1 Title: Effect of topical soluble epoxide hydrolase inhibition in a preclinical diabetic

2 wound healing model.

3 Short title: EPHX2 inhibition and wound healing

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Abstract

18

19 The objective of this study was to test a topical formulation of *EPHX2* inhibitor, GSK2256294, in

20 a dermal wound diabetic (*db/db*) mouse model. Comparisons were made between groups

21   treated with *EPHX2* inhibitor, matching vehicle control and a currently approved treatment for

22   diabetic ulcers, becaplermin/Regranex, as a positive control which is highly efficacious in this

23   model. Leptin receptor-deficient (*db/db*) male mice were sequentially assigned to treatment

24   groups (N=10 per group) based on blood glucose levels and body weight. Mice were given a

25   single 8 mm diameter full thickness wound on the back. Wounds were photographed and

26   traced, and fresh test materials applied periodically with fresh dressing. Of ten mice treated

27   with GSK2256294, three had completely healed wounds at the study conclusion, and 8/10 mice

28   reached at least 50% closure. In the vehicle group, no mice achieved complete closure, and only

29   6/10 reached at least 50% closure by the study conclusion. Nine of ten wounds achieved

30   complete closure in the positive control group by 21 days. Although the EPHX2 inhibitor results

31   were indicative of efficacy, the single-sided p-value criterion of 0.05 was not met in this study.

32

Introduction

34   Soluble epoxide hydrolase (sEH, encoded by the *EPHX2* gene), a member of the α/β-hydrolase

35   fold family of epoxide hydrolases, is a critical enzyme in the metabolism of epoxyeicosatrienoic

36   acids (EETs) to dihydroxyeicosatrienoic acids (DHETs). EETs are formed by the oxidation of

37   arachidonic acid by CYP450 enzymes, primarily members of the CYP 2C and 2J classes in

38   humans. EETs are a group of eight compounds: the four regioisomers are 5,6-EET, 8,9-EET,

39   11,12-EET and 14,15-EET, with R/S and S/R enantiomers of each. *In vitro,* EETs have

40   demonstrated anti-inflammatory and potential pro-resolving effects as well as effects on airway

41   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

Animal Care

We contracted with Explora BioLabs, San Diego, CA to manage the animal study. Every effort

was made to minimize or eliminate pain and suffering of all the animals in the study. The study

was approved by the Office of Animal Welfare, Ethics and Strategy at GSK (Reference number

D012050, Submission number S003940). All studies were conducted in accordance with the GSK

Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the

Institutional Animal Care and Use Committee at Explora BioLabs. Male mice, *db/db*

(Homozygous for Lepr^{db} <BKS.Cg-Dock7^m +/+ Lepr^{db}/J>, 8-9 weeks old, were purchased from

Jackson Laboratories. Upon arrival, animals were inspected for correct age and sex, ear-tagged

and their body weights were recorded. The animals were housed 1-per cage. The animal

housing, care and husbandry were performed per the Guide for the Care and Use of Laboratory

Animals; 8th Edition; Institute of Laboratory Animal Resources; U.S. Department of Health and

Human Services; National Institutes of Health Publication No. 85-23, Revised 2011.

Temperature in the animal holding room was maintained between 20 and 26°C (68-79°F). Room

humidity was monitored (40-70% is recommended in the Guide). Temperature and humidity

values were recorded daily. A 12-hour light/12-hour dark cycle illumination period was

maintained. Animals were housed using the dual HEPA-filtered ventilated Innovive disposable

IVC cage system 3.0, which provides 60 fresh-air changes per hour for animal cages. The

animals had ad libitum access to standard diet (2920X.10, Global 18% Protein Rodent Diet from

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

Topical sEH Formulation

119   Topical gel formulations for GSK2256294 (2) were developed by Dermatology. The solvent

120   system 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

6

125   topical formulation was shipped to the CRO weekly, for the duration of the study. Becaplermin

126   (0.01%) was purchased from Smith & Nephew (Catalogue number - REG810-15) and used as

127   directed.

128

129

130   **Study Design**

131   We requested a representative data set from the CRO to assess model variability and estimate

132   the number of mice necessary to achieve adequate power. Sample sizes of N=10 mice per

133   group were determined to achieve 80% power to detect decreases from vehicle control of 1.31

134   days in mean interpolated time to 50% wound closure when using a one-way ANOVA model

135   with five experimental groups, for a total sample size of 50 animals. The study contained five

136   experimental groups in order to investigate other chemical compounds in addition to

137   GSK2256294, although only the three previously mentioned groups are presented here.

138

139   Timeline

140   • Day -7 to Day 0   acclimation

141   • Day -3   back hair removed

142   • Day 1   wounding

143   • Day 1   pre-dose and 1 hour post-dose blood collection (N=3 mice per group)

144   • Days 1, 4, 7, 10, 13, 16, 19, 21 and 24   dosing and wound measurement

145   • Day 21   termination of positive control group

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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

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

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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**

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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.
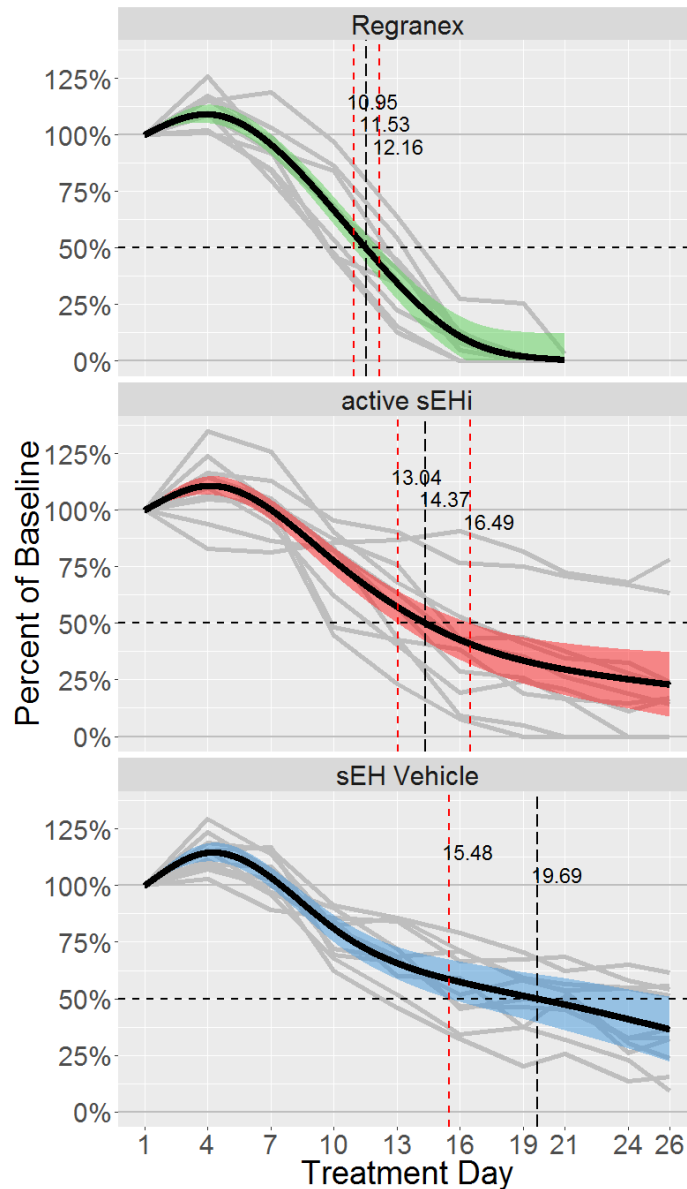
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Figure 2 shows the individual values and group means for baseline-normalized wound AUC

through Day 21, by which time the positive control group wounds had healed. The mean AUC of

the positive control group (shown in green) was significantly different (11.1 cm^2 total AUC [90%

confidence interval: 9.6-12.5] vs. 13.3 [12.8-15.8]; p-value = 0.002) than the GSK2256294 group

(shown in red). However, the GSK2256294 group was not significantly different from the vehicle

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).

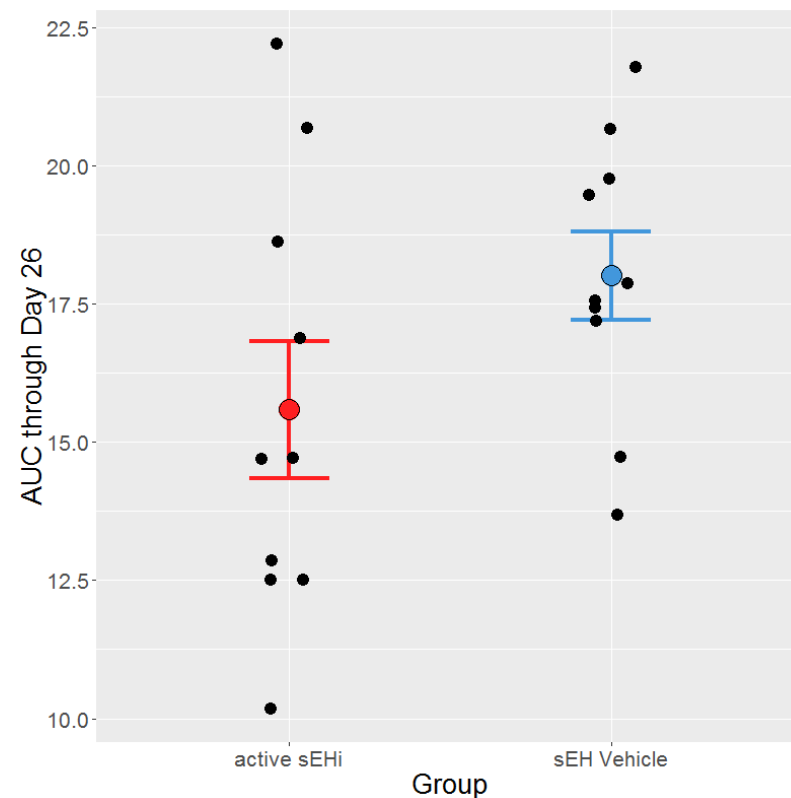229   **Fig 2. Wound AUC through day 21 for topical GSK2256294**

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.

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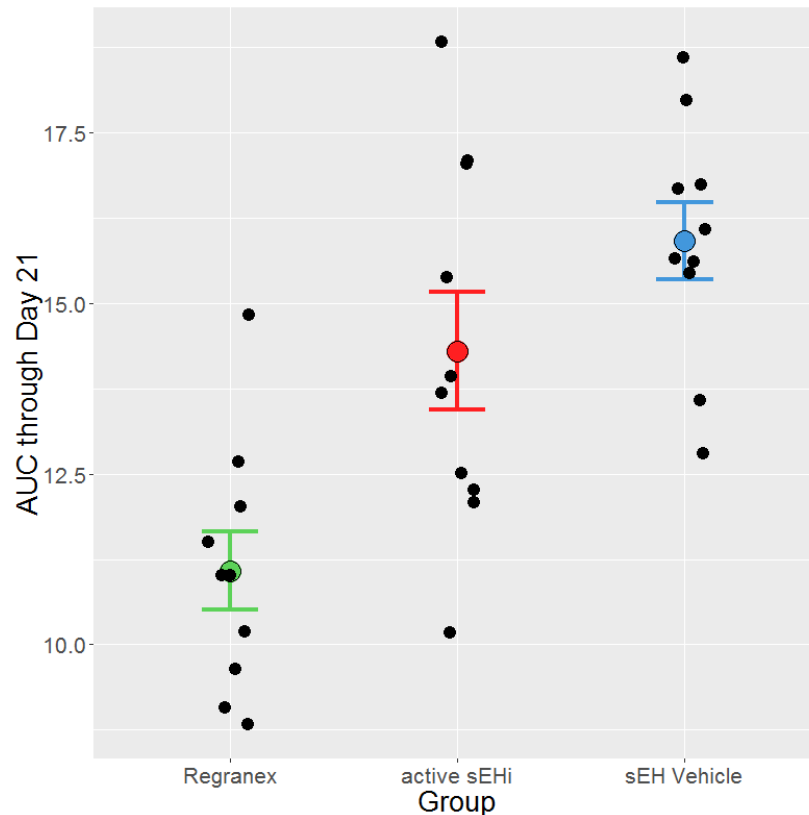
236   Because healing appeared to still be progressing at Day 21, the study was extended until Day 26

237   for the sEHi and vehicle arms, to determine if the healing trend would continue. The Day 26

238   results are shown in Figure 3. While the GSK2256294-treated group appeared to show more

239   healing than the vehicle control, the results did not meet the criterion for statistical significance

240   (15.6 [13.4-17.8] vs. 18.0 [15.8-20.2]; p-value = 0.058). Similarly, in comparison of wound AUC

241   through Day 26 with baseline area as the covariate (not shown), the mean AUC of the

242   GSK2256294 treated group was not significantly lower than that of the vehicle control group

243   (10.0 [8.6-11.5] vs. 11.6 [10.1-13.0]; p-value = 0.067).

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

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245   Data points show the computed AUC of wound area self-normalized to Day 1 baseline, for

246   individual mice in the sEHi (red) and vehicle (blue) groups. Group means and standard error

247   bars are also displayed.

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252   To summarize, 9/10 mice in the positive control group achieved wound closure by day 21, 3/10

253   mice in the GSK2256294 treatment group achieved closure by Day 26 and no mice in the vehicle

254   group achieved closure by Day 26 (Table 1). The wound area data are available in Supplemental

255   Table 1.

256

257 **Table 1 Summary of results for wound closure**

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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

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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

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Discussion

264   We were encouraged that the GSK2256294-treated group showed evidence of improved

265   healing compared to vehicle control, despite the statistical significance threshold of $P < 0.05$ not

266   being met. In part, this might be attributed to greater variability in the wound healing process

267   among the mice in this study, compared to baseline data previously seen in this model.

268   Repeating the study with larger group size would be illuminating. We also note that this study

269   was our first attempt at topical formulation of GSK2256294. It may be possible to further

270   optimize the formulation, and the dose administered, to obtain improved response.

16

271   After our study was complete, a publication on EETs and diabetic wound healing appeared (17),

272   demonstrating that 11,12-EET treatment accelerated wound healing in *ob/ob* (leptin-deficient)

273   mice. These mice also had significantly reduced mRNA and protein expression of *CYP2C65* and

274   *CYP2J2* compared to wild type. Taken together, the data strongly suggests that sEH inhibition,

275   or other strategies to raise EET levels, can potentially improve wound healing in pre-clinical

276   diabetic models.

277

278   The positive control Becaplermin is a biological agent (recombinant human platelet-derived

279   growth factor-BB), which has been approved and launched for use in treating diabetic ulcers.

280   Although it works well in the *db/db* mouse model, its efficacy in patients is less impressive. A

281   meta-analysis of five clinical trials (18) found that 205/428 (47.89%) of becaplermin-treated

282   ulcers healed completely, versus 109/335 (32.53%) of those in the placebo group. Considering

283   that becaplermin has a black box warning for malignancies in patients using more than three

284   tubes, there is clearly unmet need in the treatment of diabetic ulcers. Finally, we point out that

285   there is additional evidence from non-diabetic mouse models that pre-clinical sEH inhibitors

286   improve wound healing in that context (14-16), so applications for this therapy could be

287   extended to surgical and other types of wounds.

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289   **Acknowledgements**

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Supporting Information

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