Long-Term Dosing With the Thyroid Hormone Receptor Agonist VK0214 Reduces VLCFA Levels in Plasma and Tissue in an In Vivo Model of X-Linked Adrenoleukodystrophy

Hiroko Masamune[1], Ann Moser[2], Paul Watkins[2], Ali Fatemi[2], Xiaohai Shi[2], Brian Lian[3]

[1]Pharmaceutical Development, Viking Therapeutics, San Diego, CA, USA, [2]Kennedy Krieger Institute, Baltimore, MD, USA, [3]Executive, Viking Therapeutics, San Diego, CA, USA

Introduction

X-Linked adrenoleukodystrophy (X-ALD) is a genetic disorder characterized by adrenocortical insufficiency and neurodegeneration, along with accumulation of very long chain fatty acids (VLCFAs) in almost all tissues of the body.[1] Despite the widespread VLCFA elevation, the clinical symptoms of the disease are primarily localized to the nervous system, adrenal glands and testes. X-ALD results from mutations in the ABCD1 gene, located on chromosome Xq28,[1] which is responsible for encoding the adrenoleukodystrophy protein (ALDP). In healthy individuals, ALDP functions to transport Coenzyme A (CoA) thioesters of VLCFAs into peroxisomes for degradation via β-oxidation (Figure 1).[2] In contrast, in X-ALD patients, impaired ALDP function results in the accumulation of VLCFAs, which can disrupt cell membranes. This results in oxidative stress and a pro inflammatory state, leading to neuroinflammation and/or neurodegeneration. The reduction of VLCFA levels is thus considered an attractive goal for therapeutic approaches to treatment of the disease.[1]

There are two major phenotypes of X-ALD: the cerebral adrenoleukodystrophy (cALD) variant, characterized by a rapid inflammatory demyelinating process affecting either boys during school years or adult men, and the adrenomyeloneuropathy (AMN) variant, which is a slowly progressive distal axonopathy affecting adult men and women.

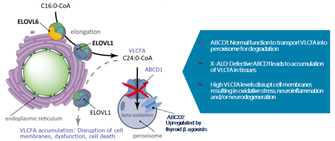


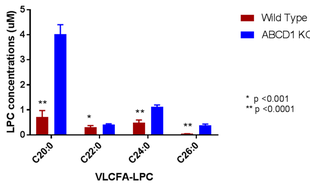
Figure 1

Over-expression of the compensatory and homologous gene, ABCD2, has previously been shown to increase β oxidation capacity and reduce VLCFAs in various models.[3,4] The β isoform of the thyroid hormone receptor (TRβ) has been shown to bind a thyroid hormone-response element on the ABCD2 promoter, mediating the gene's T3 responsiveness.[3,4] T3 and thyromimetic-mediated induction of ABCD2 has been shown to lead to normalization of VLCFA β-oxidation in human and mouse ABCD1-deficient fibroblasts, and prevent the accumulation of VLCFAs.[3,4,5] Thus, pharmacological induction of ABCD2 may provide therapeutic benefit in X-ALD patients by compensating for the effects of ABCD1 mutation.

The ABCD1 knock-out (KO) mouse model, while not displaying the inflammatory characteristics of the more severe forms of ALD, nevertheless develops a phenotype similar to AMN with advanced age and exhibits a similar biochemical profile to the human disease, in that there is an accumulation of certain VLCFAs in tissues and plasma.[6] In this model, the measurement of VLCFAs in lysophosphatidylcholine (lyso-PC or LPC) in blood samples, has been demonstrated to be more reliably quantified than direct measurement of total lipid VLCFAs.[7,8] Indeed, 1-hexacosanoyl-2-lyso-sn-3-glycerophosphorylcholine (26:0-lyso-PC, C26:0 LPC) is a diagnostic marker for X-ALD in humans.[7] Figure 2 shows comparative historical VLCFA-LPC levels from wild type mice versus levels measured from ABCD1 KO mice. As expected, ABCD1 KO mice demonstrate elevated blood levels of various LPC-derived VLCFAs compared with wild type mice.

VK0214 is a novel, orally available, small molecule prodrug of a potent TRβ agonist that is currently in preclinical development. The objectives of the current study were to determine the effect of long-term dosing (25 wks) of VK0214, the prodrug of the potent TRβ agonist VK0214A, on plasma and tissue VLCFAs in the ABCD1 KO mouse model of X-ALD. We previously reported encouraging results from a 6 wk study in this model. In the present study, the duration of treatment and cohort sizes were increased, and tissue VLCFA effects and ABCD2 induction were evaluated.

Figure 2. Comparison of LPC Levels in Blood of Wild Type vs ABCD1 KO Mice



Materials and Methods

Animals

Male ABCD1-/- mice were developed in the laboratory of Dr. Kirby Smith at the Kennedy Krieger Institute using the Taconic 129SvEv background strain.[6] This colony is currently maintained by the laboratory of Dr. Paul Watkins. Animals were housed 4-5/cage under a 12–hour lighting cycle (7 AM – 7 PM light) and controlled temperature (22 °C) in the rodent facility at the Johns Hopkins University School of Medicine. They were fed standard mouse chow and had access to drinking water ad libitum. Mice were at least 3 months of age at the beginning of this study.

Study Protocol

Because of the long duration of this study, making intraperitoneal dosing impractical, VK0214 was adsorbed onto regular chow, and initial feeding studies were conducted to determine that the mice were eating regularly. Food consumption was monitored throughout the study. Controls were fed standard unadulterated chow. A cohort of 26 mice were randomized 1:1 to receive VK0214-laced chow or regular chow, for 25 weeks (14 weeks at approximately 10 mg/kg/day, and 11 weeks at approximately 30 mg/kg/day).

For blood collection, ~50 µL of blood was obtained by facial vein puncture using a sterile lancet. Plasma was prepared and stored at -20 °C. Plasma was obtained in this manner following 2, 4, 6, 9, 12, 20, and 25 weeks of treatment. Following blood collection at 25 weeks, animals were sacrificed by cervical dislocation.

Brain, spinal cord, and liver were excised and snap frozen in liquid nitrogen for LPC and total fatty acid (TFA) analyses, and ABCD2 expression studies. Tissue was homogenized using a Qiagen TissueLyser II homogenizer. Lipids were extracted with chloroform/methanol (2:1).[9] For brain and spinal cord, lipids were further separated using silicic acid chromatography and a phospholipid- and sphingolipid-enriched fraction prepared.[10] Total fatty acids were quantified as their pentafluorobenzoyl derivatives by GC-MS.[11] Plasma and tissue LPCs were quantitated by LC-MS/MS.[7]

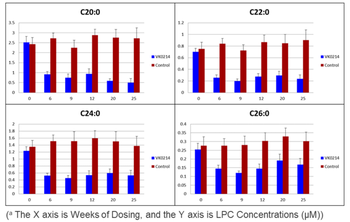
Quantitative polymerase chain reaction (qPCR) was used to quantitate ABCD2 expression in brain, spinal cord, and liver tissue. Immediately following homogenization, RNA was extracted with Trizol-LS and reverse-transcribed. PCR was performed on a Bio-Rad CFX Connect Real-Time system. GAPDH expression was used for normalization. Results were calculated using the ΔCt method with Bio-Rad software.

Results

Plasma LPCs

Plasma LPC levels were measured throughout the study, and at termination, and the results are presented in Figure 3. Results for the C26:0-LPC ester are also summarized in Table 1. As seen in our earlier 6 week study, mice receiving VK0214 demonstrated rapid reductions in plasma LPC levels within the first timepoint following initiation of dosing. Treated animals continued to experience progressive declines in C20:0-LPC through C24:0-LPC throughout the study, compared to control cohorts (Figure 4 and Table 2). The greatest effects were seen on the shorter chain lengths. Reductions in C26:0-LPC levels were sustained, relative to controls, through the course of exposure to VK0214, including following an increase in dose at week 14. Control animals, by comparison, demonstrated increases in mean LPC levels at week 25 vs. baseline.

Figure 3. Mean Plasma Levels of VLCFA-LPC Subtypes[a]



([a] The X axis is Weeks of Dosing, and the Y axis is LPC Concentrations (µM))

Table 1. Mean Plasma Levels of C26:0-LPC

	C26:0-LPC Levels (µM)					
Week	**0**	**6**	**9**	**12**	**20**	**25**
Control	0.28	0.28	0.28	0.30	0.33	0.30
VK0214	0.25	0.15	0.12	0.14	0.19	0.17
% CFB versus Control	-8%	-47%	-57%	-52%	-42%	-45%
p-Value versus Control	NS	<0.005	<0.0001	<0.0001	<0.0001	<0.0001

CFB = change from baseline; NS = not significant

Figure 4. Mean Plasma Reductions of VLCFA-LPC Subtypes at 12 and 25 Weeks, as Compared to Control

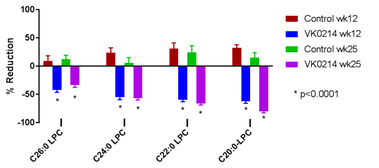


Table 2. Mean Percent Reductions in Plasma VLCFA-LPC Levels[a]

VLCA-LPC Subtype	12 Weeks	25 Weeks
C26:0-LPC	-48%	-45%
C24:0-LPC	-51%	-61%
C22:0-LPC	-55%	-74%
C20:0-LPC	-57%	-82%

[a] All values statistically significant (p<0.0001) over control values

Based on the encouraging results from the plasma LPC measurements, liver, brain, and spinal cord tissues were processed and evaluated for LPC and TFA levels.

Tissue Analyses

Liver total fatty acid analysis was performed to evaluate levels of C26:0. For spinal cord, a phospholipid-enriched fraction was prepared prior to measurement of total fatty acids. Figure 5 illustrates the change in the liver and spinal cord C26:0 as a percent of total fatty acids, respectively. In mice treated with VK0214, the levels of C26:0 decreased by 18.6% and 14.8% in liver and spinal cord tissue, respectively, compared with vehicle-treated controls. Table 3 outlines the effects on all key VLCFAs in the total lipid extract (liver) or phospholipid fraction (spinal cord). Given the robust and broad reductions in overall lipid levels among VK0214-treated animals, it is not inconceivable that the magnitude of the observed effects could be understated in this analysis.

Figure 5. Change in VLCFA in Liver and Spinal Cord

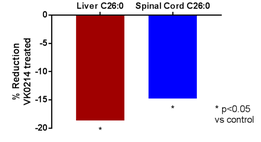


Table 3. Percent Change in Liver and Spinal Cord VLCFAs

	Liver		Spinal Cord	
	Percent Change	**p Value**	**Percent Change**	**p Value**
C20:0	-59%	<0.0001	-9%	<0.05
C22:0	-22%	NS	-12%	NS
C24:0	-49%	<0.0001	-11%	NS
C26:0	-19%	<0.05	-15%	<0.05

NS = not significant

LPC-derived VLCFAs were significantly reduced in brain tissue following exposure to VK0214 for 25 weeks (Table 4 and Figure 6). The levels of C20:0 and C22:0 LPC esters declined by 34% and 12%, respectively (p<0.0001 and p<0.05, respectively), while C26:0-LPC levels declined by 11% (p=0.07).

Figure 6. Change in VLCFA-LPC in Brain

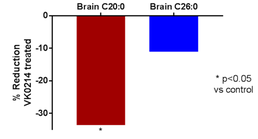


Table 4. Percent Change in Brain VLCFA-LPC

	Brain	
	Percent Change	**p Value**
C20:0-LPC	-34%	<0.0001
C22:0-LPC	-12%	<0.05
C24:0-LPC	-7%	NS
C26:0-LPC	-11%	0.07

NS = not significant

qPCR Results

Quantitative PCR (qPCR) analysis was undertaken to explore the mechanistic aspects of VK0214 action. Induction of ABCD2 would provide further evidence of the efficacy of thyroid beta receptor activation as an approach to addressing the loss of ABCD1 activity in tissue in X-ALD. Figure 7 illustrates the fold change in expression of ABCD2 (expressed as $2^{-\Delta Ct}$) comparison between control and VK0214 in the liver (262% increase), while Figure 8 provides the same analysis for the samples from the cortex (35% increase).

Figure 7. Relative ABCD2 Expression in Liver

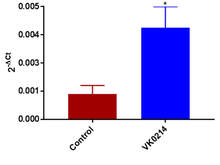
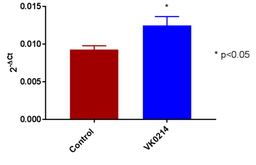


Figure 8. Relative ABCD2 Expression in Cortex



Discussion

Lowering the levels of VLCFAs, especially C26:0, continues to be an attractive therapeutic target for the treatment of X ALD.[12] Although the exact mechanism(s) of how elevated levels of VLCFAs are related to the pathogenesis of X-ALD have not been fully elucidated, there is ample evidence of the putative toxicity of these biomarkers. We have illustrated that with increased dosing duration, VK0214 demonstrates robust and durable reductions in plasma VLCFA-LPCs, including the benchmark C26:0-LPC (45% decrease). The effects on shorter chain VLCFA-LPCs (C20:0 through C24:0-LPCs) are even more dramatic, demonstrating an up to 82% decline vs. control. This is notable as shorter-chain VLCFAs serve as precursors to the highly toxic C26:0 species via iterative chain elongation. Thus, prolonged dosing may enhance the already significant direct reduction of C26:0 via depletion of the substrate pool. This may also lead to durability of effect following withdrawal of therapy.

In these studies, we also extended our evaluation to tissues, examining both the total fatty acids, fractionated to examine key VLCFA levels specifically, as well as the VLCFA-LPC esters. Statistically significant reductions were observed in the TFA C26:0 levels in both liver (-19%) and spinal cord (-15%). In addition, LPC-ester levels were also significantly reduced in brain tissue. Whilst the liver is readily exposed to VK0214A levels, access to both the spinal cord and brain is limited since both reside behind the blood brain barrier. Thus, the results in the spinal cord and brain are particularly noteworthy since it was not expected that VK0214A would demonstrate benefits across the blood-brain barrier. It is possible that the reductions in peripheral tissue and plasma VLCFA levels translate to a concomitant effect in the CNS tissues. If true, even this indirect effect may be highly beneficial, since in general, a lack of CNS penetration of the drug would minimize any potentially detrimental CNS side effects.

Presumably, TRβ agonists reduce VLCFA levels by upregulating genes for ABCD2, the compensatory VLCFA transporter into the peroxisomes. We have demonstrated herein that VK0214 produces statistically significant increases in ABCD2 expression in both liver and cerebral cortex. These data align with our earlier work where VK0214 was shown to increase ABCD2 expression levels in human X-ALD fibroblasts (unpublished results).

These results are supportive of the underlying hypothesis that stimulation of the thyroid receptor β may lead to an improved metabolic state with respect to certain problematic VLCFAs in X-ALD. The observed reductions in VLCFA levels in not only the plasma, but liver, spinal cord, and brain tissue, presumably derive from increased expression of the ABCD2 transporter, facilitating improved VLCFA transport and degradation. In addition, other compensatory mechanisms, such as inhibition of elongase enzymes or stimulation of peroxisome proliferation, may be contributing to the observed effects. Further work to characterize VK0214's impact on inflammatory aspects of X-ALD is underway.

Conclusions

- The selective TRβ agonist VK0214 represents a novel drug candidate for the treatment of X-ALD.
- Studies in ABCD1 KO mice have demonstrated the activity of VK0214 in ameliorating the accumulation of VLCFAs, a potential biomarker for therapeutic effects in X-ALD.
- Treatment with VK0214 resulted in robust and durable plasma C26:0-LPC reductions of 40% to 52% compared with vehicle, throughout the study.
- Treatment with VK0214 resulted in substantial vehicle-adjusted reductions in additional tissue total fatty acid levels and tissue LPC levels of C20:0, C22:0, C24:0, and C26:0.
- Gene expression of ABCD2 was significantly upregulated in liver and cerebral cortex, with 262% and 35% increases, respectively.
- Treatment with VK0214 has the potential to be useful in reducing blood and tissue levels of problematic VLCFAs in humans with X-ALD, which may pave the way for development of a disease-modifying therapy. Clinical studies are currently in the planning stages; additional preclinical experiments are underway.

References

1. Moser HW, Smith KD, Watkins PA, Powers J, and Moser AB. X-linked Adrenoleukodystrophy, in The Metabolic and Molecular Bases of Inherited Disease. (Scriver CR, Beaudet AL, Sly WS, and Valle D. eds.), 8th Ed. (2001) McGraw-Hill, New York, 3257-3301.
2. Adapted from http://www.x-ald.nl/origin-and-metabolism-of-vlcfa/
3. Fourcade S, Savary S, Gondcaille C, Berger J, Netik A, Cadepond F, El Etr M, Molzer B, and Bugaut M. Thyroid Hormone Induction of the Adrenoleukodystrophy-related Gene (ABCD2). Mol Pharmacol (2003) 63: 1296-1303.
4. Genin EC, Gondcaille C, Trompier D, Savary S. Induction of the Adrenoleukodystrophy-related Gene (ABCD2) by Thyromimetics. J. Steroid Biochem Molec Bio (2009) 116, 37-43.
5. Netik A, Forss-Petter S, Holzinger A, Molzer B, Unterrainer G, Berger J. Adrenoleukodystrophy-related Protein can Compensate Functionally for Adrenoleukodystrophy Protein Deficiency (X-ALD): Implications for Therapy. Hum. Mol. Genet (1999) 8: 907–913.
6. Lu JF, Lawler AM, Watkins PA, Powers JM Moser AB, Moser HW, Smith KD. A Mouse Model for X-Linked Adrenoleukodystrophy. Proc. Natl. Acad. Sci. USA (1997) 94: 9366-9371.
7. Hubbard WC et al. Newborn Screening for X-Linked Adrenoleukodystrophy (X-ALD): Validation of a Combined Liquid Chromatography – Tandem Mass Spectrometric (LC-MS/MS) Method. Molec Genetics Metab (2009) 97: 212-220.
8. Van de Beek MC et al. C26:0-Carnitine Is a New Biomarker for X Linked Adrenoleukodystrophy in Mice and Man. PLOS ONE (2016) 11: 1-19.
9. Folch J, Lees M, Sloane Stanley GH. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J Biol Chem (1957) 226: 497-509.
10. Vance DE, Sweeley CC. Quantitative Determination of the Neutral Glycosyl Ceramides in Human Blood. J Lipid Res (1967) 8: 621-30.
11. Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP. Quantitative Determination of Plasma C8-C26 Total Fatty Acids for the Biochemical Diagnosis of Nutritional and Metabolic Disorders. Mol Genet Metab (2001) 73, 38-45.
12. Berger J, Pujol A, Aubourg P, Forss-Petter S. Current and Future Pharmacological Treatment Strategies in X-Linked Adrenoleukodystrophy. Brain Pathol (2010) 20: 845-856.