01500 from urine and bile was approximately 0.120 ± 0.0400%, suggesting that ON 01910.Na is not extensively converted to ON 01500 in vivo.

Pharmacokinetic studies in dogs

Following IV bolus administration, ON 01910.Na has a short plasma elimination half-life in dogs (0.798 h, Table 2). Similar to results in other species, ON 01910.Na clearance and volume of distribution decreased with increasing dose in the canine model.

Pharmacokinetic parameters of ON 01910.Na following a 3-day continuous IV infusion are listed in Table 4. The results were consistent between dosing groups (108.4 and 325.0 mg/kg/day) with ON 01910.Na displaying linear pharmacokinetics. As demonstrated in Fig. 5,

Table 2 Pharmacokinetic parameters of ON 01910.Na single dose administration

Species (*n*)	Dose (mg/kg)	Half-life (h)	$AUC_{0-\infty}$ (μg h/mL)	Clearance (mL/h/kg)	C_{max} (μg/mL)	Volume of distribution (mL/kg)
Mouse (3)	100	0.496 ± 0.0579	142 ± 22.9	718 ± 127	137 ± 27.9	520 ± 157
Rat (4)	30	0.474 ± 0.0555	37.6 ± 11.3	855 ± 253	34.0 ± 12.8	336 ± 132
	75	0.431 ± 0.0436	187 ± 98.3	474 ± 198	197 ± 102	116 ± 63.9
	150	0.404 ± 0.0173	541 ± 51.9	279 ± 25.3	574 ± 50.7	64.4 ± 12.7
Dog (6)	10	0.870 ± 0.0831	10.3 ± 2.56	1030 ± 262	7.92 ± 2.00	696 ± 206
	25	0.730 ± 0.135	40.1 ± 10.6	664 ± 189	32.4 ± 12.6	402 ± 175
	50	0.793 ± 0.161	143 ± 30.6	365 ± 91.7	132 ± 39.2	117 ± 18.2

Data presented as mean ± standard deviation. For mouse studies, the dose was administered by IP injection. For rat and dog studies, ON 01910.Na was administered as IV bolus dose

Table 3 Plasma and tissue distribution of ON 01910.Na in mice

Sample	Time post dosing[a]		
	0.25 h	2 h	8 h
Plasma (ng/g)[b]	1370 ± 141	56.9 ± 47.1	17.7 ± 4.55
Brain (ng/g)[b]	16.2 ± 1.77	11.8 ± 9.95	3.91 ± 1.41
B:P[c]	0.0118 ± 7.11E-05	0.361 ± 0.440	0.242 ± 0.148
Heart (ng/g)[b]	298 ± 128	161 ± 128	1.63 ± 2.83
H:P[c]	0.224 ± 0.117	7.79 ± 10.8	0.125 ± 0.216
Kidney (ng/g)[b]	1060 ± 197	169 ± 156	99.3 ± 52.6
K:P[c]	0.787 ± 0.226	4.81 ± 3.71	6.18 ± 2.04
Liver (ng/g)[b]	12900 ± 2450	1960 ± 622	5410 ± 1230
L:P[c]	9.44 ± 0.816	32.1 ± 25.5	310. ± 50.2

[a] Data presented as mean ± standard deviation; $n = 2$ for 0.25 h; $n = 3$ for 2 h and 8 h

[b] Concentration expressed as nanograms (ng) of ON 01910.Na per gram (g) of matrix (plasma, brain, heart, kidney, or liver)

[c] Indicates ratio between organ tissue concentration and plasma concentration

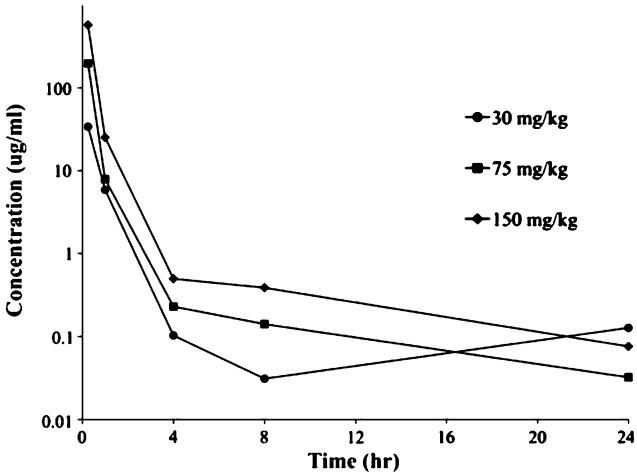

Fig. 3 Plot of mean ON 01910.Na plasma concentrations versus time following IV bolus administration to rats

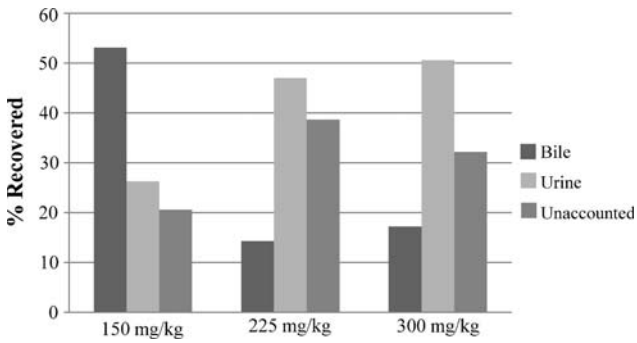

Fig. 4 Recovery of ON 01910.Na in bile and urine following IV dosing to rats (150–300 mg/kg)

ON 01910.Na plasma levels decline rapidly following infusion termination, consistent with IV bolus data.

Pharmacodynamics: In vitro activity of ON 01910.Na

The effect of drug exposure time and protein binding on the in vitro cytotoxicity of ON 01910.Na on DU145 cells is presented in Fig. 6. Increased activity was observed upon lengthening the duration of exposure. In these studies, the presence of protein, such as FBS, adversely affected the activity of the drug, indicating high protein binding potential of ON 01910.Na. In order to determine the effect of human albumin on the cell killing activity of ON 01910.Na, short-term exposure studies (1 h) with ON 01910.Na in the presence of a physiological concentration of human albumin (2.5 g/dl) were performed, followed by cytotoxicity testing. ON 01910.Na activity was reduced under these conditions (Fig. 6), indicating that the drug was sequestered by protein in the incubation medium. The mean unbound fraction of ON 01910.Na in serum-free medium, FBS, and FBS/HSA was 98, 52, and 2%, respectively (concentration range 1–5 μg/ml).

Table 4 Pharmacokinetic parameters of ON 01910.Na following IV infusion in Dog

Dose (mg/kg/day)	Half-life (h)	C_{max} (μg/mL)	C_{ss} (μg/mL)	$AUC_{0-\infty}$ (μg h/mL)	Clearance (mL/h/kg)	Volume of distribution (mL/kg)
108.4	1.38 ± 0.200	4.75 ± 1.81	3.46 ± 1.29	249 ± 93.4	1410 ± 413	2910 ± 1160
325.0	1.14 ± 0.330	17.1 ± 1.89	11.08 ± 1.32	796 ± 96.1	1230 ± 134	2030 ± 639

Data presented as mean ± standard deviation; $n = 6$ for each dose

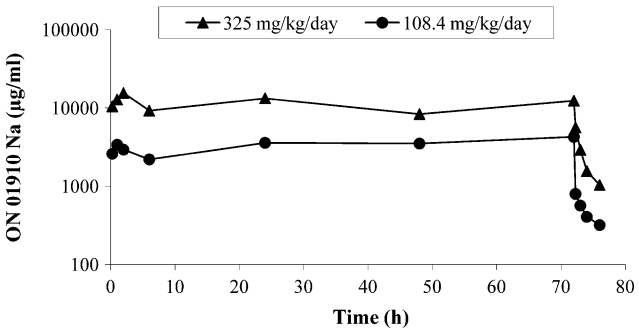

Fig. 5 Plot of mean ON 01910.Na plasma concentrations versus time following continuous IV infusion (72 h) to dogs

Discussion

ON 01910.Na is a small molecule multi-kinase mitotic inhibitor that causes mitotic arrest by creating spindle abnormalities. The cell cycle arrest leads to apoptosis in a broad range of human tumor cells [1, 5]. Recent studies have demonstrated the ability of ON 01910.Na to cause cytotoxicity and enhanced radiosensitization in a number of tumor cells [2, 6, 7]. Additionally, other studies have also revealed the synergistic effect of ON 01910.Na when used in combination with other anti-cancer agents including oxaliplatin, doxorubicin, and gemcitabine [3]. Collectively, these data suggest multiple potential uses of ON 01910.Na in anticancer therapy and have provided the motivation to advance this drug to clinical trials in cancer patients.

ON 01910.Na is a potent cytotoxic (IC_{50} values ranging from 50 to 250 nM) against a spectrum variety of human tumor cell lines in culture. The compound has been tested against more than 100 cell lines including: breast BT20 and MCF-7; prostate DU145 and LnCAP; pancreatic MIA-PaCa2; brain U87; lung H157, H187, and N417; gastric AGS, RF1, RF48; colon DLD-1; uterine MES-SA; hepatoma BEL-7404, and in the entire NCI cancer screening panel of 60 cell lines. The spectrum of activity and the potential mode of action of ON 01910.Na appear to be different from other agents as indicated by employing the NCI's "Compare Analysis". The panel results and other cytology studies suggest that ON 01910.Na is a novel

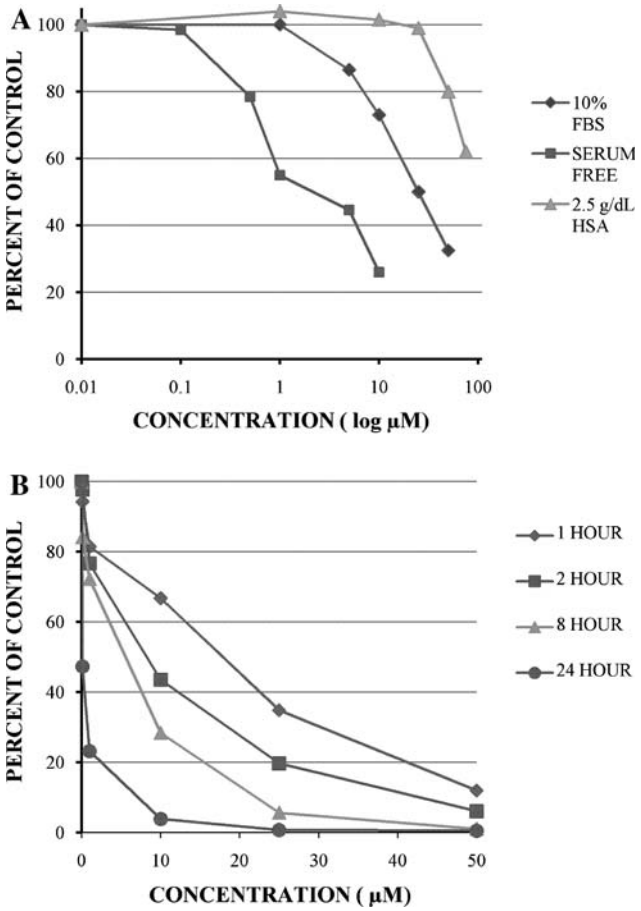

Fig. 6 Effect of FBS and human serum albumin (HSA) binding (**a**) and duration of exposure (**b**) on ON 01910.Na anti-tumor activity in DU145 cells. The data is plotted as the average percent compared to vehicle treated control cells (duplicate independent counts). Panel A represents data collected following 1 h exposure. Panel B contains data from studies with incubation medium containing FBS

antimitotic agent with many desirable properties. These results also promote the potential utility of ON 01910.Na as an anti-tumor medication in single agent- or -in combination therapy regimens.

Since all novel anticancer agents are first tested in single agent Phase I clinical trials in advanced cancer patients, pharmacokinetic and pharmacodynamic non-clinical studies are essential to help guide dose selection, choice of administration schedule, and design of appropriate dose escalation schemes. These studies can also contribute to the

design of optimal combination therapy regimens and schedules. The studies summarized in this report were carried out to meet these objectives.

Evaluation of the in vitro cancer cell cytotoxicity of ON 01910.Na was carried out following the protocol of the NCI 60 human tumor cell line anticancer drug screen developed in the late 1980s [8, 9]. The objective of this protocol is to elucidate the mechanisms of growth inhibition and tumor cell kill. The GI50 and LC50 values determined from those experiments reflect drug that exists predominantly in its unbound form. Since this assay uses 10% FBS in the medium, the results could potentially exaggerate the activity of a drug that is highly protein bound. Protein binding could also influence clearance from the circulation. The circulating free fraction of drug in plasma would not only have a direct effect on drug efficacy, but would also influence drug toxicity. Hence, it is important to relate the pharmacokinetics, plasma protein binding behavior (circulating free fraction of drug), and pharmacologic activity of the drug during the preclinical development phase. Such integration could be valuable in the choice of dose and schedule of administration of the drug in the clinical trials.

ON 01910.Na exhibits extensive protein binding in rat, dog, and human plasma, with lower binding in the mouse. Extensive plasma binding in vivo may impair drug distribution to the tissue of interest in the patient. Human plasma binding studies were conducted over a wide range of plasma concentrations of ON 01910.Na. The data from this saturation study was best described by a two-site binding model, with sites representing high affinity/low capacity binding and low affinity/high capacity binding. Based on molecular weight of ON 01910.Na (473 g/mol), the maximum binding capacity of human plasma is ~6 mM. Assuming an average plasma albumin level of 4 g/dL [0.6 mM], this corresponds to a high drug: albumin binding ratio (10:1). Characterization of protein binding is an important consideration in the development and evaluation of a highly plasma-bound anti-cancer agent as it helps establish target plasma concentrations for clinical efficacy.

Given the high plasma binding exhibited by ON 01901.Na, a systematic development program was initiated to evaluate the effects of protein binding and duration of exposure on in vitro activity against a representative human prostate cell line, DU145, which is part of the NCI panel. These studies demonstrated a significant decrease in drug activity as a function of protein binding in FBS and human serum albumin. For anti-cancer agents that exhibit significant plasma binding, in vitro cytotoxicity results will be influenced by protein binding, and this issue must be considered in the design of treatment schedules. For ON 01910.Na, which is ~98% bound in human plasma, activity is highly influenced by protein binding. In experiments, where the incubation medium was supplemented with human serum albumin, LC_{50} values increased 20-fold compared to serum-free conditions. Accordingly, this factor was incorporated into the clinical trial design for the compound.

Likewise, in vitro studies clearly demonstrate reduced cytotoxic activity of ON 01910.Na under conditions of limited drug exposure (e.g., <8 h). However, this reduced cytotoxicity at limited time exposure could be compensated by achieving higher drug concentrations (i.e., higher doses). A similar LC_{50} value is obtained by exposing cells to a 1 μM concentration of drug for 8 h compared to 0.1 μM of drug exposure for 24 h. This dose schedule flexibility could be useful in the clinical setting provided the drug has a relatively wide margin of safety.

The results of pharmacokinetic studies provide an overview of the disposition behavior of ON 01910.Na in animal models. Following intravenous dosing to mice, rats, and dogs, ON 01910.Na is rapidly eliminated from the plasma within the first several hours, with half-life estimates ranging from 0.404 to 0.870 h over this period. This is followed, however, by a second elimination phase, where plasma concentrations decline slowly, as shown in Fig. 3. The concentrations in this phase are low, representing <1% of the observed maximum concentration (C_{max}). Thus, there is minimal contribution of the terminal phase to the systemic exposure of ON 01910.Na. Furthermore, as demonstrated in Fig. 5, steady state plasma levels were reached within a few hours following continuous infusion of ON 01910.Na to dogs, and concentrations decline rapidly once the infusion is terminated. This behavior is indicative of a compound with a short plasma half-life. Therefore, while ON 01910.Na shows biphasic elimination across all species tested, the half-life estimates reported in the tables are based on data collected during the first several hours after drug administration, as this represents the functional half-life of ON 01910.Na [10].

Overall, ON 01910.Na pharmacokinetics was comparable across the three species tested. Clearance, the critical connection between the administered dose and drug exposure (AUC), was consistent among the mouse, rat, and dog. With increasing dose administered, both clearance and volume of distribution values decreased, contributing to non-linear pharmacokinetics. Although half-life was unaffected by dose, this is not unexpected as it is a dependent parameter; that is, half-life depends on both clearance and volume of distribution. In situations, where clearance and volume both decrease with increased dose, half-life will remain essentially unchanged.

Tissue distribution studies in mice indicate high drug accumulation in the liver relative to kidney, heart, and

brain. The tissue: plasma ratio for the brain, kidney, and liver increased over time following drug administration, suggesting a slow equilibration rate between plasma and these tissues, perhaps influenced by high plasma protein binding. Additionally, there was extensive hepatic sequestration of ON 01910.Na up to 8 h following drug administration, despite nearly complete elimination from the plasma during that time period.

Although these results suggest that ON 01910.Na is extracted mainly by the liver, biliary, and urinary excretion studies in rats showed renal excretion to be predominant at higher doses of ON 01910.Na, with hepatic clearance, the main route of drug elimination at lower doses. Since the dose of ON 01910.Na used in the mouse tissue distribution studies was relatively low (12.5 mg/kg), the finding of extensive hepatic uptake of ON 01910.Na in those experiments is consistent with the rat excretion studies. While extensive hepatic uptake of ON 01910.Na was identified, there is little evidence of in vivo conversion of ON 01910.Na to its potential metabolite ON 01500. Additional detailed metabolism studies are now underway to characterize the in vivo disposition of the drug.

Given the rapid elimination of ON 01910.Na noted across various species following IV bolus dosing, the pharmacokinetics of this compound were also evaluated following continuous IV infusion to dogs. The results confirm that ON 01910.Na is rapidly cleared from the plasma following administration by both IV bolus and long-term infusion. Whereas non-linear kinetics was noted upon administration of high IV bolus doses, linear kinetics was observed for longer infusions at the doses tested. Based on the short plasma elimination half-life of ON 01910.Na, drug administration via IV infusion will likely be needed to sustain adequate plasma levels in patients, which in turn will ensure sufficient drug exposure for cytotoxic activity.

In a recent Phase I dose escalation trial, patients were administered ON 01910.Na via IV infusion over a period of 2 h. The range of doses used in this study was between 80 and 4370 mg [11]. The maximum plasma concentrations of ON 01910.Na for the 80 and 4370 mg dose were 4.03 and 414 μg/mL, with clearance estimates of 9.1 and 3.7 L/h. The results of this clinical study are similar to preclinical data in dogs. When ON 01910.Na is administered to dogs at IV bolus doses from 10 to 50 mg/kg, mean estimates for C_{max} (7.92–192 μg/ml) and Cl (3.7–10 L/h, Table 2 assuming body weight of 10 kg) are consistent with the clinical data.

In conclusion, the relationship between pharmacokinetic and pharmacodynamic properties have been studied for ON 01910.Na, a novel, small-molecule anti-cancer compound with broad-spectrum anti-tumor activity against various tumor cells in vitro. These studies were carried out in support of a clinical testing program based on intravenous administration of this experimental drug. The results of preclinical pharmacokinetic studies indicate that ON 01910.Na is rapidly cleared from the plasma following IV administration with a short elimination half-life. ON 01910.Na shows extensive liver uptake, and biliary excretion is the predominant route of elimination. However, ON 01910.Na does not appear to be extensively metabolized in vivo. Due to the short half-life and rapid clearance of the drug, administration of ON 01910.Na by continuous IV infusion is a likely treatment option for cancer patients. Phase I clinical trials are being conducted to assess the effect of dosing schedule, ranging from 2 to 72 h of continuous intravenous infusion, on the pharmacokinetics of ON 01910.Na and its potential clinical benefit. Additionally, studies are underway to establish the extent of plasma protein binding at relevant dosing levels, and to further probe the hepatobiliary disposition of ON 01910.Na.

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