1   **Hyaluronidase impacts exposures of long-acting injectable paliperidone palmitate in rodent**

2   **models**

3

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24   **Abstract**

25   A significant challenge in the development of long-acting injectable drug formulations,

26   especially for anti-infective agents, is delivering an efficacious dose within a tolerable injection

27   volume. Co-administration of the extracellular matrix-degrading enzyme hyaluronidase can

28   increase maximum tolerable injection volumes but is untested for this benefit with long-acting

29   injectable formulations. One concern is that hyaluronidase could potentially alter the tissue

30   response surrounding an injection depot, a response known to be important for drug release

31   kinetics of long-acting injectable formulations. The objective of this pilot study was to evaluate

32   the impact of co-administration of hyaluronidase on the drug release kinetics, pharmacokinetic

33   profiles, and injection site histopathology of the long-acting injectable paliperidone palmitate for

34   up to four weeks following intramuscular injection in mouse and rat models. In both species, co-

35   administration of hyaluronidase increased paliperidone plasma exposures the first week after

36   injection but did not negate the overall long-acting release nature of the formulation.

37   Hyaluronidase-associated modification of the injection site depot was observed in mice but not

38   in rats. These findings suggest that further investigation of hyaluronidase with long-acting

39   injectable agents is warranted.

40

41   **Keywords** (max 6)

42   hyaluronidase, paliperidone palmitate, pharmacokinetics, histopathology, mouse model, rat

43   model

1. Introduction

Long-acting injectable (LAI) formulations of anti-infective medications hold great promise for

simplifying administration and improving adherence, especially for indications in which

preventive or therapeutic regimens require prolonged durations (weeks to months) of drug

administration, including HIV prevention and treatment [1], malaria prophylaxis [2], viral

hepatitis treatment [3, 4], and tuberculosis prevention and treatment [5]. A significant challenge

in development of anti-infective LAI formulations is achieving efficacious drug exposure

profiles within a tolerable injection volume. A recommended maximum intramuscular (IM)

injection volume in adults is 5 mL, although IM injections greater than 3 mL are rare and thus

many health care professionals are not familiar with administering larger injection volumes [6].

This is reflected in proposed target product profiles for LAI formulations for malaria and

tuberculosis indications, which suggest that, as a minimum requirement, IM injection volume

should not exceed 2 mL, and ideally would be <2 mL in adults [5, 7].

One component of the extracellular matrix and interstitial space that contributes to these volume

limitations is hyaluronic acid, which is a viscous disaccharide polymer that retains water

molecules and prevents sudden bulk fluid flow through the extracellular matrix. This property of

hyaluronic acid allows the extracellular matrix to resist compressive forces, such as those created

by an LAI drug depot. However, hyaluronic acid can be transiently depolymerized by

hyaluronidases, which are enzymes that function naturally as spreading agents to aid in the

dispersion of venoms, toxins, and sperm. A recombinant human hyaluronidase (rHuPH20,

Hylenex®) has been approved by the United States Food and Drug Administration for use as an

adjuvant to increase the dispersion and absorption of drugs [8], and the manufacturer of

67   rHuPH20, Halozyme, has developed ENHANZE® drug delivery technology for co-formulation

68   with [non-long-acting] medications to permit significantly larger subcutaneous injection volumes

69   [9]. Therefore, we hypothesized that co-administration of a hyaluronidase could also increase the

70   tolerable IM injection volume for LAI formulations.

71

72   The nature of LAI drug formulations is that the drug is slowly released over time from the

73   injected drug depot. Preclinical studies of the LAI antipsychotic agent paliperidone palmitate in a

74   rat model demonstrated that following injection into the thigh muscle, a granuloma forms around

75   the depot site, and co-administration of agents that interfere with the host inflammatory response

76   and abrogate granuloma formation can alter the paliperidone release pharmacokinetics (PK) and

77   lead to lower drug exposures [10, 11]. Although not directly anti-inflammatory, co-

78   administration of hyaluronidase could potentially influence the nature of the immune response

79   following injection of an LAI formulation by affecting immune traffic through the extracellular

80   matrix and interstitial space. The objective of this study was to evaluate the paliperidone release

81   kinetics and PK profile following IM injection with paliperidone palmitate with or without co-

82   administration of hyaluronidase in both mouse and rat models. Paliperidone palmitate was used

83   as a representative approved and marketed LAI formulation for which PK and drug depot

84   histopathology data in rat models were published [10, 11]. In this study, we also included

85   assessment in a mouse model, as mouse models are often used in pre-clinical treatment models

86   of infectious diseases, including mouse models of tuberculosis used to evaluate developmental

87   LAI formulations of anti-tuberculosis drugs [12-14].

88

89 **2. Materials and Methods**

90 *2.1. Mice (sourcing, husbandry, and other general information)*

91 All studies using mice were conducted at the Johns Hopkins University Center for Tuberculosis

92 Research, and all study procedures were approved by the Institutional Animal Care and Use

93 Committee of the Johns Hopkins University. Adult (6-8 weeks old) female BALB/c mice,

94 purchased from Charles River, were used in all studies. All mg/kg dosing of mice was based on

95 an average mouse body weight of 20 g. Mice were housed in individually ventilated cages with 4

96 mice (one sampling group) per cage with access to food and water *ad libitum* and with sterile

97 shredded paper for bedding. Room temperature was maintained at 22-24°C, and a 12-hour

98 light/12-hour dark cycle was used. Mice were killed by exsanguination via cardiac puncture

99 under inhalation anesthesia with isoflurane, followed by cervical dislocation.

100

101 *2.2. Rats (sourcing, husbandry, and other general information)*

102 All studies using rats were conducted at the University of Liverpool, under the UK Home Office

103 Project License PP9284915 in accordance with the Animals Scientific Procedures Act (ASPA)

104 1986 and approved by the local Animal Welfare and Ethical Review Board (AWERB). Adult

105 (6-8 weeks old) male Sprague Dawley rats, purchased from Charles River, were used in all

106 studies. All mg/kg dosing was based on an average body weight of 250 g. Rats were housed in

107 individually ventilated cages with 4 rats per cage and access to food and water *ad libitum*. Sterile

108 wood chippings, nesting material and enrichment was readily available in all cages. Room

109 temperature was maintained at 20-24°C, 45-65% humidity and a 12-hour light/dark cycle was

110 used. Rats were killed by exsanguination via cardiac puncture under inhalational anesthesia,

111 followed by cessation of breathing as confirmation of death.

112

113   *2.3. Sourcing and preparation of paliperidone palmitate and hyaluronidase for IM*

114   *administration and of paliperidone for IV administration to mice*

115   The paliperidone palmitate LAI formulation, manufactured by Janssen Pharmaceutica NV and

116   sold under the brand name INVEGA SUSTENNA®, was supplied at 156 mg/mL in an aqueous

117   IM-injectable suspension containing the following inactive ingredients: polysorbate 20,

118   polyethylene glycol 4000, citric acid monohydrate, sodium dihydrogen phosphate monohydrate,

119   sodium hydroxide, and water for injection. INVEGA SUSTENNA® pre-filled syringes were

120   purchased from the Johns Hopkins Hospital Pharmacy. Allometric scaling based on the standard

121   rat dose of 20 mg/kg [10, 11] and standard first human dose (234 mg or about 4 mg/kg) [15]

122   suggested a dose of 40 mg/kg in mice. However, administration of this dose would require

123   injecting only 5 µL of the INVEGA SUSTENNA® suspension. We were concerned about

124   accuracy in injecting such a small volume into mouse thighs. However, as we did have previous

125   experience with 8 µL IM injections in mice [12, 13], a pragmatic decision to dose with 8 µL of

126   the INVEGA SUSTENNA® suspension was made, resulting in a 62.4 mg/kg dose in the mice.

127

128   Hyaluronidase powder was purchased from Millipore Sigma, product number H3506

129   (hyaluronidase from bovine testes); this specific product has been previously used for IM

130   injection in mice [16]. The powder was dissolved in normal saline at concentrations of 2.5 and

131   15 enzyme units (U) per microliter, resulting in doses of 5 U and 15 U, respectively, in a 2 µL

132   volume. These doses of hyaluronidase had been previously administered to mice by IM injection

133   [16-18]. The solutions were filter-sterilized before use.

134

135   For administration of the paliperidone palmitate alone or with buffer (normal saline with or

136   without hyaluronidase), the INVEGA SUSTENNA® pre-filled syringe was vigorously shaken for

137   10 seconds to ensure a homogeneous suspension, according to the prescribing information [15].

138   The suspension was then carefully expelled and aliquotted into four sterile microcentrifuge

139   tubes. In one tube, the suspension was used directly for injection. In the remaining three tubes

140   the paliperidone palmitate suspension was mixed by gentle pipetting with buffer (normal saline)

141   alone or buffer containing hyaluronidase at 2.5 or 15 U/µL, with buffer representing 20%

142   (vol/vol) of the final volume. For IM injection of mice, 8 µL of paliperidone palmitate

143   suspension alone or 10 µL of the paliperidone palmitate suspension plus buffer (with or without

144   hyaluronidase) were injected into the right hind thigh using a BD Veo insulin syringe with a BD

145   Ultra-Fine 3/10 mL 6-mm by 31-gauge needle with a half-unit scale. This syringe/needle

146   combination had been previously used by our group to successfully administer 8 µL IM injection

147   volumes [12, 13].

148

149   Paliperidone powder was purchased from Carbosynth (now Biosynth), product number FP2572.

150   For intravenous (IV) administration, the paliperidone powder was dissolved at a concentration of

151   0.70 mg/mL in 10 mM sodium acetate-acetic acid buffer pH 4.0 [11], resulting in a dose of

152   3.5 mg/kg in a 100 µL injection volume. The solution was filter-sterilized before use. This dose

153   was selected to maximize the chances of detection while avoiding the maximum IV dose

154   (10 mg/kg) that Janssen reported testing in mouse toxicity studies [19]. A paliperidone IV

155   injection group was included to enable calculation of the absolute bioavailability of paliperidone

156   in mice and confirm any "flip-flop" kinetics [20] associated with slow depot release.

157   Paliperidone was used for IV administration instead of paliperidone palmitate because the

158   palmitate ester is hydrolyzed as part of overall depot release, freeing active paliperidone moiety

159   systematically for pharmacological effect and quantification. Thus, even when it is paliperidone

160   palmitate that is administered by IM injection, the disposition being monitored and quantified is

161   actually that of paliperidone, which therefore must be compared with the IV disposition of the

162   same.

163

164   *2.4. Sourcing and preparation of paliperidone palmitate and hyaluronidase for IM*

165   *administration to rats*

166   The paliperidone palmitate LAI suspension INVEGA SUSTENNA® was purchased from Royal

167   Liverpool Hospital Pharmacy. Based on a previous publication of this formulation by Janssen

168   researchers [10, 11], a standard dose of 20 mg/kg was administered to the rats. Hyaluronidase

169   powder was purchased from Sigma. The powder was dissolved in phosphate buffered saline to

170   generate doses of 5 U and 15 U of hyaluronidase per 2 μL. Rats were administered a 32 μL or

171   34 μL volume of INVEGA SUSTENNA® or INVEGA SUSTENNA® with hyaluronidase

172   respectively, via IM injection.

173

174   *2.5. Initial toxicity studies in mice*

175   Prior to conducting the PK study in mice, we first assessed the overt tolerability/toxicity in mice

176   following administration of paliperidone palmitate or paliperidone by IM or IV injection,

177   respectively. A total of 10 mice were used for these studies. Five mice were administered

178   paliperidone palmitate alone at 62.4 mg/kg by IM injection. Following injection, the mice were

179   observed every 1-2 hours for the first 8 hours and then daily for up to two weeks, at which point

180   the study was terminated. Five mice were administered paliperidone at 3.5 mg/kg by IV

181   injection. Following injection, the mice were observed every 1-2 hours for the first 8 hours and

182   then checked again at 24 hours, at which point the study was terminated.

183

184   *2.6. PK study in mice*

185   The primary study outcome was plasma paliperidone concentrations. Mice (n = 64) were

186   randomly assigned to one of the following five groups: (1) paliperidone palmitate alone at

187   62.4 mg/kg by IM injection (12 mice); (2) paliperidone palmitate 62.4 mg/kg plus buffer without

188   hyaluronidase by IM injection (12 mice); (3) paliperidone palmitate 62.4 mg/kg plus 5 U

189   hyaluronidase by IM injection (12 mice); (4) paliperidone palmitate 62.4 mg/kg plus 15 U

190   hyaluronidase by IM injection (12 mice); (5) paliperidone solution at 3.5 mg/kg by IV injection

191   (16 mice). For mice receiving paliperidone palmitate alone or with buffer/hyaluronidase by IM

192   injection, blood samples were obtained from mice at 0.25, 1, 2, 4, 7, 10, 24, 48, 72, 96, 168, 336,

193   and 672 hours post injection. For mice receiving paliperidone by IV injection, blood samples

194   were obtained from mice at 0.1, 0.25, 0.5, 1, 2, 4, 7, 10, and 24 hours post injection. For each

195   group at each time point, blood was sampled from four mice via in-life mandibular bleeding,

196   except for the final time point, when blood was drawn by cardiac puncture. The study design,

197   including how the mice were serially sampled and at what time point each group of mice

198   received cardiac punctures, is detailed in **Supplemental Table S1**. Blood was collected into BD

199   vacutainer plasma separation tubes with lithium heparin, and plasma was separated by

200   centrifugation at $15,000 \times g$ for 10 min at room temperature. Plasma was transferred to 1.5-mL

201   O-ring screw-cap tubes and stored at -80°C. Frozen samples were shipped on dry ice to the

202   University of Liverpool for processing and determination of paliperidone concentration.

203

204 *2.7. PK study in rats*

205 Because there are multiple published reports for IM administration of paliperidone palmitate and

206 IV administration of paliperidone in rats [10, 11, 21], initial tolerability studies in rats were not

207 deemed necessary in this project. The primary study outcome was plasma paliperidone

208 concentrations. Rats (n = 12) were randomly assigned to one of the following three groups: (1)

209 paliperidone palmitate alone at 20 mg/kg by IM injection (4 rats); (2) paliperidone palmitate 20

210 mg/kg plus 5 U hyaluronidase by IM injection (4 rats); (3) paliperidone palmitate 20 mg/kg plus

211 15 U hyaluronidase by IM injection (4 rats). Serial blood samples were obtained from each rat at

212 0.25, 1, 2, 4, 7, 10, 24, 48, 72, 96, 168, 360, 504, and 672 hours post injection. For each group at

213 each time point, blood was sampled via lateral tail vein bleed, except for the final time point,

214 when blood was drawn by cardiac puncture. The study design, including how the rats were

215 serially sampled, is detailed in **Supplemental Table S2**. Blood was collected into 1 mL syringes

216 via a 25-gauge needle and transferred to an Eppendorf tube containing heparin (Starstedt, UK).

217 Plasma was separated by centrifugation at $13,000 \times g$ for 3 min at room temperature. Plasma was

218 transferred to 1.5-mL Eppendorf tubes and stored at -80°C until analysis.

219

220 *2.8. Determination of paliperidone plasma concentrations*

221 The plasma concentration of paliperidone in both rats and mice was quantified using a liquid

222 chromatography tandem mass spectrometric method, which conforms to United States Food and

223 Drug Administration bioanalytical development guidelines [22]. A stock solution of 1 mg/mL

224 paliperidone was prepared in methanol and stored at 4°C until used for preparation of a standard

225 curve from 500 ng/mL to 1.9 ng/mL in either rat or mouse plasma by serial dilution. Paliperidone

226 was extracted from blood plasma samples via protein precipitation: in glass tubes, 100 µL of

227   sample (blank matrix, calibrator standard, quality control, or unknown) was combined with

228   450 μL acetonitrile and 50 μL internal standard stock solution (50 ng/mL sofosbuvir). Following

229   centrifugation at $3,000 \times g$ for 5 minutes, 80 μL of the supernatant fraction and 20 μL H_2O were

230   placed in chromacol vials for analysis. Quantification was then performed using a SCIEX 6500+

231   QTRAP (SCIEX MA, USA) operating in positive mode (m/z 427 > 207 and 110).

232   Chromatographic separation was achieved using a multi-step gradient with a Kinetex 2.6 μm

233   C18 column (Phenomenex, Cheshire, UK) using mobile phases A (100% H_2O, 0.05% formic

234   acid) and B (100% acetonitrile, 0.05% formic acid). Chromatography was conducted over 3.5

235   minutes at a flow rate of 400 μL/min. Concentrations obtained were expressed in ng/mL.

236

237   *2.9. PK analyses*

238   Plasma concentration-time profiles were analyzed with non-compartmental PK analysis in the

239   R data analysis environment [23] and Microsoft Excel for calculation of terminal half-lives ($T_{1/2}$)

240   and other PK parameters including maximum plasma concentration (C_{max}) and its time of

241   occurrence (T_{max}) and area under the time-concentration curve for partial sections of the time

242   course profile (AUC_{0-t}), the full profile (AUC_{0-last}) and the full profile extrapolated to infinity

243   (AUC_{inf}). For mouse data, where a maximum of three plasma samples were taken from any

244   individual mouse over the course of the study, the composite mean PK profile from all mice was

245   analyzed and PK parameters calculated. For rat data where full time courses could be sampled in

246   each individual animal, mean PK parameter values and their standard deviations were calculated

247   following from analysis of individual PK profiles. For calculation of bioavailability (F) the

248   contribution of the palmitate ester to the molecular weight of paliperidone palmitate (such that

249   paliperidone palmitate is 64.1% paliperidone by mass) was accounted for in dose normalization

250 such that 62.4 and 20 mg/kg doses of paliperidone palmitate are equivalent to 40 and 12.8 mg/kg

251 doses of paliperidone, respectively. Plasma concentrations following IV injection of paliperidone

252 in rat and calculation of bioavailability was sourced from the published data of Sun *et al*. [21].

253

254 *2.10. Histopathology assessment*

255 A secondary study outcome was a descriptive assessment of the histopathology surrounding the

256 drug depot following intramuscular injection. For both mice and rats, the hind thighs that

257 received IM injections were removed following cardiac puncture and confirmation of death and

258 fixed in formalin. Fixed rat tissues were shipped to Johns Hopkins University for analysis. Fixed

259 mouse and rat thigh samples were leveled at 60 µm and sectioned at a thickness of 5-6 µm for

260 hematoxylin and eosin staining. Sections were processed, stained, and scanned by the Johns

261 Hopkins University Oncology Tissue Services.

262

263 **3. Results and discussion**

264 *3.1. Initial toxicity studies of paliperidone palmitate (IM) and paliperidone (IV) in mice.*

265 Because the proposed 62.4 mg/kg IM dose of paliperidone palmitate was based on allometric

266 scaling while using a practical injection volume, we did not know if this dose would be tolerated

267 by the mice. Therefore, prior to conducting the full PK study, we first assessed the overt toxicity

268 in mice following IM injection of paliperidone palmitate at 62.4 mg/kg. None of the five mice

269 injected exhibited any signs of toxicity at any time during the two-week follow-up period post

270 injection. Although our proposed dose of 3.5 mg/kg of paliperidone in solution for IV injection

271 was selected to stay below the maximum IV dose (10 mg/kg) reported to have been evaluated in

272 mouse toxicity studies according to a regulatory report [19], we also wanted to first assess the

273   tolerability of this dose in mice before initiating our full PK study. Immediately following IV

274   injection, four out of the five mice became lethargic and exhibited ptosis, which was consistent

275   with the toxicity information in the regulatory report. By five hours post injection, the symptoms

276   had noticeably improved, and by 24 hours post injection, the mice were fully recovered. Based

277   on these observations, we determined that these IM and IV doses of paliperidone palmitate

278   suspension and paliperidone solution, respectively, were suitable without an overt toxicity issue.

279

280   *3.2. Evaluation of the impact of hyaluronidase on paliperidone plasma exposure profiles*

281   *following IM injection of paliperidone palmitate LAI formulation in mice*

282   Visual inspection of the mouse plasma PK profile time courses indicated a multiphasic release

283   profile for paliperidone following IM dosing as paliperidone palmitate in mice (**Fig. 1A**), with at

284   least 2 or 3 release fraction processes leading to local maxima (peaks) in the concentration-time

285   profile at time points as follows: paliperidone palmitate alone: 7 h, 1 day and 1 week;

286   paliperidone palmitate + buffer: 2 h, 2 days and 2 weeks; paliperidone palmitate + 5 U

287   hyaluronidase: 2 h, and 1 week; paliperidone palmitate + 15 U hyaluronidase, 10 h and 1 week.

288   Because of the limitations of sparse sampling, the full shape of the complex PK profile and the

289   true C_{max} may not have been observed with the time points taken. All individual mouse data are

290   available in **Supplementary Data File S1**.

291

292   Co-administration of hyaluronidase increased exposure in the first 4 days of the time course with

293   AUC_{0-4d} values approximately 3.5 to 4x greater in groups receiving paliperidone palmitate with

294   hyaluronidase compared to the group receiving paliperidone palmitate without hyaluronidase

295   (**Table 1**). A similar effect was observed with either 5 or 15 U hyaluronidase. Although there

296   were multiple local concentration in the profiles, the overall C_{max} was also approximately 2 to 3x

297   greater when hyaluronidase was co-administered (**Table 1**; **Fig. 1A**). Co-administration of

298   hyaluronidase did not compromise the long-acting exposures from IM administration of this

299   formulation to mice, as prolonged exposures were still maintained for 28 days, with similar

300   AUCs, bioavailability (F ~30%.), and terminal half-lives of (100-250 h) in the presence or

301   absence of hyaluronidase (**Table 1**). In all cases the terminal half-lives of paliperidone following

302   IM dosing as the palmitate ester were confirmed as "flip-flop" [20] in nature when taken in

303   comparison to the IV disposition half-life of paliperidone in mice of 4.5 h (**Table 2**;
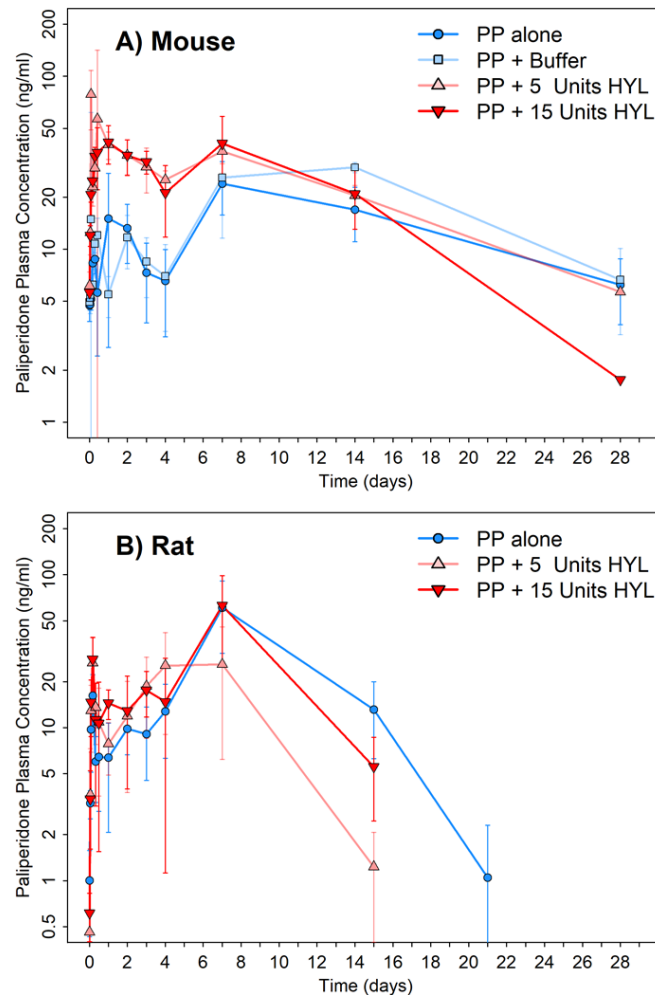
304   **Supplementary Fig. S1**).

Figure 1. Plasma concentration-time PK profiles for paliperidone in A) mice and B) rats following IM injection with paliperidone palmitate (PP) with or without hyaluronidase (HYL). Data points represent mean values (n = 4 samples per time point for mice; n = 4 samples per time point for rats), and error bars represent +/- standard deviation. All individual mouse and rat data are available in **Supplementary Data Files S1 and S2**, respectively.

311 **Table 1. PK parameters for paliperidone following IM injection as paliperidone palmitate**

312 **with or without hyaluronidase in mice and rats.** For the mouse data, the composite mean

313 profile parameters (n=12 mice) are presented. For the rat data, the mean and [standard deviation]

314 from n = 4 individual rats are provided for each parameter.

Species	Study group	AUC_{0-96} (ng·h/mL)	AUC_{0-last} (ng·h/mL)	AUC_{0-Inf} (ng·h/mL)	F (%)	Terminal $T_{1/2}$ (h)	C_{max} (ng/mL)	T_{max} (h)
Mouse	1) IM paliperidone palmitate 62.4 mg/kg alone	967	9391	11693	25	256	23.9	168
	2) IM paliperidone palmitate 62.4 mg/kg + saline buffer	851	12820	14316	30	156	29.7	336
	3) IM paliperidone palmitate 62.4 mg/kg + 5 U hyaluronidase	3720	15143	16661	35	186	96.5	10
	4) IM paliperidone palmitate 62.4 mg/kg + 15 U hyaluronidase	3165	14396	14671	31	108	41.5	24
Rat	1) IM paliperidone palmitate 20 mg/kg alone	864 [292]	11679 [5109]	12412 [5130]	52	78 [27]	60.8 [30]	168 [0]
	2) IM paliperidone palmitate 20 mg/kg + 5 U hyaluronidase	1425 [711]	5763 [3423]	7289 [2344]	31	48 [10]	33.6 [16]	86 [95]
	3) IM Paliperidone Palmitate 20 mg/kg + 15 U hyaluronidase	1516 [307]	11285 [4633]	11879 [4210]	50	63 [31]	50.3 [39]	127 [82]

315

316

317 **Table 2. PK parameters for paliperidone following IV doses in mice and rats.**

Species	Paliperidone dose for IV injection	AUC_{0-last} (ng·h/mL)	AUC_{0-inf} (ng·h/mL)	CL (L/h/kg)	Vss (L/kg)	Terminal $T_{1/2}$ (h)	C_{max} (obs) (ng/mL)	T_{max} (obs) (h)
Mouse (this study)	3.5 mg/kg	4094	4153	0.84	4.40	4.5	1604.9	0.1
Rat (Sun *et al.* 2010) [21]	2.5 mg/kg	4594	4627	0.54	0.87	1.1	2058.2	0.17

318

3.3. Evaluation of the impact of hyaluronidase on paliperidone plasma exposure profiles

following IM injection of paliperidone palmitate LAI formulation in rats

Because the IM dose of paliperidone palmitate suspension had been used in previously published studies with Janssen researchers, it was not necessary to conduct an initial toxicity study in rats. As a PK profile following IV injection with paliperidone solution was also already published [21], it was also not necessary to include such a group in this study. To further reduce the number of rats required for this arm of the study, we omitted the paliperidone palmitate plus buffer group, as data from the mouse PK study indicated that adding buffer alone (without hyaluronidase) did not impact outcome, and furthermore the concentration of buffer used with hyaluronidase was proportionally much smaller in the preparations for injections in rats compared to the preparations for injections in mice. All individual rat data are available in **Supplementary Data File S2**.

Similarly for mice, visual inspection of the rat plasma PK profile time courses also indicated a multiphasic release profile for paliperidone following IM dose as paliperidone palmitate, with two apparent release fraction processes leading to local maxima (peaks) at 4h and 1 week in mean concentration-time profiles for all three study groups, with or without co-administration of hyaluronidase (**Fig. 1B**). Also as in mice, co-administration of hyaluronidase increased exposure in the first 4 days of the time course but with reduced apparent effect compared to mice, with AUC_{0-4d} ~1.6x greater with hyaluronidase present (**Table 1**) with similar effect for either 5 or 15 U hyaluronidase. The reduced apparent effect of hyaluronidase may likely be attributed to the rats receiving a lower weight normalized dose of hyaluronidase (*i.e.*, 5 or 15 U per 0.25 kg typical body weight in rats vs per 0.02 kg typical body weight in mice). Inter-individual

342   variability across animals obscured somewhat the potential effect on overall C_{max} in rats, with

343   outlier individuals with low exposure and truncated profiles due to sampling difficulties

344   (**Supplementary Data File S2**). Similarly, as in mice, limitations of sparse sampling in the study

345   design for welfare/blood volume sampling restriction (and logistical) reasons, may also have

346   meant that the full shape of the complex profile and the true C_{max} may not have been observed

347   with the time points taken.

348

349   Co-administration of hyaluronidase again did not appear to compromise the long-acting

350   exposures from IM administration of this formulation to rats, and prolonged exposure was still

351   maintained for ~20 days (noting the rats received a lower weight normalized dose compared to

352   mice), with similar AUCs, % F (of ~30-50%.), and terminal half-lives of (50-75 h) in the

353   presence or absence of hyaluronidase. As for mice, the terminal half-lives of paliperidone

354   following IM dosing as the palmitate ester in rat were confirmed as "flip-flop" [20] in nature

355   when taken in comparison to the IV disposition half-life of paliperidone in rats of 1.1 h based on

356   the PK data of Sun *et al.* [21] (**Table 2**).

357

358   *3.4. Histopathological assessment*

359   A secondary outcome in this study was assessment of the histopathology surrounding the

360   injection site. Because a limited number of animals were used in this study (as serial, in-life

361   blood sampling used for assessment of the primary outcome) and also because animals were only

362   sacrificed at the final PK time points, sample numbers for histopathological assessment were low

363   and thus only used for a descriptive, non-quantitative assessment. Additionally, histopathological

364   assessment was not always able to locate the injection site. However, assessment was possible

365   for at least one animal in each study arm. In animals where a clear injection site was found, there

366   was variability in the location of the depot: sometimes the medication was located in the muscle,

367   between the myocytes of the thigh muscles, while in other animals, the medication was found

368   against the muscle, in the nearby adipose tissue (see example in **Supplementary Fig. S2**). It is

369   not clear from pathological assessment whether this variation originates from variation in the

370   injection site, or whether the medication might have been displaced due to the higher pressure in

371   the muscle compared to the adipose tissue (*i.e*., during contraction of the muscle).

372

373   In the mouse thighs, it appeared that macrophages had phagocytized almost all the injected

374   material after 168 hours, as seen by the foamy aspect of the cytoplasm of the macrophages

375   (**Fig. 2A-B**; **Fig. S2**). There was no central cavitation, and a very limited amount of other

376   inflammatory cells were present. In mice injected with paliperidone palmitate without

377   hyaluronidase (*i.e*., paliperidone palmitate alone or with buffer only), the collections of foamy

378   macrophages formed a mass with a pushing, smooth border, and very little infiltration between

379   the surrounding individual myocytes (**Fig. 2A**). However, when paliperidone palmitate was co-

380   administered with hyaluronidase, the macrophages showed infiltration between individual

381   myocytes (**Fig. 2B**), suggesting a possible role for hyaluronidase to digest the extracellular

382   matrix and allow the medication to disperse between individual myocytes. A dose-effect with

383   hyaluronidase was not detectable in this small sample set.

384

385   There was a clear difference between mice and rats regarding the immune reaction to the

386   injection of paliperidone palmitate. In rats, granulomas formed with a central cavity and necrosis,

387   surrounded by a wall of macrophages and fibroblasts, and the cavitating granulomas had smooth

388   borders and showed little infiltration between the individual myocytes of the thigh muscle (**Fig.**

389   **2C-D**). This observed granuloma structure aligns well with that previously described by Darville

390   *et al.* following intramuscular injection of paliperidone palmitate in rats [11]. Also in contrast to

391   the mice, co-administration of hyaluronidase did not discernably affect the granulomas or the

392   surrounding myocytes (**Fig. 2C** versus **Fig. 2D**). This apparent lack of impact by hyaluronidase

393   could be due to the hyaluronidase dosing in rats, which, as already discussed, received a lower

394   weight normalized dose than what was used in the mice. This aligns with the reduced impact of

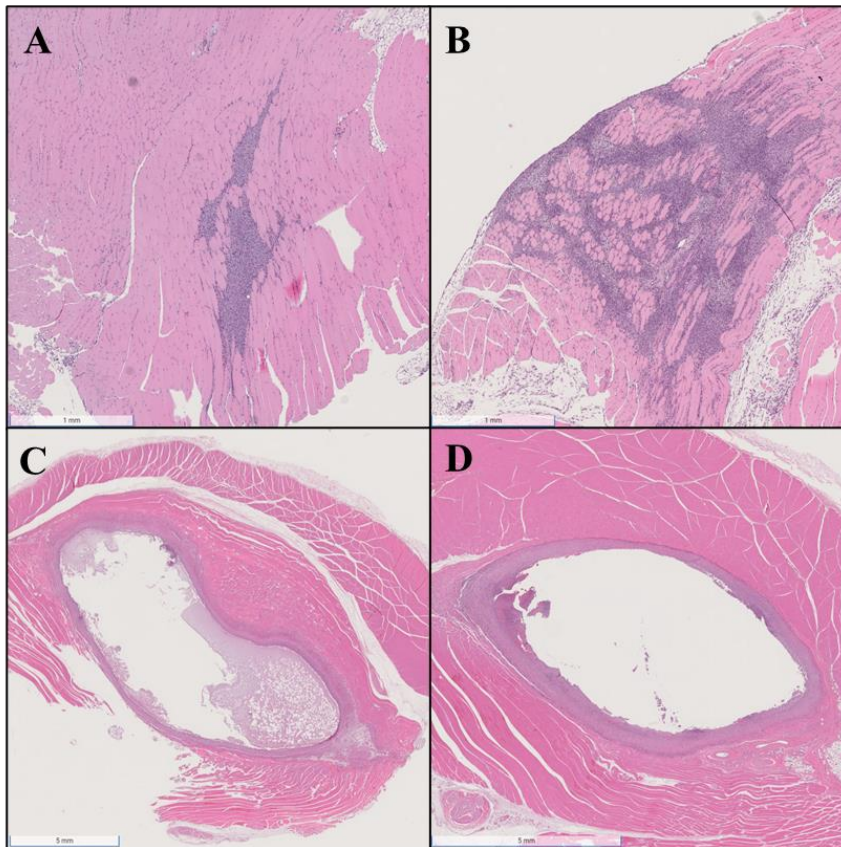395   hyaluronidase that was observed in the PK results (**Fig. 1**).

396

Figure 2. Representative hematoxylin and eosin stained sections showing the paliperidone palmitate depot site in the thigh muscles of mice (A-B) and rats (C-D). Panel A shows the depot site in a mouse thigh that was injected with paliperidone palmitate with buffer only (no hyaluronidase), 336 hours (2 weeks) post injection. The depot is located in macrophages, which form a mass with little infiltration between surrounding myocytes. Panel B shows a mouse thigh that was injected with paliperidone palmitate plus 15 U hyaluronidase, 168 hours (1 week) post injection. The depot is located in macrophages, which show infiltration between surrounding myocytes. Panel C shows a rat thigh that was injected with paliperidone palmitate alone (no buffer, no hyaluronidase), 672 hours (4 weeks) post injection. The depot is surrounded by a wall of macrophages and fibroblasts, forming a cavitating granuloma. No infiltrations are seen between surrounding cells. Panel D shows a rat thigh that was injected with paliperidone

408   palmitate plus 5 U hyaluronidase, 672 hours (4 weeks) post injection. The depot is surrounded by

409   a wall of macrophages and fibroblasts, forming a cavitating granuloma. No infiltrations are seen

410   between surrounding cells.

411

412   **4. Conclusions**

413   Co-administration of hyaluronidase altered depot release of paliperidone in the first four days

414   (96 h) following IM injection to BALB/c mice and Sprague-Dawley rats, increasing plasma

415   exposures. The higher exposures early in the profile may necessitate further evaluation of the

416   safety and tolerability, however, these data support further investigation of hyaluronidase as an

417   approach to increase injection volumes of other LAIs, particularly those of interest in the anti-

418   infectives therapeutic area such as cabotegravir and rilpivirine.

419

420   Multiphasic release processes suggested by the data, with potential modulation by hyaluronidase,

421   are consistent with potential mechanistic descriptions of depot release processes involving

422   resident phagocytic immune cells [11, 24], but this remains to be confirmed.

423

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