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ORIGINAL ARTICLE

Murine toxicology and pharmacokinetics of novel retinoic acid metabolism blocking agents

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Abstract
Purpose Novel potent C-4 azolyl retinoic acid metabolism blocking agents (RAMBAs)—VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1, have been synthesized and investigated for their in vitro and in vivo effects against breast and prostate cancers. These RAMBAs, in addition to being potent inhibitors of all-*trans*-retinoic acid (ATRA) metabolism have potent anti-cancer properties and in vivo anti-tumor efficacies as characterized in breast and prostate cancer models. Here we determined the toxicity and pharmacokinetics (PK) of these various RAMBAs.
Methods Preliminary acute toxicity studies of these RAMBAs were carried out using Swiss NIH mice. The toxicity profile of the RAMBAs was evaluated relative to ATRA. Three different doses (8.3, 33, and 100 μmol/kg/day) of ATRA and RAMBAs were administered on a daily basis subcutaneously for 14 days to the mice. Clinical signs of toxicity alopecia, scaly skin, and loss of body weight in the mice were observed during the study and the maximum tolerated dose was determined. PK of selected agents (VN/14-1, VN/50-1, and VN/66-1) was studied in Balb/C mice after a single dose subcutaneous administration. Plasma concentrations of the agents were quantitatively determined using a high-performance liquid chromatographic method with ultraviolet detection. Plasma concentration versus time profiles were fit to various PK structural models and relevant PK parameters were estimated.
Results VN/66-1 and VN/69-1 were found to be the least toxic even at the highest doses when compared to the other RAMBAs and ATRA. VN/66-1 had the longest half-life, the slowest clearance, and the greatest exposure.
Conclusions Based on PK characteristics and toxicity studies, VN/66-1 appeared to be the most favorable agent. However, both VN/14-1 and VN/66-1 are our leads based on the fact that VN/14-1 has been found to be highly effective in endocrine-sensitive and -resistant breast cancer cells and tumors with little toxicity. Our findings provide valuable information that will be used to select RAMBAs and establish therapeutic regimens that provide optimal efficacy with minimal toxicity.

Jyoti B. Patel and Aakanksha Khandelwal contributed equally to this work.

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Keywords Retinoic acid metabolism blocking agents · Retinoids · Anti-cancer agents · Pharmacokinetics · Toxicology

Introduction

The ability of all-*trans*-retinoic acid (ATRA) and other retinoids to modulate a variety of important functions like cell growth and differentiation, induction of apoptosis and prevention of angiogenesis is well documented [2, 5, 7, 8]. They are effective in the prevention and therapy of a number of proliferative diseases including breast and prostate cancers. However, the clinical use of ATRA in the treatment of malignancies is significantly hindered by the prompt emergence of resistance, which is believed to be caused at least in part by increased ATRA metabolism [9, 15, 17, 18]. The use of high dose ATRA is limited due to toxic and tetratogenic effects [1, 6]. Others [15] and ourselves [16–19] have suggested the use of retinoic acid metabolism blocking agents (RAMBAs) as a viable strategy to increase the endogenous levels and thus potentiate the effect of ATRA without the need for high exogenous doses of ATRA. Indeed, RAMBAs may be used alone or in combination with low doses of ATRA. RAMBAs may prove useful for the chemoprevention and/or treatment of different cancers and also for the treatment of dermatological diseases [15, 18].

Few groups have synthesized and studied non-retinoidal RAMBAs [24–28] and research on retinoidal RAMBAs is even less. Our RAMBAs are structural analogues of ATRA (VN/14-1, VN/50-1), 13-*cis* RA (VN/67-1, VN/69-1) and 4-HPR [*N*-(4-hydroxyphenyl) retinamide; fenretinide] (VN/66-1; Fig. 1). An analog of 4-HPR was made based on previous studies which have found that 4-HPR possesses a longer half-life than either ATRA or 13-*cis* RA [14]. Furthermore, 4-HPR has been previously determined to have lower toxicity than ATRA, thus making it a favorable compound to optimize. These RAMBAs, which have been synthesized in our laboratory, differ from each other by the structural modification at the end of the side chain [16–19]. We have reported previously that these RAMBAs, like ATRA appear to have pleiotropic properties apart from their main mode of action of inhibiting ATRA metabolism in intact breast and prostate cancer cells and microsomes. RAMBAs are able to inhibit breast and prostate cancer cell growth, induce differentiation and apoptosis and have anti-tumor efficacies in vivo in mice bearing human breast and prostate cancer xenografts [3, 11, 19, 20].

For clinical therapeutic and chemopreventive use of any compound, it must not only be effective, but also safe. Therefore, it is essential that we determine the toxicity profiles of compounds early in the drug discovery stage to enable the development of those which are non-toxic. The pharmacological disposition and metabolism of an agent are important in determining its therapeutic activity and helps to decide the frequency of dosing. In this paper we discuss the murine preliminary acute toxicological profiles of several RAMBAs and pharmacokinetic (PK) parameters of three RAMBAs. The data obtained from this study will allow us to optimize the structures, disposition profiles and in vivo activities of promising RAMBAs.

Materials and methods

Chemicals and reagents

All-*trans*-RA and 13-*cis* RA (internal standard) were purchased from LKT Laboratories Inc., St Paul, MN, USA. The RAMBAs, VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1 and VN/2-1 (internal standard) were synthesized in our laboratory as previously described [19]. Methanol, acetonitrile, ethyl acetate,

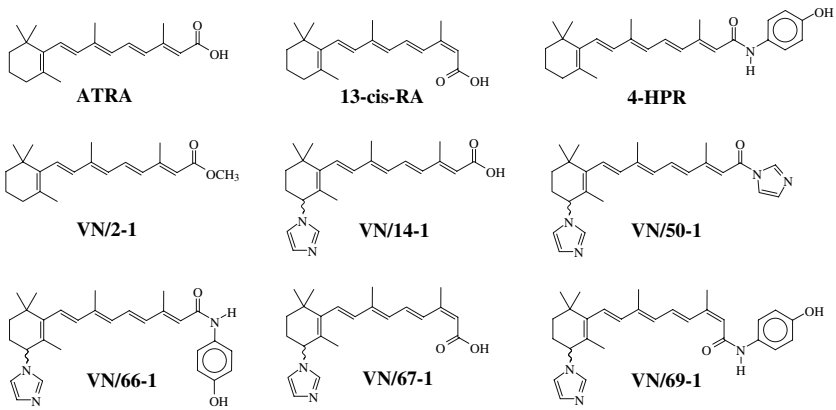

Fig. 1 Chemical structures of retinoids and RAMBAs, ATRA, 13-cis-RA, 4-HPR, VN/2-1, VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1

ammonium acetate, and water were purchased from Fisher Scientific, Fair Lawn, NJ, USA. Formic acid, butylated hydroxyl anisole, and hydroxypropyl-β-cyclodextrin (HPβCD) were purchased from Sigma-Aldrich, St Louis, MO, USA. All chemical and solvents were of analytical or high-performance liquid chromatographic (HPLC) grades.

Although the retinoidal compounds and RAMBAs appeared to be relatively stable to light, precautions were taken to minimize exposure to any light source and to the atmosphere. Thus, all operations were performed in dim light, with reaction vessels wrapped with aluminum foil. All compounds were stored in an atmosphere of argon and in the cold (−20 or −80°C) and dark without significant decomposition.

Animal handling

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland School of Medicine and followed the NIH guidelines as well. Female NIH Swiss mice (4–6 weeks old) and female Balb/c mice (4–6 weeks old, weighing 20–25 gm) were obtained from NCI (Frederick, MD, USA) and were maintained in a controlled environment of light, humidity, and temperature and were given food and water ad libitum.

Toxicology study

Female NIH Swiss mice were used for a 14 day toxicity study in which the mice were given different doses of ATRA or RAMBAs (VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1) (Fig. 1). Three different doses, i.e., 8.3, 33, and 100 μmol/kg/day (formulated in 0.3% hydroxypropyl cellulose in saline) of each compound were administered on a daily basis subcutaneously for 14 days. Each group consisted of five mice. Clinical signs of toxicity (alopecia, scaly skin, and loss of body weight), were observed during the 14 days of dosing and the maximum tolerated dose (MTD) was determined following established procedures [12, 13, 23]. Dosing with ATRA served as a reference. The MTD was essentially the highest dose at which no mortality was observed. Toxicities observed during the course of the study were scored according to their severities. The degrees of ATRA/RAMBA toxicities in each animal at weighing were scored using the rating scale described by Bollag [4]. Three physical parameters were scored on a scale of 0–4 as follows: (1) Weight loss: (10 g = 4, 7–9 g = 3, 4–6 g = 2, 1–3 g = 1, <1 g = 0; (2) hair loss (alopecia): 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. The total score for each animal was obtained by adding the three individual scores. Scores are ranked as none (score = 0), mild (score = 1–3), moderate (4–6), and severe (7+).

Pharmacokinetic study

Dosing and sampling

Female Balb/c mice were used for PK studies ($n = 2$ per time point). Mice were administered a single 10 mg/kg dose subcutaneously of RAMBA formulated in 45% HPβCD in water, and blood was collected at different time points ranging from 5 min to 12 h after drug administration. Blood was collected in heparinized tubes after cardiac puncture (VN/14-1) or retro-orbital puncture (VN/50-1 and VN/66-1) 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/1-2 (for VN/14-1) or 13-*cis* RA (for VN/50-1 and VN/66-1) as an internal standard. Two hundred microliters of plasma sample was spiked with 1 μg/ml (VN/1-2) or 0.5 μg/ml (13-*cis* RA) of internal standard and extracted with 2 ml of ethyl acetate + 10% methanol + 0.05% butylated hydroxyl anisole. Samples were vortexed and supernatant was transferred to another tube and dried under nitrogen gas. The samples were reconstituted in 100 μl of methanol, passed through 0.22 μm syringe filters and 50 μl was injected onto the HPLC system. Calibration samples were prepared by spiking control mice plasma with various concentrations of agents (1–10 μg/ml) and processed and analyzed in the same manner as described above.

HPLC bioanalytical conditions

The HPLC system consisted of a—1535 pump, 717 autosampler, and 996 detector (Waters, Miliford, MA, USA). Chromatographic separation was achieved on a reverse phase C_{18} column (3.9 mm × 150 mm × 5 μm) (Novapak) using a gradient mobile phase of various combinations of 20 μM ammonium acetate buffer and methanol at a flow rate of 0.8 ml/min for detection of VN/14-1. A mobile phase of 75% acetonitrile, 25% water, 0.5% formic acid, and 1 mM ammonium acetate at a flow rate of 1.2 ml/min was use for detection of VN/50-1 and VN/66-1. The eluate was monitored with

a UV detector set at an absorption maximum of 350 nm. Calibration curves were prepared by plotting peak area versus spiked concentration. Concentration of analytes in PK samples was obtained by simple linear regression analysis of calibration samples.

Data analysis

The lower limit of quantification (LLOQ) of the assay was determined from calibration samples. Concentrations below the LLOQ were not considered for PK modeling. Compartmental modeling was performed using non-linear regression software, WinNonlin (ver. 4.1, Pharsight Corporation, Mountain View, CA, USA). Various weighting schemes were applied to determine the best model. The goodness of fit of competing models was assessed by the Akaike information criterion (AIC), diagnostic plots, variance, and random distribution of residuals. Weighting schemes were assessed in a similar manner except that weighted sum of squares residual was examined in place of the AIC. The values for area under the curve (AUC) and other PK parameters such as clearance (CL), volume of distribution (V_d), and half-life ($t_{1/2}$) were obtained from the WinNonlin output.

Results

Toxicological evaluations

Six compounds were examined for toxic effects in female Swiss mice. Three different doses (8.3, 33, and 100 μmol/kg/day) were examined for each compound with each dosage group consisting of five mice. It is important to note that the number of moles administered was taken into account when determining the dose for each compound. Each compound was subcutaneously administered daily for 14 days, during which clinical signs of toxicity were recorded. Alopecia, scaly skin, mean change in body weight, and mortality were assessed. At the completion of the study the MTD was determined. ATRA, which has known toxic effects, was used as a reference for which to compare the five RAMBAs (VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1). Toxic events were scored on the basis of severity with 1–3 being mildly severe, 4–6 being moderately severe and above 6 as being very severe. A total score was then computed based on the occurrence of individual toxic events at each dose, with a higher score corresponding to higher toxicity. As expected, a greater score was seen with increasing doses for each of the compounds examined. Two compounds which did not exhibit any toxic or deleterious effects at the 8.3 and 30 μmol/kg/day, did exhibit skin scaling at the highest dose, 100 μmol/kg/day (VN/66-1 and VN/69-1) as shown in Table 1. Furthermore, it is important to note that there was a lack of weight loss even after administration of the highest dose with VN/66-1 and VN/69-1, where all the other compounds induced some loss in weight. Alopecia was seen in the two higher doses of ATRA and VN/50-1 and in the 100 μmol/kg/day dose of VN/14-1 and VN/67-1. Scaly skin was found to be the predominant form of toxicity in all of the groups tested. In the various dosage groups, if one mouse exhibited scaly skin or alopecia the others were observed to do the same. To clarify, there was no partial response among the groups, for example for these two toxic events either 0/5 or 5/5, but never 1, 2, 3, or 4, out of 5 mice were observed to have the condition, however, the severity did differ among the groups. The greatest severity of skin scaling and weight loss was seen in the VN/14-1 and VN/50-1 treated mice. Mortality at the highest dose was also seen in these two groups resulting in the death of all five mice at 100 μmol/kg/day. It was found that VN/14-1 and VN/50-1 were the most toxic of the five RAMBAs tested and VN/66-1 and VN/69-1 were the least toxic.

Pharmacokinetics

The pharmacological disposition and metabolism of an agent are important determinants of its pharmacodynamic activity and play a critical role in the development of an optimal dosing regimen. Different PK parameters such as $t_{1/2}$, CL, V_d, and AUC for the plasma concentration versus time profile of different RAMBAs were determined. Three RAMBAs were used for the PK studies, VN/14-1, VN/50-1, and VN/66-1. These three were chosen out of the six compounds examined for toxicity based on previous research done in our lab showing that these three compounds were most potent in vitro in various breast and prostate cancer cell lines.

Balb/c mice were administered 10 mg/kg dose of RAMBA subcutaneously. The dose was determined from the toxicity studies, at this dose there were minimal or no toxic effects of the compounds. Blood plasma was collected and reverse phase HPLC analysis was performed to obtain the PK profile of the various RAMBAs All compounds fit a one compartment model with first order elimination and flip-flop kinetics, where the rate of absorption is equal to the rate of elimination. The mean plasma concentration–time profile of VN/14-1 is shown in Fig. 2a. Following subcutaneous administration of VN/14-1, there was an initial increase in plasma

Table 1 Effects of ATRA and RAMBAs on body weight and clinical observations at termination of study

Compound	Dose level		Clinical observations[a]			Mean body weight change (g)	Total score
	Mg/kg/day	μmol/kg/day	Mortality	Alopecia	Scaly skin		
Vehicle	0	0	–	–	–	+0.11	0
ATRA	2.5	8.3	–	–	5/5 (1)[b]	−0.12	1
	10.0	33.0	–	5/5 (2)	5/5 (2)	+0.004	4
	30.0	100.0	–	5/5 (3)	5/5 (3)	−5.28 (2)	8
VN/14-1	3.1	8.3	–	–	–	+1.04	0
	12.2	33.0	–	–	5/5 (2)	−1.26 (1)	3
	36.6	100.0	5/5	5/5 (4)	5/5 (4)	−6.39 (3)	11
VN/50-1	3.5	8.3	–	–	–	+1.04	0
	13.9	33.0	–	5/5 (2)	5/5 (2)	−1.26 (1)	5
	41.6	100.0	5/5	5/5 (4)	5/5 (4)	−5.68 (3)	11
VN/66-1	3.8	8.3	–	–	–	+1.13	0
	15.2	33.0	–	–	–	+0.37	0
	45.7	100.0	–	–	5/5 (1)	+2.13	1
VN/67-1	3.1	8.3	–	–	–	+1.08	0
	12.2	33.0	–	–	5/5 (1)	−0.22	1
	36.6	100.0	–	5/5 (2)	5/5 (2)	−3.09 (1)	2
VN/69-1	3.8	8.3	–	–	–	+0.95	0
	15.2	33.0	–	–	–	+0.06	0
	45.7	100.0	–	–	5/5 (1)	+1.09	1

– indicates that no death or signs of toxicity were found in any animal within the group of five

[a] Data are presented as the number of mice exhibiting clinical observations/number of mice in the dose group

[b] The numbers in parentheses indicate the range of severity of the indicated lesion, according to the scale indicated above

concentration with a maximum plasma concentration (C_{max}) of 4.3 μg/ml at a time taken to achieve maximum plasma concentration (t_{max}) of 30 min (Table 2). After 30 min the plasma concentration declined exponentially with a mean $t_{1/2}$ of 0.34 h and CL of 1,720 ml/h/kg. VN/50-1 on the other hand was not absorbed as fast and to the same extent as VN/14-1 (Fig. 2b). VN/50-1 also had a faster rate of CL (1,924 ml/hr/kg) and a longer $t_{1/2}$ (0.57 h) thus is not as favorable as VN/14-1. Furthermore, between 0.5 and 2 h (Fig. 2b), the plasma concentration of VN/50-1 appears to have reached a pseudo steady-state condition because the concentrations appear to be saturated and not undergoing elimination. At these time points the concentrations are not near the LLOQ, thus we can assume that the model is accurately predicting the kinetics of VN/50-1. VN/66-1 was found to possess the most favorable PK out of the three compounds examined (Fig. 2c). VN/66-1 had the greatest exposure, having a much higher C_{max} (12.32 μg/ml) and greatest AUC (41.3 h·μg/ml) compared with VN/14-1 and VN/50-1, which both had AUC values less than 6 h·μg/ml. VN/66-1 also showed the slowest elimination as measured by CL and $t_{1/2}$, 242 ml/hr/kg and 0.85 h, respectively. The relative highness of the last two points (6 and 12 h) was very close to the LLOQ and was most likely measured with a fair amount of background noise. Thus, the prediction is not highly influenced by these two points for VN/66-1.

Discussion

Toxicity as determined by alopecia, skin scaling, loss of body weight and ultimate mortality in mice was lowest for VN/66-1 and VN/69-1. VN/14-1 and VN/50-1 had the highest toxicity scores and displayed equal toxicity

Table 2 Pharmacokinetic parameters of various RAMBAs in mice after a single s.c dose of 10 mg/kg

Compound	C_{max} (μg/ml)	t_{max} (h)	$t_{1/2}$ (h)	AUC (h·μg/ml)	V_d (ml/kg)	CL (ml/h/kg)
VN/14-1	4.32	0.50	0.34	5.81	852	1,720
VN/50-1	2.31	0.83	0.57	5.20	1,594	1,924
VN/66-1	12.32	1.23	0.85	41.30	299	242

C_{max} maximum plasma concentration, t_{max} time taken to achieve C_{max}, $t_{1/2}$ elimination half-life, AUC area under the curve, V_d volume of distribution, CL clearance

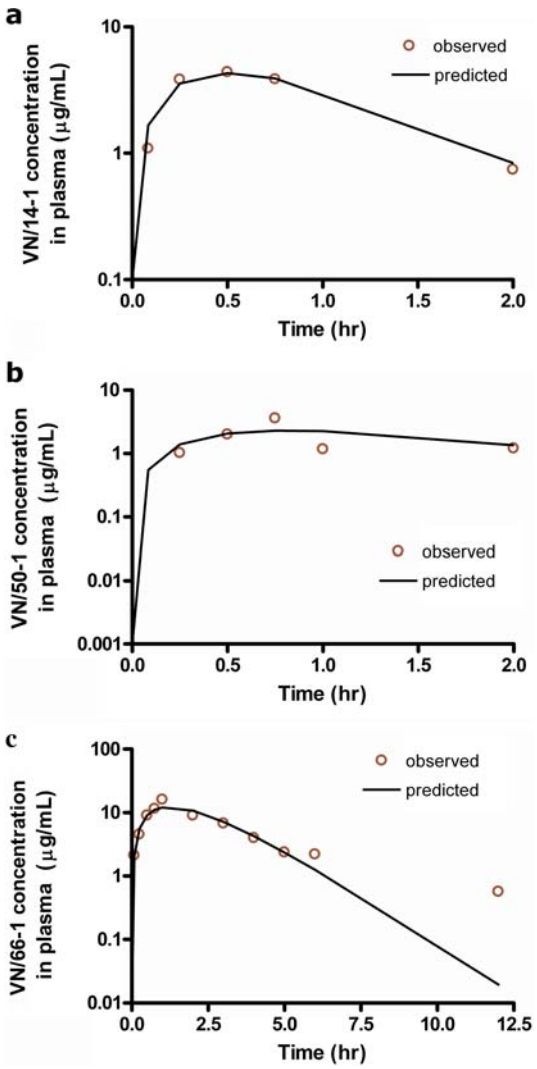

Fig. 2 Plasma concentration–time profiles of VN/14-1 (**a**), VN/50-1 (**b**), and VN/66-1 (**c**) after s.c. administration of a 10 mg/kg dose in Balb/c mice. Observed values are presented with the predicted values. Values represent the mean plasma concentrations from two mice per time point

for each clinical observation, including weight loss. Interestingly, both of these compounds displayed higher toxicity than their parent compound ATRA, which was used as a reference. VN/67-1 displayed relatively low toxicity and was very similar but not as good as VN/66-1 and VN/69-1. These results suggest that analogs of 4-HPR exhibit the lowest toxicity. Furthermore, the two most toxic compounds, VN/14-1 and VN/50-1 and the least toxic compound, VN/66-1 are the three which showed the greatest ability to inhibit breast and prostate cancer cell proliferation as determined by previous experiments in our lab. It is for this reason that VN/14-1, VN/50-1, and VN/66-1 were chosen for the PK study. At the conclusion of the toxicity study, it was found that ATRA, VN/66-1, VN/67-1, and VN/69-1 all had a MTD of 100 μmol/kg/day. VN/14-1 and VN/50-1, the most toxic RAMBAs, were found to have a MTD of 33 μmol/kg/day. However, it should be noted that we have shown in recent anti-tumor xenograft studies in nude mice that VN/14-1 up to 66 μmol/kg/day was not toxic [3, 19, 20]. In contrast, R116010, a non-retinoidal RAMBA in clinical develop was found to have a MTD of 5 mg/kg or 13.25 μmol/kg in syngeneic A/J mice [25].

Pharmacokinetic studies were carried out with VN/14-1, VN/50-1, and VN/66-1 after a single subcutaneous dose of 10 mg/kg. Though VN/14-1 and VN/50-1 showed high toxicity, we wanted to examine the difference in the PK of these two compounds and VN/66-1, a non-toxic compound. The PK parameters for VN/14-1 in mice observed in this study were similar to those reported previously by us in Sprague–Dawley rats [29]. Elimination as measured by CL and $t_{1/2}$ in Balb/c mice was found to be the most favorable for VN/66-1, an analog of 4-HPR. This compound not only had the longest half-life and slowest clearance it also had the greatest exposure when compared to VN/14-1 and VN/50-1. VN/66-1 also showed one of the lowest levels of toxicity out of all the compounds. This result is expected, because 4-HPR itself shows lower clinical toxicity than ATRA [22], thus its analog is also expected to have lower toxic effects than analogs of ATRA and 13-*cis* RA which have very similar structures.

Determining both the toxicity and PK parameters of VN/14-1, VN/50-1, and VN/66-1 will allow us to optimize these compounds in terms of their chemistries, in vivo action and dosing schedules. This comparison would not have been as strong if the PK of only the non-toxic compounds was determined. The toxicological profiles of these RAMBAs appear to be related to the chemical nature of the terminal polar groups of the retinoid side chain. The particularly toxic nature of VN/50-1 may be attributed to the propensity for imidazole amides to undergo facile nucleophilic substitution reactions [21]. Based on these results it would be beneficial to examine the effect of VN/66-1 in various cancer xenograft models and carry out further experiments to determine its mechanism of action. As stated above, VN/14-1 has been shown to be non-toxic up to 66 μmol/kg/day. We also envision that an isosteric replacement for the carboxylic acid moiety of VN/14-1 with 5-substituted-1H-tetrazole would yield its metabolism-resistant analog that is expected to be significantly less toxic than VN/14-1 [10].

In conclusion, a combined evaluation of toxicity and PK of selected RAMBAs has allowed us to select lead candidates for further development as anti-cancer

agents. VN/14-1 and VN/66-1 have been chosen for further studies for the reasons stated above. That is, their significantly superior efficacies in breast or prostate in vitro and in vivo cancer model systems.

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