Author's response to reviews

Title: (R)-[11C]Verapamil PET studies to assess changes in P-glycoprotein expression and functionality in rat blood-brain barrier after exposure to kainate-induced status epilepticus

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Version: 2 Date: 29 September 2010

Author's response to reviews: see over
Reviewer: David Reutens

Reviewer’s report:
This is a study aiming to examine the function of P-gp at the blood-brain barrier with PET and the tracer [C11]verapamil. Animals were studied a week after treatment with kainate or saline and with and without co-administration of the P-gp inhibitor tariquidar. A range of analytic methods were used and reported. P-gp expression was also studied in the brain post-mortem using immunohistochemistry.

The histological studies showed a non-significant trend in P-gp area. PET showed significant differences between kainate and saline treated animals only with tariquidar administration. In both groups tariquidar increased the brain-blood concentration ratio. Both K1 and k2 were reduced and Vbr1 increased in the kainate group.

MINOR ESSENTIAL REVISIONS
The authors indicate that the kainate model is one of pharmacoresistance, but the development of chronic epilepsy was not able to be examined in the animals used in this study. Outcome in this epilepsy model is not invariable in terms of development of spontaneous seizures or pharmacoresistance. This should be reflected in the introduction / discussion.

Authors’ response: We fully agree. However, we should point out here that in this study it has not been our intention to use the kainate model as a model of pharmacoresistance, but as an animal model for human mesial temporal lobe epilepsy in which increase in P-gp expression occurs. In the present study the aim was to investigate increase in P-gp expression and functionality with PET and for that we needed a time point at which increase in P-gp expression was expected to be prominent. In subsequent studies we intend to study the relation between altered P-gp expression and pharmacoresistance, identify subjects (animal/human) that are prone to develop pharmacoresistance, etc.

We have modified the Introduction and Discussion to emphasize this point more clearly.

The overall conclusion is that P-gp expression and functionality ‘do not seem to change’ at the time studied in this model. The conclusion does not take into account the positive findings in this study. What do the authors conclude from the differential effects of tariquidar on K1 and k2 in the kainate-treated animals using several of the analytic methods? What does the differential response indicate about differences in P-gp function in the kainate-treated group?

Authors’ response: We have rewritten the Conclusion section accordingly

Analyses are carried out at whole brain level. While I understand the limitations in terms of resolution of the tomograph, some comment should be made in relation to the impact of this on the results. Could this have obscured significant regional changes?
Authors’ response: This is a very relevant comment. We also looked at the cerebellum, but the trend was the same there, i.e. no differences between kainate and control animals. At the time of this study, we could only define regions of interest over the whole brain and cerebellum.

We have added a comment about possible regional differences in the manuscript in the Discussions section.

DISCRETIONARY REVISIONS
In general the paper is well written. However, it is a complex paper with many analyses and results. It would be easier to read if the key findings are summarized at the end of the results section.

Authors’ response: We have added this to the manuscript

Reviewer’s report
Title: (R)-[11C]Verapamil PET studies to assess changes in P-glycoprotein expression and functionality in rat blood-brain barrier after exposure to kainate-induced status epilepticus
Version: 1 Date: 27 August 2010
Reviewer: Mark Muzi
Reviewer’s report:
Comments on the manuscript “(R)-[11C]Verapamil PET studies to assess changes in P-glycoprotein expression and functionality in rat blood-brain barrier after exposure to kainate-induced status epilepticus”. Reviewed by Mark Muzi and Sara Eyal.
Synopsis: The authors describe a PET and IHC study of P-gp expression and function at an early time point in the kainate model for spontaneous seizures in rats. NONMEM analysis detected small differences between kainate-treated and control rats in verapamil BBB kinetics when P-gp was inhibited. The manuscript is concisely written and the results are of interest, but there are concerns about the modeling approach and the major conclusion, as described below.

Major comments:
1. The paper evaluates too many models in light of the available biological data to select among them. Previous publications indicate that a simple one-tissue compartment model for the initial 10-15 minutes determining the transfer constant K1 assess P-gp activity with verapamil and avoids contamination of brain regions with radiometabolites (Muzi 2009, Ikoma 2006, both in J Nucl Med, and the review by Kannan 2009 in Clin Pharmacol Ther). Measures of verapamil retention in tissue (Logan distrubution volume, for example) may have nothing to do with P-gp activity at the surface of the BBB, unless there is significant and persistent binding (infinite binding) of verapamil entering brain tissue throughout the 60 minutes of scanning to track the transfer into brain. Please indicate the biological basis for selecting each of the models presented in this paper and justify the use of 60 minutes of imaging data overall, but specifically for the 1C model.
Authors’ response: The reviewers are correct that a 1-compartment model is enough for analysing $[^{11}\text{C}]$verpamil data in humans without complete P-gp inhibition. When P-gp is inhibited several studies have shown that a 1-compartment model is not sufficient, at least not in rats (Bankstahl et al. 2008; Syvänen et al. 2008). This was also shown in this study, and hence the 2-compartment model was preferred over the 1-compartment model.

Logan analysis was carried out to give a robust estimate of the brain-to-plasma partition coefficient and to enable comparison to other $[^{11}\text{C}]$verpamil studies in rats.

We think that the approach to study changes in K1 is a very good option in for example clinical studies in which one only wants to have a “simple” estimate of the P-gp function. In this study we wanted to investigate both the tariquidar effect and differences between epileptic and control animals. As shown in this paper, tariquidar had mainly an effect on K1 (Qin), and in this case a K1-analysis as suggested by the reviewers would have been enough. Also, the most significant difference between kainate and saline rats was found for K1. We did re-run the population model using data obtained only during the first 30 min or 15 min. The estimates were very similar to the estimates obtained with data from 60 min. What probably is in line with the reviewer’s comments is that the covariate effect for tariquidar on Qin is larger when only early data is used.

<table>
<thead>
<tr>
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<th>60 min (from article)</th>
<th>30 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>$V_c$ (mL)</td>
<td>22.8</td>
<td>21.6</td>
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<td>393</td>
<td>721</td>
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<tr>
<td>$V_{p2}$ (mL)</td>
<td>70.2</td>
<td>55.7</td>
<td>55.3</td>
</tr>
<tr>
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<td>14.7</td>
<td>15.8</td>
<td>10.5</td>
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<tr>
<td>$Q_1$ (mL·min$^{-1}$)</td>
<td>16.1</td>
<td>16.4</td>
<td>22.8</td>
</tr>
<tr>
<td>$Q_2$ (mL·min$^{-1}$)</td>
<td>22.7</td>
<td>20.2</td>
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<tr>
<td><strong>Brain</strong></td>
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<td>$V_{br2}$ (mL)</td>
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<td>0.877</td>
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<td>1.20</td>
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<td>0.128</td>
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<tr>
<td>brain</td>
<td>0.226</td>
<td>0.239</td>
<td>0.255</td>
</tr>
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</table>

Tariquidar also affected the volume of distribution in the brain which probably relates to the fact that P-gp is not only distributed at the BBB but also within the brain. Further, this study also indicated that kainate treatment increased the volume of distribution in the brain. This could also potentially be related to P-gp expressed on intrabrain targets. This has also been shown by Liefaard et al using $[^{11}\text{C}]$flumazenil in...
epileptic animals (Liefgaard et al. 2009). The effects on the volume of distribution inside the brain would probably not have been picked up by a K1-analysis.

In addition, we think that it is a simplification to only expect an effect on K1 after inhibiting P-gp. Studies have shown that when P-gp inhibition is achieved through pretreatment with a P-gp inhibitor the P-gp effect is mainly (sometimes only) observed as an increase in K1. However, when the inhibitor is given after the radiotracer, a decrease in k2 is also observed (Tunblad et al. 2003; Syvänen et al. 2006; Bankstahl et al. 2008; Syvänen et al. 2008; Bartmann et al. 2010). We believe that although P-gp mainly acts as a “gate-keeper”, it can also pick molecules from the BBB and extrude them. Thus, when P-gp substrate radiotracer is already inside the brain when the P-gp inhibitor is given, there will also be an effect on k2, since the radiotracer molecules will by passive diffusion pass into the BBB endothelial cells.

2. The study evaluated P-gp expression and function at a single time point after kainate injection. The lack of change in P-gp expression / functionality on day 7 may reflect selection on a non-representative time point or inability of the study methodology to detect a change in P-gp rather than a stable level of expression (in the absence of a positive control). Therefore, the results appear to be preliminary and do not necessarily lead to the general conclusion that P-gp expression and activity do not seem to change at an early stage after kainate treatment.

Authors’ response: This is true. It might be that [11C]verapamil is not sensitive enough to detect a small difference between the two groups. This is especially the case for the baseline studies (no tariquidar). This is discussed in the second section of the Discussion. We have also discussed the time point (7 days) in the Discussion. We fully agree that there may be changes either earlier or later. We have added some more discussion regarding this in the Discussion.

3. Could regional changes in P-gp activity (such as in the hippocampus) be detected when whole brain VOIs are evaluated? The major concern is that significant changes in P-gp activity in relevant brain regions may be masked by the overall unaffected P-gp activity in the rest of the brain.

Authors’ response: This is a very good comment. We have discussed this more extensively in the Discussion section. The HRRT has a resolution of 2.3-3 mm and at the time of the study we were not able to define small regions of interest (as the hippocampus). We could only investigate whole brain and cerebellum.

4. P. 8, second paragraph: The 10 blood samples add up to 1 mL for sample activity plus 0.9 mL from three blood samples for metabolite analysis, and assuming some waste in catheterization and clearing lines between samples this totals potentially 3-4 mL of lost blood from the animal, which is significant for a 200g rat. Assuming about 12 mL of blood in a 200g rat, how does a loss of 20-30% blood volume affect delivery and metabolism of verapamil.

Authors’ response: The rats were 200-224 g at arrival, but at the actual time of the PET scan they were on average around 300 g (Table 1), and hence had around 19.5 mL (6.5 mL per 100 g) blood. We tried to minimize the waste by first withdrawing
some blood into one syringe and then changing to another for the actual blood sample. The blood in the first syringe was then pushed back into the animal. The only waste was what was left in the blood sample syringe. However it is true that we probably took in total around 2.5 mL blood. Of this 0.4 ml was obtained at 60 min, and hence did not affect distribution of verpamil.

5. P. 11, second paragraph: Vb (vascular volume of brain) should be floated in compartmental models and not subtracted prior to curve fitting, as this would likely affect K1 determination.

Authors’ response: It is quite common that a fixed value is used. We agree that the optimal way would be to add it as an extra parameter, but since the model already was on the limit to be over-parameterised we chose to fix it.

6. P. 13, lines 13-14: AIC comparisons cannot be made due to violation of the assumptions of the Akaike critera (differences among the models in parameter numbers). Additionally, the AIC does not indicate the biological basis for model selection.

Authors’ response: in AIC calculation the number of parameters is taken into account:

\[ \text{AIC}=2k+n[\ln(\text{RSS})] \]

k is the number of parameters, n is the number of observations and RSS is the residual sum of squares. The AIC penalizes free parameters less strongly than does the related Schwarz criterion and thus it will sometimes favor models with higher number of parameters. In this study it was used just as one of many criteria to select between models, because just as the reviewers point out, it does not indicate any biological basis for model selection.

7. The Methods section does not describe the statistical tests used for between-group comparisons. Given the 4 treatment groups, the appropriate test is ANOVA and not t-test.

Authors’ response: ANOVA does not describe between which groups there is a significant difference. To determine this, t-tests between groups were performed. We have included a correction for multiple comparisons (bonferroni). The change from simple t-tests to t-test with bonferroni correction did not change the outcome of the analysis, except that in the 1T2k model difference in k2 became non-significant. Table 2 has been modified accordingly.

8. Background: although it has been established that P-gp is over-expressed at the BBB of patients with pharmacoresistant epilepsy and in animal models of epilepsy, there is no clear evidence that p-glycoprotein limits brain uptake of antiepileptic drug or contributes to AED resistance. Also, the association between increased functionality of BBB efflux transporters and disease development or severity (as described in the abstract) is not supported by experimental data from the references cited in the Background section.
Authors’ response: We believe that there are studies that indicate that AEDs have limited brain uptake due to being actively effluxed. See for examples the following references: (Lösch et al. 2005; Brandt et al. 2006; Hocht et al. 2007; van Vliet et al. 2009). These in vivo studies have shown that Phenobarbital and phenytoin have limited brain uptake due to being P-gp substrates. In vitro studies have also shown that other AEDs are interacting with P-gp. A recent review by Potschka is addressing this question (Potschka).

We agree that there is limited evidence for disease development and severity and we have rewritten this section in the Background.

9. Consider citing the recent article by Bartmann et al. (Epilepsia 2010 Jul 14).

Authors’ response: Thank you for pointing this out. We have added the reference.

Minor comments:
1. Title: Please delete [0] between “expression” and “and” in the title.

Authors’ response: Has been removed. Thank you for pointing it out.

2. Abstract, Methods: verapamil is the most established PET ligand for determining BBB P-gp functionality, but not necessarily the best, in particular when studying over-expressed BBB P-gp.

Authors’ response: we completely agree. We have added a comment about desmethyl-loperamide in the discussion section

3. Background, P. 4, line 6: patients are normally treated at maximal tolerated dose and not level.

Authors’ response: Has been changed according to the comment

4. Background, P. 4, line 5 from page end: the BBB P-gp expression is upregulated.

Authors’ response: Has been changed according to the comment

5. Methods, What was the PET image resolution?

Authors’ response: 2.3-3 mm and this has been added.

6. Methods, P. 8, lines 13-15: For iterative reconstructions, like OSEM, the authors should indicate the number of subsets (s) and iterations per subset (i) performed or the number of updates (s * i = update number). Please provide the post-reconstruction filtering parameters.

Authors’ response: Has been added.

7. Methods, Data analysis, P. 11: it would help the reader if the sentence beginning with “liver, obtained from the PET images” is re-written such that the
content is more clear.

**Authors’ response:** Has been changed according to the comment

8. Methods, P. 10, line 15: I assume that Milli Q is ultrapurified water from Millipore’s Milli Q system, but that should be stated more directly.

**Authors’ response:** Has been changed according to the comment

9. Results, P. 13, line 4: models are fitted to the data and not vice versa.

**Authors’ response:** Has been changed according to the comment

10. Discussion, P. 21, second paragraph: the words “of (R)-[11C]Verapamil” should follow “co-administration”.

**Authors’ response:** Has been changed according to the comment

11. Please check the equations and parameter definitions. For example, Vbr2 and Q2 (Fig. 3 and text) are not defined. Is the determination of Kp for Logan, and the other models identical? All parameters used in the paper should be defined. Consider a table of parameter definitions and formulas.

**Authors’ response:** Has been changed according to the comment

12. Fig. 5: Consider deleting the figure because it does not add to the data described in the text or on Fig. 6. In addition, the legend does not specify the treatment group or the brain region.

**Authors’ response:** We like to keep it as it shows the quality of our P-gp staining. We have added more information to the legend.


