Author’s response to reviews

Title: ATR-101, a selective ACAT1 inhibitor, decreases ACTH-stimulated cortisol concentrations in dogs with naturally occurring Cushing’s syndrome

Authors:

Daniel Langlois (langlo21@cvm.msu.edu)
Michele Fritz (fritzmi2@msu.edu)
William Schall (schall@cvm.msu.edu)
N. Olivier (olivier@cvm.msu.edu)
Rebecca Smedley (smedley1@dcpah.msu.edu)
Paul Pearson (pearson@p3pharma.com)
Marc Bailie (marc.bailie@inds-inc.com)
Stephen Hunt III (hunt@millendo.com)

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Author’s response to reviews:

The author's response to reviewer 1 has been included as a supplementary file. Our response to Reviewer 2 is below.

Robert J. Kemppainen (Reviewer 2): General Comments: This report describes use of a novel small molecule ATR-101 in canine Cushing's disease. ATR-101 is a selective inhibitor of ACAT1 and as such reduces formation of cholesterol ester accumulation and storage in steroid secreting cells; in this case, targeting those in the adrenal cortex. The drug has been studied in healthy dogs and this report extends examination of its effects to dogs with either pituitary dependent Cushing's disease or with functional adrenocortical tumors.

1. From the introduction, ATR-101 has differing effects depending on dose; that is reduction of cortisol and other adrenal steroids in circulation when administered at low doses to adrenal cell apoptosis when given at high doses. What is the ultimate goal, in the author's opinion, when using the drug to treat hyperadrenocorticism? Is it simply to reduce steroid secretion or is it to kill adrenal cells? This also relates to lines 341-354, the description of adrenal histology in the 3 dogs with adrenal tumors.
This is an excellent question. A primary goal of using ATR-101, or any drug for that matter, to treat hyperadrenocorticism is to reduce the increased cortisol levels, which are directly responsible for the much of the morbidity and mortality associated with the disease, to normal levels. As the reviewer has astutely noted, ATR-101 is quite unique in that it has different effects at different doses/exposures. In studies in normal dogs, we have seen dose- and time-dependent decreases in levels of basal and post ACTH stimulation serum cortisol (and indeed all steroids and steroids intermediates) and adrenal weights. These findings are believed to result first from adrenocortical cells that lack the necessary reservoir of esterified cholesterol required to produce steroid hormones after low-dose ATR-101 exposure (d 1–7), and subsequently adrenocortical cells that have undergone cell death and are no longer capable of producing hormones (d 14)a,b. Certainly, we would expect there would be a transition phase in which both effects would be active. Thus, we believe ATR-101 doses can be adjusted to dial-in the appropriate level of cortisol reduction required and have the ability to treat CS due to any etiology (for example, the induction of apoptosis seen at higher doses would obviously be important when treating adrenocortical neoplasia). We have modified the portion of the introduction concerning ATR-101 to provide further clarification. Please see lines 94-99.


2. What is the relative tissue distribution of ACAT1? Specifically, is it present in hepatocytes? Could this explain the effects on liver enzymes?

ACAT1 is known to be expressed throughout the body including liver, however, most studies in humans show the levels in adrenals to be >15X higher than other tissues. This is one of the reasons we believe the effects of ATR-101 to be selective for the adrenal. Previous studiesa have looked, specifically, and not seen toxic effects of ATR-101 on hepatocytes unless the cells were pre-treated with agents to block glycolysis or cytochrome P450-mediated metabolism. As ATR-101 is highly metabolized in the liver, we believe the liver effects are most likely due to hepatic enzyme inductionb, a well-known adaptive response to xenobiotics associated with increases in liver weight, induction of gene expression and morphological changes in hepatocytes without histological correlates. We have now included some of this information and associated references in the discussion. Please see lines 464-469 and 474-475.


Specific Comments:

1. Lines 164-166. What happened to dogs with pituitary disease after discontinuance of ATR-101? Did they start on trilostane?

Following completion of the study, owners were instructed to allow a washout period of 4 weeks prior to initiating trilostane therapy. Five of the 6 dogs with PDH were started on trilostane therapy. One of the dogs was not immediately started on therapy due to owner financial concerns despite the presence of clinical signs of CS. A statement about the washout period has been added to the manuscript. Please see lines 170-172.

2. Lines 222-229. Please provide citations for validations of the hormone assays.

References containing validation data from our laboratory for cortisol, aldosterone, and ACTH are now provided. Please see lines 234-237 and references 33-35.

ACTH:


Cortisol:


Aldosterone:

3. Lines 300-310. Was there any difference in ACTH stimulated cortisol responses comparing dogs with pituitary disease versus those with adrenal tumors?

We apologize for not addressing this in the original submission. The one dog not experiencing a decrease in post-ACTH stimulated cortisol concentrations was a dog with adrenal-dependent disease. Overall, the post-ACTH stimulated cortisol concentrations at any time point (days 0, 7, 14, 21, 28) were not different between ADH and PDH dogs, albeit the small sample size is small. In dogs with adrenal-dependent CS, the post-ACTH-stimulated cortisol concentrations at day 14 were increased by 43.2% in one dog and decreased by 36.1% and 84.2% in the other two dogs as compared to baseline (day 0) concentrations. In dogs with pituitary-dependent CS, the mean ± SD percent reduction in post-ACTH-stimulated cortisol concentration at day 14 was 49.8 ± 12.8%. This information has been added to the results. Please note, day 14 data was utilized for the percent change statement in order to maintain a total of 10 dogs (as opposed to n=5 at day 28). Please see lines 312-319.

4. Line 332. Did the ACTH stimulated cortisol decline (as much) in the 2 dogs whose clinical signs were unchanged or the dog whose signs worsened? Or were they outliers?

The only dog to experience worsening clinical signs was also the same dog in which reductions in post-ACTH cortisol concentrations were not observed. This has been added to the manuscript. Please see lines 351-352.

As for the 2 dogs with unchanged clinical signs, they did experience reductions in post-ACTH cortisol concentrations, and they were not of any lesser magnitude than the dogs in which clinical improvements were reported. The reasons for this are unclear. It is possible they represent outliers. It is also possible that mild improvements did occur, but they may not have been apparent to owners. The USG in these 2 dogs (1.025 to 1.031 and 1.044 to 1.051) did increase slightly. However, the USG in these dogs was unlikely to be associated with severe PU/PD. As such, slight changes may not have been apparent. Alternatively, they may represent day to day fluctuations. Given the speculative nature of the above statements, we have not added additional information to the manuscript as it pertains to these 2 dogs. However, we do not object to adding such information if the reviewer thinks it appropriate to do so.

5. Lines 443-444. "...biochemical parameters associated with liver function remained normal". What parameters are the authors referring too?

This statement was referring to bilirubin, BUN, and glucose. In addition, the cholesterol concentrations remained static. Albumin did decrease slightly, but this is difficult to interpret given the concurrent proteinuria in many dogs, and the mean value remained within a normal
reference interval throughout the study. We have now specifically referred to bilirubin, glucose, and BUN in the manuscript. Please see lines 461-462.