Title: Effect of topical soluble epoxide hydrolase inhibition in a preclinical diabetic wound healing model.

Short title: EPHX2 inhibition and wound healing

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Abstract

The objective of this study was to test a topical formulation of *EPHX2* inhibitor, GSK2256294, in a dermal wound diabetic (*db/db*) mouse model. Comparisons were made between groups

treated with *EPHX2* inhibitor, matching vehicle control and a currently approved treatment for diabetic ulcers, becaplermin/Regranex, as a positive control which is highly efficacious in this model. Leptin receptor-deficient (*db/db*) male mice were sequentially assigned to treatment groups (N=10 per group) based on blood glucose levels and body weight. Mice were given a single 8 mm diameter full thickness wound on the back. Wounds were photographed and traced, and fresh test materials applied periodically with fresh dressing. Of ten mice treated with GSK2256294, three had completely healed wounds at the study conclusion, and 8/10 mice reached at least 50% closure. In the vehicle group, no mice achieved complete closure, and only 6/10 reached at least 50% closure by the study conclusion. Nine of ten wounds achieved complete closure in the positive control group by 21 days. Although the EPHX2 inhibitor results were indicative of efficacy, the single-sided p-value criterion of 0.05 was not met in this study.

Introduction

Soluble epoxide hydrolase (sEH, encoded by the *EPHX2* gene), a member of the α/β-hydrolase fold family of epoxide hydrolases, is a critical enzyme in the metabolism of epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acids (DHETs). EETs are formed by the oxidation of arachidonic acid by CYP450 enzymes, primarily members of the CYP 2C and 2J classes in humans. EETs are a group of eight compounds: the four regioisomers are 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, with R/S and S/R enantiomers of each. *In vitro,* EETs have demonstrated anti-inflammatory and potential pro-resolving effects as well as effects on airway and vascular smooth muscle, and vascular endothelial cells. The actions shown by EETs in

42 animal models and human cellular assays include vasodilatory effects, ion channel modulation,
43 mitogenesis and modulation of endothelial cell survival and vascular smooth muscle cell
44 proliferation.
45
46 Although the molecular mechanisms of action of EETs have not been completely elucidated, it
47 is hypothesized that the therapeutic effects of sEH inhibition are mediated by the accumulation
48 of EETs and that many potentially beneficial actions of EETs are attenuated by the conversion of
49 EETs to the less active DHETs. Therefore, sEH inhibition is a potential therapeutic target to
50 enhance the beneficial effects of EETs (1). GSK2256294 is a potent and selective soluble epoxide
51 hydrolase (sEH) inhibitor (2) which has previously been studied in preclinical models (3, 4) and
52 in phase I clinical trials (5, 6).
53
54 Chronic wounds, including pressure ulcers, leg ulcers and diabetic foot ulcers are an area of
55 significant unmet need. Thousands of lower limb amputations are performed annually on
56 diabetic patients. One estimate of the direct cost of diabetes treatments in the US in 2007 was
57 $116 billion, of which one third was linked to treating foot ulcers (7). Recognizing that diabetic
58 wound healing is a very challenging paradigm, we hypothesized that inhibition of sEH would
59 result in improved resolution of chronic wounds and could lead to a potentially
60 transformational medicine for patients. We therefore proceeded to test GSK2256294 in a
61 validated diabetic wound healing model to investigate this hypothesis.
62

63 Although there have been no GWAS focused on chronic wound healing, there are some
64 interesting Mendelian skin disorders involving other enzymes of arachidonic acid metabolism.
65 Autosomal recessive congenital ichthyosis type 1 (ARCI1, OMIM #242300) is caused by loss of
66 function (LoF) mutation in *TGM1* (transglutaminase 1), ARCI2 (OMIM #242100) is caused by LoF
67 in *ALOX12B* (arachidonate 12-lipoxygenase, 12R type) and ARCI3 (OMIM #606545) is caused by
68 LoF in *ALOXE3* (arachidonate lipoxygenase 3). All three are keratinization disorders,
69 characterized by abnormal skin scaling over the whole body (8). Mice with knockout expression
70 of *TGM1, ALOX12B* or *ALOXE3* have impaired skin barrier function (9-11). EETs have been
71 shown to activate *TGM1* in human and mouse keratinocytes (12), and *TGM1* mRNA facilitated
72 wound healing in neonatal mouse skin (13).

73 Several published reports implicate direct involvement of sEH in wound healing in non-diabetic
74 models. A tool sEH inhibitor (t-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-
75 benzoic acid) significantly accelerated wound closure, epithelialization and neovascularization
76 in an ear wound model in SKH-1 hairless mice (14), as did administration of sEH substrates
77 11,12-EET and 14,15-EET. The same research group showed accelerated wound closure in
78 *EPHX2* KO mice (15). An independent study using knockout mice, sEH substrates and a different
79 tool inhibitor (TUPS, 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea)
80 has also shown similarly enhanced wound healing (16). In addition, it was demonstrated that
81 increased expression of *CYP2C8* or *CYP2J2* also increased EET levels and promoted wound
82 healing, whereas increased expression of sEH itself suppressed wound healing.

83 # Materials and methods

84 ## Animal Care

85 We contracted with Explora BioLabs, San Diego, CA to manage the animal study. Every effort
86 was made to minimize or eliminate pain and suffering of all the animals in the study. The study
87 was approved by the Office of Animal Welfare, Ethics and Strategy at GSK (Reference number
88 D012050, Submission number S003940). All studies were conducted in accordance with the GSK
89 Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the
90 Institutional Animal Care and Use Committee at Explora BioLabs. Male mice, *db/db*
91 (Homozygous for $Lepr^{db}$ <BKS.Cg-$Dock7^{m}$ +/+ $Lepr^{db}$/J>, 8-9 weeks old, were purchased from
92 Jackson Laboratories. Upon arrival, animals were inspected for correct age and sex, ear-tagged
93 and their body weights were recorded. The animals were housed 1-per cage. The animal
94 housing, care and husbandry were performed per the Guide for the Care and Use of Laboratory
95 Animals; 8th Edition; Institute of Laboratory Animal Resources; U.S. Department of Health and
96 Human Services; National Institutes of Health Publication No. 85-23, Revised 2011.
97 Temperature in the animal holding room was maintained between 20 and 26°C (68-79°F). Room
98 humidity was monitored (40-70% is recommended in the Guide). Temperature and humidity
99 values were recorded daily. A 12-hour light/12-hour dark cycle illumination period was
100 maintained. Animals were housed using the dual HEPA-filtered ventilated Innovive disposable
101 IVC cage system 3.0, which provides 60 fresh-air changes per hour for animal cages. The
102 animals had ad libitum access to standard diet (2920X.10, Global 18% Protein Rodent Diet from
103 Harlan, San Diego, CA) and acidified water (pH 2.7-3.0) throughout the study period. The

104 bedding material was hardwood chips (Sani-Chips, Cat # 7115, Harlan, CA, USA) and was
105 changed weekly to ensure the ammonia levels within each cage were below 24 ppm.
106
107 Animals were acclimated to the housing environment for 7 days prior to the initiation of the
108 study. During the acclimation period, the general health of the animals was monitored daily.
109 Animals appeared normal and did not exhibit signs of poor health. No animals were excluded
110 from this study. Glucose and body weight were measured on Day -1, and treatment groups
111 were assigned sequentially to animals ordered by their Day -1 glucose levels (e.g., 1, 2, 3, 4, 5,
112 5, 4, 3, 2, 1, etc.) to obtain similar average blood glucose and body weight values among groups.
113 The treatment group assignment was performed on Study Day -1 prior to test article
114 administration. Animals were weighed once per week, and body weights were recorded. The
115 general health condition and attitude of each animal was monitored daily throughout the
116 study. Clinical signs indicative of poor health, stress and pain were noted.
117

118 Topical sEH Formulation

119 Topical gel formulations for GSK2256294 were developed by Dermatology. The solvent system
120 was decided based on solubility of GSK2256294. Gel formulation prototypes were then
121 prepared with the solvent system using various combinations of polymers and pH adjusters.
122 The formulation used in the animal study comprised of 1% (%w/w) GSK2256294, 20% PEG400,
123 25% propylene glycol, 25% transcutol, 1% Ultrez-10, 1.5% triethanolamine in water. Vehicle
124 control lacked GSK2256294 (q.s. with water) but was otherwise identical. Fresh supply of the

topical formulation was shipped to the CRO weekly, for the duration of the study. Becaplermin (0.01%) was purchased from Smith & Nephew (Catalogue number - REG810-15) and used as directed.

Study Design

We requested a representative data set from the CRO to assess model variability and estimate the number of mice necessary to achieve adequate power. Sample sizes of N=10 mice per group were determined to achieve 80% power to detect decreases from vehicle control of 1.31 days in mean interpolated time to 50% wound closure when using a one-way ANOVA model with five experimental groups, for a total sample size of 50 animals. The study contained five experimental groups in order to investigate other chemical compounds in addition to GSK2256294, although only the three previously mentioned groups are presented here.

Timeline

- Day -7 to Day 0 acclimation
- Day -3 back hair removed
- Day 1 wounding
- Day 1 pre-dose and 1 hour post-dose blood collection (N=3 mice per group)
- Days 1, 4, 7, 10, 13, 16, 19, 21 and 24 dosing and wound measurement
- Day 21 termination of positive control group

146 • Day 26 termination of sEHi and vehicle test groups, wound measurement

147 On Day -3, all animals were anesthetized via inhaled isoflurane and the entire back hair shaved
148 with clippers. The remaining hair was removed with depilatory cream (Nair,
149 unscented/sensitive skin). On Day 1, animals were anesthetized via inhaled isoflurane and a
150 single 8mm diameter full thickness (skin and the underlying panniculus carnosus) wound
151 created starting 1cm below the scapula bones running parallel at 0.5cm lateral from the spine.
152 Animals received ~50-100µL of Positive Control or Test Article or Vehicle spread out to create
153 an even and consistent layer over the wound making sure to cover all the wound area. Wounds
154 were covered with an occlusive dressing [High MVP Transparent Dressing (Smith + Newman)
155 and Mastisol liquid adhesive (Eloquent Healthcare)] and changed on dosing days following
156 surgery. Animals were weighed once per week, and body weights recorded. Weight loss in
157 excess of 20% compared to Day 0 was to be reported, but no mice reached this threshold
158 during the study. On Study Day 21, the animals in the positive control group were euthanized
159 by isoflurane overdose. On Study Day 26, the animals in the sEHi GSK2256294 treatment and
160 vehicle control groups were euthanized. Wounds were collected into 10% formalin for storage.
161 Each wound was excised completely to include underneath muscle and 5mm of surrounding
162 tissue. The personnel conducting the animal wounding, dosing, and measuring of wound area
163 were blinded to the identity of each test article, although the laboratory was aware of this
164 information as part of study planning and reporting. The dosing, photographing, and measuring
165 of wound area were each conducted sequentially by group and not in a random order.
166
167

168 ## Data Analysis

169 Wounds were photographed, traced and analyzed using the computer program Image J
170 software from digital photographs (www.rsb.info.nih.gov/ij/). Analysis was performed using R
171 package *lme4* version 1.1-18-1 and SAS/STAT version 12.1. The tests of statistical significance
172 are reported for a single sided test in the direction of reduced wound size or time to smaller
173 wound size. All endpoints for statistical analysis were derived from the wound size
174 measurements and were based on individual mice being the experimental unit. Data from all
175 five experimental groups were included in these analyses. A variety of alternative statistical
176 approaches were considered instead of the one-way ANOVA used for the earlier power analysis
177 due to some mice not achieving 50% closure during the study.
178 Time to 50% closure (T_{50}) was estimated using a spline model to estimate the wound closure
179 percent at timepoints between the days of measurement. The spline model incorporated a
180 forced intercept of 100% at Day 1 to account for the normalization of wound area to the
181 baseline measurement. It also involved knots at Days 4, 10, 16 and 21, a term for the
182 interaction between group and the spline, and a random coefficient for Day that varied by
183 animal as repeated measurements were observed for each mouse. Group means and 90%
184 confidence intervals were computed. For each group, the T_{50} mean estimate and its confidence
185 intervals were calculated from the intersection of the spline model estimates with 50% closure.
186 Time to 50% closure was also evaluated using survival analysis due to some mice not achieving
187 50% closure during the study. The time of event of 50% closure was taken as the observed
188 Study Day when a wound was at or below half its baseline size. A separate analysis calculated

189 the time of event using linear interpolation connecting Study Days between which 50% closure
190 was achieved. Wounds which remained larger than 50% of their baseline on all observed Study
191 Days were considered censored (no event). Time to complete closure was similarly analyzed
192 using the first observed Study Day when the wound was completely closed as the time to event.
193 The log-rank test was used to compare treatment groups by their survival.

194 Baseline-normalized wound area under the curve (AUC) was also computed for each mouse,
195 and group means and standard errors were reported, using a one-way ANOVA. Similarly,
196 wound area AUC was calculated without normalization and was modeled by an ANCOVA model,
197 with baseline wound area as the covariate.
198

199 Results

200 Results for time to 50% closure using the spline model are shown in Figure 1. The positive
201 control 0.01% becaplermin (Regranex) group showed complete healing in 9/10 animals over the
202 course of 21 days. The T_{50} (the time to 50% closure estimate) was 11.5 days, with 90%
203 confidence limits of approximately 11 and 12 days. The positive control arm was terminated at
204 Day 21. The GSK2256294 treatment group showed healing, but the process was slower than for
205 the positive control group and not all animals responded: T_{50} = 14.4 days, 90% confidence limits
206 of 13 and 16.5 days. The vehicle group also showed some healing over time, T_{50} = 19.7 Days,
207 90% confidence lower time 15.5 days (insufficient data to estimate the upper confidence limit),
208 but the progress was somewhat slower than the GSK2256294 group.

209 **Fig 1. Time to 50% wound closure (T_{50}) for topical GSK2256294**

210 *db/db* mice (N=10 per treatment group) were treated with topical GSK2256294, vehicle control,
211 or positive control (0.01% becaplermin). Wound areas were determined by tracing the wound
212 boundary at each time point and compared to the wound area on Day 1 (baseline = 100%). A
213 spline model was used to interpolate between the discrete wound area measurements. The
214 dashed horizontal line indicates 50% closure. The dashed black vertical line shows the
215 treatment group estimate of mean T_{50}, and the red dashed vertical lines show the 90%
216 confidence interval for T_{50}. Grey lines represent data from individual animals.

217

218 Figure 2 shows the individual values and group means for baseline-normalized wound AUC
219 through Day 21, by which time the positive control group wounds had healed. The mean AUC of
220 the positive control group (shown in green) was significantly different (11.1 cm^2 total AUC [90%
221 confidence interval: 9.6-12.5] vs. 13.3 [12.8-15.8]; p-value = 0.002) than the GSK2256294 group
222 (shown in red). However, the GSK2256294 group was not significantly different from the vehicle
223 control group (shown in blue; 13.3 [12.8-15.8] vs. 15.9 [14.5-17.4]; p-value = 0.061). The same
224 conclusion was reached when wound AUC through Day 21 was modeled with baseline as the
225 covariate (not shown): the mean AUC of the GSK2256294 treated group was significantly higher
226 than that of the positive control group (9.3 [8.4-10.2] vs. 7.2 [6.3-8.1]; p-value = 0.001) but not
227 significantly lower than the vehicle control group (9.3 [8.4-10.2] vs. 10.3 [9.3-11.2], respectively;
228 p-value = 0.075).

229 **Fig 2. Wound AUC through day 21 for topical GSK2256294**

230 Data points show the computed AUC of wound area self-normalized to Day 1 baseline, for
231 individual mice in the sEHi (red), vehicle (blue) and positive control (green) groups. Group
232 means and standard error bars are also displayed.

233

234 Because healing appeared to still be progressing at Day 21, the study was extended until Day 26
235 for the sEHi and vehicle arms, to determine if the healing trend would continue. The Day 26
236 results are shown in Figure 3. While the GSK2256294-treated group appeared to show more
237 healing than the vehicle control, the results did not meet the criterion for statistical significance
238 (15.6 [13.4-17.8] vs. 18.0 [15.8-20.2]; p-value = 0.058). Similarly, in comparison of wound AUC
239 through Day 26 with baseline area as the covariate (not shown), the mean AUC of the
240 GSK2256294 treated group was not significantly lower than that of the vehicle control group
241 (10.0 [8.6-11.5] vs. 11.6 [10.1-13.0]; p-value = 0.067).

242 **Fig 3. Wound AUC through day 26 for topical GSK2256294**

243 Data points show the computed AUC of wound area self-normalized to Day 1 baseline, for
244 individual mice in the sEHi (red) and vehicle (blue) groups. Group means and standard error
245 bars are also displayed.

246

247 To summarize, 9/10 mice in the positive control group achieved wound closure by day 21, 3/10
248 mice in the GSK2256294 treatment group achieved closure by Day 26 and no mice in the vehicle
249 group achieved closure by Day 26 (Table 1). The wound area data are available in Supplemental
250 Table 1.

Table 1 Summary of results for wound closure

Day 21			
Treatment Group	**% wound closure (median)**	**N 100% closure**	**N 50% closure**
becaplermin (positive control)	100	9/10	10/10
Active sEHi	76	2/10	8/10
Vehicle control	48	0/10	6/10

Day 26			
Treatment Group	**% wound closure (median)**	**N 100% closure**	**N 50% closure**
Active sEHi	83	3/10	8/10
Vehicle control	65	0/10	6/10

Discussion

We were encouraged that the GSK2256294-treated group showed evidence of improved healing compared to vehicle control, despite the statistical significance threshold of $P < 0.05$ not being met. In part, this might be attributed to greater variability in the wound healing process among the mice in this study, compared to baseline data previously seen in this model. Repeating the study with larger group size would be illuminating. We also note that this study

264 was our first attempt at topical formulation of GSK2256294. It may be possible to further
265 optimize the formulation, and the dose administered, to obtain improved response.

266 After our study was complete, a publication on EETs and diabetic wound healing appeared (17),
267 demonstrating that 11,12-EET treatment accelerated wound healing in *ob/ob* (leptin-deficient)
268 mice. These mice also had significantly reduced mRNA and protein expression of *CYP2C65* and
269 *CYP2J2* compared to wild type. Taken together, the data strongly suggests that sEH inhibition,
270 or other strategies to raise EET levels, can potentially improve wound healing in pre-clinical
271 diabetic models.

272

273 The positive control Becaplermin is a biological agent (recombinant human platelet-derived
274 growth factor-BB), which has been approved and launched for use in treating diabetic ulcers.
275 Although it works well in the *db/db* mouse model, its efficacy in patients is less impressive. A
276 meta-analysis of five clinical trials (18) found that 205/428 (47.89%) of becaplermin-treated
277 ulcers healed completely, versus 109/335 (32.53%) of those in the placebo group. Considering
278 that becaplermin has a black box warning for malignancies in patients using more than three
279 tubes, there is clearly unmet need in the treatment of diabetic ulcers. Finally, we point out that
280 there is additional evidence from non-diabetic mouse models that pre-clinical sEH inhibitors
281 improve wound healing in that context (14-16), so applications for this therapy could be
282 extended to surgical and other types of wounds.

283

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288 comments on earlier drafts of this manuscript.

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292 # References

293

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343

344 Supporting Information

345 S1 Table. Wound healing time-course measurements for individual mice in each treatment
346 group.

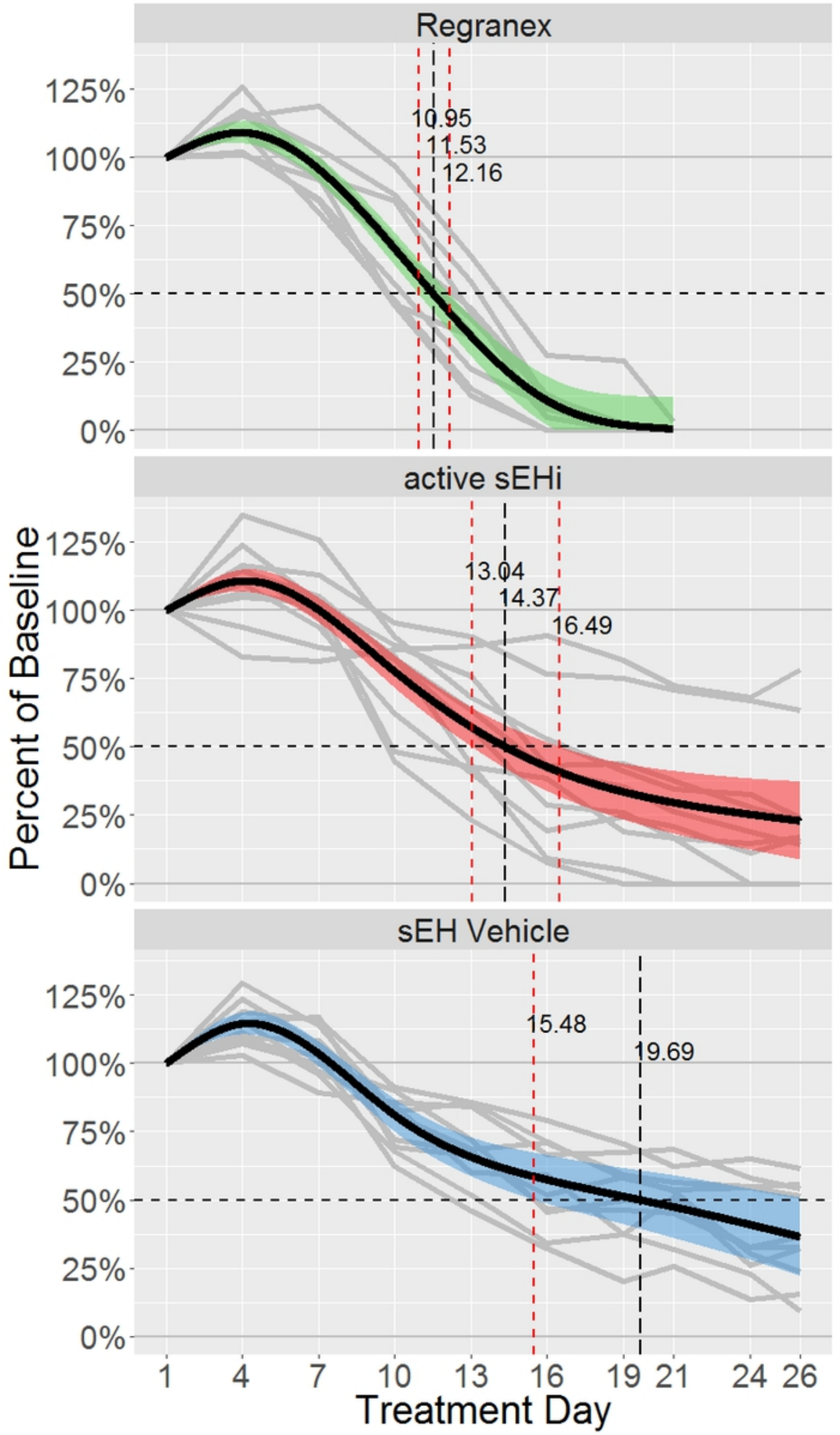
Regranex
10.95
11.53
12.16
active sEHi
13.04
14.37
16.49
sEH Vehicle
15.48
19.69
125%
100%
75%
50%
25%
0%
Percent of Baseline
1
4
7
10
13
16
19
21
24
26
Treatment Day

Figure1

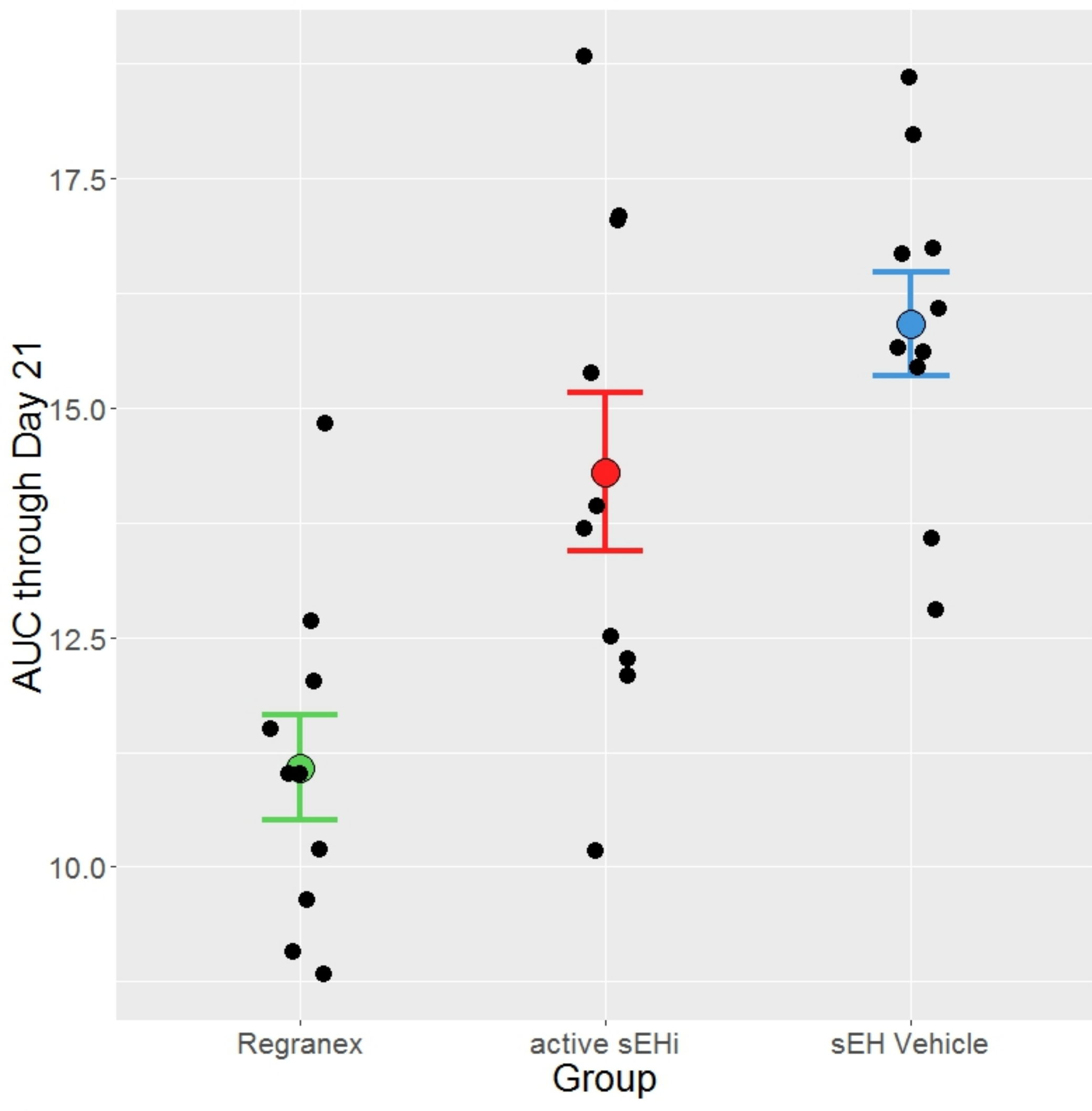
17.5
15.0
12.5
10.0
AUC through Day 21
Regranex
active sEHi
sEH Vehicle
Group

Figure2

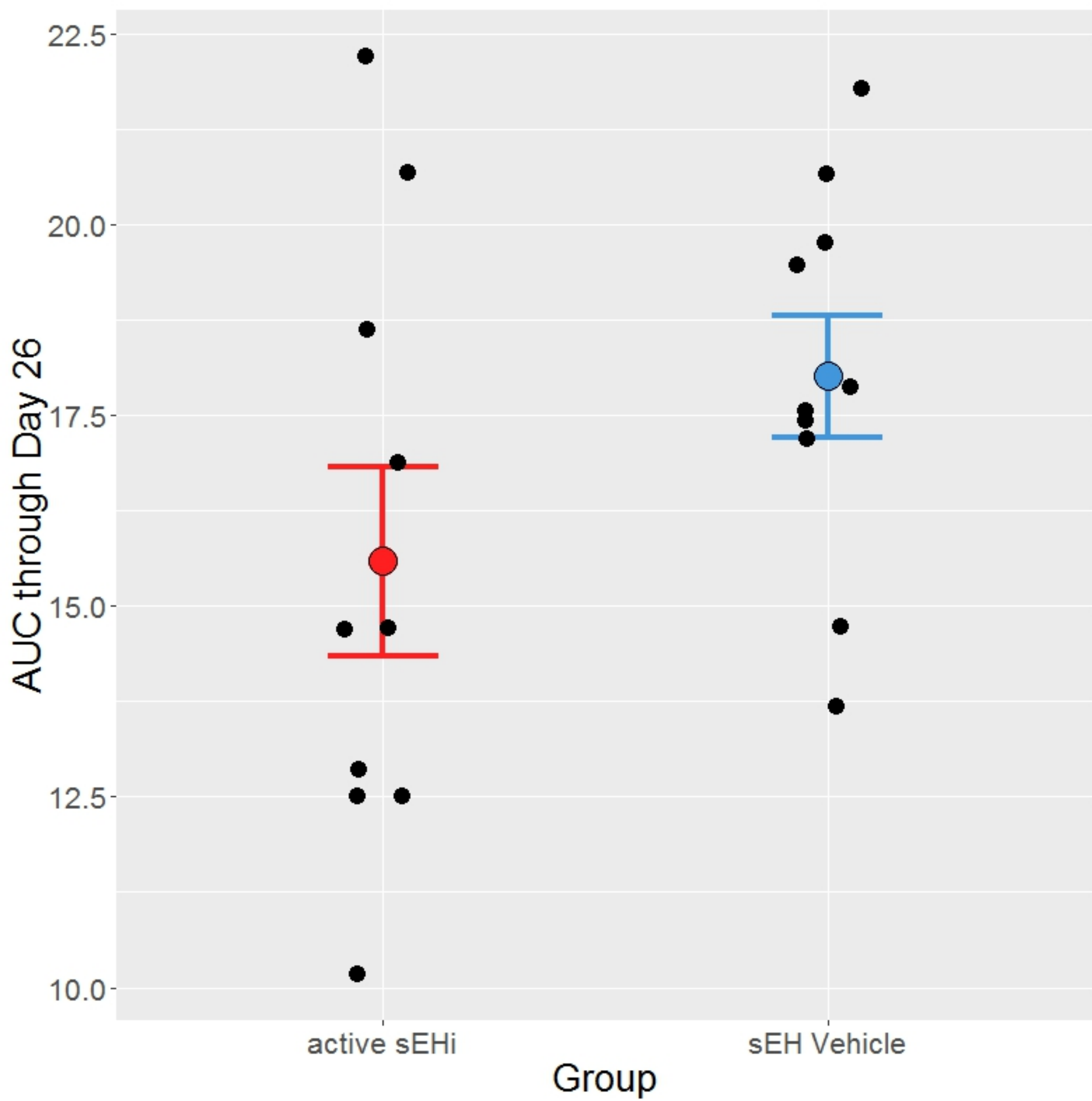
22.5
20.0
17.5
15.0
12.5
10.0
AUC through Day 26
active sEHi
sEH Vehicle
Group

Figure3