1
2 **Full title:**
3
4 Drug reformulation for a neglected disease.
5
6 The NANOHAT project to develop a safer more effective sleeping sickness drug.
7

8 **Short title:**

9 Pentamidine reformulation to improve safety and efficacy against Human African Trypanosomiasis

10

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34 **Author Contributions for uploading during submission**

35 **LS: PDRA** *in vivo* BBB (PK and In situ brain perfusion technique) and *in vitro* BBB studies. Data analysis.
36 Result writing.

37 **GNS:** PhD Student *in vitro* BBB studies, haemolysis assay, membrane integrity assay. Data analysis

38 **RCB:** *in vitro* BBB studies.

39 **MF:** PhD Student contributed to the initial concept and data sets with P85.

40 **MAS:** PDRA insulin secretion and cell viability studies, physical chemistry, data analysis result writing.

41 **SAT:** Introduction, method, result and discussion writing, paper collation, co-ordination. CNS drug
42 delivery expert. Obtained funding, concept, project management and experimental design, data
43 analysis and interpretation.

44 **HBS:** *in vitro* anti-trypanosomal assays.

45 **CD, CL, SP, MC, SC, SAT:** obtaining funding, concept, project management and experimental design,
46 data analysis and interpretation.

47

Abstract

Human African trypanosomiasis (HAT or sleeping sickness) is caused by the parasite *Trypanosoma brucei sspp.* The disease has two stages, a haemolymphatic stage after the bite of an infected tsetse fly, followed by a central nervous system stage where the parasite penetrates the brain, causing death if untreated. Treatment is stage-specific, due to the blood-brain barrier, with less toxic drugs such as pentamidine used to treat stage 1. The primary objective of our research programme was to develop an intravenous formulation of pentamidine which increases CNS exposure by some 10-100 fold, leading to efficacy against a model of stage 2 HAT. This target candidate profile is in line with drugs for neglected diseases inititative (DNDi) recommendations. To do this, we evaluated the physicochemical and structural characteristics of Pluronic-pentamidine formulations, selected appropriate candidates for efficacy and toxicity evaluation *in vitro*, quantified pentamidine CNS delivery of a sub-set of formulations *in vitro and in vivo*, and progressed one pentamidine-Pluronic formulation for further evaluation using an *in vivo* single dose brain penetration study. Screening pentamidine against 40 CNS targets did not reveal any major neurotoxicity concerns, however, pentamidine had a high affinity for the imidazoline$_2$ receptor. The observed reduction in insulin secretion with pentamidine in MIN6 β-cells maybe secondary to pentamidine-mediated activation of β-cell imidazoline receptors and impairment of cell viability. F68 (0.01%w/v)-pentamidine formulation had a similar inhibitory effect on insulin secretion as pentamidine alone and an additive trypanocidal effect *in vitro*. However, all Pluronics tested (P85, P105 and F68) did not significantly enhance brain exposure of pentamidine. We therefore closed the study before progressing to *in vivo* efficacy and toxicity studies. Importantly, this MRC DPFS funded study has resulted in the generation of a set of results which are relevant to further developing block-copolymers as nanocarriers, improving BBB drug penetration and understanding the side effects of pentamidine.

Abbreviations: blood-brain barrier (BBB), critical aggregation concentration (CAC), critical micellar concentration (CMC); circumventricular organs (CVO), developmental pathway funding scheme (DPFS), Drugs for Neglected Diseases initiative (DNDi), dynamic light-scattering (DLS), human African

74   trypanosomiasis (HAT); hydrophilic-lipophilic balance (HLB), molecular weight (MW), multi-drug

75   resistance associated protein (MRP), Medical Research Council (MRC), pentamidine isethionate (PTI),

76   P-glycoprotein (Pgp), plasma free haemoglobin (PFH), poly(ethylene oxide) (PEO), poly(propylene

77   oxide) (PPO), scattering length density (SLD).

78

Introduction

79

80 Human African trypanosomiasis (HAT or sleeping sickness) is a potentially fatal disease caused by the

81 parasite *Trypanosoma brucei sspp*. Recent epidemiological studies in 30 of the 36 African countries

82 listed as endemic for the disease indicate that, whilst the number of disease cases has been decreasing

83 since 1990, there are still ~4,000 new infections/year, and ~15,000 cases worldwide [1][2].

84 Furthermore, there is a substantial unreported burden of HAT [3].

85 The disease has two stages – a haemolymphatic stage after the bite of an infected tsetse fly, followed

86 by a central nervous system (CNS) stage when the parasite penetrates the brain, causing death if left

87 untreated. The blood-brain barrier (BBB) makes the CNS stage difficult to treat because it prevents

88 99% of all known compounds from entering the brain, including most anti-HAT drugs[4][5][6][7].

89 Those that do enter the brain are toxic compounds, can have serious side effects, are complex to

90 administer and/or are expensive. Pentamidine is a less toxic blood stage drug, which is known to treat

91 early-late (transition) stage HAT[8], but cannot treat stage 2 disease as it does not sufficiently

92 penetrate the BBB[7] and causes peripheral side effects (e.g. hypoglycaemia (incidence 5-40%) and

93 diabetes mellitus (incidence: occasional but irreversible)[9] which preclude increasing the dose to

94 overcome this limitation.

95 Research has shown pentamidine has a limited ability to cross the BBB and reach the brain due to it

96 physicochemical characteristics and its removal by the efflux transporters P-glycoprotein (Pgp) and

97 multi-drug resistance associated protein (MRP) [7](Fig S1). Furthermore, transporters are considered

98 essential in the mode of action of pentamidine against trypanosomes.

99

100 Poloxamers, commercial trademark Pluronics® (BASF) or Synperonics® (CRODA), are triblock

101 copolymers made of two poly(ethylene oxide) (PEO) blocks interspaced by a poly(propylene oxide)

102 (PPO) block and follow the general basic formula: PEO_x-PPO_y-PEO_x, where x and y are the size of PEO

103 and PPO blocks, respectively (Table 1). In an aqueous environment and above the critical micelle

104 concentration (CMC), the copolymers self-assemble into micelles, with the PEO chains forming a

105   hydrophilic shell around a PPO hydrophobic core, within which lipophilic drugs can be solubilised,

106   drug-free fraction decreased and circulation time increased [10]. Furthermore, combining different

107   Pluronics® can enhance drug/micelle interactions and drug loading[11][12]. The PEO shell serves as a

108   stabilizing layer between the hydrophobic core and the external medium, and prevents aggregation,

109   plasma protein adsorption and opsonization and therefore recognition by the macrophages of the

110   reticuloendothelial system [13]. Pluronic copolymers are also endowed with low cytotoxicity and

111   weak immunogenicity in topical and systemic administration. Even though PEO–PPO–PEO materials

112   are non-degradable, molecules with a molecular weight (MW) <7 kDa will be filtered by the kidney

113   and cleared in urine[14]. A variety of Pluronic block copolymers differing in the lengths of the EO and

114   PO blocks are available for formulation with pharmaceutical drugs. Importantly the size of the

115   hydrophobic block affects micellization and drug solubilisation[15]. In addition, Pluronics are

116   recognised pharmaceutical excipients listed in the US and British Pharmacopoeia so have an

117   established safety profile.

118   Thus Pluronics have attracted a great deal of attention in pharmaceutical applications as drug

119   solubilisers [13] or controlled drug-release agents[16][12][17]. Notably, Pluronic P85, P105, F68 and

120   L61 have been shown to inhibit efflux transporters (including P-gp and MRP1-2) and have potential to

121   enhance drug passage across the BBB [18] [19][20] [21] [22][23][24][16][25][26][27]. They have all

122   been approved as cosmetic ingredients [14] with F68 having being utilized as a blood substitute

123   component[28]. Transporter-targeting Pluronics® (L61 and F127) have successfully completed a phase

124   2 clinical trial for the intravenous treatment of adenocarcinoma of the upper gastrointestinal tract

125   [29][30][30]. Interestingly, F127-based amphotericin B-containing micelles have been shown to be

126   highly effective in treating *Leishmania amazonensis*-infected BALB/c mice [31], demonstrating that

127   Pluronics have potential beyond the traditional role of simple micellar vessels for drug encapsulation

128   and longer circulation, but are also active agents with key biological functions [32].

129

130   In this Medical Research Council (MRC) developmental pathway funding scheme (DPFS) multi-

131   disciplinary study our team developed a milestone driven progression strategy (Fig 1) in order to

132   assess the potential of pentamidine-Pluronic formulations to effectively treat stage 2 disease, reduce

133   the major known side effect of pentamidine on the pancreas and shorten the length of treatment

134   required to treat stage 1 disease. It was anticipated that the benefits of this approach would be a

135   combined pentamidine-Pluronic formulation which would provide a single therapeutic entity for safer,

136   simpler and more cost-effective treatment of all HAT stages using an established drug with a known

137   safety profile. Four Pluronic were selected for evaluation based on their block-copolymer architecture,

138   established safety profile and known ability to inhibit Pgp. These were P85, P105, F68 and L61 (Table

139   1). An iterative approach was utilized as illustrated in Fig 1.

140

141   **Fig 1. NANOHAT project progression strategy.**

142
143   We screened approximately 30 pentamidine/Pluronic® formulations during this project using a

144   rational, iterative approach. The three milestones were intended to ensure that the most appropriate

145   formulations, on the basis of *in silico* and *in vitro* data, are taken forward to the *in vivo*

146   pharmacokinetic studies and that the formulations with the greatest likelihood of success are tested in

147   the whole animal efficacy studies as outlined in the progression strategy.

148
149

2. Methods

2.1a Materials

Pentamidine (1,5-bis-4ρ-amidinophenoxypentane) isethionate salt (MW 592.68; 98% purity; catalogue number P0547) and Pluronic P105 (batch number BCBP8915V) were purchased from Sigma Aldrich (Poole, Dorset, UK). Pluronic P85 (mat 30085877 batch number: WPYE5378) was a kind donation from BASF plc (Cheshire, UK). Pluronic F68 (medical grade Catalogue number 2750016; batch numbers M7102 and MR29468) was purchased from MP Biomedicals, LLC (Illkirch Cedex, France). L61 was purchased from Aldrich (catalogue number 435422; batch number MKBH8737V).

2.1b Purity of excipients

All Pluronic formulations (F68, P105, P85 and L61) were tested for possible contamination due to the production process and met specifications established by the US Pharmacopeia convention NF32 monograph for polaxamers [14], in that the EO, PO and p-dioxane in the Pluronic were below 1, 5 and 5 parts per million (ppm), respectively. This analysis was performed by an external specialist laboratory (Butterworth laboratories, Teddington, UK).

Table 1. Pluronic used in this Study, with their Name, Block Composition, Hydrophilic-Lipophilic Balance (HLB) and General Formula. L, F, or P, Refers to Liquid, Flake, or Paste Physical Forms, respectively.

Poloxamer	Pluronic	MW	Number of EO blocks	Number of PO blocks	HLB	Formula
235	P85	4600	52.27	39.66	16	$EO_{26.13}PO_{39.66}EO_{26.13}$
335	P105	6500	73.86	56.03	15	$EO_{36.93}PO_{56.03}EO_{36.93}$
188	F68	8400	152.73	28.97	29	$EO_{76.37}PO_{28.97}EO_{76.37}$
181	L61	1950	4.55	31.03	3	$EO_2PO_{30}EO_2$

2.2 Evaluation of potential neurotoxicity of pentamidine

8

171   New toxicities may arise following pentamidine's improved access to the CNS.   The potential of

172   pentamidine to cause neurotoxicity was evaluated by a brief review of the literature together with a

173   neurological profiling screen and ion channel activity screens. The biological screens were performed

174   by external specialist laboratories.

175

176   **2.2a Neurological profiling screen.** A CNS side effect panel was custom designed and binding assays

177   performed by Perkin-Elmer Science Discovery Systems.   Testing was performed at a single

178   concentration of 1 μM (100-times the trypanocidal concentration), with follow up concentration-

179   response curves in any assay where there was greater than 70% inhibition to determine an inhibition

180   constant (K_i)

181

182   **2.2b Ion channel (hKir2.1) activity screens.** The *in vitro* effects of pentamidine isethionate on cloned

183   hKir2.1 potassium channels (encoded by the human KCNJ2 gene) responsible for the I_{K1}, inwardly

184   rectifying potassium current, were examined by ChantTest Corporation (Cleveland Ohio 44128, USA)

185   to industry standards (Chantest FastPatch Assay; study number. 130827.DCC).   Human epithelial

186   kidney 293 (HEK293) cells (ATCC, Manassas VA USA) were stably transfected with the appropriate ion

187   channel cDNA encoding the pore-forming channel unit. Cells were cultured in Dulbecco's Modified

188   Eagle Medium / Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100

189   U/mL penicillin G sodium, 100 μg/mL streptomycin sulphate and 500 μg/mL G418. Cultured cells were

190   maintained in a tissue culture incubator set at 37°C in a humidified 95% air and 5% CO_2 atmosphere.

191   Pentamidine was dissolved in HEPES-buffered physiological saline containing 0.3% DMSO and

192   sonicated (Model 2510/5510, Branson Ultrasonics, Danbury, CT) at room temperature for at least 20

193   minutes. A glass-lined 96 well compound plate was loaded with the appropriate amount of test (five

194   different concentrations) and positive control (100μM $BaCl_2$) solutions, and placed in the plate well of

195   the QPatchHT (Sophion Bioscience A/S, Denmark).   All experiments were performed at room

196   temperature. Each cell acted as its own control. Vehicle was applied to naïve cells for a 5-10 minute

9

197 exposure interval. The test solution applied for a minimum of three minutes via the QPatch robot

198 pipetting system to naïve cells (n≥2, where n=the number of cells/concentration). Each solution

199 exchange on the QPatch, performed in quadruplicate, consisted of a 5 µl exchange through the

200 microfluidic flow channel, resulting in 100% replacement of the compound in the QPlate. Intracellular

201 solution was loaded into the intracellular compartments of the QPlate planar electrode (130mM K-

202 Asp, 5mM MgCl$_2$, 5 mM EGTA, 4mM Tris-ATP and 10 mM HEPES). Cell suspension was pipetted into

203 the extracellular compartments of the QPlate planar electrode.

204

205 Onset and steady state block of hKir2.1current was measured using a ramp protocol with fixed

206 amplitudes (hyperpolarization: -110 mV, 200 ms duration, followed by a 1-second ramp from -110 mV

207 to +50 mV) repeated at 10 s intervals from a holding potential of −70 mV. Current amplitude was

208 measured at the end of the step to -110 mV. Leak current was calculated and subtracted from the

209 total membrane current record.

210

211 **2.3 Determination of the micellar aggregation properties of Pluronic**

212 As a first step towards the biological evaluation of the polymers, we evaluated the micellar

213 aggregation properties of P85, P105, F68 and L61. In particular, we determined the CMC, micellar size

214 and aggregation number, in different solvents, using a unique combination of light and neutron

215 scattering and atomistic simulations. We also measured the partitioning of pentamidine isethionate in

216 selected Pluronic and its *in vitro* release profile.

217

218 **2.3a Preparation of solutions for physicochemical measurements** Unless stated, F68, P85,

219 P105 or L61 were either dissolved in water (aqueous) or saline solution (0.9% w/v sodium

220 chloride solution). Pluronics mixtures were also prepared either with a fixed mass ratio of 1:1

221 (F68-P105 or F68-P85) or in the specific case of L61, 0.01%. Samples were left to equilibrate

222   for at least 3 hours prior to any measurement. Ultra-pure water (18.2 MΩ·cm - Millipore-

223   filtered) was used throughout the experiments.

224

225   **2.3b: Phase behaviour.** In this study, L61 alone and in mixtures with one or two other

226   Pluronic in both water (aqueous) and saline mediums were visually assessed from 20°C to

227   50°C in 5°C steps, plus 37°C, to assess the impact of mixtures on L61 cloud point (24°C for a

228   1% solution) [33].

229

230   **2.3c CMC determination by fluorescence spectroscopy.** The CMC determines thermodynamic

231   stability of the micelles during dilution of the drug delivery system in body fluids[17][15].

232   Furthermore, CMC is an important parameter in view of the biological response modifying

233   effects of Pluronic block copolymers since it is needed to determine the maximum achievable

234   concentration of the polymer single chains ("unimers") [20]. For measurement of the CMC,

235   pyrene (Sigma catalogue number 82648; pyrene puriss p.a. for fluorescence, ≥99%) was used

236   as a probe. A stock solution of pyrene in acetone (1.7×10^{-2} M) was initially prepared. A 35 μL

237   aliquot of this solution was placed in a 100 mL volumetric flask and the solvent was

238   evaporated to air. The residue was then dissolved in either ultra-pure water (18.2 MΩ·cm -

239   Millipore-filtered) or 0.9% w/v sodium chloride solution, resulting in a final concentration of

240   pyrene of 6×10^{-6} M. These solutions were then subsequently used as the solvent for the

241   polymer solutions. Stock solutions of each Pluronic in water and saline solution were

242   prepared. An appropriate aliquot of these solutions were dissolved in the pyrene/H_2O or

243   pyrene/saline solution. Solutions of different polymer concentration were obtained by diluting the

244   stock polymer solution with the appropriate solvent. Mixed samples of two Pluronic were also

11

245 prepared either with a fixed ratio of 1:1 or containing 0.01% L61. Samples were left to equilibrate for

246 at least 3 hours prior to the experiment.

247 The fluorescence emission spectra were recorded on a Cary Eclipse fluorescence spectrophotometer

248 (Varian, Oxford, UK) with λ_{exc} = 340 nm. For the CMC, fluorescence intensities at 373, 384, 393 nm and,

249 when it appeared, also at the excimer band centred at 490 nm, were measured. For each polymer, the

250 critical aggregation concentration value was determined by using the intensity of the best resolved

251 peak. At least two repeats were performed for each sample. Measurements were performed at 20°C

252 and 37°C.

253

254 **2.3d Stability testing** The purpose of stability testing is to provide evidence of how the quality of

255 pentamidine varies with time under the influence of a variety of environmental factors such as

256 temperature, humidity and light (Climatic zone IV, 30°C and 65-75% relative humidity) [34].

257

258 In our initial 7 day assessment we also considered interaction of pentamidine with pluronic as

259 product-related factors may also influence its quality. A 5% or more change in initial content of

260 pentamidine was considered significant. Pentamidine concentration at day 0, 10 and 7 was assessed

261 by NMR.

262 A Bruker Advance 400 MHz spectrometer was used for recording the one-dimensional (1D) 1H NMR.

263 Solutions of PTI, PTI/P85, pentamidine PTI/P105 and PTI/F68 were prepared in D_2O (≥99.85% in

264 deuterated component). Data were collected at days 0, 1 and 7. Samples were stored in amber NMR

265 tubes at 37°C.

266

267 **2.3e Partition coefficient determination.** The partitioning coefficient, *P*, determines the portion of

268 drug incorporated into the micelle and provides thermodynamic characterization for the stability of

269 the drug-micelle complex during dilution within the body fluids[17][15].

270   The partition coefficient of pentamidine in the micellar core and bulk solvent, as described by

271   Kabanov and co-workers [15], was measured for F68, P105 and mixtures of P105 and F68 (1:1), in both

272   saline and aqueous solutions and at 20°C and 37°C. This method assumes that the fluorescence signal

273   of the molecular probe, in our case pentamidine isethionate (PTI), can be described as a linear

274   combination of the intensity of the free probe in the bulk solvent (I_0) and the intensity of the probe

275   within the micellar core (I_{max}), weighted by the concentration of the probe in the bulk solvent and the

276   micellar core, respectively. Therefore, it is necessary to obtain both values for the calculation of the

277   partition coefficient (P). I_{max} cannot be directly measured, as there always exists an equilibrium

278   between the probe solubilised in the micellar core and in the bulk solvent. A good approximation is to

279   measure the fluorescence intensity at a micellar concentration high enough that the intensity no

280   longer changes with micelle concentration. In this situation, the equilibrium is forced towards having

281   the probe within the micellar core and the dominant contribution to the fluorescence will be from the

282   probe within the micelle. This is a good approximation for hydrophobic probes as the equilibrium

283   tends to favour dissolution of the probe in the micellar core. For water-soluble probes, the amount of

284   polymer required to reach I_{max} can be very high, and possibly not reachable experimentally.

285   The partition coefficient was obtained following the procedure described by Kabanov and co-

286   workers [Kozlov, 2000], which is described briefly below. The partition coefficient is defined as:

287    $$P = \frac{c_m}{c_{bulk}}$$ Eq. 1

288   where c_m is the PTI concentration in the micelle core and c_{bulk} is the PTI concentration in the

289   bulk solvent.

290   The partition coefficient P can be obtained from [Kozlov, 2000]:

291    $$P = \frac{1}{0.01 * \alpha * v}$$ Eq. 2

292   where, v (cm³/g), the partial molar specific volume, is defined as:

293    $$v = \frac{1}{\rho_0} * \frac{1 - (\rho_s - \rho_0)}{c}$$ Eq. 3

294   with ρ_0 (g·cm^{-3}) the solvent density, ρ_s (g·cm^{-3}) the density of the solution and C the polymer

295   concentration (g·mL^{-1}).

296   α is defined as the angular coefficient of the plot $(\frac{I-I_0}{I_{max}-I_0})^{-1}$ vs. (Pluronic concentration − cac)$^{-1}$, where I

297   is the fluorescence intensity of the solution, I_0 is the intensity with no Pluronic present and I_{max} is the

298   intensity at saturating concentration of the Pluronic, cac the critical aggregation concentration.

299

300   For the measurement of pentamidine isethionate salt (PTI) partition coefficient, a PTI 1×10^{-6} M in both

301   water and saline solutions were initially prepared. These solutions were then subsequently used as the

302   solvent for the polymer solutions and the preparation followed a similar method as for the CMC

303   measurements. Samples were left to equilibrate for at least 3 hours prior to the experiment.

304

305   The fluorescence emission spectra were recorded on a Cary Eclipse fluorescence spectrophotometer

306   (Varian, Oxford, UK) with $\lambda_{exc} = 260$nm, for pentamidine. The fluorescence emission intensity at ca

307   340 nm was followed.

308   **2.3f Drug release.** In a successful drug delivery system, the rate at which the drug is released from
309   preparations is important.

310

311   Solutions of Pluronic (1% F68 and 1% P105) with 10 mM PTI and PTI alone in water (2 mL) were loaded

312   into 2 mL mini-dialysis tubes with 1 kDa molecular weight cut-off (GE Healthcare Bio-sciences Corp.

313   USA). The tube was immersed in a 200 mL closed Duran flask which was placed in a water bath at 37°C

314   for the duration of the experiment. Aliquots were collected from the immersion water (ultra-pure

315   water (18.2 MΩ·cm - Millipore-filtered) in the flask every 30 min for the first 2 hours, every hour for

316   the next 5 hours and then once more after 1 week. At the end of the experiment, an aliquot was

317   collected from the dialysis cell. PTI concentrations were determined by UV spectroscopy (wavelength

318   260 mm).

319   The data was fitted to Ritger-Peppas model[35].

320

$$\frac{M}{M_\infty} = kt^n \qquad\qquad\qquad \text{Eq. Drug release 1}$$

322

Where M and M_∞ are the cumulative amounts of drug released at time t and at infinite time,

respectively; k, the reaction constant, t the time, n, the diffusional exponent describing the type of

regime type: n=1, case II transport, n=0.5, Fickian diffusion, 0.5<n<1 non-Fickian diffusion.

326

2.3g Dynamic light scattering (SLS/DLS).

Light scattering allows detection of unimers and/or micelles.

Dynamic light-scattering (DLS) were performed with a photon correlation spectrometer Malvern

Zetasizer Nano with a laser wavelength of 633nm. For obtaining the reduced scattered intensity,

toluene was used as the standard and the increment in the refractive index, $\partial n/\partial c$, was assumed to be

independent on the temperature and taken as 0.133 ± 0.002 mL·g^{-1} [Gaitano, 1997]. The samples, of

concentrations ranging between 1 to 5% w/v, were filtered prior to the measurements by 0.22 μm

Millex syringe PVDF filters onto semi-micro glass cells. The temperature of the sample was controlled

with 0.1°C accuracy by the built-in Peltier in the cell compartment. Size distributions were obtained for

each sample from the analysis of the intensity autocorrelation function, which was performed with the

Zetasizer software in the high resolution mode to distinguish overlapping distributions.

338

2.3h Small-Angle Neutron Scattering (SANS). The architecture of the nanocarriers was measured by

SANS. The aggregation number (N_{agg}) and radius micellar size, including volume of core and shell

region, correlates directly with are relevant to properties such as drug loading encapsulation

efficiency, stability, half-life and hence and circulation time[36].

The pluronic molar volume can be determined using small-angle neutron scattering (SANs).

Small-angle neutron scattering experiments were performed on the LOQ instrument at ISIS

15

345 pulsed neutron source (ISIS, Rutherford-Appleton Laboratory, STFC, Didcot, Oxford). LOQ uses

346 incident wavelengths from 2.2 to 10.0 Å, sorted by time-of-flight, with a fixed sample-

347 detector distance of 4.1 m, which provided a range of scattering vectors (q) from 0.009 to

348 0.29 Å^{-1}.

349 The samples used in the SANS experiments were prepared in D_2O to optimise the contrast with the

350 protonated polymer. The samples were placed in clean disc-shaped quartz cells (Hellma) of 1 and

351 2 mm path length and the measurements were carried out at 25°C and 37°C. All scattering data were

352 first normalised for sample transmission and then background-corrected using a quartz cell filled with

353 D_2O (this process also removes the inherent instrumental background arising from vacuum and

354 windows) and finally corrected for the linearity and efficiency of the detector response using

355 instrument-specific software package. The data were then converted to the differential scattering

356 cross-sections (in absolute units of cm^{-1}) using the standard procedures at ISIS [Heenan, 1989].

357 The curves were fitted to a core-shell sphere (CSS) model combined with a structure factor for hard

358 spheres, implemented in the Sasview software (SasView, http://www.sasview.org/) accessed

359 23/11/2014. The initial fitting assumptions were a dry PPO core, i.e., the scattering length density

360 (SLD) for the core was set to the SLD of PPO (3.44×10^{-7} Å^{-2}), the solvent as D_2O (SLD of 6.38×10^{-6} Å^{-2})

361 and the shell as dry PEO (SLD 6.4×10^{-7} Å^{-2}). The SLD of core and solvent were kept fixed while the shell

362 was left to vary as a high level of hydration of PEO is expected, as a result, the returned SLD should be

363 an intermediate value between D_2O and PEO. After obtaining a stable set of parameters, the SLD of

364 the core was also let free to vary. Input values for the core radius and shell thickness were based on

365 hydrodynamic radius values obtained by DLS. A term to compensate for polydispersity was included

366 for Pluronics (around 0.20), as well as a structure factor ($S(q)$), corresponding to a hard sphere model,

367 in order to account for intermicellar interactions.

368 Assuming that the shell SLD is a linear combination of the EO SLD and D_2O SLD, i.e., the densities are

369 additive, it is possible to calculate the solvent volume fraction in the shell using the following

370 expression:

16

371
$$a. \quad \chi_{\text{solv}} = \frac{SLD_{shell} - SLD_{EO}}{SLD_{D_2O} - SLD_{EO}}$$
Eq. SANS_1

372 The amount of solvent molecules in the shell than can be obtained from the ratios of the shell volume

373 by the solvent molecular volume weighted by the solvent volume fraction:

374
$$b. \quad n_{D_2O} = \frac{\chi_{solv} \cdot V_{shell}}{V_{D_2O}}$$
Eq. SANS_2

375 Furthermore, the total micelles volume is the sum of both Pluronics and D_2O molecular volumes

376 weighted by their respective volume fractions. Therefore, combining the total micelle volume (shell +

377 core) minus the solvent contribution, it is possible to obtain the amount of Pluronic molecules present

378 in the micelle and calculate the aggregation number of the micelles.

379
$$N_{agg} = \frac{V_{micelle} - \chi_{solv} V_{shell}}{V_{pluronic}}$$
Eq. SANS_3

380 The molecular volume of the Pluronic was approximated from the Pluronic molar volume ($v_{pluronic}$) in

381 water.

382
$$V_{pluronic} = \frac{v_{pluronic}}{N_{AV}}$$
Eq. SANS_4

383

384 **2.3i Simulations of Pluronic self-assembly and pentamidine encapsulation.** During the course of this

385 project, we worked to develop a model of the Pluronic and pentamidine systems that would allow us

386 to simulate the self-assembly of the polymers and the encapsulation of the drugs. In order to simulate

387 the timescales and system sizes required to study these systems, we utilized a coarse-grain approach,

388 which is called dissipative particle dynamics (DPD)[37]. This method has been used to study Pluronics

389 before and has been shown to represent expected phenomena well. So we used the simulation

390 parameters from [38].

391
392 **2.4 Evaluation of potential peripheral toxicity of pentamidine ± Pluronic**

393 The toxicity of pentamidine in the presence of the Pluronic was explored using a variety of assays.

394 These included haemolysis, insulin secretion and membrane integrity assays.

395

17

396 **2.4a Haemolytic effects on human blood samples**

397 The proposed route of administration for our Pluronic formulations with pentamidine was intravenous,

398 hence the propensity for Pluronics to lyse red blood cells was studied using a haemolytic assay.

399

400 **2.4ai Chemicals.** Cyanmethaemoglobin (CMH) reagent, haemoglobin standard, Ca^{2+}/Mg^{2+} free

401 Dulbecco's Phosphate Buffered Saline (DPBS), DMSO, and Triton X-100 were purchased from Sigma-

402 Aldrich, Dorset, UK.

403

404 **2.4aii Research Donor Blood.** Healthy volunteer blood samples were drawn under the guidelines of

405 the Research Ethics Committee South East London REC 4 (10/H0807/99). Blood was collected in BD

406 vacutainer tubes containing lithium heparin as anticoagulant.   Blood samples from two healthy

407 volunteers were collected and kept separate.

408

409 **2.4aiii Haemolysis Assay.** The assay is based on the protocol detailed by [39]. In order to determine

410 the total blood haemoglobin, the CMH method was used to map a standard curve based on the

411 absorption wavelength at 540 nm. Nine calibration standards were produced by preparing a stock

412 solution of 5 mg/ml (standard 1) using haemoglobin standards in CMH reagent and serially diluting it

413 to produce further standards of concentrations 2.5, 1, 0.80, 0.40, 0.20, 0.10, 0.05, and 0.025 mg/ml.

414 10% Triton X-100 (v/v) was prepared using distilled water to be used as the positive control. A 0.05%

415 solution of DMSO was prepared using distilled water and used as one of the negative controls. 0.9%

416 saline was another negative control. Pluronic samples were prepared in 0.9% saline.   The test

417 concentrations of Pluronic (P85, P105 or F68) used were 0.01%, 0.1%, 0.25%, 1% or 5%.

418

419 A 5 ml vial of whole blood was centrifuged at 800 g for 15 minutes at room temperature. The

420 supernatant was removed and the remainder was used to determine the plasma free haemoglobin

421 (PFH) concentration.

422

423 200 µl x 2 of each of the calibration standards prepared were then transferred onto the 96-well plate

424 and 200 µl x 4 of CMH reagent (control 1). Total blood haemoglobin (TBH) was then prepared by

425 adding 20 µl of the whole blood with 5 ml of CMH reagent. 200 µl x 6 of TBH was transferred to the

426 plate. 6 other wells were filled with 100 µl of plasma to which 100 µl of CMH reagent was added. After

427 shaking it gently for a few minutes, the absorbance was read at 540 nm. Once the total haemoglobin

428 concentration was adjusted to 10 mg/ml using Ca^{2+}/Mg^{2+} free DPBS, 20 µl x 3 of the blank (control 2

429 0.9% saline), positive control, negative control, or Pluronic samples were added to Eppendorf tubes.

430 160 µl of Ca^{2+}/Mg^{2+} free DPBS and 20 µl of whole blood were then added to each tube except for one.

431 20 µl of Ca^{2+}/Mg^{2+} free DPBS was added instead. These served as control 3 without any blood enabling

432 us to determine any interactions from Pluronic with the assay. These tubes were incubated for 3 hours

433 ± 15 minutes in a temperature-controlled shaker (THERMOstar, BMG labtech, Offenburg, Germany) at

434 37 °C and 120 rpm. After the incubation, these were centrifuged at 800 × g for 15 minutes at room

435 temperature. 100 µl of the test samples and the controls were transferred to the 96-well plate. 100 µl

436 of CMH reagent was added to all these before measuring the absorbance spectrophotometrically at

437 540 nm. Using the calibration curve mapped earlier, the haemoglobin concentration in each of the

438 wells was determined. The dilution factor of 18 was also considered when calculating the

439 haemoglobin concentration. Haemoglobin concentration was converted to percentage haemolysis

440 compared to the negative control 0.05% DMSO. A significant increase compared to control at the 5%

441 level was taken as positively haemolytic.

442

443 **2.4b Membrane integrity/cytotoxicity.**

444 Method is described as part of 2.5b and 2.6.

445

446 **2.4c Insulin secretion and beta-cell viability.**

447 All tissue culture reagents were purchased from Sigma Aldrich (Poole, Dorset, UK).

19

448   Peripheral toxicity of pentamidine/Pluronic formulations to the endocrine pancreas was evaluated by

449   quantifying β-cell viability and insulin secretion from the mouse MIN6 β-cell line [40].

450

451   MIN6 β-cells were maintained in culture at 37°C (95% air/5% CO_2) in DMEM supplemented with 10%

452   fetal bovine serum, 2mM L-glutamine and $100U.ml^{-1}/0.1mg/ml^{-1}$ penicillin / streptomycin, with a

453   change of medium every 3 days.   Cell were trypsinised (0.1% trypsin, 0.02% EDTA) when

454   approximately 70% confluent and seeded into 96 well plates at a density of $30x10^3$ cells/well. After a

455   24 hour culture period to allow cells to adhere, the wells were washed with PBS and cells were pre-

456   incubated for 2 hours in DMEM supplemented with 2mM glucose after which the medium was

457   replaced with DMEM supplemented with Pluronic, pentamidine and Pluronic/pentamidine solutions.

458

459   The following formulations were evaluated: F68/PTI, P85/PTI and P105/PTI with Pluronics

460   concentrations of 0, 0.01, 0.025, 0.1 and 0.5% w/v and PTI concentrations of 0, 1, 10 and 100 μM (20

461   formulations in total, including controls, Pluronics only, PTI only and solvent only were used). The cells

462   were incubated under each treatment condition for 24 hours and then evaluated for their capacity to

463   secrete insulin in response to 30 minute stimulation in the presence of 10 μM forskolin and 100 μM

464   IBMX. Insulin secretion was measured by RIA [41]. The effect of the formulations on β-cell viability

465   was assessed by determining the access of trypan blue to the cell interior, indicative of a compromised

466   plasma membrane[42].

467

468   **2.5 Blood-brain barrier studies**

469   **2.5a Radiochemicals**

470   [^{3}H(G)]pentamidine (specific activity, 31.9 Ci/mmol; concentration, 10.74 μg/ml; radiochemical purity,

471   99.4%; MW 342.64) was custom synthesized and [^{14}C(U)]sucrose (specific activity, 536 mCi/mmol;

472   concentration, 67.07 μg/ml; radiochemical purity, 98.7%) was purchased from Moravek Biochemicals,

473   California, USA.

474

475 **2.5b** *In vitro* **permeability assays**

476 Several *in vitro* BBB models were evaluated for this study including Caco2 (permeability format),

477 hCMEC-D3 (accumulation format), bEnd-3 (accumulation format) and MDCK-MDR (accumulation

478 format) cell lines, before selecting the MDR1-MDCK cells (permeability format) as the most

479 appropriate tool to address our objectives. MDR1-MDCK cells originate from transfection of Madin-

480 Darby canine kidney (MDCK) cells with the MDR1 gene, the gene encoding for the human efflux

481 protein, P-glycoprotein (P-gp). Using MDR1-MDCK cells avoids the complexities of multiple

482 transporters by focusing specifically on P-gp.

483 **2.5bi Preparation of formulation** 1% (w/v) stock solutions of each Pluronic and 10 mM pentamidine

484 isethionate were prepared in Hank's Balanced Salt Solution (HBSS) containing 25 mM HEPES and 4.45

485 mM glucose, at pH 7.4. These were further diluted to give final concentrations of 0.01, 0.1 or 0.5%

486 (w/v) Pluronic containing 10 μM pentamidine isethionate. Formulations were stored at room

487 temperature for 2-4 days prior to use.

488 **2.5bii** *In vitro* **permeability assays.** MDR1-MDCK cells (NIH, Rockville, MD, USA) were maintained and

489 permeability assays were performed at both Cyprotex (Macclesfield, Cheshire, UK) and King's College

490 London. Analysis was by UPLC-MS/MS or liquid scintillation counting as appropriate.

491 Transmission electron microscopy confirmed appropriate cell morphology of a monolayer with

492 microvilli on the apical membrane and Western blot confirmed expression of P-gp (data not shown).

493 3.4×10^5 cells/cm^2 were seeded on Multiscreen™ plates with 0.4 μ polycarbonate Isopore™

494 membranes (Millipore, MA, USA) in DMEM/High glucose (Sigma-Aldrich, UK , D6429) media containing

495 1% Non-Essential Amino Acids and 10% foetal calf serum (both from Sigma-Aldrich, UK). Plates were

496 maintained at 37°C/5% CO$_2$ for 4 days before use. On the day of the assay, DMEM was removed and

497 both the apical and basolateral surfaces of the cell monolayer were washed twice with transport

21

498   medium consisting of HBSS containing 25 mM HEPES and 4.45 mM glucose, (pH 7.40; 37°C). Plates

499   were incubated for 40 minutes at 37°C/5% CO_2 to stabilize physiological conditions. Transport buffer

500   was removed from the apical or basolateral chamber and replaced with the formulation to be tested.

501   Samples were taken from the apical and basolateral compartments after 1 hour of incubation at 37

502   °C/5% CO_2. Samples, including the test formulation added to the apical chamber at t=0 were analysed

503   at Cyprotex using UPLC-MS-MS method to quantify the pentamidine isethionate content or were

504   analysed for radioactivity using a Tricarb 2900TR liquid scintillation counter.

505   **2.5biii UPLC-MS/MS.**   Quantification of pentamidine isethionate was carried out on an ABSciex

506   API5500 QTrap triple quadrupole mass spectrometer coupled to an Agilent 1290 Infinity UPLC system.

507   A 10μl sample from the apical or basolateral chamber was diluted with 80 μl buffer and 160 μl

508   methanol and centrifuged for 20 minutes at 1,400 × g. After centrifugation 90 μl of this was removed

509   and diluted with 10 μl of the internal deuterated standard to give a final concentration of 10 nM. This

510   was injected onto an Acquity™ HSS T3 (1.8 μm) column 2.1 x 50 mm (Waters Ltd, Herts, UK),

511   equilibrated at 70 °C. Separation was carried out by gradient elution using a mixture of 0.1% formic

512   acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B), at a flow rate of 600 μL/min.

513   Upon injection (10 μl), the mobile phase was held at initial conditions of 100% solvent A for 0.05

514   minutes, the concentration of solvent B was then increased to 95% at 1.00 minute post-injection using

515   a linear gradient and held for a further 0.40 minutes. The mobile phase was then returned to 100%

516   solvent A at 1.41 minutes and held for a further 0.39 minutes. The eluent was analysed by MS/MS

517   under positive ion electrospray mode and the multiple reaction monitoring transitions for

518   pentamidine and deuterated pentamidine were 341.174 to 119.984 m/z and 345.212 to 120.028 m/z

519   respectively. Calibration curves were prepared using pentamidine and the internal standard over a

520   concentration range of 0.0078 to 12 μM. The column was washed with a weak wash: 9:1 H_2O: MeCN

521   and strong wash: 4:3:3 MeOH: isopropyl alcohol: acetone + 1% acetic acid between samples.

522

523 **2.6 Sensitivity of MDCK-MDR cells to Pluronic**

524 Permeability assay with 5 µCi (0.9 µM) [^{14}C(U)]sucrose alone in the presence of varying concentrations

525 of Pluronics was used to further assess potential cytotoxicity of Pluronics. Sucrose is a paracellular

526 permeability marker and therefore any cytotoxic effects of Pluronics will lead to increased diffusion of

527 sucrose from apical to basolateral chambers as a result of compromised BBB integrity. The assay was

528 carried out for an hour in the apical to basolateral direction only and the P_{app} of radiolabelled sucrose

529 determined at the end of the assay. Control for the assay was HBSS buffer alone.

530

531 *2.7 In situ perfusions*

532 The *in situ* brain/choroid plexus perfusion method for examination of the distribution of molecules

533 into the brain and CSF is an established technique in the rat, guinea-pig and mouse [43][6][44]. It

534 allows the passage of slowing moving molecules across the blood-brain and blood-CSF barriers to be

535 examined and quantified in brain, capillary endothelial cells and choroid plexus tissue for perfusion

536 periods up to 30 minutes.

537

538 **2.7a Preparation of formulation:** All formulations were prepared on the day of experiment at a

539 Pluronic concentration of 0.1, 1.0 or 5% (w/v) using artificial plasma as a diluent. The artificial plasma

540 consisted of a modified Krebs-Henseleit mammalian Ringer solution containing; 117 mM NaCl, 4.7 mM

541 KCl, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 24.8 mM NaHCO$_3$,1.2 mM KH$_2$PO$_4$, 39 g dextran (MW 40,000), 1 g/L

542 of bovine serum albumin and 10mM glucose. [^{3}H(G)]pentamidine was added to give a final

543 concentration of 157nM (equivalent to 5 µCi/ml). All formulations were stirred at room temperature

544 for at least 1 hour to allow any chemical interactions and micelle formation to stabilize.

545 **2.7b Animal studies:** Adult male BALB/c mice were purchased from Harlan UK Ltd (Oxon, UK). All

546 animals were maintained under standard temperature/lighting conditions and given food and water

547 *ad libitum.* Only mice above 23g in weight were used for experiments which were carried out within

23

548   the framework of the Animals Scientific Procedures Act (1986) and Amendment Regulations 2012 and

549   with consideration to the ARRIVE guidelines. The study was approved by the King's College London

550   Animal Welfare and Ethical Review Body.

551

552   **2.7c *In situ* perfusions.** [^{3}H(G)]pentamidine formulations were delivered to the brain using an *in situ*

553   brain perfusion technique as previously described [6]. Briefly, mice were anaesthetized (mixture of 2

554   mg/Kg Domitor™/150 mg/Kg ketamine administered via the intraperitoneal route) and heparinized

555   (100 U ip.). Oxygenated artificial plasma (described above) at 37°C was pumped via a 25 gauge

556   cannula into the left ventricle of the heart, with the right atrium severed to prevent recirculation.

557   Pumps were calibrated to deliver an overall flow rate of 5 ml/min from the cannula.

558   [^{3}H(G)]pentamidine formulations (maintained at room temperature) were fed into the flow line from a

559   dual syringe infusion pump (Harvard Apparatus, UK), at a rate of 0.5 ml/min such that the formulation

560   was diluted 1/10 immediately prior to entering the heart. 11 μM [^{14}C(U)]sucrose in artificial plasma

561   (equivalent to 5 μCi/ml) was simultaneously fed into the flow line from a second identical syringe

562   using the same pump set at 0.5 ml/min (equivalent to 1.1 μM or 0.5 μCi/ml entering the heart from

563   the cannula). The perfusion was terminated at 10 minutes or 30 minutes, and the brain was sectioned

564   as previously described [45]. Samples taken were those known to be invaded by parasites during

565   second stage sleeping sickness and/or those which control mechanisms that are disrupted by the

566   disease such as the sleep/wake cycle[6].   All samples were solubilized with 0.5 ml Solvable™

567   (PerkinElmer Life and Analytical Sciences, Buckinghamshire, UK) for 48 hours. Scintillation fluid (3.5 ml

568   Luma Safe, PerkinElmer Life and Analytical Sciences) was added and radioactivity (^{3}H and ^{14}C) was

569   counted on a Packard Tri-Carb2900TR scintillation counter in dual-label mode.

570   2.7d **Capillary depletion.** After the required samples were taken, the remaining brain tissue was

571   homogenized and analyzed by the capillary depletion method described by Thomas & Segal [46]. This

572   method uses dextran density gradient centrifugation to produce a vascular endothelial cell-enriched

573 pellet and a brain parenchyma-containing supernatant from homogenized brain tissue. Thus the

574 partitioning of a drug between the endothelial cells and the post-vascular brain parenchyma can be

575 examined [47]. Briefly, the brain tissue was weighed and homogenized in a Dounce homogenizer with

576 3 ml/g capillary depletion buffer [47] and 4 ml/g 26% dextran. The homogenate was separated by

577 centrifugation at 5,400g and 4°C for 15 minutes. Homogenate, pellet and supernatant samples were

578 solubilized and counted for radioactivity using the method described above.

579 **2.7e Expression of results.** The radioactivity (either ^{3}H or ^{14}C) present in tissue samples (dpm/g) was

580 expressed as a percentage of that measured in the artificial plasma (dpm/ml) and was termed R_{TISSUE}%,

581 as previously described [45]. Where stated, measurements for [^{3}H(G)]pentamidine were corrected for

582 the contribution of drug present in the vascular space by subtraction of the R_{TISSUE}% for [^{14}C(U)]sucrose

583 from the R_{TISSUE}% of [^{3}H(G)]pentamidine and these corrected values were termed $R_{CORR\ TISSUE}$%.

584 **2.8 Pharmacokinetic brain distribution experiments**

585 *2.8a In vivo pharmacokinetic experiments with [^{3}H(G)]pentamidine.* Formulations containing 0.025%

586 F68 with 8 μM [^{3}H(G)]pentamidine, 0.5% F68 with 8 μM [^{3}H(G)]pentamidine and 8 μM

587 [^{3}H(G)]pentamidine alone were prepared in 0.9% sterile saline and allowed to equilibrate at room

588 temperature for at least 1 hour before use. A 200 μl bolus of the formulation to be tested (equivalent

589 to 15 μCi [^{3}H(G)]pentamidine) was administered to mice via the tail vein. At 2 hours post-injection,

590 mice were exsanguinated via the right atrium of the heart into a heparinised syringe then perfused for

591 2.5 minutes with [^{14}C(U)]sucrose (1.1 μM, 0.5 μCi/ml) via the left ventricle, (all mice were

592 anaesthetised with Domitor™/ketamine and heparinised 20 minutes prior to exsanguination). Whole

593 blood samples were immediately centrifuged for 15 minutes at 5,400 × g to remove red blood cells

594 and the resulting plasma was placed on ice. A CSF sample was taken from the cisterna magna, the

595 IVth ventricle choroid plexus and pituitary gland were collected and the brain was sectioned into right

596 brain and left brain (both comprising frontal cortex and caudate putamen), cerebellum and midbrain

597 (including pons and hypothalamus). The remaining brain (including occipital cortex and hippocampus)

598 was used for capillary depletion analysis and all brain, circumventricular organs (CVO) and plasma

599 samples were solubilized and subjected to dual label (^{3}H/^{14}C) scintillation counting as previously

600 described.

601 **2.8b In vivo pharmacokinetic experiments with pentamidine isethionate.** Adult female CD1 mice

602 (20-25g) were injected intravenously with pentamidine isethionate (4 mg/kg in 0.9% physiological

603 saline) in the absence and presence of concomitant dosing with F68 (initial plasma concentration,

604 calculated by estimating plasma volume at 10% of body weight) at 0.025%. Each group had an n = 3.

605 Blood (<10 µl) was collected using a heparinized syringe at 1, 30, 120, 600 minutes post-injection and

606 plasma prepared. Both blood and plasma samples were snap frozen on dry ice and stored at -80°C

607 before analysis. After the last blood sample, the mice were perfused with sterile 0.9% physiological

608 saline (via the hepatic portal vein), the brains removed, weighed and snap frozen. Analysis of samples

609 was by a validated weak cation exchange solid phase extraction (WCX-SPE) approach performed by a

610 specialist contract research organization (Cyprotex). Briefly samples were diluted with water, WCX-

611 SPE sorbent was primed with MeOH and then water (to ensure phase was fully ionised). Samples

612 were then loaded onto sorbent and washed with pH7 buffer and MeOH. Pentamidine was then

613 washed off sorbent by eluting with a combination of MeOH/H_2O + 5% v/v formic acid. If necessary

614 samples were then evaporated to dryness and reconstituted in injection solvent. Samples were

615 analysed by UPLC-MS/MS as described above. LLOQ in plasma samples was 2 ng/ml and in brain

616 samples was 80 ng/ml.

617 Additional experiments revealed that intravenous administration of 10mg/kg pentamidine isethionate

618 plus or minus 0.5% F68 was toxic to the mice and the experiment was terminated.

619 **Data analysis.** All data are presented as means ±S.E.M and statistical analysis was carried out using

620 Sigma Stat software, version 12.0 (SPSS Science Software UK Ltd, Birmingham, UK).

621   2.9 **Trypanocidal activity** *in vitro*

622   *In vitro* activity of drug formulations against *Trypanosoma brucei* blood stream form trypomastigotes

623   was determined *in vitro* using Alamar Blue (resazurin: Bio-Source, Camarillo, CA) as described by [48].

624   Prior to determination of the trypanocidal activity of pluronic-pentamidine combinations, the IC_{50}

625   values of the Pluronic alone was established. Each Pluronic was tested in at 12-serial dilutions in

626   triplicate and in three separate experiments (n=3). The diluent was HMI-9 media (Invitrogen, UK).

627   Blood stream form *T. b. brucei* (strain S427) trypomastigotes, cultured in modified HMI-9 media

628   supplemented with 10% v/v heat-inactivated foetal calf serum, (hi-FCS, Gibco, Life Technologies,

629   UK), were incubated (37°C; 5% CO_2) at a density of 2 x 10^4/ml in the presence of pentamidine alone or

630   pentamidine-Pluronic formulations for 66h. Resazurin (20 µl 0.49mM in PBS) solution was then added

631   to each well and incubation continued for 6 hours. After incubation, samples were removed and

632   fluorescence was measured using excitation 530nm and emission 590nm on a Spectramax M3 plate

633   reader (Molecular Devices, USA). IC_{50} values were determined (where appropriate) using GraphPad

634   Prism.

635

636 3. Results

637 3.1 Evaluation of potential neurotoxicity of pentamidine

638 *3.1a Literature review.* We conducted a brief review of the literature to assess the potential

639 neurotoxicity of pentamidine. Information was considered relevant to the NanoHAT project if it

640 described an activity that could be detected in a simple profiling screen, rather than secondary

641 readouts (e.g. hERG-mediated, downstream effects on cardiomyocyte [Ca^{2+}]i). Table 2 lists the known

642 pharmacology and approximate affinities of the interaction that have been reported for this

643 compound.

644 As the trypanocidal activity of pentamidine occurs at around 10 nM *in vitro*, we considered that any

645 affinity greater than 1 µM (i.e. more than 100-fold greater than the trypanocidal concentration) was

646 unlikely to be relevant.

647

648 **Table 2. Reported Pharmacology of Pentamidine *in vitro*.**

Property	Affinity (µM)	Comments	Reference
Trypanocidal	0.01	Time-dependent	[49]
Imidazoline₂ receptor	0.014	3H-idazoxan binding	[50]
Potassium channel expression/function	0.17	K(v)11.1(hERG) expression, K(IR)2.1 block	[51]; [52]
NMDA (Ionotropic) glutamate receptor	0.2	Voltage dependent	[53]
Human anti-platelet	1.1	Inhibits fibrinogen binding to GP11b/IIIa	[54]
Rat NMDA receptor	1.8	Rat brain membrane 3H-dizocilpine binding	[55]
PRL phosphatases	3	Oncology target	[56]
Delta2glutamate receptor	5	Voltage independent	[53]
Calmodulin antagonist	30	Inhibits nNO synthase	[57]

28

		in vitro	
Acid sensing ion channels (ASIC)	38	Potency 1b>3>2a>or=1a	[58]
Serine proteases	4000		[59]

649

650 There are 3 major target families for which pentamidine has significant affinity (<20 fold above

651 trypanocidal range) that were of concern; the imidazoline$_2$ receptor (responsible for effects on central

652 blood pressure control and pancreatic beta cells); inward rectifying potassium channels particularly

653 blockade of Kir2.1 (this is more likely cardiac than CNS-relevant) and NMDA glutamate receptors.

654

655 **3.1b A neurological profiling screen**

656 A wide ligand profiling screen was carried out against 40 CNS targets (Perkin Elmer customised CNS

657 screening; listed in Table S1), testing at a single concentration of 10 μM (1000-times the trypanocidal

658 concentration), with follow up concentration-response curves in any assay where there was greater

659 than 70% inhibition. Pentamidine was inactive at 29 out of 40 CNS targets (including 5 glutamate

660 receptor binding sites) at 10 μM, and was re-tested against the remaining targets at a range of

661 concentrations to generate an inhibitory constant, K_i. These results, together with the calculated

662 relative selectivity values compared with trypanocidal affinity, are listed in Table 3.

663

664 **Table 3. K_i Values for Pentamidine Determined for Selected CNS targets.**

Target	K_i (μM)	Relative to trypanocidal activity
Trypanocidal Activity	0.01	1.0
Imidazoline 1$_2$	0.001	0.1
Monoamine oxidase B	0.181	18
Monoamine oxidase A	0.217	22
Adrenergic alpha1	0.273	27
Muscarinic (central)	0.281	28

Histamine H2	7.21	721
Opioid	1.41	141
DA transporter	2.11	211
Adrenergicalpha2	10	1000 Estimate from single-point screen
Adrenergic β	10	1000 Estimate from single-point screen
5HT transporter	10	1000 Estimate from single-point screen

665

666 Selectivity screening of pentamidine identified 5 targets (imidazoline I_2 receptor; monoamine oxidase

667 A and B; adrenergic α_1 receptor; muscarinic receptor) for which it has significant affinity, and which

668 should be monitored as we progressed through the screening cascade. In particular, pentamidine's

669 high affinity for the imidazoline receptor may explain the cardiovascular adverse events associated

670 with this drug. The project team considered that remaining targets were of minor concern, as the

671 adverse events of drugs targeting the adrenergic monoamine oxidase and muscarinic systems are

672 reasonably well described. The relatively low affinity of pentamidine for the remaining targets

673 (histamine H_2 receptor; opioid receptor; adrenergic α_2, β receptors; 5HT transporter) indicated that

674 the drug was unlikely to have significant effects until plasma/brain levels exceeded ~ 100-fold the

675 trypanocidal concentration.

676

677 *3.1c* **Ion channel screen**

678 We carried out ion channel screening at Chantest in order to investigate the potential potassium

679 (K(IR)2.1) blocking liability reported by de Boer et al., (2010) (Table 2). Pentamidine isethionate salt

680 was evaluated at 0.001, 0.01, 0.1, 1 and 10 µM (Table S2). The IC_{50} value for pentamidine isethionate

681 salt was unable to be calculated as the highest tested concentration resulted in hKir2.1 inhibition less

682 than 50% (i.e. 12.3±1.3%). The IC_{50} is estimated to be greater than 10 µM. The positive control (100

683 µM barium) confirms the sensitivity of the test system to ion channel inhibition.

684

685 **3.2 Formulation Development**

686   As this was a milestone driven project an iterative, dynamic approach was utilized to select the lead

687   formulation to take forward as quickly as possible in the screening cascade (Fig 1), hence not all

688   Pluronic formulations were assessed with each of the methods.

689

690   **3.2a Phase Behaviour**

691   L61 phase diagrams were evaluated by visual inspection from 20°C to 50°C for L61 alone and in

692   mixtures with P105 and/or F68 in water and saline solutions. F68 does not improve L61 solubility,

693   while P105 does (Tables S3 and S4).

694

695   **3.2b. Critical micelle concentration (CMC) by fluorescence spectroscopy**

696   CMC were measured for individual Pluronic and mixtures of F68, P85, P105 and L61 at 20°C and 37°C,

697   both in aqueous and saline (0.9 wt%) solutions, using the intensity of pyrene fluorescence emissions

698   (Table 3). Mixtures of two Pluronics in both aqueous and saline mediums were prepared in either a

699   fixed mass ratio of 1:1 or with the addition of 0.01% w/v L61 and the CMC determined. All CMC curves

700   show two inflection points, a feature widely reported in the literature; the first corresponds to the

701   onset of aggregation and was chosen as the CMC (Fig S2; Table 4), giving the following values in saline

702   solution at 37°C (g/L): $P85_{sal}=0.042\pm0.018$; $F68_{sal}=0.048\pm0.012$ and $P105_{sal}=0.069\pm0.020$. Overall, these

703   CMC values are fairly similar and do not allow a prioritisation based on CMC alone. The CMC of F68

704   and P85 mixtures (1:1 mass ratio) is about double the CMC , when expressed in total Pluronic mass, of

705   the individual polymers suggesting the absence of mixed micelles in these mixtures. Small amounts of

706   L61 (0.01%w/v) does not affect the CMC of F68 or P85 or P105 under the conditions tested.

707

708   **Table 4. CMC Values of Pluronic Dissolved in Pure Water (aq) or Saline (sal) at 20°C and 37°C**
709   **Determined Using Pyrene Fluorescence Intensity. Values Mean ± S.D. Saline (0.9 wt%).**
710

Temperature	20°C	37°C
Sample	g/L	g/L
	CMC	CMC

P85$_{aq}$	0.3197±0.0067	0.0432±0.0075
P85$_{sal}$	0.1460±0.0311	0.0424±0.0178
F68$_{aq}$	0.2737±0.0311	0.0606±0.0045
F68$_{sal}$	0.2730±0.0029	0.0476±0.0119
P105$_{aq}$	0.2429±0.0140	0.0730±0.0136
P105$_{sal}$	0.1905±0.0093	0.0686±0.0191
L61$_{aq}$	0.0299±0.0320	n.a.
L61$_{sal}$	0.0240±0.0236	n.a.
Fixed ratio 1:1 mixtures		
P85+F68$_{aq}$	0.7415±0.0000	0.0951±0.0000
P85+F68$_{sal}$	0.6777±0.0000	0.0993±0.0000
P85+L61$_{aq}$	0.2678±0.0000	n.a.
P85+L61$_{sal}$	0.3024±0.0000	n.a.
Sample + L61 (0.01w/v%)		
P85$_{aq}$	0.1135±0.0035	0.0508±0.0264
P85$_{sal}$	0.2836±0.1278	0.0734±0.0316
F68$_{aq}$	0.2010±0.0042	0.0510±0.0177
F68$_{sal}$	0.2060±0.0283	0.0430±0.0000
P105$_{aq}$	0.2421±0.0298	0.0705±0.0237
P105$_{sal}$	0.1938±0.0139	0.0833±0.0475

711   *L61 has a very low cloud point of 24°C and therefore it is not soluble at 37°C. CMC is not available
712   (n.a.)[60]. Also see Fig S2.
713

714   **3.2c Stability of the formulations**:

715   Pentamidine stability in solution was followed by NMR. Pentamidine and pentamidine/Pluronics

716   solutions prepared in D_2O were kept in amber NMR tubes at 37°C. Spectra were measured at days 0, 1

717   and 7. As a control, pentamidine in D_2O was left at 4°C and measured at day 0 and 7. NMR data

718   showed no significant change on peak position or peak intensity when compared to day 0

719   measurements or to control samples, confirming no thermal degradation of pentamidine after 7 days

720   at 37°C.

721

722 ***3.3d Partition***

723 Partition of PTI in the micelles was measured by fluorescence spectroscopy for P105 and F68.

724 Pentamidine has a slightly larger partition coefficient in F68 than in P105 (Table 5). Measurements in

725 mixtures (F68/L61, P105/L61 and F68/P105, 1:1 mass ratio in all cases) do not significantly change the

726 partition coefficient (data not shown).

727 The values of Log P obtained in saline and aqueous solutions are rather similar, suggesting that

728 pentamidine partition is not sensitive to the saline levels used here.

729 The effect of temperature is quite weak (Table 5), and does not follow the same trend with the two

730 Pluronic studied: values of Log Ps for P105 are lower at 20°C than at 37°C (but still very close); instead,

731 for F68 the partition of PTI decreases slightly at higher temperature.

732 At biologically relevant concentrations, 0.5 wt% Pluronics and 1.0×10^{-6} M PTI, extrapolation of the Log

733 P data suggest that ca. 0.1 PTI molecules would be incorporated in one P105 micelle, and 0.01 PTI

734 molecules in one F68 micelle. At the concentrations used for SANS (5 wt% Pluronic and 1 wt% PTI), we

735 expect to have 166 PTI molecules in the micellar core per P105 micelle and 15 for F68 micelle.

736 The relative low values of log P for PTI/Pluronics system, for instance log P for pyrene/Pluronics is ca.

737 2.5 and 3.5 for F68 and P105, respectively [15], is not surprising given the high water solubility of

738 pentamidine, and helps to explain the drug release profile for PTI / Pluronics systems discussed next.

739 Overall, this means that Pluronic have a limited capacity to interact with pentamidine and prolong its

740 circulation.

741

742 **Table 5.** Partition of the Tested Pluronic Dissolved in Pure Water (Aqueous) or Saline (Saline) at
743 20°C and 37°C. (Also see Fig S3).
744

Pluronic	Solvent	Temperature °C	Log P
P105	saline	20	1.06
		37	1.15

33

P105	water	20	0.99
		37	1.09
F68	saline	20	1.67
		37	1.47
F68	water	20	1.67
		37	1.46

745

746   *e.   Drug release*

747   Solutions of 10 mM pentamidine or 10 mM pentamidine plus 1 % F68 or 1% P105 were loaded in

748   dialysis cells and the amount of pentamidine eluting from the cells into water at 37°C were measured

749   over time (Fig S4). Both reaction type and reaction constant, for PTI alone and PTI/Pluronic were in a

750   similar range. ca. 0.5 (Fickian diffusion) for reaction type and ca 0.3 for reaction constant. No

751   significant difference was observed between PTI/Pluronics and PTI/water systems. Thus in the

752   conditions tested, pentamidine release seems to be dominated by diffusion and Pluronics micelles

753   were not a barrier for drug release.

754

755   *f.   Aggregation number/Micellar size:*

756   Pluronic micelles can be reasonably described as a compact core formed by a dry PPO block

757   surrounded by a highly hydrated shell formed by the two PEO blocks [Mortensen, 1993;

758   Hammouda, 2010]. The core-shell model was thus used to provide a more detailed

759   characterisation of the morphology of the Pluronic micelles in D_2O and how it is affected by the

760   presence of PTI, using input values for the core radius and shell thickness were based on

761   hydrodynamic radius values obtained by DLS (Table 6). A term to compensate for polydispersity

762   was included for both Pluronics, as well as a structure factor ($S(q)$), corresponding to a hard sphere

763   model, in order to account for intermicellar interactions. A summary of the main parameters

764   obtained from the analysis of the data is present in Table 7.

765

766   ==Table 6==. Stokes Radii of P105, P85 and F68 Micelles Obtained from DLS (1% w/w, 37°C).

Pluronic$_{(aq)}$	Radius (nm)	Pluronic$_{(saline)}$	Radius (nm)
F68	2.8	F68	2.5
P85	6.6	P85	6.5
P105	8.1	P105	8.0
[PTI]= 10^{-6} M		[PTI]= 10^{-6} M	
F68	2.8	F68	2.6
P85	6.6	P85	7.8
P105	7.7	P105	6.7
PTI - 1:3 mass ratio		PTI - 1:3 mass ratio	
F68	2.3	F68	3.1
P85	6.8	P85	6.5
P105	8.1	P105	8.0

767

768   ==Table 7.== Geometric parameters from model-fitting of the SANS Pluronic data at 37°C,
769   including core and shell micellar sizes, fraction of solvent in the corona (χ_{solv}) and
770   aggregation number (N_{agg}).

Sample	Core radius (Å)	Shell thickness (Å)	Total radius (Å)	χ_{solv}	N_{agg}
F68 5%	15.4	36.5	52.0	0.99	2.37
F68 5%/ PTI 1%	15.1	34.7	49.8	0.98	2.25
F68 5%/ PTI 3%	15.5	33.9	49.4	0.99	2.38
P85 5%	42.9	31.4	74.3	0.95	35.4
P85 5%/ PTI 1%	41.5	30.5	72.0	0.95	32.4
P85 5%/ PTI 3%	41.0	30.5	71.5	0.99	31.6

771

772   A direct comparison of F68 and P85 micelles in D_2O shows that both have similar shell thickness, with

773   F68 showing values slightly larger, 36.5 Å vs 31.4 Å, respectively. It is worth noting that F68 EO blocks

774   have on average 76.4 EO units while P85 blocks are only 26.1 units long. The core of F68 micelles are

775   significantly smaller than P85 micelles, 15.4 vs 42.9 Å. In terms of PO content, the F68 PO block is 29

776   units long while P85 is 40 units long. Overall, P85 micelles are larger than F68 micelles, 74.3 vs 52.0 Å,

777   respectively.

778

779   The simulation work agrees well with these experimental results. The average aggregation number

780   per micelle (N_{agg}) and the average number of micelles (N_{mic}) were calculated once the systems had

781   equilibrated have been measured. Fig 2 shows plots of N_{agg} and N_{mic} as a function of pluronic

782   concentration for both the F68 and P105 Pluronics. We carried out simulations over a range of

783   Pluronic concentrations that span the CAC and the CMC values observed experimentally to validate

784   the models (at least qualitatively). From Fig 2, one can see that in both systems, once we have passed

785   the CAC the number of micelles remains more or less constant but they continue to grow in size as the

786   concentration increases until we reach the CMC at which point the size of the micelles more or less

787   plateaus. Also, when comparing the behavior of the F68 and P105 Pluronics, we found that the P105

788   Pluronics form larger aggregates when near the CMC as compared to that for the F68 Pluronics, and

789   therefore fewer micelles. Note, we have also simulated mixtures of F68 and L61 Pluronics, and the

790   results of those systems are presented in Fig S5.
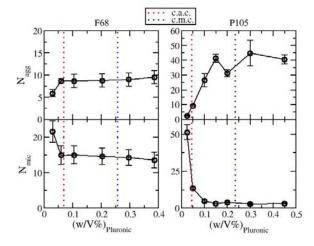

791

792   **Fig 2. The average number of Pluronic molecules found in a micelle (N_{agg}) and the**
793   **number of micelles in our system (after they have equilibrated) (N_{mic}) as a function of**
794   **the concentration of the Pluronics in the system for both the F68 (left) and P105 (right)**
795   **Pluronics**. Additionally, we have compared the findings from the simulations with the
796   identified values (dashed lines) of the CAC and CMC from the experimental systems.

797

798   In the presence of 1% PTI, a small reduction in size was observed for both Pluronics, ca. 2 Å in both

799   cases. The increase to 3% PTI does not cause further changes.

800   The coronas were highly hydrated, as reported for these polymers [61] Puig-Rigall JCIS 2018]. F68

801   micelles were more hydrated than P85: for each EO unit in the shell, there were are 17 D_2O molecules

802   in a F68 micelle but only 3.4 in a P85 micelle.

803   The addition of pentamidine leads to a subtle, but perceptible, reduction of the number of water

804   molecules in the F68 micelle shell. For P85, no measurable changes were observed.

805

806   **3.3 Peripheral Toxicity**

807   Pluronic concentrations used in the biological assays were based on the CMC measurements.

808   Peripheral toxicity of the individual polymers was assessed. L61 was not studied at this stage due to

809   its limited solubility.

810

811   **a. Haemolysis assay**

812   No haemolysis was observed for any of the Pluronics (P85, F68 and P105) at the tested concentrations

813   (0.01%, 0.1%, 0.25%, 1% or 5%) or the negative controls 0.05% DMSO or 0.9% saline. Positive control

814   10% Triton X-100 showed significantly more haemolysis (2025% ***$p < 0.001$).

815

816   **b. Permeability assay (MDCK-MDR) to assess membrane integrity/cytotoxicity**

817   [^{14}C(U)]sucrose is an inert, polar molecule which normally does not cross cell membranes, but may

818   cross between cells through the paracellular cleft. Significant differences in [^{14}C(U)]sucrose P_{app} values

819   existed in the presence of all tested concentrations of P85, and 0.5% and 0.01% P105 compared to

820   [^{14}C(U)]sucrose P_{app} in the absence of Pluronic (Fig S6). No tested concentration of F68 significantly

821   affected the radiolabelled sucrose P_{app} values.

822

37

823   c.   Peripheral toxicity to the endocrine pancreas

824   Exposure of MIN6 β-cells to 1 and 100μM pentamidine for 24 hours caused a concentration-

825   dependent inhibition of acute insulin secretion in response to the cyclic AMP elevating agents

826   forskolin and IBMX (Fig 3). Surprisingly, P85 and 105 were significantly more effective than

827   pentamidine in inhibiting insulin secretion in response to forskolin/IBMX, such that insulin release was

828   fully inhibited by these Pluronics in the absence of pentamidine at all concentrations tested (0.01-0.5%

829   w/v) (Fig 3A-D). Low concentrations of F68 (0.01 and 0.025% w/v) generated similar inhibitory effects

830   on insulin secretion as unformulated pentamidine (Fig 3A-B) and increased toxicity was observed with

831   higher concentrations of F68 (Fig 3C-D).

832

833   **Fig 3**. The effect of Pluronic on insulin secretion from MIN6 β-cells.
834   (A-D) P85 and P105 induced a strong suppression of insulin secretion from MIN6 β-cells even at low
835   concentrations. (C-D) F68 only induced insulin secretion suppression at concentrations ≥0.1% w/v.
836

837   Trypan blue staining indicated that the MIN6 β-cells were able to tolerate pentamidine concentrations

838   of 1 and 10 μM, but 100 μM pentamidine, which induced maximal inhibition of insulin secretion, was

839   accompanied by a large number of cells taking up Trypan blue (Fig S7 and S8). These micrographs are

840   indicative of the suppression of insulin secretion by pentamidine being associated with marked

841   reductions in β-cell viability, but the plasma membrane was largely intact as there was no leakage of

842   insulin, a 5.5 kDa peptide, from the cell interior. The combination of 100 μM pentamidine with 0.5%

843   w/v F68, which caused maximal suppression of insulin release (Fig 3), led to the highest proportion of

844   cells that showed Trypan blue staining.

845

846   **3.4 *Trypanocidal activity in vitro***

847   *The In vitro* activity of Pluronic drug formulations alone against *T. b. brucei* blood stream form

848   trypomastigotes was determined showing low trypanocidal activity of F68 compared to high activity of

849   P85 and P105 (Table 8).

850 **Table 8. The Inhibitory Concentration (IC$_{50}$) required to reduce number of bsf trypomastigotes by**
851 **50%. Pluronic were tested at 12 serial dilutions in triplicate and repeated in 3 separate experiments**
852 **(n=3) to produce IC$_{50}$ values.**

w/v %	F68	F68/0.01% L61	P85	P105
IC$_{50}$	0.48%	0.46%	0.00021%	0.00084%
95% CI	0.38 - 1.35	0.027 - 0.94	0.00056 - 0.0014	0.00070 - 0.0012

853

854

855 In further studies the anti-trypanosomal activity of combinations of F68 and pentamidine were

856 assessed (Table 9). The IC$_{50}$ (± 95% CI) values of pentamidine were $2.11 \times 10^{-5} \pm (1.79 \times 10^{-5} - 2.50 \times$

857 $10^{-5})$ µM alone, 6.36×10^{-6} (± $4.43 \times 10^{-6} - 9.12 \times 10^{-6}$) µM with 0.01% F68 and $3.25 \times 10^{-6} \pm (3.13 \times 10^{-7}$

858 $- 3.38 \times 10^{-5})$ µM with 0.001% F68.

859

860 **Table 9. The % of bsf trypomastigotes inhibited by pentamidine/pluronic combinations. The**
861 **combination formulation was tested in triplicate and repeated in 3 separate experiments (n=3).**

862

	Pentamidine (µM)					
	1	0.3	0.000152	5.1×10^{-5}	1.7×10^{-5}	5.7×10^{-6}
F68 (w/v %)						
0.5%	99.5%	98.6%	98.6%	98.3%	98.3%	99.2%
0.1%	98.5%	97.7%	97.1%	97.1%	97.3%	97.7%
0.025%	98.3%	97.5%	97.0%	96.9%	97.0%	90.6%
0.01%	98.4%	97.6%	96.4%	95.1%	82.8%	3.4%
0.001%	98.3%	97.4%	96.4%	91.9%	73.1%	1.8%
0%	98.3%	97.4%	92.7%	65.3%	35.0%	4.1%

863

864 To determine if the addition of Pluronic to pentamidine had an additive effect on the trypanocidal

865 activity of pentamidine, it was decided that work should focus on F68 rather than the other pluronics.

866 Although IC$_{50}$ values could only be determined for two combinations, in part due to the high starting

867 concentration of pentamidine used, a limited interaction between Pluronic F68 and pentamidine was

868 observed at the lowest F68 concentrations (Table 9 boxes shaded in red), suggesting that the addition

869 of Pluronic had an additive effect on the trypanocidal activity.

870

871 **3.5 Blood-brain barrier: *In vitro* permeability assays**

872 We examined the ability of different pentamidine-Pluronic formulations to cross the BBB using the

873 MDR1-MDCK cell line. Two analytical methods were applied: one detected pentamidine isethionate

874 using UPLC-MS/MS (Table S5) and the other detected radiolabelled pentamidine using liquid

875 scintillation counting (Table 9).

876 The ability of pentamidine isethionate to cross the MDR1-MDCK cell monolayers in the apical (luminal)

877 to basolateral (abluminal) direction was limited as the concentration of pentamidine isethionate in the

878 basolateral chamber were below the limits of UPLC-MS/MS detection, even when the pentamidine

879 isethionate concentration was increased to 20 μM (data not shown). The lower limit of quantification

880 (LLQ) for UPLC-MS/MS method was ≤ 7.8 nM which is equivalent to <0.039% of the dose in the donor

881 (apical) chamber. These results would suggest that pentamidine is a 'CNS negative' drug. The mass

882 balance (% recovery) results suggest that up to 25% may have been trapped either in the endothelial

883 cells or by non-specific binding to the plastic plate or the polycarbonate membrane and this may

884 contribute to the reduced recovery. It is important to note that the two amine groups of pentamidine

885 are ionized at physiological pH (pKa is 11.4) and are likely to react with static charges on the surface of

886 plastics. Interestingly the presence of Pluronic increased the mass balance results by 10-20%. In

887 contrast the movement of pentamidine isethionate (20μM) across the monolayer in the basal to apical

888 direction was measurable, the calculated P_{app} being 0.418 x 10^{-6} cms^{-1}. Taken together with the

889 absence of a detectable movement of pentamidine isethionate in the apical to basolateral direction

890 these data would indicate the presence of an efflux mechanism for this molecule, likely MDR1. The

891 presence of Pluronics (F68, P105 or P85 at concentrations of 0.01%, 0.1% or 0.5%) did not affect the

892 distribution of pentamidine isethionate across the monolayer in either direction.

893   MDR1-MDCK permeability assay experiments were also performed using radiolabelled pentamidine

894   and liquid scintillation counting. The rate of transport of [^{3}H(G)]pentamidine (9 nM) across MDR1-

895   MDCK monolayer was examined and in contrast to the results achieved with the pentamidine

896   isethionate was measurable in both directions (Table S5). In these experiments the highest

897   concentrations of P85 (0.5%) and P105 (0.5%) affected the integrity of the cell monolayer and the

898   Papp for [^{14}C(U)]sucrose was increased to 4.8 and 4.3 x 10^{-6} cm/s respectively. The [^{14}C(U)]sucrose P$_{app}$

899   for all other experiments was 1.61±0.15 x 10^{-6} cm/s. The presence of the Pluronics (F68, P105 or P85)

900   at concentrations of 0.01% and 0.1% did not significantly increase the distribution of

901   [^{3}H(G)]pentamidine across the MDR1-MDCK monolayer measured over 60 minutes confirming the

902   results obtained using pentamidine isethionate and the UPLC-MS/MS detection method.

903   In conclusion, our target formulation characteristics of at least a 2-fold increase in pentamidine /

904   pentamidine isethionate movement across the monolayer, compared with unformulated pentamidine,

905   was not observed using these *in vitro* models of BBB permeability.

906   **Table 9**. The Effect of P85, F68 and P105 on the Apparent Permeability of [^{3}H(G)]pentamidine (9 nM)
907   MDR1-MDCK Cell Monolayers in the Apical to Basolateral Direction and the Basolateral to Apical
908   Direction. The percentage recovery of pentamidine is also shown. All the data has been corrected
909   for extracellular space by subtracting [^{14}C(U)]sucrose (5.5 μM) P$_{app}$ values which ranged from 0.89
910   to 2.00 x 10^{-6} cm/s. Each value represents three replicates for each n and n =3. n.d. = not
911   determined as integrity of the barrier compromised.

[^{3}H(G)]Pentamidine (9 nM)	Pluronic Concentration (%)	P$_{app}$ A2B (10^{-6} cm/s)	P$_{app}$ B2A (10^{-6} cm/s)	A2B (%)	B2A (%)
		Mean±SEM	Mean±SEM	Mass balance	Mass balance
	0	0.678±0.025	0.776±0.062	84	85
	0.01% P85	0.310±0.142	0.431±0.161	86	87
	0.1% P85	0.561±0.0.172	0.227±0.081	89	89
	0.5% P85	n.d.	n.d.	90	90
	0.01% P105	0.577±0.0710	0.818±0.086	86	89
	0.1% P105	0.898±0.161	0.776±0.054	89	88

	0.5% P105	n.d.	n.d.	91	91
	0.01% F68	0.200±0.115	0.106±0.061	95	83
	0.1% F68	0.221±0.067	0.033±0.019	98	87
	0.5% F68	0	0	98	84

912

913   **3.6: Blood-brain barrier *In situ* brain perfusion**

914 ***3.6a* PLURONIC P85**

915 Co-formulation of 15.7 nM [^{3}H(G)]pentamidine with Pluronic P85 did not significantly increase

916 [^{3}H(G)]pentamidine accumulation in any of the brain regions examined (Table S6).

917   In fact, overall there was a decrease in the [^{14}C(U)]sucrose-corrected uptake of [^{3}H(G)]pentamidine

918 with P85 at 0.01% ($p<0.001$) and at 0.1% ($p<0.01$) (Two-Way ANOVA), but these decreases were not

919 statistically significant when individual brain regions were examined using Bonferroni's pairwise

920 comparisons.

921 Table S6 shows the results of capillary depletion analysis of the brain tissue after 10 minutes of

922 perfusion in the presence or absence of the Pluronic, P85. [^{3}H(G)]pentamidine accumulated in the

923 capillary endothelial cells (pellet) of control mice while less than 2% of the plasma concentration

924 crossed the basolateral membrane to reach the parenchyma (supernatant). These results are in good

925 agreement with our previously published data[45]. There appeared to be an overall reduction in this

926 accumulation of [^{3}H(G)]pentamidine into the endothelial cells and consequently a reduction in the

927 parenchyma when [^{3}H(G)]pentamidine was co-formulated with 0.01% and 0.1% P85, but the reduction

928 did not attain statistical significance (Two-Way ANOVA with Bonferroni's pairwise comparisons).

929 Whilst there appeared to be an overall inhibitory effect of Pluronic on the transport of

930 [^{3}H(G)]pentamidine across the BBB, there was a significant, 3-fold increase in the uptake of

931 [^{3}H(G)]pentamidine into the pituitary gland after 10 minutes of perfusion with 0.1% and 0.5% P85

932 ($p<0.05$ at both concentrations; Two-way ANOVA with Bonferroni's pairwise comparisons). This

933   enhanced uptake of pentamidine appeared to be associated with an approximate 2-fold increase in

934   accumulation of [^{14}C(U)]sucrose from 20.5±3.9% ([^{14}C(U)]sucrose alone) to 35.0±5.5% (+0.01%P85),

935   43.0±4.0% (+0.1% P85) and 34.3±7.9% (+0.5% P85). A similar effect was observed in the choroid

936   plexus sampled from a few individual mice that were perfused with pentamidine co-formulated with

937   P85 at concentrations above the CMC. This resulted in a 2-fold increase in the mean uptake of

938   [^{3}H(G)]pentamidine which was not statistically significant. P85 did not affect accumulation of

939   [^{3}H(G)]pentamidine or [^{14}C(U)]sucrose by the pineal gland.

940   **3.6b PLURONIC P105**

941   An overall decrease in the [^{14}C(U)]sucrose-corrected uptake of [^{3}H(G)]pentamidine into brain

942   parenchyma was observed when 15.7nM [^{3}H(G)]pentamidine was co-formulated with 0.1% (p<0.001)

943   and 0.5% (p<0.001) P105, as shown in Table S7, but (like P85) these data did not reach statistical

944   significance in any of the individual regions sampled (Two-Way ANOVA with Bonferroni's pairwise

945   comparisons).

946   In contrast, there was a 33% increase in the [^{14}C(U)]sucrose-corrected uptake of [^{3}H(G)]pentamidine

947   into the endothelial cell pellet when it was co-formulated with 0.1% P105 (p=0.027; Two- way ANOVA

948   with Bonferroni's pairwise comparisons). This increase was apparent in only 3 out of 6 mice, and was

949   associated with penetration of the brain tissue by the vascular space marker [^{14}C(U)]sucrose, perhaps

950   indicating an increase in the permeability of the apical/luminal endothelial cell membrane.

951   No significant differences were observed in the uptake of either the vascular space marker

952   [^{14}C(U)]sucrose or [^{3}H(G)]pentamidine into the pineal gland, choroid plexus or pituitary gland after 10

953   minutes of perfusion with [^{3}H(G)]pentamidine co-formulated with P105 as shown in Table S7 (p>0.05

954   for each Pluronic concentration and circumventricular organ; Two-way ANOVA with Bonferroni's

955   pairwise comparisons).

956   **3.6c PLURONIC F68**

43

957 **10 minute perfusions**

958 Co-formulation of [^{3}H(G)]pentamidine with F68 resulted in an overall decrease in accumulation of

959 [^{3}H(G)]pentamidine into brain parenchyma after 10 minutes of perfusion (p=0.002 for 0.1% and p=0.03

960 for 0.5% respectively; Two-way ANOVA with Bonferroni's pairwise comparisons) (Table S8). A

961 decrease in vascular space as measured by accumulation of [^{14}C(U)]sucrose was also measured when

962 0.01 or 0.1% F68 (but not 0.5%) was present in the artificial plasma (p=0.042 for 0.01% and p=0.004

963 for 0.1% respectively; Two-way ANOVA with Bonferroni's pairwise comparisons) (Table S9).

964 F68 did appear to increase accumulation of [^{3}H(G)]pentamidine into the endothelial cell pellet at

965 concentrations of 0.01% and 0.1%, but these results did not attain significance. This increase in

966 [^{3}H(G)]pentamidine, did not appear to be associated with a concomitant increase in uptake of

967 [^{14}C(U)]sucrose (p>0.05) and might have been due, at least in part, to a small decrease in the amount

968 of drug crossing the basolateral membrane to enter the brain parenchyma, as indicated by a marginal

969 reduction of [^{3}H(G)]pentamidine in the supernatant (Table S8).

970 Co-formulation of [^{3}H(G)]pentamidine with 0.5% F68 resulted in a 2-fold increase in uptake into the

971 pituitary gland after 10 minutes of perfusion (p=0.017; 1-way ANOVA with Bonferroni's pairwise

972 comparisons). A similar, but not statistically significant increase was observed in uptake of

973 [^{14}C(U)]sucrose into this organ over the same time period.

974 **30 minute perfusion**

975 Accumulation of [^{14}C(U)]sucrose measured in brain parenchyma, as a percentage of concentration in

976 the artificial plasma (R$_{TISSUE/PLASMA}$%), ranged from 1.3% in the hippocampus to 4.3% in the pons after 30

977 minutes of perfusion. These values are almost identical to our previously published data for BALB/c

978 male mice (1.6 and 4.5% respectively)[7]. Accumulation of [^{3}H(G)]pentamidine, when corrected for

979 vascular space ranged from 6.9% in the hippocampus to 15% and 10.9% in the more highly

980 vascularized regions of the hypothalamus and pons respectively. These values were slightly higher

981   than our previously published data (4.3% for hippocampus, 7.6% for hypothalamus and 8.2% for pons)

982   and might reflect changes in expression of transporters due to differences in environment/diet or

983   selective pressures during breeding.

984   Formulation of 15 nM [^{3}H(G)]pentamidine with 0.01% or 0.1% F68 did not affect [^{14}C(U)]sucrose brain

985   space (p=0.139 and 0.460 respectively; 2-way ANOVA with Bonferroni's post-tests). No significant

986   differences were observed in [^{3}H(G)]pentamidine accumulation at these concentrations (p=0.120 and

987   1.000 respectively; 2-way ANOVA with Bonferroni's post-tests). Similarly, F68 had no significant effect

988   on [^{14}C(U)]sucrose or [^{3}H(G)]pentamidine accumulation in the capillary depletion samples after 30

989   minutes of perfusion (p>0.05 for each concentration tested for each isotope; 2-Way ANOVA) nor in

990   the circumventricular organs (p>0.05 for each concentration tested for each isotope; 2-Way ANOVA).

991   There was an approximate 2-fold increase in accumulation of both [^{3}H(G)]pentamidine and the

992   vascular space marker [^{14}C(U)]sucrose in the brain parenchyma of mice that were perfused with

993   formulations containing 0.5% F68, (p=0.003 and p <0. 001 respectively; 2-way ANOVA with

994   Bonferroni's post-tests), as shown in Table S10A and S10B. Visible signs of damage to the BBB

995   including permeation and staining with Evans blue (MW 961), were also observed in some mice. The

996   results from the capillary depletion analysis after 30 minutes of perfusion would also appear to reflect

997   damage to both the apical and basolateral endothelial cell membranes, with a tendency for increased

998   permeation of [^{14}C(U)]sucrose into the brain parenchyma, as demonstrated by a small, though not

999   statistically significant rise in this isotope being detected in the supernatant (Table S10).

1000   Co-formulation of [^{3}H(G)]pentamidine and [^{14}C(U)]sucrose with 0.5% F68 resulted in an increase into

1001   the pituitary gland and the choroid plexus when the perfusion time was extended to 30 minutes,

1002   although these results were not statistically significant.

1003   *3.7 In vivo* **pharmacokinetic experiments with pentamidine isethionate or [^{3}H(G)]pentamidine.**

1004   F68 at the 0.025% does not increase the accumulation of pentamidine in the plasma, brain

1005   parenchyma or blood in the mouse up to 10 hours post-dosing (Fig 4). There might be a late-onset

1006   increase in brain concentrations in the pentamidine alone group, but as the standard deviations for

1007   this group at this time-point are large this is unlikely to be significant.

1008   **Fig 4. The effect of Pluronic F68 on pentamidine concentrations in mouse plasma, blood and brain**
1009   **after an intravenous dose**. Each point represents an n of 3. 4mg/kg pentamidine ± 0.025% F68 i.v.
1010   Values ± SD.
1011
1012   Table 12 shows the mean plasma and CSF (corrected for blood/sucrose contamination) concentrations

1013   for [^{3}H(G)]pentamidine and/or its metabolites, measured at 2 hours after intra-venous injection. No

1014   significant differences were observed when [^{3}H(G)]pentamidine was co-formulated with either 0.025%

1015   or 0.5% F68 (p >0.05 for plasma and CSF; One-way ANOVA). Similarly, no significant differences were

1016   observed in uptake of [^{3}H(G)]pentamidine or the vascular space marker [^{14}C(U)]sucrose, into the brain

1017   parenchyma, capillary depletion samples or the circumventricular organs when [^{3}H(G)]pentamidine

1018   was injected in the presence or absence of F68 (p>0.05; 2-way ANOVA with Bonferroni's pairwise

1019   comparisons) as shown in Table 13.

1020   **Table 12. Concentration of [^{3}H(G)]pentamidine/metabolites in the plasma and**
1021   **CSF at 2 hours post-injection.**
1022

Group	mean concentration in plasma ng/ml (± SEM)	mean concentration in CSF pg/ml (± SEM)
control	0.343 (± 0.061)	2.669 (± 0.765)
0.025% F68	0.345 (± 0.013)	1.948 (± 0.826)
0.5% F68	0.356 (± 0.026)	3.592 (± 1.932)

1023

1024   **Table 13. Uptake of [^{3}H(G)]pentamidine into brain tissue (corrected for vascular space) at 2 hours**
1025   **post-injection. Data is presented as the tissue/plasma ratio (a) and converted into concentrations in**
1026   **ng/g of tissue (b).** A limitation of measuring pentamidine by scintillation counting is that any
1027   metabolites produced during the 2 hours that have retained the radiolabel, will be counted as
1028   [^{3}H(G)]pentamidine. These metabolites may have different transport characteristics and may or may
1029   not be active against trypanosomes.

(a)	$R_{TISSUE/PLASMA}$% (mean±SEM)		
Region	Control	0.025% F68 +	0.5% F68 +

	(15.7 nM pentamidine) (n=6)	(15.7 nM pentamidine) (n=6)	(15.7 nM pentamidine) (n=5)
Right brain	115.52 (± 12.46)	120.29 (± 17.14)	87.36 (± 20.36)
Left brain	152.29 (± 33.48)	111.85 (± 19.15)	106.10 (± 12.92)
Cerebellum	204.02 (± 35.28)	208.87 (± 28.81)	172.48 (± 30.34)
Midbrain	181.18 (± 45.30)	254.02 (± 35.48)	180.00 (± 32.83)
Homogenate	249.41 (± 35.59)	184.18 (± 35.22)	293.81 (± 122.95)
Supernatant	123.35 (± 28.45)	99.72 (± 9.02)	98.66 (± 9.47)
Pellet	479.72 (± 72.50)	310.63 (± 38.62)	536.52 (± 212.72)
Choroid plexus	24666.66 (± 4928)	19628.89 (± 4672)	20463.70 (± 1827)
Pituitary gland	15053.41 (± 3598)	11285.42 (± 2008)	15061.87 (± 5321)

1030

(b) Region	Mean concentration (ng/g or ng/ml for the supernatant ±SEM)		
	Control (15.7 nM pentamidine) (n=6)	0.025% F68 (15.7 nM pentamidine) (n=6)	0.5% F68 + (15.7 nM pentamidine) (n=5)
Right brain	0.363 (± 0.035)	0.417 (± 0.061)	0.302 (± 0.058)
Left brain	0.472 (± 0.084)	0.383 (± 0.063)	0.375 (± 0.048)
Cerebellum	0.607 (± 0.032)	0.719 (± 0.097)	0.591 (± 0.084)
Midbrain	0.494 (± 0.075)	0.866 (± 0.115)	0.614 (± 0.072)
Homogenate	0.820 (± 0.183)	0.643 (± 0.132)	0.988 (± 0.375)
Supernatant	0.363 (± 0.037)	0.345 (± 0.035)	0.351 (± 0.043)
Pellet	1.482 (± 0.151)	1.067 (± 0.125)	1.827 (± 0.662)
Choroid plexus	74.68 (± 11.48)	84.04 (± 5.78)	72.20 (± 7.60)
Pituitary gland	43.76 (± 3.82)	37.58 (± 6.54)	68.13 (± 15.05)

1031

1032   **Discussion**

1033   In this study we generated pentamidine/Pluronic® formulations and prioritised 18 formulations using

1034   a rational, iterative approach (Fig 1). The milestones were intended to ensure that the most

1035   appropriate formulations, on the basis of *in silico* and *in vitro* data, were taken forward to the *in vivo*

1036   pharmacokinetic studies and that the formulations with the greatest likelihood of success would be

1037   assessed for toxicity issues *in vivo* and tested in animal efficacy models of stage 1 and stage 2 HAT. An

1038   ideal formulation for injection should be equipped with characteristics that improved the stability and

1039   safety profile of pentamidine, enhanced therapeutic effect, and accelerated the absorbance of drugs.

1040   Since increasing the concentration of pentamidine in the brain may cause an intractable neurotoxicity

1041   and serious adverse events our starting point was a customised, wide ligand profiling screen carried

1042   out against 40 CNS targets (Tables 2 and S1). Five targets (imidazoline I_2 receptor; monoamine

1043   oxidase A and B; adrenergic α_1 receptor; muscarinic receptor) were identified to have significant

1044   affinity for pentamidine (Table 3). All but one of these (imidazoline I_2 receptor) had a 20-1000 fold

1045   lower affinity than the relative trypanocidal activity and did not generate major concern[55]. The

1046   activity against the imidazoline I_2 receptor may explain the cardiovascular adverse events with this

1047   drug. We were unable to reproduce the result of De Boer et al., 2010 in a recombinant human system

1048   indicating that pentamidine was without effect (at up to 10 µM) on the hKir2.1 potassium channel-

1049   induced inward rectifying current (Tables 2 and S2). Thus progression could continue through the

1050   screening cascade.

1051   For the Pluronics tested in this study (P85, P105, F68 and L61), phase behaviour [62][33] and cloud

1052   points [63] are well established. P85, P105 and F68 are soluble in water and saline solutions at both

1053   24°C and 37°C. L61 has a very low cloud point of 24°C and is not soluble at 37°C. Pure L61 therefore

1054   has limitations as a formulation for drug delivery. Our phase diagrams revealed that F68, which is

1055   highly hydrated, is unable to solubilise highly hydrophobic L61 to a great extent, so it was not possible

48

1056   to pursue a 1:1 mixture of L61:F68 in the assays (Table S3 and S4). Although F68 formulations

1057   containing small concentrations of L61 (i.e. 0.01%) could be assessed.

1058

1059   Using molecular dynamics (MD) simulations and physical techniques, we elucidated many of the

1060   structural properties of Pluronic P85, P105, F68 and L61 micelles, and were able to extract

1061   fundamental parameters required for biological evaluation of the formulations. For example, the CMC

1062   were measured for F68, P85 and P105 at 20°C and 37°C both in aqueous as well as saline (0.9 wt%)

1063   solutions. Several values for the CMC of Pluronics can be found in the literature [64][65][66][15][67].

1064   These values tend to vary widely, showing as much as one order of magnitude differences for the

1065   same Pluronic[68]. This has been attributed to several reasons: difference in molecular weight

1066   distribution between batches [69][67], presence of impurities such as diblocks[69][70] slow micellar

1067   formation leading to non-equilibrium states[71][72], differences inherent to the technique employed

1068   [Loh, 2002]. In addition, for some Pluronic systems, two critical concentrations are detected, both in

1069   surface tension and spectroscopic experiments [69][65]. This behaviour has been ascribed to

1070   formation of premicellar aggregates occurring before full micelle formation[73][74][64][65][75].

1071   Overall, an unambiguous determination of the CMC for Pluronics is not a trivial task – despite decades

1072   of data on the topic – and remains very dependent on the technique used, the experimental

1073   conditions and batch variations within the same type of Pluronic. In this work, which used the

1074   intensity of pyrene fluorescence emission, two critical concentrations were also detected (Fig S2). In

1075   this study, the CMC was taken as the first critical concentration, whch may reflect a more efficient

1076   drug delivery vehicle with greater permeability characteristics and longer circulation times

1077   (10.1021/la4022273). Therefore, the CMC values presented here (Table 4) are taken from the first

1078   break point. The CMC values achieved for F68, P85 and P106 were fairly similar and did not allow a

1079   prioritisation of a specific formulation based on CMC alone. However. the concentrations of Pluronic

1080   (0.001 to 0.025%) used in the biological assays were based on the CMC values and were selected on

1081   the basis that they would be likely to consist of mainly unimers (0.001-0.025%); a mixture of unimers

1082   and micelles (0.1%) and mostly micelles (0.5%) respectively.

1083

1084   F68 micelles have a relatively small radius of 52.0 Å (Table 7). This attribute will increase stability, half-

1085   life and therefore circulation time of this Pluronic, since small micelles evade detection and

1086   destruction by the reticuloendothelial system. However, F68 also has a small core volume which will

1087   directly correlate to low drug loading/partioning (Table 5; Figure S3). In addition, the fact that

1088   pentamidine release from the F68 and P105 micelles is by diffusion would indicate that these Pluronics

1089   are unlikely to significantly prolong the circulation time of pentamidine (Figure S4).

1090

1091   Haemolysis of human red blood cells was not observed in the presence of 0.5%, 0.1%, 0.025%, 0.01%,

1092   and 0.001% P85, P105 or F68. The results being comparable to the negative control (0.05% DMSO).

1093   This suggests that an intravenous formulation containing P85, P105, or F68 would not lead to

1094   haemolysis at the tested concentrations, supporting the safety profile of Pluronic polymers for medical

1095   use[14]. In agreement, no differences were reported in the terminal haematological values (including

1096   haemoglobin, packed cell volume, number of erythrocytes, total number of leukocytes) and blood-

1097   chemical values (including urea, total protein, alkaline phosphatase) obtained from rats who had

1098   received once daily intravenous doses of F68 (doses ranging from 10-1000 mg/kg body weight) and

1099   from rats who had been administered physiological saline for one month [76]. No morphological

1100   abnormalities were detected in the rats which received the 0-50 mg/kg daily dose of F68, however,

1101   rats which received the higher doses had detectable alterations i.e. the presence of foam cells in the

1102   lungs (dose was 500-1000 mg/kg) and focal cortical degenerative changes in the kidneys (dose was

1103   100-1000 mg/kg).

1104   Pentamidine caused a concentration-dependent inhibition of insulin secretion from MIN6 β-cells

1105   suggesting that this is one mechanism through which it could induce diabetes[9]. Pentamidine is

1106   known to be an agonist at imidazoline receptors [81], but it is unlikely that this explains its inhibitory

1107   effects on insulin secretion since β-cell imidazoline receptors are coupled to increased insulin

1108   release[82].   However, the imidazoline ligand idazoxan is reported to cause a concentration-

1109   dependent inhibition of β-cell viability[83], similar to the effects observed here with pentamidine, so it

1110   is possible that the reduction in insulin secretion is secondary to pentamidine-mediated activation of

1111   β-cell imidazoline receptors and impairment of cell viability. Pentamidine induced diabetes is not

1112   thought to be reversible [9], and so testing for a marker of pancreatic toxicity occurred early in the

1113   screening cascade.   Importantly, a number of Pluronic formulations (P85, P105) were shown to

1114   increase the peripheral toxicity of pentamidine. In a human tissue cell model (HEK-293) P105 has

1115   previously been shown to cause dose dependent changes in cell viability[16]. However, a lead pluronic

1116   (F68) was identified which demonstrated equivalent toxicity to unformulated pentamidine, on β-cell

1117   viability and insulin secretion. Supporting this formulation selection our studies also revealed that P85

1118   and P105 at 0.01% and 0.5% concentrations caused loss of MDCK-MDR monolayer integrity, whereas

1119   F68 at concentrations up to 0.5% had no effect (Figure S6). A correlation between HLB and

1120   cytotoxicity has previously been observed with low cytotoxicity being guaranteed when the HLB of the

1121   polymer is ≥10 (Table S1)[28].

1122   Importantly, all formulations tested did not prevent pentamidine killing blood *Trypanosoma brucei*

1123   blood stream form trypomastigotes. In fact, pure P85 and P105 were highly trypanocidal and F68-

1124   pentamidine formulations had a slight synergistic effect.

1125   *In vitro* BBB studies indicated that there was an efflux process for pentamidine as also demonstrated

1126   in P-gp knockout mice studies [7].   However, we were unable to demonstrate an increase in

1127   pentamidine movement across the barrier in either direction, compared with unformulated

1128   pentamidine in any of our *in vitro* systems.

1129   Further studies utilizing the *in situ* brain perfusion technique confirmed that the Pluronics (P85, P105

1130   or F68) did not increase pentamidine delivery to the brain, including the choroid plexus, after either 10

51

1131   or 30 minutes exposure.   In fact our studies using *in situ* brain perfusions over 10 minutes in mice

1132   have shown that the P85, P105 and F68 formulations have a tendency to actually prevent uptake of

1133   pentamidine into brain tissue and/or vascular endothelial cells, which constitute an intact BBB. This

1134   may be related to interactions of the Pluronics with influx transporters for pentamidine (e.g. OCT1),

1135   although our *in vitro* BBB studies did not indicate that the pentamidine permeability was affected by

1136   the presence of F68, P85 and P105 (0.01% and 0.1%) in either direction. Importantly, a similar P85

1137   induced reduction in BBB permeability was observed by [77] who noted a reduction in the rate of

1138   uptake into brain tissue of P85-leptin conjugates during the first 90 minutes after iv injection

1139   compared with native leptin.   Despite this initial inhibition of P85-leptin influx, a greater overall

1140   concentration of the conjugate was measured in brain tissue after 4 hours, an observation that the

1141   authors ascribed to improved pharmacokinetic properties. Digoxin delivery to the brain has previously

1142   been determined 1 - 10 hr post-injection in mice and found to be significantly enhanced when Pluronic

1143   85 is present [78].

1144   Sucrose does not cross phospholipid membranes and was used in the brain perfusion experiments as a

1145   vascular space marker.   An increase in [^{14}C(U)]sucrose would indicate that the integrity of the

1146   membrane or the tight junctions between cells had been compromised. Conversely, a decrease would

1147   suggest that the proportionate volume of tissue occupied by blood vessels had been reduced.   It is

1148   therefore interesting that F68 has previously been shown to interact with the mechanisms that control

1149   vasoconstriction and vasodilation[79][80] and could lead to the observed reduction in vascular space.

1150

1151   Interestingly, the *in vivo* mouse PK study revealed that the concentrations of pentamidine in brain

1152   parenchyma in this species seem high compared with data from man (using CSF rather than brain

1153   parenchyma) which indicated that less than 1% of the plasma pentamidine concentration is detected

1154   in CSF[84]. Furthermore, assessment of this lead formulation in an *in vivo* PK study confrmed that F68

1155   did not increase pentamidine delivery to the brain under the conditions studied. This is not linked to

52

1156   partitioning of pentamidine inside the micelles as this is low, hence the use of Pluronic micelles to

1157   protect this drug after administration and extend its circulation time is probably limited. Although it

1158   may be related to the fact that F68 is hydrophilic and prefers to remain in the plasma than be

1159   distributed to organs [17].

1160

1161   Whilst there are limitations to all assay systems, the package of data generated by the team provided

1162   a compelling and robust data set.   The screening cascade has successfully identified pluronic-

1163   pentamidine formulations that harbour trypanocidal activity and do not increase the safety concerns

1164   centrally or peripherally (over unformulated pentamidine). However, the data suggested that we will

1165   not be able to significantly enhance brain exposure of pentamidine using the Pluronic (F68 , P85 or

1166   P105) within a reasonable time frame and existing budget. We therefore drew the study to a close at

1167   milestone 2. Importantly a significant body of high-quality data has been generated as part of this

1168   project which may be highly relevant to other teams looking to understand block-copolymer

1169   architecture, further develop block-copolymers as nanocarriers, improve BBB penetration of drugs or

1170   to those looking to understand toxicity of pentamidine.

1171

1172

Acknowledgements

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1197
1198   **References**

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1373 **Supporting information captions**

1374 **Fig S1. Pentamidine is returned to the blood from the capillary endothelial cell by P-gp and MRP. Pluronic®P85 inhibits-mediated efflux (e.g. P-gp and**

1375 **MRP transport) by two mechanisms; the first through membrane fluidisation and the second through transient ATP depletion**. These effects are believed

1376 to be mediated by unimers (single polymer chains) (Shaik *et al.* 2009)(Batrakova *et al.* 1998). Inhibition of efflux should facilitate the accumulation of

1377 pentamidine in the human cerebral capillary endothelium and the murine choroid plexus epithelium, leading to higher concentrations of pentamidine.

1378 **Fig S2. Pyrene fluorescence intensity dependence on pluronic concentration for F68, P85 and P105**. The CMC was determined using 18 different

1379 concentrations (range 0.0001 to 1 w/v%) of pure P85, P105 and F68. The value at each concentration is the mean of two samples, each prepared from a

1380 separate preparation of the stock solution. As expected the curves show two inflection points. The first was taken as the CMC.

1381

1382 **Fig S3. Typical partition data for PTI fluorescence as a function of F68 and P105 concentration.**

1383

1384 **Fig S4. Drug release from dialysis cells measured over time. The experiments were conducted in water at 37°C for concentrations as close as possible to**

1385 *in vitro* **conditions, within experimental limitations, namely, 1% w/v of Pluronics and 10mM PTI**. No significant differences between the Pluronics were

1386 observed and drug release is diffusion controlled (Fickian diffusion) under the experimental conditions. Pluronics micelles are not a barrier to drug release.

1387

Fig S5. The average number of Pluronic molecules found in a micelle (N_{agg}) and the number of micelles in our system (after they have equilibrated) (N_{mic}) as a function of the concentration of the F68 Pluronic in a system that contains F68 and 0.01 w/v% of L61 Pluronic. In the both plots, the black curve represents the results when considering both the L61 and F68 polymers in the mixture, and the blue dashed curve represents the data from the pure F68 simulated systems. In the top curve, the red curve represents the number of F68 in a micelle which contains both F68 and L61, and the green curve represents the number of L61 in a micelle. The results show that as we increase the concentration of F68, and therefore make the system more and more like the pure F68 system, the number of polymer molecules in a micelle and the number of micelles converge to that observed in the pure F68 system, as expected. Interestingly, it seems that from our simulations that L61 causes the aggregation of F68 to become slightly enhanced as the number of F68 in the average micelle is always larger than that found in the pure F68 micelles, which naturally results in their being fewer micelles.

Fig S6. Apical to basolateral permeability of [^{14}C]sucrose in the presence of P85, P105, and F68 concentrations measured over 60 minutes. Significant differences compared to control (no pluronic) was observed in the presence of P85 and P105 (***p<0.001, **p<0.01). All data are expressed as mean ± S.E.M, n= 3 wells. Data were analysed using one-way ANOVA with SigmaPlot 13.0.

Fig S7. Effects of exposure of MIN6 β-cells to 0 (control), 1 or 100 µM pentamidine for 3 and 24 hours. Trypan blue uptake. Blue staining demonstrates cells of compromised viability, highlighting the toxicity of 100 µM pentamidine to these cells after 3 hours exposure.

1404 **Fig S8.** **Effects of exposure of MIN6 β-cells to 0, 1, 10 or 100 µM pentamidine and 0, 0.01, 0.025, 0.1 or 0.5% w/v% F68 for 24 hours**. Trypan blue uptake.

1405 Blue staining demonstrates cells of compromised viability, highlighting the toxicity of 100 µM pentamidine and 0.5% F68 to these cells.

1406

1407 **Table S1. Single point CNS screening of pentamidine at a concentration of $1.0E^{-5}$ M (PE study no. 13-9625).** Values are expressed as the percent inhibition

1408 of specific binding and represent the average of replicate tubes. Bolder values represent inhibition of 50% or greater.

1409 **Table S2. Inhibition of hKir2.1 potassium channel activity with pentamidine isethionate.**

1410 **Evaluated by the QPatch HT an automatic parallel patch clamp system.** The duration of exposure to each test concentration was 3 minutes.

1411

1412 **Table S3. A visual evaluation of the phase separation of Pluronics dispersions in pure water. Transparent is fully transparent. Opaque**

1413 **completely blocks light.** Slight indicates for slightly translucent (faintly white tint in the solution), and medium indicates obvious translucence.

1414 **Table S4. A visual evaluation of the phase separation of pluronics dispersions in saline. Transparent is fully transparent**. Opaque completely blocks light.

1415 Slight indicates for slightly translucent (faintly white tint in the solution), and medium indicates obvious translucence

1416 **Table S5.** **The effect of P85, F68 and P105 on the apparent permeability of pentamidine isethionate across MDR1-MDCK cell monolayers in the**

1417 **basolateral to apical direction.** The apical to basolateral movement of pentamidine isethionate was below the limits of detection. The percentage

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1418 recovery of pentamidine isethionate is also shown. Lucifer yellow permeation was below 0.5×10^{-6} cm/s in all experiments confirming the integrity of the

1419 monolayer. Transcellular marker (propranolol) and Pgp and BCRP substrate (prazosin) apparent permeability values are also shown.

1420 **Table S6. The effect of Pluronic P85 on the accumulation of [^{3}H(G)]pentamidine (15.7 nM) into brain tissues after 10 minutes of *in situ* perfusion.** All

1421 values have been corrected for vascular space by subtraction of the R_{TISSUE}% for [^{14}C(U)]sucrose from the R_{TISSUE}% for [^{3}H(G)]pentamidine. All values mean ±

1422 SEM.

1423 **Table S7. The effect of Pluronic P105 on the accumulation of [^{3}H(G)]pentamidine (15.7 nM) into brain parenchyma after 10 minutes of *in situ***

1424 **perfusion.** All values have been corrected for vascular space by subtraction of the R_{TISSUE}% for [^{14}C(U)] sucrose from the R_{TISSUE}% for [^{3}H(G)]pentamidine.

1425 **Table S8. Accumulation of ^{3}H-pentamidine (15.7 nM) after 10 minutes perfusion with or without pluronic F68 (not corrected for vascular space; Control A**

1426 **and 0.01% and 0.1% F68 experiments were carried out using MP Biomedicals dextran.** Control B and 0.5% F68 experiments were carried out

1427 using VWR dextran).

1428 **Table S9. Accumulation of [^{14}C]sucrose after 10 minutes perfusion with or without Pluronic F68; Control A and 0.01% and 0.1% F68 experiments were**

1429 **carried out using MP Biomedicals dextran.** Control B and 0.5% F68 experiments were carried out using VWR dextran).

1430 **Table S10A. Accumulation of [^{3}H]pentamidine after 30 minutes perfusion with or without pluronic F68**. (Not corrected for vascular space).

1431 **Table S10B. Accumulation of [^{14}C]sucrose (B) after 30 minutes perfusion with or without pluronic F68.** (Not corrected for vascular space).

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