Published in final edited form as:

Cancer Chemother Pharmacol. 2012 August ; 70(2): 339–344. doi:10.1007/s00280-012-1877-z.

Murine toxicology and pharmacokinetics evaluation of Retinoic Acid Metabolism Blocking Agent (RAMBA), VN/12-1

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Abstract

Purpose—Novel retinoic acid metabolism blocking agent (RAMBA), VN/12-1, is a highly potent anti-cancer agent which induces autophagy. Its combination with autophagy inhibitor chloroquine (CHL) has been shown to synergistically enhance apoptosis in breast cancer cells. The purpose of this study was to determine the toxicity and pharmacokinetic profile of VN/12-1 and its combination with CHL.

Methods—Preliminary toxicology of VN/12-1 was determined using female SCID mice (n=4 for each group). ATRA was used for comparison. We selected four different doses of VN/12-1 and ATRA. Two of the doses were low and less frequent (2.5 and 5 mg/kg twice a week) and the remaining doses were high and more frequent (10 and 20 mg/kg every day). The dose of CHL was 50 mg/kg twice a week. For pharmacokinetic (PK) study, 20 mg/kg of VN/12-1 was injected subcutaneously (s.c.) into the mice and their plasma was collected at various intervals (n=2) and analyzed by HPLC.

Results—The lower and less frequent doses of VN/12-1 and ATRA were found to be least toxic. However, high and more frequent doses of these compounds were toxic to the mice. PK results showed that VN/12-1 has a half-life of 6 hours. The area under the curve (AUC) for VN/12-1 was 83.78 hr*μg/ml.

Conclusions—VN/12-1 and ATRA are non-toxic when used as 5 mg/kg twice a week as single agents or in combination with CHL. The favorable PK properties of VN/12-1 can potentially be used for its further advanced pre-clinical and clinical development.

Keywords

VN/12-1; toxicology; pharmacokinetics; chloroquine; RAMBAs

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Disclosure of Potential Conflict of Interest

Vincent C. O. Njar holds an ownership interest in the RAMBAs patents and technologies thereof. The other authors declare no potential conflict of interest

Introduction

All-*trans* retinoic acid (ATRA) is an effective differentiating agent that has proven to be useful against cancer progression. It has been tested as an anticancer agent for various cancers including breast cancer [1]. However, several factors compromise the extensive clinical use of ATRA, such as low *in vitro* anticancer potency, limited bioavailability, and unfavorable pharmacokinetic profiles due to rapid metabolism by cytochrome P450 enzymes [2, 3]. Accordingly, the structural modification of ATRA to develop novel retinoic acid metabolism blocking agents (RAMBAs) with improved potency and metabolic stability has been the focus of our group [4-12].

VN/12-1, a novel RAMBA discovered in our laboratory, shows enhanced cancer cell growth inhibition over ATRA against both estrogen receptor α (ER- α) positive and negative breast cancer cell lines. The structures of VN/12-1 and ATRA are shown in **Fig. 1a**. We recently showed that VN/12-1 has dual effects on the cancer cells based on the dose [13, 14]. Low dose ($< 10 \mu\text{M}$) of VN/12-1 induces autophagy, endoplasmic reticulum stress (ERS) and cell cycle arrest as an immediate protective response in SKBR-3 cells and xenograft tumors. Importantly, we demonstrated that inhibition of autophagy using either pharmacological inhibitors (e.g. CHL) or RNA interference of essential autophagy gene Beclin-1 potentiates apoptotic cell death induced by VN/12-1. Notably, VN/12-1 produced impressive tumor inhibitory effects when used alone or in combination with CHL ($p < 0.001$). These findings strongly suggested that inhibition of autophagy may enhance the therapeutic efficacy of VN/12-1 in the treatment of breast cancer. This study provided the first evidence that a RAMBA was effective against ER α deficient breast cells and tumors, activates autophagy in breast cancer cells and that such RAMBA-induced autophagy can be exploited as a target to achieve potent anti-cancer activity in drug-resistant breast cancer [13, 14].

With such impressive results, suggesting that impeding autophagy enhances the therapeutic efficacy of VN/12-1 in the treatment of breast cancer, we deemed it necessary to determine the toxicological and pharmacokinetic effects of VN/12-1 alone and in combination with CHL. Ideal results would yield a non-toxic, yet, effective dosage of VN/12-1 with CHL that has an acceptable maximum plasma concentration, time to achieve the maximum plasma concentration, and elimination half-life. Therefore, it is imperative to define the toxicity profile and pharmacokinetic parameters so that therapeutic activity and dosing frequency are established early in anti-cancer drug development process.

In this study, we discuss the murine preliminary acute toxicological profiles of VN/12-1 and ATRA and also pharmacokinetic (PK) parameters of VN/12-1. The data obtained from this study will allow us to optimize further development of VN/12-1.

Methods and Materials

Chemicals and reagents

ATRA was purchased from LKT Laboratories Inc. (St Paul, MN, USA). Chloroquine was purchased from Sigma Aldrich. VN/2-1 (internal standard) and VN/12-1 were synthesized in our laboratory as previously described [10].

Animal handling

All animal studies were performed according to the guidelines and approval of the Institutional Animal Care Committee of the Thomas Jefferson University, Philadelphia. Indeed, all animals were handled in accordance with the Principles of Laboratory Animal care (<http://www.history.nih.gov/laws>). Female SCID mice (4–6 weeks old) weighing 20–25 g were obtained from NCI (Frederick, MD, USA) and were maintained in a controlled

environment of light, humidity, and temperature and were provided food and water ad libitum.

Toxicological Study

We selected four different doses and administration schedules of VN/12-1 and ATRA. Two of the doses were low and less frequent (2.5 and 5 mg/kg twice a week) and the remaining doses were high and more frequent (10 and 20 mg/kg every day). The dose of CHL was 50 mg/kg twice a week, based on previous studies by several research groups [15, 16]. Each group consisted of four mice. All the doses were administered subcutaneously (s.c.) for 14 days. Clinical signs of toxicity (mortality, alopecia at injections sites, scaly skin, and weight loss) were observed and the maximum tolerated dose (MTD) was determined following established procedures [11, 17]. Toxicities observed during the course of study were scored according to severity. Dosing with ATRA served as a reference. The degree of ATRA or VN/12-1 toxicities in each animal at weighing was scored using the rating scale described before [11, 16, 17]. As shown in **Table 1**, three physical parameters were scored on a scale of 0-4 as follows: (1) weight loss: 10g = 4, 9-7g = 3, 6-4g = 2, 3-1g = 1, <1g = 0; (2) alopecia at injection site: very severe = 4, severe = 3, moderate = 2, slight = 1, none = 0; (3) skin scaling: very severe = 4, severe = 3, moderate = 2, slight = 1, none = 0.

Pharmacokinetic study

Dosing and sampling—Female SCID mice were used for PK studies (n = 2 per time point). Mice were administered a single 20 mg/kg dose subcutaneously of VN/12-1 formulated in 3% hydroxypropylcellulose and blood was collected at different time points ranging from 30 min to 21 hours after drug administration. Blood was collected in heparinized tubes after retroorbital puncture using light halothane for anesthesia. The plasma was separated and stored at -20°C until HPLC analysis.

Sample preparation—Sample preparation for various agents involved a liquid-liquid extraction method using VN/2-1 as an internal standard. Two hundred microliters of plasma sample was spiked with 15 µg/ml of internal standard and extracted with 300 µl of ethyl acetate + 10% methanol. Samples were vortexed and supernatant was transferred to another tube and dried under nitrogen gas. The samples were reconstituted in 50 µl of methanol, passed through 0.22 µm syringe and 10 µl was injected onto the HPLC system. Calibration samples were prepared by spiking control mice plasma with various concentrations of agents (5–30 µg/ml) and processed and analyzed in the same manner as described above.

HPLC bioanalytical conditions—The HPLC system from Waters, Milford, MA, USA was used for *in vivo* sample analysis. Chromatographic separation was achieved on a reverse phase C18 column (3.5 µm × 4.6 mm × 75 mm) (Novapak) using the gradient solvent system mobile phase of methanol: water (60:40) with 20 mM ammonium acetate buffer (100 to 0%) at 0.8ml/min for 20min. For the next 10 min the mobile phase was 100% methanol at 1ml/min, during which time retinoids were eluted. For the remaining 5 min the mobile phase was methanol: water (60:40, v/v) with 20 mM ammonium acetate (0 to 100%) at a flow rate of 0.8 ml/min. The wavelength for detection had a lambda max at 350 nM. The analysis of the pharmacokinetics was done using non-compartmental WinNonLin model.

Results

Toxicological evaluations

The compounds evaluated and their respective doses are described in the experimental section [15, 16]. There was no mortality in the groups treated with ATRA. Groups which were administered 10mg/kg and 20mg/kg of VN/12-1 had mortality rates of 75% and 100%

(Table 1). However, the lower doses of VN/12-1 did not result in any mortality as single agent or in combination with CHL. There was grade 1 and grade 3 alopecia at the injection site and skin scaling for 10 mg/kg and 20 mg/kg doses of daily ATRA treatment respectively. Severe alopecia (grade 3 and grade 4) at the injection site and skin scaling (grade 2 and grade 4) was observed with the 10 mg/kg and 20 mg/kg daily doses of VN/12-1 treatment. No significant signs of toxicity were observed in mice injected with 2.5mg/kg of VN/12-1 twice per week and there was minor alopecia at the injection site and scaly skin in mice treated with 5mg/kg of VN/12-1. CHL alone did not contribute to signs of toxicity in the mice. The combination of CHL with ATRA or with 2.5 mg/kg twice a week of VN/12-1 resulted in no significant toxicity. The combination of CHL with 5 mg/kg VN/12-1 twice a week resulted in grade 1 minor skin toxicity. 10 and 20 mg/kg every day treatment of ATRA and VN/12-1 resulted in loss of body weight. However, the lower doses did not result in any significant loss of weight.

In summary, ATRA and VN/12-1 when used in high (10 and 20 mg/kg) and more frequent dosing (daily) resulted in toxicity in the mice. However, the lower doses and less frequent administration of both ATRA and VN/12-1 did not produce significant toxicity either alone or in combination with CHL.

Pharmacokinetics

The pharmacological disposition and metabolism of an agent are important determinants of its pharmacodynamics activity and play a critical role in the development of an optimal dosing regimen. In our preliminary PK studies, female SCID mice were administered 20 mg/kg dose of RAMBA subcutaneously. The dose was determined based on the apoptotic dose of VN/12-1 in the xenograft studies [13, 14]. Plasma was collected and reverse phase HPLC analysis was performed to obtain the PK profile of VN/12-1. Non-compartmental WinNonLin model was used for the analysis of PK properties.

Various PK parameters such as $t_{1/2}$, t_{max} , C_{max} and AUC for the plasma concentration *versus* time profile of VN/12-1 (retention times (t_R = 15.2 min)) were determined. Following subcutaneous administration of VN/12-1, there was an initial increase in plasma concentration with a maximum concentration (C_{max}) of 41.38 μ g/ml. The time taken to achieve maximum plasma concentration (t_{max}) was 2 hours (Fig. 1b). After 2 hours the plasma concentration declined exponentially with a mean $t_{1/2}$ of 6 hours. A $t_{1/2}$ as high as 6 hours after subcutaneous injection suggests that VN/12-1 stays in the body of the mice for a long time. The plasma concentration-time curves after s.c. administration of VN/12-1 to female mice and polar metabolites thereof are shown in Fig. 1b. Whether this is due to increase in tissue binding or adipose tissue absorption of the compound will be explored in our future studies. PK profile of the three polar metabolites (retention times (t_R) of 9.5, 8.4 and 7.5 minutes, respectively) was also determined. The metabolites rose to a level of 11.98 μ g/ml in 3 hours post-injection. Their $t_{1/2s}$ were comparable to that of VN/12-1. The area under the curves (AUCs) of VN/12-1 and the polar metabolites were 83.78 and 89.24 hr* μ g/ml, respectively. Of the three metabolites peaks, one has a retention time of 9.5 min which is identical to that of VN/14-1, the corresponding carboxylic acid of VN/12-1. We will attempt to identify these VN/12-1 metabolites using rigorous LC-MS techniques in future studies.

Discussion

Due to the heterogeneity and potential for development of resistance in breast cancer, effective treatment should consist of combination therapies or multi-targeted agents. We previously demonstrated that autophagy serves as a protective mechanism in SKBR-3 cells and its inhibition can be exploited to enhance apoptosis. Discovering that our novel RAMBA, VN/12-1, significantly decreases breast cancer cell proliferation alone and in

combination with CHL *in vitro* encouraged us to conduct an *in vivo* study. In this study, we performed preliminary toxicological analysis and determined the pharmacokinetic profile of VN/12-1.

The lower and less frequent doses (2.5 mg/kg and 5 mg/kg twice a week) of VN/12-1 were found to be less toxic as single agent or in combination with CHL. However, the higher and more frequent doses (10 mg/kg and 20 mg/kg everyday) resulted in significant toxicity in mice. The maximum plasma concentration, 41.38 µg/ml was obtained after two hours. Importantly, VN/12-1 and its metabolites were obtained from the serum samples even after 21 hours of injection which indicates that VN/12-1 has very long $t_{1/2}$ and it stays in the body for a long time. The relative stability of VN/12-1 may be due to its steric and/or electronic nature of the polyene retinoidal moiety. Indeed, there are literature precedents of unique *in vitro* and *in vivo* stability of alkyl esters. Our literature survey revealed that the structurally similar synthetic aromatic retinoid, etretinate (ethyl ester prodrug of clinically used acitretin) is reported to possess *unexpected long half-life* in animals [18] and also in humans [19]. In addition, Sporn and colleagues recently reported of the unique stability of the *methyl ester* of 2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oic acid (CDDO-Me), a compound currently in several clinical trials [20].

In summary, the present study describes the toxicology and pharmacokinetics of VN/12-1, a novel RAMBA in mice. Although high doses of the compound were toxic, lower doses and less frequent dosing were found to be safe. After s.c. administration, plasma concentration-time profile obtained clearly shows that VN/12-1 has an excellent pharmacokinetic profile. The anti-tumor efficacy of VN/12-1 (*vide supra*) and this preliminary toxicity/pharmacokinetic evaluation provide a strong rationale for further development of VN/12-1 as single agent or in combination with autophagy inhibitors as potential new therapy for breast cancer.

Acknowledgments

This research study was supported by grants from the US Department of Defense under the Peer Reviewed Medical Research Program (PRMRP, W81XW-04-1-0101, Njar, VCO), the US National Institutes of Health and National Cancer Institute (NIH/NCI, 1R01 CA129379-01A2 and 5R01 CA129379-02, Njar, VCO). We thank all these agencies for their generous support.

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Fig 1a.
Chemical structures of ATRA, VN/2-1 (internal standard), VN/12-1, VN/14-1 and chloroquine.

Fig.1b.

Pharmacokinetic profiles of VN/12-1 and metabolites following a single subcutaneous bolus dose (20 mg/kg) to female SCID mice.

Toxicological evaluation

Table 1

Compound	Dose		Clinical Observations			Mean change in body weight	Total score
	Mg/kg	Frequency	Mortality	Alopecia at injection site	Scaly skin		
Vehicle	-	daily	- ^A	-	-	+1.28	0
ATRA	2.5	twice/week	-	-	-	+2.4	0
	5	twice/week	-	-	-	+1.5	0
	10	daily	-	4/4 (1)	4/4 (1)	-1.6 (1)	1
	20	daily	-	4/4 (3)	4/4 (3)	-3.2 (2)	8
VN/12-1	2.5	twice/week	-	-	-	+2.43	0
	5	twice/week	-	4/4 (1)	2/4 (1)	-0.23	2
	10	daily	3/4	4/4 (3)	4/4 (2)	-2.6 (1)	6
	20	daily	4/4	4/4 (4)	4/4 (4)	-4.2 (2)	10
CHL	50	twice/week	-	-	-	+1.03	0
	50	daily	-	-	-	+1.5	0
ATRA + CHL ^B	5	mice/week	-	-	-	+0.43	0
VN/12-1	2.5	mice/week	-	-	-	+1.5	0
+CHL ^B	5	mice/week	-	4/4 (1)	2/4 (1)	-1.43 (1)	3

Effects of VN/12-1, ATRA and CHL on body weight and clinical observations at day 14 of the study

Data are presented as the number of mice exhibiting clinical observations/number of mice in the dose group. The numbers in parentheses indicate the range of severity of the indicated lesion, according to the scale indicated above.

^A Indicates that no death or signs of toxicity were found in any animal within the group of four.

^B CHL (chloroquine) dose = 50 mg/kg.