Involvement of mitogen activated kinase kinase 7 intracellular signalling pathway in Sunitinib-induced cardiotoxicity

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1   **Involvement of mitogen activated kinase kinase 7 intracellular signalling pathway in**

2   **Sunitinib-induced cardiotoxicity**

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21

22   **Abbreviations:**

23   ASK1, apoptosis signal-regulating kinase 1; ASK2, apoptosis signal-regulating kinase 2;

24   DMSO, dimethyl sulphoxide; hERG, human ether-a-go-go-related gene; JNK, c-Jun N-

25   terminal kinase; MKK7, mitogen activated kinase kinase 7; MTT, 3-(4,5-dimethylthiazol-2-yl)-

26   2,5-diphenyltetrazolium bromide; NQDI-1, 2,7-dihydro-2,7-dioxo-3*H*-naphtho[1,2,3-

27   *de*]quinoline-1-carboxylic acid ethyl ester; TTC, 2,3,5-Triphenyl-2H-tetrazolium chloride.

28 **Abstract**

29 The tyrosine kinase inhibitor Sunitinib is used to treat cancer and is linked to severe adverse

30 cardiovascular events. Mitogen activated kinase kinase 7 (MKK7) is involved in the

31 development of cardiac injury and is a component of the c-Jun N-terminal kinase (JNK)

32 signal transduction pathway. Apoptosis signal-regulating kinase 1 (ASK1) is the upstream

33 activator of MKK7 and is specifically inhibited by 2,7-dihydro-2,7-dioxo-3*H*-naphtho[1,2,3-

34 *de*]quinoline-1-carboxylic acid ethyl ester (NQDI-1). This study investigates the role of ASK1,

35 MKK7 and JNK during Sunitinib-induced cardiotoxicity.

36

37 Infarct size were measured in isolated male Sprague-Dawley rat Langendorff perfused

38 hearts treated for 125 min with Sunitinib in the presence and absence of NQDI-1. Left

39 ventricular cardiac tissue samples were analysed by qRT-PCR for MKK7 mRNA expression

40 and cardiotoxicity associated microRNAs (miR-1, miR-27a, miR-133a and miR-133b) or

41 Western blot analysis to measure ASK1/MKK7/JNK phosphorylation.

42

43 Administration of Sunitinib (1 µM) during Langendorff perfusion resulted in increased infarct

44 size, increased miR-133a expression, and decreased phosphorylation of the

45 ASK1/MKK7/JNK pathway compared to control. Co-administration of NQDI-1 (2.5 µM)

46 attenuated the increased Sunitinib-induced infarct size, reversed miR-133a expression and

47 restored phosphorylated levels of ASK1/MKK7/JNK. These findings suggest that the

48 ASK1/MKK7/JNK intracellular signalling pathway is important in Sunitinib-induced

49 cardiotoxicity. The anti-cancer properties of Sunitinib were also assessed using the 3-(4,5-

50 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Sunitinib

51 significantly decreased the cell viability of human acute myeloid leukemia 60 cell line (HL60).

52 The combination of Sunitinib (1 nM - 10 µM) with NQDI-1 (2.5 µM) enhanced the cancer-

53 fighting properties of Sunitinib. Investigations into the ASK1/MKK7/JNK transduction

54 pathway could lead to development of cardioprotective adjunct therapy, which could prevent

55 Sunitinib-induced cardiac injury.

1. Introduction

The tyrosine kinase inhibitor Sunitinib is used in the treatment of renal cell carcinoma and in gastrointestinal stromal tumours (Faivre et al. 2007). Sunitinib prevents tumour cell survival and angiogenesis by inhibiting a variety of growth factor and cytokine receptors, including platelet derived growth factor receptors, vascular endothelial growth factor receptors and proto-oncogenes c-Kit and RET. However, Sunitinib unfortunately is also associated with a lack of kinase selectivity resulting in the cardiotoxic adverse effects (Force et al. 2007). In the clinic, Sunitinib causes QT prolongation (Bello et al. 2009),left ventricular dysfunction (Shah and Morganroth 2015) and heart failure (Ewer et al. 2014). These findings are consistent with many other successful chemotherapy agents linked with severe drug-induced cardiotoxicity (Hahn et al. 2014), including electrophysiological changes and left ventricular dysfunction which can cause heart failure in some patients (Aggarwal et al. 2013). Intracellular studies using animals have revealed that Sunitinib causes mitochondrial injury and cardiomyocyte apoptosis through an increase in caspase-9 and cytochrome C release in both mice and in cultured rat cardiomyocytes (Chu et al. 2007). Other indicators of apoptosis, such as an increase in caspase-3/7, have also been detected after Sunitinib treatment in rat myocytes (Hasinoff et al. 2008).

83

84 MKK7 is a member of the mitogen-activated protein kinase kinase super family, which allows

85 the cell to respond to exogenous and endogenous stimuli (Foltz et al. 1998), and furthermore

86 MKK7 has shown to demonstrate a key role in protecting the heart from hypertrophic

87 remodelling, which occurs via cardiomyocyte apoptosis and heart failure (Liu et al. 2011).

88 The MKK7 activation of JNK results in many cellular processes including: proliferation,

89 differentiation and apoptosis (Chang and Karin 2001; Schramek et al. 2011; Sundarrajan et

90 al. 2003), and JNK signalling is vital for the maintenance and organisation of the

91 cytoskeleton and sarcomere structure in cardiomyocytes (Windak et al. 2013). Interestingly,

92 Sunitinib is an ATP analogue and competitively inhibits the ATP binding domain of its target

93 proteins (Roskoski 2007; Shukla et al. 2009). MKK7 also contains a highly conserved ATP

94 binding domain (Song et al. 2013). It is possible that Sunitinib binds as a ligand in the MKK7

95 ATP binding pocket, and thereby Sunitinib inhibits the MKK7/JNK transduction pathway, and

96 as a result this could potentially cause myocardial injury. It is important to determine the

97 relationship between MKK7 expression and Sunitinib induced cardiotoxicity by measuring

98 the alteration of MKK7 mRNA and phosphorylated MKK7 levels in the presence of Sunitinib.

99 Unravelling the relationship between Sunitinib and MKK7 could lead to a greater

100 understanding of its off-target mechanism of action and lead to the improvement in the

101 development of future drug discovery programmes or novel cardioprotective adjunct

102 therapies.

103

104 Short non-coding RNA microRNAs carry out the negative regulation of mRNA transcripts by

105 repressing translation (Bagga et al. 2005). Specific microRNAs expression patterns have

106 been linked to cardiomyocyte differentiation and in response to stress (Babiarz et al. 2012)

107 and have also been shown to be differentially expressed during the development of heart

108 failure (Thum et al. 2007). The microRNAs miR-1, miR-27a, miR-133a and miR-133b

109 produce differential expression patterns during the progression of heart failure (Akat et al.

110 2014; Tijsen et al. 2012). It is important to identify microRNA expression profiles in response

111 to drug-induced cardiotoxicity as similar patterns in microRNA expression to those identified

112 during heart failure may indicate the early onset of cardiotoxicity at a molecular level.

113

114 As MKK7 has no direct inhibitor, we have chosen to look at the upstream kinase ASK1

115 linked to MKK7 activation (Ichijo et al. 1997). ASK1 is activated in response to oxidative

116 stress-induced cardiac vascular endothelial growth factor suppression in the heart (Nako et

117 al. 2012). Izumiya et al. 2003 used ASK1 deficient transgenic mice to assess the role of

118 ASK1 in angiotensin II induced hypertension and cardiac hypertrophy. Both the wild type and

119 ASK1 deficient mice developed hypertension when stimulated with angiotensin II, however,

120 the ASK1 deficient mice lacked cardiac hypertrophy and remodelling and activation of ASK1,

121 p38 and JNK was severely attenuated, thus emphasising the importance of ASK1 in cardiac

122 hypertrophy and remodelling signalling (Izumiya et al. 2003). ASK1 is selectively inhibited by

123 NQDI-1 with high specificity with a K_i of 500nM and IC_{50} of 3µM (Volynets et al. 2011),

124 however, as this is a relatively new drug, a complete pharmacological profile has not yet

125 been fully characterised. ASK1 inhibition has previously been shown to offer protection

126 against ischemia reperfusion injury (Toldo et al. 2012) and has also been shown to suppress

127 the progression of ventricular remodelling and fibrosis in hamsters expressing severe

128 cardiomyopathy phenotypes (Hikoso et al. 2007). These findings highlight the potential of

129 NQDI-1 as a valuable asset to inhibit cardiac injury via the ASK1/MKK7/JNK pathway.

130

131 This novel study investigated the involvement of the ASK1/MKK7/JNK pathway in the

132 Sunitinib-induced cardiotoxicity via the assessment of cardiac function and infarct in

133 conjunction with relevant intracellular signalling mediators. Furthermore, we assessed the

134 anti-cancer properties of Sunitinib and determined whether co-administration of Sunitinib

135 with NQDI-1 affected the anti-cancer/apoptotic effect of Sunitinib in HL60 cells.

136

5

2. Materials and Methods

2.1. Main reagents and kits

Sunitinib malate and NQDI-1 were purchased from Sigma Aldrich (UK). Both drugs were dissolved in dimethyl sulphoxide (DMSO) and stored at -20 °C. Krebs perfusate salts were from either VWR International (UK) or Fisher Scientific (UK). Total ASK1 (Catalogue no ab131506) was purchased from Abcam (UK). Phospho-ASK1 (Thr 845) (Catalogue no 3765S), Phospho-MKK7 (Ser271/Thr275) (Catalogue no 4171S), Total MKK7 (Catalogue no 4172S), Phospho-SAPK/JNK (Thr183/Tyr185) (Catalogue no 9251), Total SAPK/JNK rabbit mAb antibody (Catalogue no 9252), anti-rabbit IgG, HRP-linked antibody and anti-biotin, HRP-linked antibody were purchased from Cell signalling technologies (UK). All the primary antibodies were from a rabbit host, and MKK7 and JNK were monoclonal antibodies, whereas ASK1 was polyclonal (all antibodies were validated by the manufacturers). The Ambion MicroPoly(A)Puris kit, Ambion mirVana miRNA Isolation Kit and Reverse Transcription Kit were from Life Technologies (USA). The mRNA primers and the Applied Biosystems primers assays (U6, rno-miR-1, hsa-miR-27a, hsa-miR-133a, and hsa-miR-133b) were purchased from Invitrogen (UK). The iTaq Universal SYBR Green Supermix was purchased from BioRad (UK). The HL60 cell line were obtained from European Collection of Cell Culture (UK) (catalogue no. 98070106).

2.2. Animals

Adult male Sprague-Dawley rats (300-350 g in body weight); were purchased from Charles River UK Ltd (UK) and housed suitably. They received humane care and had free access to standard diet according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Animals were selected at random for all treatment groups and the collected tissue was blinded for infarct size assessment. The experiments were performed following approval of the protocol by the Coventry University Ethics Committee. All efforts were made to minimise animal suffering and to reduce the number of animals used in the experiments. Rats were sacrificed

165 by cervical dislocation (Schedule 1 Home Office procedure). A total of 80 animals were used

166 for this study and the data from 68 rats were included, while data from 12 rats were excluded

167 from analysis due to the established haemodynamic exclusion criteria. A total of 16 animals

168 were included for Langendorff perfusion experiments per main groups (Control, Sunitinib,

169 Sunitinib+NQDI-1, and NQDI-1, where 6 of the animals were used for measurement of the

170 area of infarct and the area of risk and the left ventricular tissue from another 10 animals

171 was used for real time PCR and Western blot analysis). Furthermore, an additional 4

172 animals were used for Langendorff perfusion experiments with Sorbitol as a positive control

173 for p-MKK7 Western blot analysis. No animals were culled due to ill health.

174

2.3. Langendorff perfusion model

176 The hearts were rapidly excised after the rats were culled and placed into ice-cold Krebs

177 Henseleit buffer (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM

178 KH_2PO_4, 1.7 mM $CaCl_2$, and 12 mM glucose, pH7.4). The hearts were mounted onto the

179 Langendorff system and retrogradely perfused with Krebs Henseleit buffer. The pH of the

180 Krebs Henseleit buffer was maintained at 7.4 by gassing continuously with 95 % O_2 and 5 %

181 CO_2 and maintained at 37 ± 0.5 °C using a water-jacketed organ chamber. Each

182 Langendorff experiment was carried out for 145 minutes: a 20 minute stabilisation period

183 and 125 minutes of drug or vehicle perfusion in normoxic conditions. Hearts were included in

184 the study with a CF between 3.5-12.0 ml/g (weight of the rat heart) during the stabilisation

185 period. Sunitinib malate (1 µM) was administered throughout the perfusion period in the

186 presence or absence of NQDI-1 (2.5 µM).

187

188 The clinically relevant dose of 1 µM Sunitinib was chosen in line with previous studies by

189 (Henderson et al. 2013). Additionally, it has been reported that the plasma concentration of

190 Sunitinib has a C_{max} in the rage of 0.5–1.4µM (Doherty et al. 2013). While, the dose of 2.5

191 µM NQDI-1 was chosen following a thorough literature review (Eaton et al. 2014; Song et al.

192 2015; Volynets et al. 2011). NQDI-1 is not yet used in the clinic, therefore a clinically relevant

193 dose has not been reported.

194

195 Langendorff perfused hearts treated with vehicle were analysed as the control group. The

196 hearts were then weighed and either stored at -20 °C for 2,3,5-triphenyl-2H-tetrazolium

197 chloride (TTC) staining or the left ventricular tissue was dissected free and immersed in

198 RNAlater from Ambion (USA) for qRT-PCR or snap frozen by liquid nitrogen for Western blot

199 analysis.

200

201 **2.4. Infarct size analysis**

202 Frozen whole hearts were sliced into approximately 2 mm thick transverse sections and

203 incubated in 0.1 % TTC solution in phosphate buffer (2 ml of 100 mM $NaH_2PO_4.2H_2O$ and 8

204 ml of 100 mM NaH_2PO_4) at 37 °C for 15 minutes and fixed in 10% formaldehyde (Fisher

205 Scientific, UK) for 4 hours. The risk zone and infarct areas were traced onto acetate sheets.

206 The tissue at risk stained red and infarct tissue appeared pale. The acetate sheet was

207 scanned and ImageTool from UTHSCSA (USA) software was used to measure the area of

208 infarct and the area of risk. A ratio of infarct to risk size was calculated as a percentage for

209 each slice. An average was taken of all of the slices from each heart to give the percentage

210 infarct size of the whole heart. The mean of infarct to risk ratio for each treatment group and

211 the mean ± SEM was plotted as a bar chart. The infarct size determination was randomised

212 and blinded.

213

214 **2.5. Analysis of microRNA expression profiles**

215 The microRNA was isolated from left ventricular tissue using the *mir*Vana™ miRNA Isolation

216 Kit from Ambion (UK). The microRNA quantity and quality was measured by NanoDrop from

217 Nanoid Technology (USA). A total of 500 ng microRNA was reverse transcribed into cDNA

218 using primers specific for housekeeping reference RNA U6 snRNA and target microRNAs:

219 hsa-miR-155, hsa-miR-15a, hsa-miR-16-1, rno-miR-1, hsa-miR-27a, hsa-miR-133a or hsa-

8

220 miR-133b (please note all human hsa-miR assays are compatible with rat samples) from

221 Applied Biosystems (USA) using the MicroRNA Reverse Transcription Kit from Applied

222 Biosystems (USA) according to the manufacturer's instructions. The reverse transcription

223 quantitative PCR reaction was performed with the following setup: 16 °C for 30 min, 42°C for

224 30 min and 85 °C for 5 min and ∞ at 4°C. The qRT-PCR was performed using the TaqMan

225 Universal PCR Master Mix II (no UNG) from Applied Biosystems (USA) protocol on the 7500

226 HT Real Time PCR sequence detection system from Applied Biosystems (USA). A 20µl

227 reaction mixture containing 100 ng cDNA, specific primer assays mentioned above from

228 Applied Biosystems (USA) and the TaqMan Universal PCR Master Mix was used in the qRT-

229 PCR reaction in triplicates. A non-template control was included in all experiments. The real

230 time PCR reaction was performed using the program: 1) 2 minutes at 50°C, 2) 10 minutes at

231 95°C, 3) 15 seconds at 95°C, 4) 1 minute at 60°C. Steps 3) and 4) were repeated 40 times.

232

233 Analysis of qRT-PCR data of microRNAs were performed using the Ct values for U6 snRNA

234 as reference for the comparison of the relative amount of microRNAs (rno-miR-1, hsa-miR-

235 27a, hsa-miR-133a and hsa-miR-133b). The values of each of the microRNAs were

236 calculated to compare their ratios. The formula used was $X_0/R_0=2^{CTR-CTX}$, where X_0 is the

237 original amount of target microRNA, R_0 is the original amount of U6 snRNA, CTR is the CT

238 value for U6 snRNA, and CTX is the CT value for the target microRNAs (rno-miR-1, hsa-

239 miR-27a, hsa-miR-133a and hsa-miR-133b) (Sandhu et al. 2010). Averages of the Ct values

240 for each sample group (Control and Sunitinib treated hearts) and each individual primer set

241 were calculated, and bar charts were plotted with mean ± SEM. The mean of the control

242 group was set as 1 for all microRNAs.

243

244 **2.6. Measurement of MKK7 mRNA expression**

245 Total mRNA was extracted from left ventricular tissue using The Ambion MicroPoly(A)Purist

246 kit from Ambion (USA). Extracted mRNA was processed directly to cDNA by reverse

247 transcription using Reverse Transcription Kit from Applied Biosystems (USA) with the

9

248 respective primers for MKK7 (MKK7 forward primer: CCCCGTAAAATCACAAAGAAAATCC

249 and MKK7 reverse primer: GGCGGACACACACTCATAAAACAGA) and GAPDH (GAPDH

250 Forward primer: GAACGGGAAGCTCACTGG and GAPDH Reverse primer:

251 GCCTGCTTCACCACCTTCT) according to the instructions from the manufacturer Invitrogen

252 (UK). The reverse transcription PCR reaction was performed with the following setup: 16 °C

253 for 30 minutes, 42°C for 30 minutes and 85 °C for 5 minutes. The qRT-PCR reactions were

254 performed with the iTaq Universal SYBR Green Supermix from BioRad (UK), GAPDH and

255 MKK7 mRNA primer sets on the 7500 HT Real Time PCR machine from Applied Biosystems

256 (USA) using the program: 1) 2 minutes at 50°C, 2) 10 minutes at 95°C, 3) 15 seconds at

257 95°C, 4) 1 minute at 60°C. Steps 3) and 4) were repeated 40 times.

258

259 Analysis of qRT-PCR data of MKK7 mRNA were performed using the Ct values for GAPDH

260 mRNA as reference for the comparison of the relative amount of MKK7 mRNA. The value of

261 mRNA was calculated to compare the ratios using the formula $X_0/R_0 = 2^{CTR-CTX}$, where X_0 is

262 the original amount of target mRNA, R_0 is the original amount of GAPDH mRNA, CTR is the

263 CT value for GAPDH mRNA, and CTX is the CT value for the MKK7 mRNA (Sandhu et al.

264 2010). Averages of the Ct values for each sample group (control and Sunitinib treated

265 hearts) and each individual primer set were calculated, and bar charts were plotted with

266 mean ± SEM. The mean of the control group was set as 1 for the MKK7 mRNA.

267

268 **2.7. Western blot detection of ASK1, MKK7 and JNK**

269 A total 45-55 mg of the frozen left ventricular tissue was lysed in lysis buffer (NaCl 0.1 M,

270 Tris base 10 µM, EDTA 1 mM, sodium pyrophosphate 2 mM, NaF 2 mM, β-glycaophosphate

271 2 mM, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (0.1 mg/ml, 1/1.5 of protease

272 cocktail tablet) using a IKA Overtechnical T25homogeniser at 11,000 RPM. The

273 supernatants were measured for protein content using NanoDrop from Nanoid Technology

274 (USA). Then 80 µg of protein was loaded to 4–15 % Mini-Protean TGX Gel from BioRad

275 (UK) and separated at 200 V for 60 minutes. After separation, the proteins were transferred

10

276 to the Bond-P polyvinylidene difluoride membrane from BioRad (UK) by using the Trans-Blot

277 Turbo transfer system from BioRad (UK) and probed for the phosphorylated forms

278 Phospho(Thr^{845})-ASK1 (p-ASK1), Phospho(Ser^{271}/Thr^{275})-MKK7 (p-MKK7) and

279 Phospho(Thr^{183}/Tyr^{185}) -SAPK/JNK (p-JNK), and total forms of ASK1(Thr^{845}),

280 MKK7(Ser^{271}/Thr^{275}) and JNK(Thr^{183}/Tyr^{185}). The p-MKK7 and p-JNK blots were stripped by

281 boiling and the PVDF membrane was used for total MKK7 and total JNK analysis,

282 respectively. According to recommendations from Cell signalling technologies (UK) total

283 ASK1 analysis had to be performed on a separate Western blot, as the stripping procedure

284 would remove total ASK1 protein. The relative changes in the p-ASK1, p-MKK7 and p-JNK

285 protein levels were measured and corrected for differences in protein loading as established

286 by probing for total ASK1, MKK7 and JNK respectively.

287

288 For Western blot analysis phosphorylated antibody levels were normalised to total antibody

289 levels in order to correlate for unequal loading of protein and differential blot transfer and to

290 identify the level of active vs inactive protein levels. Results were expressed as a percentage

291 of the density of phosphorylated protein relative to the density of total protein using Image

292 Lab 4.1 from BioRad (UK). The phosphorylated antibody levels determination was

293 randomised and blinded.

294

295 **2.8. MTT assay assessment of HL60 cell viability in the presence of Sunitinib with and**

296 **without NQDI-1**

297 The HL60 cell line were maintained in in RPMI 1640 medium supplemented with L-

298 Glutamine (2 mM) and 10 % heat-inactivated fetal bovine serum and antibiotics mix at 37 °C

299 in a humidified incubator under 5 % CO_2/95 % air. Cells were split in a 1:5 ratio every 2-3

300 days. Cells were incubated with Control, increasing concentrations of Sunitinib (1nM – 10

301 µM), Sunitinib (0.1 – 10 µM) + NQDI-1 (2.5 µM), or increasing concentrations of NQDI-1

302 (0.2-200 µM) for 24 h. Both Sunitinib and NQDI-1 were dissolved in DMSO. The DMSO

303 concentration was < 0.05 % (v/v) during the *in vitro* studies.

11

305 Cells were plated at a cell density of 10^5 cells/ml in 96-well plates and the above indicated

306 concentration of the drug was added. The plate was then incubated at 37°C for 24hrs. After

307 drug incubation, 50 µl of MTT solution (5 mg MTT/ml H_2O) was added and the cells were

308 incubated for a further 24 h. Next, 50 µl of DMSO was added to each well and mixed by

309 pipetting to release reduced MTT crystals from the cells. Relative cell viability was obtained

310 by scanning with an ELISA reader (Anthos Labtech AR 2001 Multiplate Reader, *Anthos*

311 *Labtec* Instruments, Austria) with a 490 nm filter. Results were expressed as a percentage of

312 viable cells relative to untreated cells/control. Experiments were performed in triplicates and

313 repeated ≥ 3 times.

2.9. Statistical analysis

316 Results are presented as mean ± standard error of the mean (SEM). Significance of all data

317 sets was measured by one-way ANOVA analysis with the Tukey post hoc test using the

318 Matlab prism program. The following groups were compared during ANOVA analysis:

319 Control versus Sunitinib, Control versus Sunitinib and NQDI-1, Control versus NQDI-1 (all

320 statistically significant data compared to control marked with *), and Sunitinib versus

321 Sunitinib and NQDI-1 (all statistically significant data compared to Sunitinib marked with #).

322 For MKK7 mRNA some data was evaluated by using student's t-test. P-values <0.05 were

323 considered statistically significant.

3. Results

3.1. Sunitinib treatment induces cardiac injury

327 The effect of Sunitinib (1µM) administration on myocardial infarction development was

328 investigated by TTC staining. The hearts were stabilised for a period of 20 minutes, followed

329 by 125 minutes of drug perfusion. Administration of Sunitinib (1µM) for 125 minutes resulted

330 in a significant increase in infarct size compared with non-treated controls (Control: 7.81 ±

331 1.16 %; Sunitinib: 41.02 ± 1.23 %, p<0.001) (Fig 1). This demonstrated that Sunitinib

12

332 treatment of the Langendorff perfused hearts results in a drastic increase in cardiac injury.

333 The infarct was globally distributed in all groups investigated in this study (i.e. Control,

334 Sunitinib ± NQDI-1, and NQDI-1).

335

3.2. Sunitinib and NQDI-1 co-treatment alleviate cardiac injury

337 The effect of ASK1 inhibition by NQDI-1 on cardiac function and infarction was investigated.

338 Co-administration of Sunitinib (1 µM) with NQDI-1 (2.5 µM) significantly decreased infarct

339 size compared to Sunitinib treated hearts (Sunitinib: 41.02 ± 1.23 %; Sunitinib + NQDI-1:

340 17.54 ± 2.97 %, $p<0.001$). However, administration of NQDI-1 alone for 125 minutes of

341 perfusion significantly increased infarct size compared with control hearts (Control: 7.81 ±

342 1.16 %; NQDI-1: 16.68 ± 2.66 %, $p<0.05$) (Fig 1).

343

3.3. Sunitinib treatment modulates expression of microRNAs involved in cardiac injury

346 The expression of cardiac injury specific microRNAs during Sunitinib-induced cardiotoxicity

347 was determined by qRT-PCR assessment. The microRNAs miR-1, miR-27a, miR-133a and

348 miR-133b have been shown to produce differential expression patterns during the

349 progression of heart failure. The ratio of target microRNA normalised to U6 was set to 1 in

350 the control group for easier comparison of microRNA ratio values between the various drug

351 therapy groups. There was a significant increase in miR-133a when hearts were perfused

352 with Sunitinib (1 µM) compared to control hearts (Ratio of target microRNA normalised to U6

353 in Sunitinib treated hearts: miR-133a: 535.78 ± 61.27, $p<0.001$). Co-administration of NQDI-

354 1 (2.5 µM) along with Sunitinib reversed this miR-133a expression trend by decreasing the

355 miR-133a expression when compared to Sunitinib perfused hearts (Ratio of target microRNA

356 normalised to U6 in Sunitinib and NQDI-1 treated hearts: miR-133a: 52.76 ± 28.30,

357 $p<0.001$). Hearts perfused with NQDI-1 alone showed an increase in miR-1, miR-27a and

358 miR-133b expression compared to control hearts (Ratio of target microRNA normalised to

359 U6 in NQDI-1 treated hearts: miR-1: 32.33 ± 16.47, $p<0.01$; miR-27a: 11.27 ± 2.86, $p<0.001$;

13

360　miR-133b: 167.85 ± 58.13, p<0.001). The expression of miR-1, miR-27a, miR-133a and

361　miR-133b was increased in the Sunitinib and NQDI-1 co-treated hearts when compared to

362　Sunitinib perfused hearts (miR-1: p<0.05; miR-27a: p<0.001; miR-133a: p<0.001; miR-133b:

363　p<0.05). (Fig 2 A-D).

364

365　The results from the microRNA qRT-PCR analysis show there is a similar expression pattern

366　for miR-1, miR-27 and miR-133b, while miR-133a has its own pattern. This indicates that the

367　cardiac injury induced by Sunitinib, which is alleviated by the ASK1 inhibitor NQDI-1, triggers

368　a complex alteration of these cardiac injury microRNAs. Further studies looking at the

369　altered expression profiles for these cardiac injury microRNAs have to be undertaken in

370　order to clarify the expression patterns.

371

372　3.4. MKK7 mRNA expression profile is altered by ASK1 inhibitor NQDI-1

373　As MKK7 contains an ATP binding domain (Song et al. 2013) and Sunitinib is an ATP

374　analogue and competitively inhibits the ATP binding domain of its target proteins (Roskoski

375　2007; Shukla et al. 2009). We therefore wanted to investigate the interaction between

376　Sunitinib and MKK7, and question whether Sunitinib could bind as a ligand in the ATP

377　binding pocket of MKK7. This would determine if Sunitinib might potentially have an

378　inhibitory effect on the MKK7/JNK pathway. The relationship between MKK7 expression and

379　Sunitinib-induced cardiotoxicity was assessing on transcriptional level by MKK7 mRNA qRT-

380　PCR analysis on Sunitinib (1 µM) perfused hearts, and the interaction by ASK1 specific

381　inhibitor NQDI-1 was detected to highlight if any interaction between ASK1/MKK7 and

382　Sunitinib-induced alteration of MKK7 transcription due to cardiac injury was observed to

383　impact on mRNA levels. The ratio of MKK7 mRNA normalised to GAPDH was set to 1 in the

384　control group for easier comparison of GAPDH normalised MKK7 mRNA values between the

385　various drug therapy groups. The qRT-PCR analysis of MKK7 mRNA revealed that co-

386　administration of NQDI-1with Sunitinib caused a significant increase in MKK7 mRNA

387　expression compared to Sunitinib treatment alone (p<0.01) (Ratio of MKK7 mRNA

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388 normalised to GAPDH. Sunitinib: 0.12 ± 0.03; Sunitinib + NQDI-1: 1.18 ± 0.65) (Fig 3). The

389 decrease in MKK7 mRNA observed in the Sunitinib (1 μM) perfused hearts compared to

390 control hearts was not significant, but a clear trend was observed. If the data from groups

391 control and Sunitinib were compared using a Student's t-test the decline in MKK7 mRNA in

392 Sunitinib treated hearts was statistically significant with p=0.0043.

393

394 These MKK7 mRNA qRT-PCR results clearly demonstrate that Sunitinib treatment shows a

395 tendency to decrease the MKK7 mRNA, and co-administration of ASK1 specific inhibitor

396 NQDI-1 restores the MKK7 mRNA level observed in control treated heart. This could indicate

397 a complex regulation system where Sunitinib-induced cardiac injury in directly linked with the

398 ability of Sunitinib to reduced MKK7 expression at transcriptional level, which is counteracted

399 by the ASK1 specific inhibitor NQDI-1.

400

3.5. ASK1/MKK7/JNK pathway is involved in Sunitinib-induced cardiotoxicity

402 As explained in the previous MKK7 mRNA results section we wanted to investigate the

403 interaction between Sunitinib and MKK7, as they both interact via the ATP binding pocket.

404 The key question being if Sunitinib can bind as a ligand in the ATP binding pocket of MKK7

405 and if Sunitinib is able to block the MKK7/JNK pathway. Here we investigate the role of

406 cardiotoxic Sunitinib therapy on the ASK1/MKK7/JNK pathway phosphorylation and how the

407 interaction with the cardioprotective ASK1 specific agent NQDI-1 effects the

408 ASK1/MKK7/JNK pathway phosphorylation. Following Langendorff perfusion of Sunitinib and

409 NQDI-1, p-ASK1, p-MKK7 and p-JNK levels were measured in the left ventricular tissue of

410 the hearts by Western blot analysis.

411

412 Western blot analysis showed that Sunitinib treatment decreased p-ASK1, p-MKK7 and p-

413 JNK levels significantly when compared to control (density of phosphorylated protein

414 normalised to total protein: *p-ASK1*: Control: 389.43 ± 4.18 and Sunitinib: 16.47 ± 3.56,

415 p<0.001; *p-MKK7*: Control: 55.60 ± 4.86 and Sunitinib: 23.66 ± 4.53, p<0.001; *p-JNK*:

15

416 Control: 43.66 ± 2.82 and Sunitinib: 22.52 ± 2.74, p<0.001). Co-administration with NQDI-1

417 increased the p-ASK1, p-MKK7 and p-JNK levels, and these were statistically significantly

418 elevated when compared to heart treated with Sunitinib monotherapy (density of

419 phosphorylated protein normalised to total protein: *p-ASK1*: Sunitinib + NQDI-1: 51.17 ±

420 3.66, p<0.001; *p-MKK7*: Sunitinib + NQDI-1: 38.11 ± 1.87, p<0.05; *p-JNK*: 48.95 ± 2.76,

421 p<0.001). The p-ASK1, p-MKK7 and p-JNK levels were decreased in NQDI-1 treated hearts

422 compared to control hearts (density of phosphorylated protein normalised to total protein: *p-*

423 *ASK1*: NQDI-1: 20.56 ±1.99, p<0.01; *p-MKK7*: NQDI-1: 18.40 ± 2.98, p<0.001; *p-JNK:* 28.78

424 ± 3.03, p<0.01) (Fig 4A-C).

425

426 These Western blot results show that Sunitinib decreased the phosphorylation of the

427 ASK1/MKK7/JNK pathway. The fact that Sunitinib was able to show a strong tendency of

428 decreasing the MKK7 mRNA highlights that the decreased MKK7 phosphorylation is

429 regulated at the transcriptional level, which is then affecting the post-transcriptional MKK7

430 phosphorylation levels. However, interestingly the cardiotoxic Sunitinib is having an inhibiting

431 effect throughout all three parts of the ASK1/MKK7/JNK pathway. Co-administration of

432 NQDI-1 is counteracting the Sunitinib inhibiting effect on the phosphorylation level

433 throughout the ASK1/MKK7/JNK pathway. These results show a clear indication of Sunitinib

434 interacting with ASK1, MKK7 and JNK at post-translational level and MKK7 gene expression

435 at pre-transcriptional level.

436

3.6. Cancer cell viability in response to Sunitinib with and without NQDI-1

438 The effect of Sunitinib on cell viability was examined in HL60 cells. The HL60 cells were

439 treated with Sunitinib for 24 hrs and then the level of mitochondrial metabolic-activity

440 inhibition was measured with the MTT assay. Sunitinib showed a pronounced decrease in

441 metabolic activity and a dose-dependent decrease in cell viability (Fig 5A). In particular,

442 Sunitinib significantly reduces cell viability at 1 nM (82.62 ± 6.55 %, p<0.05), 0.1 µM (85.94 ±

443 3.80 %, p<0.05), 0.5 µM (83.94 ± 3.86 %, p<0.05), 1 µM (77.28 ± 6.58 %, p<0.05), 5 µM

16

444 (58.61 ± 4.44 % p<0.001) and 10 µM (47.14 ± 6.77 %, p<0.001) concentrations of Sunitinib.

445 The IC_{50} value was 6.16 µM. All the concentrations of Sunitinib in the absence and presence

446 of NQDI-1 used during this study produced significant reductions in HL60 cell viability

447 compared to vehicle treatment.

448

449 The co-administration of NQDI-1 (2.5 µM) to increasing concentrations of Sunitinib (1 nM -

450 10 µM) enhanced the inhibition of mitochondrial metabolism shown by Sunitinib (Fig 5A).

451 Specifically, co-treatment of Sunitinib with NQDI-1 reduced cell viability at 1 µM (59.58 ±

452 6.30 %, p<0.001), 5 µM (36.62 ± 6.52 %, p<0.001) and 10 µM (15.10 ± 2.31 %, p< 0.001)

453 concentrations of Sunitinib compared to Sunitinib alone. The IC_{50} value for Sunitinib plus

454 NQDI-1 was 1.76 µM.

455

456 Interestingly, increasing concentrations of NQDI-1 alone (0.2-200 µM) only significantly

457 reduced cell viability at very high concentrations. Reductions in cell viability were significant

458 at 100 µM (55.44 ± 12.39 %, p<0.05), 200 µM (33.15± 9.67 %, p<0.001) (Fig 5B). The IC_{50}

459 value for NQDI-1 produced by the MTT assay was 130.8 µM.

460

461 **4. Discussion**

462 **4.1. Involvement of ASK1/MKK7/JNK in Sunitinib-induced cardiotoxicity**

463 The occurrence heart failure associated with anti-cancer treatment has been investigated

464 extensively (Khakoo et al. 2008), however, the underlying mechanism of cardiotoxicity is still

465 unclear. Determining cellular pathways involved in Sunitinib-induced cardiotoxicity could help

466 to develop therapies which could prevent the potential development of heart failure

467 associated with Sunitinib treatment.

468

469 Data presented in this study confirms that Sunitinib causes drug-induced myocardial injury

470 via an increase in infarct size (Fig 1). We observed that infarct size increased from ~ 8 % in

471 Control hearts to ~ 41 % in hearts perfused with Sunitinib. This observation is in accordance

17

472 with our previous study, where we investigated the involvement of A3 adenosine receptor

473 activation during Sunitinib induced cardiotoxicity in perfused rat hearts (Sandhu et al. 2017).

474 Also, the injury induced by Sunitinib administration is very similar to ischemia/reperfusion

475 injury rats investigated by the same Langendorff model (Gharanei et al. 2013). The Control

476 rat hearts do suffer from minor infarct injury during the brief period it takes from sacrificing

477 the animal and perfusing the heart with the Krebs buffer during the Langendorff model. This

478 insult accounts for the ~ 8 % infarct size we observe in Control hearts. A dose of 1 μM

479 Sunitinib increased the infarct to ~ 41 % in perfused heart, and this steady state blood

480 concentration of Sunitinib has been found in patients treated with Sunitinib (Henderson et al.

481 2013). As the rat hearts are physically much smaller than human hearts, the rat hearts may

482 have been more sensitive to the adverse effect of Sunitinib administration at the clinically

483 relevant dose of 1 μM.

484

485 Other animal studies investigating Sunitinib-induced cardiotoxicity were linked to a significant

486 decrease in left ventricular function (Henderson et al. 2013; Mooney et al. 2015), and

487 interestingly left ventricular dysfunction has also been identified in patients undergoing

488 Sunitinib chemotherapy (Di Lorenzo et al. 2009). Also, Sunitinib has also been associated

489 with electrophysiological disturbances and an increased pro-arrhythmic relative risk of

490 developing QTc interval prolongation (Ghatalia et al. 2015; Schmidinger et al. 2008).

491 Several studies show that Sunitinib could potentially block human ether-a-go-go-related

492 gene (hERG) potassium channels and cause irregular contractions (Doherty et al. 2013; Guo

493 et al. 2013). The *in vitro* study by *Thijs et al.* 2015 investigated the effect of Sunitinib on the

494 contractile force measured during normal pacing or after simulated ischemia on isolated

495 human atrial trabeculae from patients awaiting coronary artery bypass graft and/or aorta

496 valve replacement. They showed that treatment with 81.3 nM Sunitinib did not attenuate the

497 recovery in contractile force of atrial cardiomyocytes after simulated ischemia and

498 reperfusion compared to vehicle treated atrial cardiomyocytes, and thus they concluded that

499 the development of heart failure in patients treated with Sunitinib could not be explained by

18

500 an acute cardiotoxic Sunitinib stimulation of cardiomyocytes. However, it should be noted

501 that they used a 12-times lower Sunitinib dose compared to what we have used in this study,

502 and thus they might have not used a dose of Sunitinib in the toxic range (Thijs et al. 2015).

503

504 Modulation of ASK1 and its downstream targets MKK7 and JNK have been shown to play

505 important roles in regulating cardiomyocyte survival, apoptosis, hypertrophic remodeling and

506 intracellular signalling associated with heart failure (Mitchell et al. 2006). Therefore, we

507 hypothesised that successful modulation of the ASK1/MKK7/JNK pathway would produce an

508 effective cardioprotective treatment against Sunitinib induced cardiotoxicity.

509

510 As we were interested in the ASK1/MKK7/JNK signalling pathway we targeted ASK1 with

511 NQDI-1. Administration of the ASK1 specific inhibitor NQDI-1 resulted in abrogation of the

512 some cardiotoxic effects of Sunitinib. This study therefore, demonstrates the potentially

513 pivotal role that this kinase and the related pathway could play in protecting the heart from

514 Sunitinib induced cardiotoxicity. These observations are in accordance with other studies

515 that have also implicated the involvement of ASK1 in cardioprotection in other mammalian

516 models (Boehm 2015; Hao et al. 2016; He et al. 2003). Furthermore, inhibition of ASK1 with

517 thioredoxin results in reduction of infarct size compared to ischemic/reperfusion control

518 hearts (Gerczuk et al. 2012; Huang et al. 2015; Zhang et al. 2007).

519

520 ASK1 signalling pathway is facilitated through two main routes: either (i) MKK4/7 and JNK,

521 or (ii) MKK3/6 and p38 (Ichijo et al. 1997). As mentioned in the introduction the study by

522 Izumiya *et al.* 2003 showed the importance of ASK1 during angiotensin II induced

523 hypertension and cardiac hypertrophy in mice, as ASK1 knockout mice failed to develop

524 cardiac hypertrophy and remodelling through both JNK and p38 (Izumiya et al. 2003). In

525 another study by Yamaguchi *et al.* 2003 knockout of ASK1 in mice was also linked to

526 cardioprotection through the JNK pathway, as coronary artery ligation or thoracic transverse

527 aortic constriction in ASK1 deficient hearts showed no morphological or histological defects.

19

528 Both left ventricular end-diastolic and end-systolic ventricular dimensions were increased

529 less then wild-type mice, and the decreases in fractional shortening in both experimental

530 models were less when compared with wild-type mice (Yamaguchi et al. 2003). However, in

531 a study by Taniike *et al.* 2008 when ASK1 knockout mice were subjected to mechanical

532 stress it resulted in exaggerated hearth growth and hypertrophy development through p38

533 pathway (Taniike et al. 2008). It is worth noting that attenuation of ASK1 by NQDI-1 during

534 normoxic conditions and Sunitinib treatment could have affected the MKK3/6 and p38

535 signalling pathway, which would have led to the conflicting results that we observe during

536 our study: there was a significant reduction in infarct size when Sunitinib was administered in

537 the presence of the ASK-1 inhibitor NQDI-1, however administration of NQDI-1 alone also

538 increased the infarct size. The increase in infarct size with NQDI-1 was not as profound as

539 the increase observed with Sunitinib (Fig 1).

540

541 NQDI-1 is a recently discovered highly specific ASK1 inhibitor. In the presence of 25 µM

542 NQDI-1 the residual activity of ASK1 is reduced to 12.5 % using the ɣ-32P-ATP *in vitro*

543 kinase assay model. By using the same assay model Volynetys and colleagues determined

544 the residual activity of the tyrosine protein kinase fibroblast growth factor receptor 1 (FGFR1)

545 which was measured to be 44 % after 25 µM NQDI-1 exposure (Volynets et al. 2011).

546 FGFR1 as a key component involved in *in vivo* cardiomyocyte proliferation during early

547 stage heart development (Mima et al. 1995). Furthermore, FGFR1 is an essential regulator

548 of coronary vascular development through Hedgehog signalling activation, which in the adult

549 heart leads to increased coronary vessel density (Lavine et al. 2006). It is therefore possible

550 that NQDI-1 is blocking FGFR1 directly in the treated hearts of our study and thereby

551 exerting a slight increase in infarct size compared to the control.

552

553 In addition, NQDI-1's specificity towards other kinases has yet to be determined. In particular

554 its homologue apoptosis signal-regulating kinase 2 (ASK2) may potentially be inhibited by

555 NQDI-1 (Hattori et al. 2009). Both ASK1 and ASK2 is expressed in the heart (Iriyama et al.

556 2009). ASK2 is only stable and active when it forms a heteromeric complex with ASK1.

557 ASK2 mediate its stress response from the stable ASK1-ASK2 heteromeric complex

558 platform through both JNK and p38 and induce apoptosis. Furthermore, ASK1 and AS2 are

559 able to activate each other, and thus ASK1 assists ASK2 with ASK2 regulatory mechanisms

560 in addition to stabilising and activating ASK2 (Takeda et al. 2007). It is therefore very likely

561 that NQDI-1 will affect the ASK2 mediated signalling. Several studies by the team of Kataoka

562 have revealed the involvement of ASK2 during hypertension, cardiac hypertrophy and

563 remodelling development. They have shown that ASK2 deficient mice have a significantly

564 higher blood pressure and increased left ventricular weight then wild type mice, with an

565 underlying analysis revealing that perivascular and interstitial myocardial fibrosis was

566 increased (Kataoka 2008, 2009, 2010, 2011). Therefore, the relatively small adverse effect

567 of NQDI-1 administration on producing an increased infarct size could also be caused by the

568 NQDI-1 effect through attenuated ASK2 signalling.

569

570 Further studies are required to unravel the underlying mechanisms associated with the

571 cardiac tissue injury and haemodynamic responses observed upon NQDI-1 stimuli to assess

572 if p38, FGFR1, and/or ASK2 are involved. Also, other concentration of NQDI-1 treatment of

573 hearts should be investigated, as lower concentrations of NQDI-1 most likely would cause

574 less cardiac adverse effects.

575

576 **4.2. Profiling of cardiotoxicity linked microRNAs**

577 MicroRNAs have been shown to have important roles in tissue formation and function in

578 response to injury and disease. The microRNAs miR-1, miR-27a, miR-133a and miR-133b

579 have been shown to produce differential expression patterns during the progression of heart

580 failure (Akat et al. 2014; Tijsen et al. 2012). Here we investigate the altered expression

581 profiles of microRNAs miR-1, miR-27a, miR-133a and miR-133b after Sunitinib-induced

582 cardiotoxicity with or without the ASK1 inhibitor NQDI-1 (Fig 2A-D). To the best of our

583 knowledge there are no other studies showing a significant altered expression of miR-1,

584 miR-27a, miR-133a and miR-133b after Sunitinib treatment. A reduction in hERG potassium

585 channels expression causes the delayed myocyte repolarisation attributed to a long QT

586 interval and interestingly the 3' untranslated region of hERG potassium channel transcripts

587 have a partial complimentary miR-133a target site (Xiao et al. 2007). The current study

588 shows an increase in miR-133a expression following Sunitinib treatment, which was

589 attenuated with NQDI-1 co-administration (Fig 2C). This could imply that miR-133a

590 overexpression inhibits the hERG potassium channel, which would have a negative impact

591 on the electrophysiological response (Xu et al. 2007). Over expression of miR-133a has also

592 been shown to have negative effects on cardiomyocyte proliferation and survival (Liu et al.

593 2008), which corroborates the results from the current study.

594

595 In the heart miR-1 and miR-133 maintain the heart beat rhythm by regulating the cardiac

596 conduction system (Kim 2013). Furthermore, miR-1 and miR-133 are downregulated during

597 cardiac hypertrophy in both mouse and human models. *In vitro* studies have shown that the

598 overexpression of miR-133 inhibits cardiac hypertrophy, whereas inhibition of miR-133

599 induces more pronounced hypertrophy (Care et al. 2007). In addition, a decreased in cardiac

600 expression of miR-133b is sufficient to induce hypertrophic gene expression (Sucharov et al.

601 2008). In support of these studies our analysis shows that the miR-133b expression is

602 increased during NQDI-1 mono-therapy and co-administration of Sunitinib and NQDI-1 (Fig

603 2C), thus protecting the heart against hypotrophy/cardiac damage development, which is the

604 same pattern as we observe in the infarct to risk analysis.

605

606 The miR-27a expression has been observed to downregulate FOXO-1 protein, a

607 transcription factor which regulates genes involved in the apoptotic response, cell cycle, and

608 cellular metabolism (Guttilla and White 2009). Moreover, miR-27a is downregulated in

609 coronary sinus samples of heart failure patients (Marques et al. 2016). The increase in miR-

610 27a expression in our study during NQDI-1 mono-therapy and co-administration of Sunitinib

22

611 and NQDI-1 (Fig 2B) support the findings from these studies as the link increased miR-27

612 expression to reduced apoptosis, which is what we observe in the infarct to risk analysis.

613

4.3. Sunitinib treatment supresses the ASK1/MKK7/JNK pathway

4.3.1. ASK1

616 Western blot assessment of Sunitinib treated hearts showed a significant decrease in p-

617 ASK1 when compared to control perfused hearts, and this decrease in p-ASK1 levels was

618 abrogated with NQDI-1 co-administration. However, in p-ASK1 levels from hearts subjected

619 to Sunitinib and NQDI-1 co-administration were compared to control hearts we did observe a

620 significant increase in p-ASK1. Furthermore, NQDI-1 treated hearts had significantly

621 decreased p-ASK1 levels when compared to control hearts (Fig 4A).

622

623 ASK1 has multiple phosphorylation sites. The Akt/protein kinas B complex binds to and

624 phosphorylates Ser^{83} of ASK1, resulting in the inhibition of ASK1-mediated apoptosis (Kim et

625 al. 2001), the 14-3-3 interacts with phosphorylated Ser^{967} of ASK1 to block the function of

626 ASK1 (Zhang et al. 1999), protein phosphatase 5 dephosphorylates Thr^{845} within the

627 activation loop of ASK1 and thereby inhibits ASK1-mediated apoptosis (Morita et al. 2001),

628 while Ser^{1034} phosphorylation suppresses ASK1 proapoptotic function (Fujii et al. 2004).

629 ASK1 undergoes auto-phosphorylation at the Thr^{845} (Tobiume et al. 2002). It is possible that

630 auto-phosphorylation increased when Sunitinib was combined with NQDI-1, which led to the

631 increased levels of p-ASK1 identified by western blot analysis. In its inactive form, ASK1 is

632 complexed with thioredoxin (Saitoh et al. 1998). It has been proposed that auto-

633 phosphorylation at Thr^{845} is increased in response to H_2O_2 treatment, due to H_2O_2 preventing

634 thioredoxin from complexing with ASK1. This suggests that ASK1 activation is due to

635 oxidative stress (Tobiume et al. 2002). An increase in p-ASK1 could indicate increased

636 levels of oxidative stress, which potentially reduced cardiac function and generated a level of

637 infarct when NQDI-1 was administered alone compared to control.

23

638

639   Attenuation of ASK1 by the specific inhibitor NQDI-1 produces a protective role in the heart

640 compared to Sunitinib treatment alone. In this study we have focused on the ASK1 Thr^{845}

641 phosphorylation site, however, in future studies it would be interesting to investigate the

642 ASK1 Ser^{967} phosphorylation, as phosphorylation at this site has been linked to

643 cytoprotection (Kim et al. 2009). Another interesting aspect would be to identify if NQDI-1 is

644 able to inhibit the ASK1 homologue ASK2 as indicated by Nomura *et al.* 2013 (Nomura et al.

645 2013) and establish the K_i and IC_{50} values are.

646

647 ASK1 is also a key mediator of apoptotic signalling (Hattori et al. 2009; Ichijo et al. 1997) and

648 the team of Huynh *et al.* 2011 investigated the expression of ASK1 in tumours lysates from

649 mice bearing 06-0606 tumours treated with 40 mg/kg/day Sunitinib for 11 days (Huynh et al.

650 2011). Their study showed a significant increase in total ASK1 levels after Sunitinib

651 treatment compared to vehicle treated 06-0606 tumour expressing mice, however, in our

652 study the expression of total ASK1 levels in left ventricular coronary tissue did not differ in

653 hearts perfused with Sunitinib compared to vehicle treated hearts.

654

655 **4.3.2. MKK7**

656 We assessed MKK7 expression level at both transcriptional and post-translational levels in

657 hearts treated with Sunitinib with and without the upstream ASK1 inhibitor NQDI-1. There

658 was a strong tendency for MKK7 mRNA expression to be decreased in Sunitinib perfused

659 hearts compared to control, but without significance. The expression of MKK7 mRNA was

660 increased significantly in Sunitinib and NQDI-1 co-treated hearts compared to Sunitinib

661 perfused hearts, and NQDI-1 solo treatment did not alter the MKK7 mRNA expression

662 compared to control hearts (Fig 3). The p-MKK7 level was significantly decreased in

663 Sunitinib treated hearts when compared to control, and the p-MKK7 decrease is attenuated

664 by NQDI-1 co-administration. The p-MKK7 levels are significantly decreased in Sunitinib and

24

665 NQDI-1 co-treatment and NQDI-1 solo-treatment hearts compared to control hearts (Fig 4B).

666 We believe that this is the first study to investigate the expression of MKK7 after Sunitinib

667 therapy.

668

669 MKK7 has a vital role in protecting the heart from hypertrophic remodelling and

670 cardiomyocytes apoptosis during stress and therefore the transition into heart failure

671 (Mitchell et al. 2006). Studies by Liu *et al.* 2011 revealed the importance of MKK7 by

672 demonstrating that deprivation of MKK7 in cardiomyocytes provokes heart failure in mice

673 when exposed to pressure overload (Liu et al. 2011)**.** In addition to this, it has been shown

674 that inhibition and specific knockout of MKK7 increases the sensitivity of hepatocytes to

675 tumour necrosis factor alpha-induced apoptosis (Jia et al. 2015). Treatment with Sunitinib in

676 rat hearts in the current study down regulates both mRNA and phosphorylated protein levels

677 of MKK7. These studies suggest an important role of MKK7 in the maintenance of heart

678 homeostasis and expression of associated genes are important during cardiac hypertrophy

679 and heart failure.

680

681 MKK7 contains an ATP binding domain which could be inhibited by the ATP analogue,

682 Sunitinib (Roskoski 2007; Shukla et al. 2009; Song et al. 2013). It is therefore possible that

683 Sunitinib has an inhibitory effect on the MKK7/JNK signal transduction pathway. With

684 reduced MKK7 activity demonstrating both an incline towards cardiomyocyte damage and a

685 reversal of anti-tumour effects of chemotherapy, it would be interesting to assess both the

686 changes in expression levels and levels of phosphorylated MKK7 during Sunitinib treatment

687 affecting the heart in future studies. It may therefore be possible to identify a link between

688 MKK7 expression and tyrosine kinase inhibitor-induced cardiotoxicity.

689

690 Co-treatment of Sunitinib and NQDI-1 increases the level of p-MKK7 to levels compared to

691 Sunitinib treated hearts, restoring them almost back to the p-MKK7 levels observed in

692 control hearts. This could suggest that phosphorylated MKK7 is required to maintain at a

25

693 stable level to prevent damage to the heart. To illustrate this upregulation of transforming

694 growth factor beta has been found in compensatory hypertrophy, myocardial remodelling

695 and heart failure (Rosenkranz 2004). However, if MKK7 is removed entirely from the JNK

696 pathway cardiomyocyte damage ensues (Liu et al. 2011). Tang *et al.* 2012 demonstrated

697 that by causing an upregulation of MKK7 in hepatoma cells with the treatment with Alpinetin

698 it was possible to arrest cells in the G_0/G_1 phase of the cell cycle. However, by inhibiting

699 MKK7 the anti-tumour effect of the delta-opioid receptor agonist cis-diammined

700 dichloridoplatinum was reversed (Tang et al. 2012). This suggests that by agonising MKK7 it

701 may be possible to both enhance anti-cancer properties of chemotherapy agents, as well as

702 limiting apoptosis as the cell cycle is arrested.

703

4.3.3. JNK

705 Administration of Sunitinib decreased the p-JNK levels significantly when compared to

706 control hearts, and this decrease was abrogated with NQDI-1 co-treatment. Treatment with

707 NQDI-1 however also decreased the p-MKK7 levels when compared to control perfused

708 hearts (Fig 4C).

709

710 Many JNK knock out models have been used to determine the role of JNK in the

711 development of cardiac dysfunction. Kaiser *et al.* 2005 demonstrated the importance of JNK

712 in ischemia-reperfusion injury. They showed that a reduction in JNK activity in the heart

713 resulted in a reduced level of cardiac injury and cellular apoptosis. The same study

714 demonstrated an increase in JNK activity by using mouse models overexpressing MKK7 in

715 the heart, and this caused a significant protection against ischemia-reperfusion injury (Kaiser

716 et al. 2005). This highlights the complexity of JNK signalling. In this study, a significant

717 decrease in JNK phosphorylation was identified when hearts were treated with Sunitinib. In

718 addition, NQDI-1 treatment had a tendency to increase in JNK phosphorylation. It has been

719 established that a reduction in JNK activation is associated with cardiac hypertrophy and

720 cardiovascular dysfunction (Pan et al. 2014). The reduction in JNK activation caused by the

721 treatment of Sunitinib could also explain the increased infarct size and irregularities found in

722 the haemodynamic data.

723

724 Our study contradicts some previous studies, for example Wang *et al.* 1998 investigated the

725 role of MKK7 in cardiac hypertrophy in neonatal myocytes (Wang et al. 1998). Transgenic

726 neonatal rat cardiomyocytes expressing wild type MKK7 and a constitutively active mutant of

727 MKK7 were created. This study demonstrated JNK specific activation by MKK7 and showed

728 the key role of the JNK pathway in cardiac hypertrophy as cells infected with the

729 constitutively active form of MKK7 adopted characteristic features of myocardial stress

730 (Wang et al. 1998).

731

732 The study by Fenton *et al.* 2010 looked at the expression of JNK in papillary cancer cells

733 with RET/PTC1 rearrangement treated with Sunitinib (Fenton et al. 2010). Sunitinib inhibited

734 proliferation of these RET/PTC1 subcloned papillary cancer cells, and furthermore inhibited

735 the JNK phosphorylation in the cytoplasm of the papillary cancer cells. In our study the

736 expression of p-JNK levels in left ventricular coronary tissue was also reduced in hearts

737 perfused with Sunitinib compared to vehicle treated hearts.

738

739 In summary, Sunitinib administration resulted in significant reduction in p-ASK1, p-MKK7 and

740 p-JNK levels, whilst NQDI-1 co-administration counteracted this increase. It is worth noting

741 that MKK7 is activated through phosphorylation at a special site at the C-terminal kinase

742 domain core called the "Domain for Versatile Docking" (DVD), which includes serine and

743 threonine sites (Wang et al. 2007). Sunitinib does not not discriminate between inhibition of

744 tyrosine kinases or serine-threonine kinases (Karaman et al. 2008). Therefore, Sunitinib

745 might potentially inhibiting serine-threonine kinases, however, it is much more likely that

746 Sunitinib inhibits tyrosine kinases as expected, resulting in the downstream inhibition of

747 ASK1, which then results in a downstream inhibition of MKK7 and JNK. However, more

748 detailed investigations into the pathway involvement are required to fully elucidate the

27

749 intracellular signalling pathways. In addition, the increase in p-ASK1, p-MKK7 and p-JNK

750 levels that we observe in the presence of both Sunitinib and NQDI-1, when compared to

751 Sunitinib perfused hearts, could be due to the fact that we only assessed the

752 phosphorylation of ASK1 at Thr^{845}, however, it is possible that NQDI-1 blocks both Ser^{83} and

753 Thr^{845} of ASK1, and further investigations of phosphorylation activity at both sides could

754 clarify this issue. This altered pattern in ASK1/MKK7/JNK pathway phosphorylation suggests

755 that Sunitinib has a direct effect on part of the ASK1/MKK7/JNK pathway.

756

4.4. The anti-cancer properties of Sunitinib were enhanced by NQDI-1 treatment

758 It is well established that Sunitinib achieves anti-tumour effects by inhibiting tyrosine kinases,

759 which have been over-expressed in cancer cells (Krause and Van Etten 2005). Sunitinib has

760 previously been shown to directly inhibit the survival and proliferation of a variety of cancer

761 cells, including leukaemia cells (Ilyas 2016).

762

763 We demonstrated a dose dependant decline in the cell viability of HL60 cells when treated

764 with Sunitinib (Fig 5A). This produced an IC_{50} value of 6.16 µM. Our results are in line with

765 existing data on the anti-proliferative effect of Sunitinib on HL60 cells. Sunitinib has

766 previously been shown to reduce the level of HL60 cell survival in a dose dependant manor

767 using a cell-titre blue reagent proliferation assay. This produced an IC_{50} value of 5.7 µM after

768 48 hrs of Sunitinib treatment (Ilyas 2016). Another group performed an MTT assay on a

769 variety of acute myelogenous leukaemia cell lines and found Sunitinib to have an IC_{50} values

770 between 0.007-13 µM (Hu et al. 2008).

771

772 To investigate the anti-proliferative effect of inhibition of the MKK7 pathway, HL60 cells were

773 treated with Sunitinib in co-treatment with NQDI-1 and NQDI-1 alone (Fig 5A-B). NQDI-1 is a

774 selective inhibitor for ASK1, the upstream regulator of MKK7. Previous studies have shown

775 ASK1 to have a crucial role in a variety of organ systems. However, ASK1 has also been

28

776 shown to promote tumorigenesis in gastric cancer and promote the proliferation of cancer

777 cells in skin cancer (Hayakawa et al. 2012; Iriyama et al. 2009).

778

779 Inhibition of ASK1 with compound K811 has been shown to prevent cell proliferation in

780 gastric cancer cell lines and reduce the size of xenograft tumours (Hayakawa et al. 2012).

781 Recently, Luo et al. 2016, investigated the involvement of ASK1 during proliferation in

782 pancreatic tumour cell line PANC-1 (Luo et al. 2016). The knock-down of ASK1 in mice with

783 pancreatic tumours reduced tumour growth, suggesting that ASK1 has an important role in

784 pancreatic tumorigenesis. The same group also demonstrated a dose-dependent inhibition

785 of the PANC-1 cell line when cells were treated with NQDI-1 at concentrations of 10 and 30

786 μM. However, the inhibition of ASK1 did not increase levels of apoptosis.

787

788 We have shown that increasing concentrations of NQDI-1 (0.2-200 μM) significantly reduce

789 the level of viable HL60 cells at 100 and 200 μM. This could suggest that ASK1 is expressed

790 at different levels in different cell types as a higher concentration was required in HL60 cells

791 compared to PANC-1 cells. Interestingly, the increasing concentrations of Sunitinib with 2.5

792 μM NQDI-1 enhanced the level of Sunitinib induced a reduction in HL60 cell proliferation.

793 The reason for this is not yet clear and further investigation into this is required.

794

795 As mentioned before NQDI-1 blocks FGFR-1 (Volynets et al. 2011). In cancer cells FGFR1

796 inhibitors have shown to elicit direct anti-tumour effects. The FGFR-1 inhibitors being

797 investigated in clinical trials for their anti-tumour qualities effecting various cancer types

798 include AZD4547, BGJ398, Debio-1347 and dovitinib (Katoh 2016). The apoptotic effect of

799 NQDI-1 we are observing in HL60 cells can therefore be a direct consequence of FGFR-1

800 inhibition.

801

802 In conclusion, our study demonstrates the potential of NQDI-1 as a valuable asset to cardiac

803 injury through the ASK1/MKK7/JNK transduction pathway, which could potentially lead to

29

804 development of cardioprotective adjunct therapy during drug-induced cardiac injury. NQDI-1

805 was observed to be cardioprotective as it reduced the Sunitinib-induced infarct size, and

806 addition it increased the apoptotic effect of Sunitinib in HL60 cells. This could indicate that

807 NQDI-1 - or an optimised derivative - could potentially be used as cardioprotective adjunct

808 therapy in e.g. Sunitinib treated leukaemia patients, which would not only protect the

809 patients' hearts but also boost the anti-cancer abilities of Sunitinib.

810

814

Conflict of interest

816 All authors have no conflict of interest to declare.

817

Figure legends

822 **Figure 1: Infarct to whole heart ratio assessment.** The hearts were drug perfused with

823 Sunitinib and/or NQDI-1 for 125 min in an isolated Langendorff heart model. This establishes

824 that Sunitinib-induced cardiotoxicity is reduced by ASK1 inhibitor NQDI-1. Groups: Control,

825 Sunitinib (1 µM), Sunitinib (1 µM) and NQDI-1 (2.5 µM), and NQDI-1 (2.5 µM) (n=6 per

826 group). Groups were assessed for statistical significance at each time point using one-way

827 ANOVA. Control versus Sunitinib (***=$P<0.001$), Control versus Sunitinib+NQDI-1

828 (**=$P<0.01$), Control versus NQDI-1 (*=$P<0.05$), or Sunitinib vs Sunitinib+NQDI-1

829 (###=$P<0.001$).

830

831 **Figure 2: Cardiotoxicity linked microRNAs expression.** The effect of Sunitinib (1 µM) and

832 the co-administration of ASK1 inhibitor, NQDI-1 (2.5 µM), on the expression of cardiotoxicity

833 linked microRNAs following 125 minute drug perfusion in an isolated heart Langendorff

834 model. The qRT-PCR results are shown as the ratio of target microRNA normalised to U6

835 with control group microRNA ratio set as 1 of microRNAs A) miR-1, B) miR-27a, C) miR-

836 133a and D) miR-133b. The ratio of target microRNA normalised to U6 is presented on a log

837 scale. Groups: Control (n=6 for miR-1, miR-27a and miR-133a; n=5 for miR-133b), Sunitinib

838 (1 µM) (n=6 for miR-1 and miR-27a; n=5 for miR-133a and miR-133b), Sunitinib (1 µM) and

839 NQDI-1 (2.5 µM) (n=6 for miR-1, miR-27a, miR-133a and miR-133b), and NQDI-1 (2.5 µM)

840 (n=4 for miR-1, miR-27a, miR-133a and miR-133b). Groups were assessed for statistical

841 significance at each time point using one-way ANOVA. Control versus Sunitinib

842 (***=$P<0.001$), Control versus Sunitinib+NQDI-1 (*=$P<0.05$, **=$P<0.01$), Control versus

843 NQDI-1 (**=$P<0.01$, ***=$P<0.001$), or Sunitinib vs Sunitinib+NQDI-1 (#=$p<0.05$,

844 ###=$P<0.001$).

845

846 **Figure 3: MKK7 mRNA expression levels.** The qRT-PCR assessment of MKK7 mRNA

847 expression levels in an isolated heart Langendorff model. The qRT-PCR results are shown

848 as the ratio of MKK7 mRNA normalised to GAPDH with control group ratio set as 1. Groups:

849 Control (n=5), Sunitinib (1 µM) (n=6), Sunitinib (1 µM) and NQDI-1 (2.5 µM) (n=3), and

850 NQDI-1 (2.5 µM) (n=3). Groups were assessed for statistical significance at each time point

851 using one-way ANOVA. Control versus Sunitinib, Control versus Sunitinib+NQDI-1, Control

852 versus NQDI-1, or Sunitinib vs Sunitinib+NQDI-1 (#=p<0.05).

853

854 **Figure 4: ASK1/MKK7/JNK pathway western blot assessment**. A) p-ASK1, B) p-MKK7,

855 and C) p-JNK phosphorylation levels in an isolated heart Langendorff model. Sorbitol was

856 included as a positive control in p-MKK7 Western blot analysis (n=4). Groups: Control (n=6

857 for p-ASK1; n=4 for p-MKK7 and p-JNK), Sunitinib (1 µM) (n=5 for p-ASK1; n=4 for p-MKK7

858 and p-JNK), Sunitinib (1 µM) and NQDI-1 (2.5 µM) (n=6 for p-ASK1; n=4 for p-MKK7 and p-

859 JNK) and NQDI-1 (2.5 µM) (n=5 for p-ASK1; n=4 for p-MKK7 and p-JNK). Groups were

860 assessed for statistical significance at each time point using one-way ANOVA. Control

861 versus Sunitinib (***=P<0.001), Control versus Sunitinib+NQDI-1 (*=p<0.05), Control versus

862 NQDI-1 (**=P<0.01; ***=P<0.001), or Sunitinib vs Sunitinib+NQDI-1 (#=p<0.05;

863 ###=P<0.001).

864

865 **Figure 5: HL60 cell viability**. HL60 cells (10^5 cells/ml) were incubated for 24 hours with

866 control or with increasing concentrations of A) Sunitinib (0.1 – 10 µM) or Sunitinib (0.1 – 10

867 µM) + NQDI-1 (2.5 µM) or B) NQDI-1 (0.2 µM – 200 µM). Groups were assessed for

868 statistical significance at each time point using one-way ANOVA. Control versus Sunitinib

869 (*=P<0.05 and ***=P<0.001), Control versus NQDI-1 (*=P<0.05 and ***=P<0.001), or

870 Sunitinib vs Sunitinib+NQDI-1 (###=p<0.001).

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1   **Involvement of mitogen activated kinase kinase 7 intracellular signalling pathway in**

2   **Ssunitinib-induced cardiotoxicity**

3

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22   **Abbreviations:**

23   ASK1, apoptosis signal-regulating kinase 1; ASK2, apoptosis signal-regulating kinase 2;

24   DMSO, dimethyl sulphoxide; hERG, human ether-a-go-go-related gene; JNK, c-Jun N-

25   terminal kinase; MKK7, mitogen activated kinase kinase 7; MTT, 3-(4,5-dimethylthiazol-2-yl)-

26   2,5-diphenyltetrazolium bromide; NQDI-1, 2,7-dihydro-2,7-dioxo-3*H*-naphtho[1,2,3-

27   *de*]quinoline-1-carboxylic acid ethyl ester; TTC, 2,3,5-Triphenyl-2H-tetrazolium chloride.

28 **Abstract**

29 The tyrosine kinase inhibitor Sunitinib is used to treat cancer and is linked to severe adverse

30 cardiovascular events. Mitogen activated kinase kinase 7 (MKK7) is involved in the

31 development of cardiac injury and is a component of the c-Jun N-terminal kinase (JNK)

32 signal transduction pathway. Apoptosis signal-regulating kinase 1 (ASK1) is the upstream

33 activator of MKK7 and is specifically inhibited by 2,7-dihydro-2,7-dioxo-3*H*-naphtho[1,2,3-

34 *de*]quinoline-1-carboxylic acid ethyl ester (NQDI-1). This study investigates the role of ASK1,

35 MKK7 and JNK during Sunitinib-induced cardiotoxicity.

36

37 Infarct size were measured in isolated male Sprague-Dawley rat Langendorff perfused

38 hearts treated for 125 min with Sunitinib in the presence and absence of NQDI-1. Left

39 ventricular cardiac tissue samples were analysed by qRT-PCR for MKK7 mRNA expression

40 and cardiotoxicity associated microRNAs (miR-1, miR-27a, miR-133a and miR-133b) or

41 Western blot analysis to measure ASK1/MKK7/JNK phosphorylation.

42

43 Administration of Sunitinib (1 µM) during Langendorff perfusion resulted in increased infarct

44 size, increased miR-133a expression, and decreased phosphorylation of the

45 ASK1/MKK7/JNK pathway compared to control. Co-administration of NQDI-1 (2.5 µM)

46 attenuated the increased Sunitinib-induced infarct size, reversed miR-133a expression and

47 restored phosphorylated levels of ASK1/MKK7/JNK. These findings suggest that the

48 ASK1/MKK7/JNK intracellular signalling pathway is important in Sunitinib-induced

49 cardiotoxicity. The anti-cancer properties of Sunitinib were also assessed using the 3-(4,5-

50 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Sunitinib

51 significantly decreased the cell viability of human acute myeloid leukemia 60 cell line (HL60).

52 The combination of Sunitinib (1 nM - 10 µM) with NQDI-1 (2.5 µM) enhanced the cancer-

53 fighting properties of Sunitinib. Investigations into the ASK1/MKK7/JNK transduction

54 pathway could lead to development of cardioprotective adjunct therapy, which could prevent

55 Sunitinib-induced cardiac injury.

2

56

1. Introduction

The tyrosine kinase inhibitor Sunitinib is used in the treatment of renal cell carcinoma and in gastrointestinal stromal tumours (Faivre et al. 2007). ~~The tyrosine kinase inhibitor Sunitinib is used in the treatment of many soft cell cancers (Faivre et al. 2007).~~ Sunitinib prevents tumour cell survival and angiogenesis by inhibiting a variety of growth factor and cytokine receptors, including platelet derived growth factor receptors, vascular endothelial growth factor receptors and proto-oncogenes c-Kit and RET. However, Sunitinib unfortunately is also associated with a lack of kinase selectivity resulting in the cardiotoxic adverse effects (Force et al. 2007). In the clinic, Sunitinib causes QT prolongation (Bello et al. 2009),left ventricular dysfunction (Shah and Morganroth 2015) and heart failure (Ewer et al. 2014). These findings are consistent with many other successful chemotherapy agents linked with severe drug-induced cardiotoxicity (Hahn et al. 2014), including electrophysiological changes and left ventricular dysfunction which can cause heart failure in some patients (Aggarwal et al. 2013). Intracellular studies using animals have revealed that Sunitinib causes mitochondrial injury and cardiomyocyte apoptosis through an increase in caspase-9 and cytochrome C release in both mice and in cultured rat cardiomyocytes (Chu et al. 2007).

82 Other indicators of apoptosis, such as an increase in caspase-3/7, have also been detected

83 after Sunitinib treatment in rat myocytes (Hasinoff et al. 2008).

84

85 MKK7 is a member of the mitogen-activated protein kinase kinase super family, which allows

86 the cell to respond to exogenous and endogenous stimuli (Foltz et al. 1998), and furthermore

87 MKK7 has shown to demonstrate a key role in protecting the heart from hypertrophic

88 remodelling, which occurs via cardiomyocyte apoptosis and heart failure (Liu et al. 2011).

89 The MKK7 activation of JNK results in many cellular processes including: proliferation,

90 differentiation and apoptosis (Chang and Karin 2001; Schramek et al. 2011; Sundarrajan et

91 al. 2003), and JNK signalling is vital for the maintenance and organisation of the

92 cytoskeleton and sarcomere structure in cardiomyocytes (Windak et al. 2013). Interestingly,

93 Sunitinib is an ATP analogue and competitively inhibits the ATP binding domain of its target

94 proteins (Roskoski 2007; Shukla et al. 2009). MKK7 also contains a highly conserved ATP

95 binding domain (Song et al. 2013). It is possible that Sunitinib binds as a ligand in the MKK7

96 ATP binding pocket, and thereby Sunitinib inhibits the MKK7/JNK transduction pathway, and

97 as a result this could potentially cause myocardial injury. It is important to determine the

98 relationship between MKK7 expression and Sunitinib induced cardiotoxicity by measuring

99 the alteration of MKK7 mRNA and phosphorylated MKK7 levels in the presence of Sunitinib.

100 Unravelling the relationship between Sunitinib and MKK7 could lead to a greater

101 understanding of its off-target mechanism of action and lead to the improvement in the

102 development of future drug discovery programmes or novel cardioprotective adjunct

103 therapies.

104

105 Short non-coding RNA microRNAs carry out the negative regulation of mRNA transcripts by

106 repressing translation (Bagga et al. 2005). Specific microRNAs expression patterns have

107 been linked to cardiomyocyte differentiation and in response to stress (Babiarz et al. 2012)

108 and have also been shown to be differentially expressed during the development of heart

109 failure (Thum et al. 2007). The microRNAs miR-1, miR-27a, miR-133a and miR-133b

4

110 produce differential expression patterns during the progression of heart failure (Akat et al.

111 2014; Tijsen et al. 2012). It is important to identify microRNA expression profiles in response

112 to drug-induced cardiotoxicity as similar patterns in microRNA expression to those identified

113 during heart failure may indicate the early onset of cardiotoxicity at a molecular level.

114

115 As MKK7 has no direct inhibitor, we have chosen to look at the upstream kinase ASK1

116 linked to MKK7 activation (Ichijo et al. 1997). ASK1 is activated in response to oxidative

117 stress-induced cardiac vascular endothelial growth factor suppression in the heart (Nako et

118 al. 2012). Izumiya et al. 2003 used ASK1 deficient transgenic mice to assess the role of

119 ASK1 in angiotensin II induced hypertension and cardiac hypertrophy. Both the wild type and

120 ASK1 deficient mice developed hypertension when stimulated with angiotensin II, however,

121 the ASK1 deficient mice lacked cardiac hypertrophy and remodelling and activation of ASK1,

122 p38 and JNK was severely attenuated, thus emphasising the importance of ASK1 in cardiac

123 hypertrophy and remodelling signalling (Izumiya et al. 2003). ASK1 is selectively inhibited by

124 NQDI-1 with high specificity with a K_i of 500nM and IC_{50} of 3µM (Volynets et al. 2011),

125 however, as this is a relatively new drug, a complete pharmacological profile has not yet

126 been fully characterised. ASK1 inhibition has previously been shown to offer protection

127 against ischemia reperfusion injury (Toldo et al. 2012) and has also been shown to suppress

128 the progression of ventricular remodelling and fibrosis in hamsters expressing severe

129 cardiomyopathy phenotypes (Hikoso et al. 2007). These findings highlight the potential of

130 NQDI-1 as a valuable asset to inhibit cardiac injury via the ASK1/MKK7/JNK pathway.

131

132 This novel study investigated the involvement of the ASK1/MKK7/JNK pathway in the

133 Sunitinib-induced cardiotoxicity via the assessment of cardiac function and infarct in

134 conjunction with relevant intracellular signalling mediators. Furthermore, we assessed the

135 anti-cancer properties of Sunitinib and determined whether co-administration of Sunitinib

136 with NQDI-1 affected the anti-cancer/apoptotic effect of Sunitinib in HL60 cells.

137

5

2. Materials and Methods

2.1. Main reagents and kits

Sunitinib malate and NQDI-1 were purchased from Sigma Aldrich (UK). Both drugs were dissolved in dimethyl sulphoxide (DMSO) and stored at -20 °C. Krebs perfusate salts were from either VWR International (UK) or Fisher Scientific (UK). Total ASK1 (Catalogue no ab131506) was purchased from Abcam (UK). Phospho-ASK1 (Thr 845) (Catalogue no 3765S), Phospho-MKK7 (Ser271/Thr275) (Catalogue no 4171S), Total MKK7 (Catalogue no 4172S), Phospho-SAPK/JNK (Thr183/Tyr185) (Catalogue no 9251), Total SAPK/JNK rabbit mAb antibody (Catalogue no 9252), anti-rabbit IgG, HRP-linked antibody and anti-biotin, HRP-linked antibody were purchased from Cell signalling technologies (UK). All the primary antibodies were from a rabbit host, and MKK7 and JNK were monoclonal antibodies, whereas ASK1 was polyclonal (all antibodies were validated by the manufacturers). The Ambion MicroPoly(A)Puris kit, Ambion mirVana miRNA Isolation Kit and Reverse Transcription Kit were from Life Technologies (USA). The mRNA primers and the Applied Biosystems primers assays (U6, rno-miR-1, hsa-miR-27a, hsa-miR-133a, and hsa-miR-133b) were purchased from Invitrogen (UK).The iTaq Universal SYBR Green Supermix was purchased from BioRad (UK). The HL60 cell line were obtained from European Collection of Cell Culture (UK) (catalogue no. 98070106).

2.2. Animals

Adult male Sprague-Dawley rats (300-350 g in body weight); were purchased from Charles River UK Ltd (UK) and housed suitably. They received humane care and had free access to standard diet according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Animals were selected at random for all treatment groups and the collected tissue was blinded for infarct size assessment. The experiments were performed following approval of the protocol by the Coventry University Ethics Committee. All efforts were made to minimise animal suffering and to reduce the number of animals used in the experiments. Rats were sacrificed

166   by cervical dislocation (Schedule 1 Home Office procedure). A total of 80 animals were used

167   for this study and the data from 68 rats were included, while data from 12 rats were excluded

168   from analysis due to the established haemodynamic exclusion criteria. No animals were

169   culled due to ill health. A total of 16 animals were included for Langendorff perfusion

170   experiments per main groups (Control, Sunitinib, Sunitinib+NQDI-1, and NQDI-1, where 6 of

171   the animals were used for measurement of the area of infarct and the area of risk and the

172   left ventricular tissue from another 10 animals was used for real time PCR and Western blot

173   analysis). Furthermore, an additional 4 animals were used for Langendorff perfusion

174   experiments with Sorbitol as a positive control for p-MKK7 Western blot analysis.

175

2.3. Langendorff perfusion model

177   The hearts were rapidly excised after the rats were culled and placed into ice-cold Krebs

178   Henseleit buffer (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM

179   KH_2PO_4, 1.7 mM $CaCl_2$, and 12 mM glucose, pH7.4). Each Langendorff study was

180   conducted using the protocol for naïve Langendorff studies (Gharanei et al. 2013). The

181   hearts were mounted onto the Langendorff system and retrogradely perfused with Krebs

182   Henseleit buffer. The pH of the Krebs Henseleit buffer was maintained at 7.4 by gassing

183   continuously with 95 % O_2 and 5 % CO_2 and maintained at 37 ± 0.5 °C using a water-

184   jacketed organ chamber. Each Langendorff experiment was carried out for 145 minutes: a

185   20 minute stabilisation period and 125 minutes of drug or vehicle perfusion in normoxic

186   conditions. Hearts were included in the study with a CF between 3.5-12.0 ml/g (weight of the

187   rat heart) during the stabilisation period. Sunitinib malate (1 µM) was administered

188   throughout the perfusion period in the presence or absence of NQDI-1 (2.5 µM).

189

190   The clinically relevant dose of 1 µM Sunitinib was chosen in line with previous studies by

191   (Henderson et al. 2013). Additionally, it has been reported that the plasma concentration of

192   Sunitinib has a C_{max} in the rage of 0.5–1.4µM (Doherty et al. 2013). While, the dose of 2.5

193   µM NQDI-1 was chosen following a thorough literature review (Eaton et al. 2014; Song et al.

7

194 2015; Volynets et al. 2011). NQDI-1 is not yet used in the clinic, therefore a clinically relevant

195 dose has not been reported.

196

197 Langendorff perfused hearts treated with vehicle were analysed as the control group. The

198 hearts were then weighed and either stored at -20 °C for 2,3,5-triphenyl-2H-tetrazolium

199 chloride (TTC) staining or the left ventricular tissue was dissected free and immersed in

200 RNAlater from Ambion (USA) for qRT-PCR or snap frozen by liquid nitrogen for Western blot

201 analysis.

202

2.4. Infarct size analysis

204 Frozen whole hearts were sliced into approximately 2 mm thick transverse sections and

205 incubated in 0.1 % TTC solution in phosphate buffer (2 ml of 100 mM $NaH_2PO_4.2H_2O$ and 8

206 ml of 100 mM NaH_2PO_4) at 37 °C for 15 minutes and fixed in 10% formaldehyde (Fisher

207 Scientific, UK) for 4 hours. The risk zone and infarct areas were traced onto acetate sheets.

208 The tissue at risk stained red and infarct tissue appeared pale. The acetate sheet was

209 scanned and ImageTool from UTHSCSA (USA) software was used to measure the area of

210 infarct and the area of risk. A ratio of infarct to risk size was calculated as a percentage for

211 each slice. An average was taken of all of the slices from each heart to give the percentage

212 infarct size of the whole heart. The mean of infarct to risk ratio for each treatment group and

213 the mean ± SEM was plotted as a bar chart. The infarct size determination was randomised

214 and blinded.

215

2.5. Analysis of microRNA expression profiles

217 The microRNA was isolated from left ventricular tissue using the *mir*Vana™ miRNA Isolation

218 Kit from Ambion (UK). The microRNA quantity and quality was measured by NanoDrop from

219 Nanoid Technology (USA). A total of 500 ng microRNA was reverse transcribed into cDNA

220 using primers specific for housekeeping reference RNA U6 snRNA and target microRNAs:

221 hsa-miR-155, hsa-miR-15a, hsa-miR-16-1, rno-miR-1, hsa-miR-27a, hsa-miR-133a or hsa-

8

222 miR-133b (please note all human hsa-miR assays are compatible with rat samples) from

223 Applied Biosystems (USA) using the MicroRNA Reverse Transcription Kit from Applied

224 Biosystems (USA) according to the manufacturer's instructions. The reverse transcription

225 quantitative PCR reaction was performed with the following setup: 16 °C for 30 min, 42°C for

226 30 min and 85 °C for 5 min and ∞ at 4°C. The qRT-PCR was performed using the TaqMan

227 Universal PCR Master Mix II (no UNG) from Applied Biosystems (USA) protocol on the 7500

228 HT Real Time PCR sequence detection system from Applied Biosystems (USA). A 20µl

229 reaction mixture containing 100 ng cDNA, specific primer assays mentioned above from

230 Applied Biosystems (USA) and the TaqMan Universal PCR Master Mix was used in the qRT-

231 PCR reaction in triplicates. A non-template control was included in all experiments. The real

232 time PCR reaction was performed using the program: 1) 2 minutes at 50°C, 2) 10 minutes at

233 95°C, 3) 15 seconds at 95°C, 4) 1 minute at 60°C. Steps 3) and 4) were repeated 40 times.

234

235 Analysis of qRT-PCR data of microRNAs were performed using the Ct values for U6 snRNA

236 as reference for the comparison of the relative amount of microRNAs (rno-miR-1, hsa-miR-

237 27a, hsa-miR-133a and hsa-miR-133b). The values of each of the microRNAs were

238 calculated to compare their ratios. The formula used was $X_0/R_0=2^{CTR-CTX}$, where X_0 is the

239 original amount of target microRNA, R_0 is the original amount of U6 snRNA, CTR is the CT

240 value for U6 snRNA, and CTX is the CT value for the target microRNAs (rno-miR-1, hsa-

241 miR-27a, hsa-miR-133a and hsa-miR-133b) (Sandhu et al. 2010). Averages of the Ct values

242 for each sample group (Control and Sunitinib treated hearts) and each individual primer set

243 were calculated and bar charts were plotted with mean ± SEM. The mean of the control

244 group was set as 1 for all microRNAs.

245

246 **2.6. Measurement of MKK7 mRNA expression**

247 Total mRNA was extracted from left ventricular tissue using The Ambion MicroPoly(A)Purist

248 kit from Ambion (USA). Extracted mRNA was processed directly to cDNA by reverse

249 transcription using Reverse Transcription Kit from Applied Biosystems (USA) with the

250 respective primers for MKK7 (MKK7 forward primer: CCCCGTAAAATCACAAAGAAAATCC

251 and MKK7 reverse primer: GGCGGACACACACTCATAAAACAGA) and GAPDH (GAPDH

252 Forward primer: GAACGGGAAGCTCACTGG and GAPDH Reverse primer:

253 GCCTGCTTCACCACCTTCT) according to the instructions from the manufacturer Invitrogen

254 (UK). The reverse transcription PCR reaction was performed with the following setup: 16 °C

255 for 30 minutes, 42°C for 30 minutes and 85 °C for 5 minutes. The qRT-PCR reactions were

256 performed with the iTaq Universal SYBR Green Supermix from BioRad (UK), GAPDH and

257 MKK7 mRNA primer sets on the 7500 HT Real Time PCR machine from Applied Biosystems

258 (USA) using the program: 1) 2 minutes at 50°C, 2) 10 minutes at 95°C, 3) 15 seconds at

259 95°C, 4) 1 minute at 60°C. Steps 3) and 4) were repeated 40 times.

260

261 Analysis of qRT-PCR data of MKK7 mRNA were performed using the Ct values for GAPDH

262 mRNA as reference for the comparison of the relative amount of MKK7 mRNA. The value of

263 mRNA was calculated to compare the ratios using the formula $X_0/R_0=2^{CTR-CTX}$, where X_0 is

264 the original amount of target mRNA, R_0 is the original amount of GAPDH mRNA, CTR is the

265 CT value for GAPDH mRNA, and CTX is the CT value for the MKK7 mRNA (Sandhu et al.

266 2010). Averages of the Ct values for each sample group (control and Sunitinib treated

267 hearts) and each individual primer set were calculated and bar charts were plotted with

268 mean ± SEM. The mean of the control group was set as 1 for the MKK7 mRNA.

269

2.7. Western blot detection of ASK1, MKK7 and JNK

271 A total 45-55 mg of the frozen left ventricular tissue was lysed in lysis buffer (NaCl 0.1 M,

272 Tris base 10 μM, EDTA 1 mM, sodium pyrophosphate 2 mM, NaF 2 mM, β-glycaophosphate

273 2 mM, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (0.1 mg/ml, 1/1.5 of protease

274 cocktail tablet) using a IKA Overtechnical T25homogeniser at 11,000 RPM. The

275 supernatants were measured for protein content using NanoDrop from Nanoid Technology

276 (USA). Then 80 μg of protein was loaded to 4–15 % Mini-Protean TGX Gel from BioRad

277 (UK) and separated at 200 V for 60 minutes. After separation, the proteins were transferred

278 to the Bond-P polyvinylidene difluoride membrane from BioRad (UK) by using the Trans-Blot

279 Turbo transfer system from BioRad (UK) and probed for the phosphorylated forms

280 Phospho(Thr^{845})-ASK1 (p-ASK1), Phospho(Ser^{271}/Thr^{275})-MKK7 (p-MKK7) and

281 Phospho(Thr^{183}/Tyr^{185}) -SAPK/JNK (p-JNK), and total forms of ASK1(Thr^{845}),

282 MKK7(Ser^{271}/Thr^{275}) and JNK(Thr^{183}/Tyr^{185}). The p-MKK7 and p-JNK blots were stripped by

283 boiling and the PVDF membrane was used for total MKK7 and total JNK analysis,

284 respectively. According to recommendations from Cell signalling technologies (UK) total

285 ASK1 analysis had to be performed on a separate Western blot, as the stripping procedure

286 would remove total ASK1 protein. The relative changes in the p-ASK1, p-MKK7 and p-JNK

287 protein levels were measured and corrected for differences in protein loading as established

288 by probing for total ASK1, MKK7 and JNK respectively.

289

290 For Western blot analysis phosphorylated antibody levels were normalised to total antibody

291 levels in order to correlate for unequal loading of protein and differential blot transfer and to

292 identify the level of active vs inactive protein levels. Results were expressed as a percentage

293 of the density of phosphorylated protein relative to the density of total protein using Image

294 Lab 4.1 from BioRad (UK). The phosphorylated antibody levels determination was

295 randomised and blinded.

296

297 **2.8. MTT assay assessment of HL60 cell viability in the presence of Sunitinib with and**

298 **without NQDI-1**

299 The HL60 cell line were maintained in in RPMI 1640 medium supplemented with L-

300 Glutamine (2 mM) and 10 % heat-inactivated fetal bovine serum and antibiotics mix at 37 °C

301 in a humidified incubator under 5 % CO_2/95 % air. Cells were split in a 1:5 ratio every 2-3

302 days. Cells were incubated with Control, increasing concentrations of Sunitinib (1nM – 10

303 µM), Sunitinib (0.1 – 10 µM) + NQDI-1 (2.5 µM), or increasing concentrations of NQDI-1

304 (0.2-200 µM) for 24 h. Both Sunitinib and NQDI-1 were dissolved in DMSO. The DMSO

305 concentration was < 0.05 % (v/v) during the *in vitro* studies.

11

306

307 Cells were plated at a cell density of 10^5 cells/ml in 96-well plates and the above indicated

308 concentration of the drug was added. The plate was then incubated at 37°C for 24hrs. After

309 drug incubation, 50 µl of MTT solution (5 mg MTT/ml H_2O) was added and the cells were

310 incubated for a further 24 h. Next, 50 µl of DMSO was added to each well and mixed by

311 pipetting to release reduced MTT crystals from the cells. Relative cell viability was obtained

312 by scanning with an ELISA reader (Anthos Labtech AR 2001 Multiplate Reader, *Anthos*

313 *Labtec* Instruments, Austria) with a 490 nm filter. Results were expressed as a percentage of

314 viable cells relative to untreated cells/control. Experiments were performed in triplicates and

315 repeated ≥ 3 times.

316

2.9. Statistical analysis

318 Results are presented as mean ± standard error of the mean (SEM). Significance of all data

319 sets was measured by one-way ANOVA analysis with the Tukey post hoc test using the

320 Matlab prism program. The following groups were compared during ANOVA analysis:

321 Control versus Sunitinib, Control versus Sunitinib and NQDI-1, Control versus NQDI-1 (all

322 statistically significant data compared to control marked with *), and Sunitinib versus

323 Sunitinib and NQDI-1 (all statistically significant data compared to Sunitinib marked with #).

324 Control versus Sunitinib (red), Control versus versus Sunitinib and NQDI-1 (green), Control

325 versus NQDI-1 (blue) (all statistically significant data compared to control marked with *),

326 and Sunitinib versus Sunitinib and NQDI-1 (all statistically significant data compared to

327 Sunitinib marked with a purple $). For MKK7 mRNA some data was evaluated by using

328 student's t-test. P-values <0.05 were considered statistically significant.

329

3. Results

3.1. Sunitinib treatment induces cardiac injury

332 The effect of Sunitinib (1µM) administration on myocardial infarction development was

333 investigated by TTC staining. The hearts were stabilised for a period of 20 minutes, followed

12

334 by 125 minutes of drug perfusion. Administration of Sunitinib (1µM) for 125 minutes resulted

335 in a significant increase in infarct size compared with non-treated controls (Control: 7.81 ±

336 1.16 %; Sunitinib: 41.02 ± 1.23 %, p<0.001) (Fig 1). This demonstrated that Sunitinib

337 treatment of the Langendorff perfused hearts results in a drastic increase in cardiac injury.

338 The infarct was globally distributed in all groups investigated in this study (i.e. Control,

339 Sunitinib ± NQDI-1, and NQDI-1).

340

3.2. Sunitinib and NQDI-1 co-treatment alleviate cardiac injury

342 The effect of ASK1 inhibition by NQDI-1 on cardiac function and infarction was investigated.

343 Co-administration of Sunitinib (1 µM) with NQDI-1 (2.5 µM) significantly decreased infarct

344 size compared to Sunitinib treated hearts (Sunitinib: 41.02 ± 1.23 %; Sunitinib + NQDI-1:

345 17.54 ± 2.97 %, p<0.001). However, administration of NQDI-1 alone for 125 minutes of

346 perfusion significantly increased infarct size compared with control hearts (Control: 7.81 ±

347 1.16 %; NQDI-1: 16.68 ± 2.66 %, p<0.05) (Fig 1).

348

3.3. Sunitinib treatment modulates expression of microRNAs involved in cardiac injury

351 The expression of cardiac injury specific microRNAs during Sunitinib-induced cardiotoxicity

352 was determined by qRT-PCR assessment. The microRNAs miR-1, miR-27a, miR-133a and

353 miR-133b have been shown to produce differential expression patterns during the

354 progression of heart failure. The ratio of target microRNA normalised to U6 was set to 1 in

355 the control group for easier comparison of microRNA ratio values between the various drug

356 therapy groups. There was a significant increase in miR-133a when hearts were perfused

357 with Sunitinib (1 µM) compared to control hearts (Ratio of target microRNA normalised to U6

358 in Sunitinib treated hearts: miR-133a: 535.78 ± 61.27, p<0.001). Co-administration of NQDI-

359 1 (2.5 µM) along with Sunitinib reversed this miR-133a expression trend by decreasing the

360 miR-133a expression when compared to Sunitinib perfused hearts (Ratio of target microRNA

361 normalised to U6 in Sunitinib and NQDI-1 treated hearts: miR-133a: 52.76 ± 28.30,

13

362 p<0.001). Hearts perfused with NQDI-1 alone showed an increase in miR-1, miR-27a and

363 miR-133b expression compared to control hearts (Ratio of target microRNA normalised to

364 U6 in NQDI-1 treated hearts: miR-1: 32.33 ± 16.47, p<0.01; miR-27a: 11.27 ± 2.86, p<0.001;

365 miR-133b: 167.85 ± 58.13, p<0.001). The expression of miR-1, miR-27a, miR-133a and

366 miR-133b was increased in the Sunitinib and NQDI-1 co-treated hearts when compared to

367 Sunitinib perfused hearts (miR-1: p<0.05; miR-27a: p<0.001; miR-133a: p<0.001; miR-133b:

368 p<0.05). (Fig 2 A-D).

369

370 The results from the microRNA qRT-PCR analysis show there is a similar expression pattern

371 for miR-1, miR-27 and miR-133b, while miR-133a has its own pattern. This indicates that the

372 cardiac injury induced by Sunitinib, which is alleviated by the ASK1 inhibitor NQDI-1, triggers

373 a complex alteration of these cardiac injury microRNAs. Further studies looking at the

374 altered expression profiles for these cardiac injury microRNAs have to be undertaken in

375 order to clarify the expression patterns. ~~This indicates a complex alteration of these cardiac~~

376 ~~injury microRNAs by Sunitinib and ASK1 specific inhibitor, NQDI-1. This indicates an~~

377 ~~interaction between the ASK1/MKK7 pathway and Sunitinib in respect to expression of~~

378 ~~cardiotoxicity linked microRNAs during cardiac injury.~~

379

380 **3.4. MKK7 mRNA expression profile is altered by ASK1 inhibitor NQDI-1**

381 As MKK7 contains an ATP binding domain (Song et al. 2013) and Sunitinib is an ATP

382 analogue and competitively inhibits the ATP binding domain of its target proteins (Roskoski

383 2007; Shukla et al. 2009). We therefore wanted to investigate the interaction between

384 Sunitinib and MKK7, and question whether Sunitinib could bind as a ligand in the ATP

385 binding pocket of MKK7. This would determine if Sunitinib might potentially have an

386 inhibitory effect on the MKK7/JNK pathway. The relationship between MKK7 expression and

387 Sunitinib-induced cardiotoxicity was assessing on transcriptional level by MKK7 mRNA qRT-

388 PCR analysis on Sunitinib (1 μM) perfused hearts, and the interaction by ASK1 specific

389 inhibitor NQDI-1 was detected to highlight if any interaction between ASK1/MKK7 and

14

390 Sunitinib-induced alteration of MKK7 transcription due to cardiac injury was observed to

391 impact on mRNA levels. The ratio of MKK7 mRNA normalised to GAPDH was set to 1 in the

392 control group for easier comparison of GAPDH normalised MKK7 mRNA values between the

393 various drug therapy groups. The qRT-PCR analysis of MKK7 mRNA revealed that co-

394 administration of NQDI-1with Sunitinib caused a significant increase in MKK7 mRNA

395 expression compared to Sunitinib treatment alone ($p<0.01$) (Ratio of MKK7 mRNA

396 normalised to GAPDH. Sunitinib: 0.12 ± 0.03; Sunitinib + NQDI-1: 1.18 ± 0.65) (Fig 3). The

397 decrease in MKK7 mRNA observed in the Sunitinib (1 µM) perfused hearts compared to

398 control hearts was not significant, but a clear trend was observed. If the data from groups

399 control and Sunitinib were compared using a Student's t-test the decline in MKK7 mRNA in

400 Sunitinib treated hearts was statistically significant with $p=0.0043$.

401

402 These MKK7 mRNA qRT-PCR results clearly demonstrate that Sunitinib treatment shows a

403 tendency to decrease the MKK7 mRNA, and co-administration of ASK1 specific inhibitor

404 NQDI-1 restores the MKK7 mRNA level observed in control treated heart. This could indicate

405 a complex regulation system where Sunitinib-induced cardiac injury in directly linked with the

406 ability of Sunitinib to reduced MKK7 expression at transcriptional level, which is counteracted

407 by the ASK1 specific inhibitor NQDI-1.

408

409 **3.5. ASK1/MKK7/JNK pathway is involved in Sunitinib-induced cardiotoxicity**

410 As explained in the previous MKK7 mRNA results section we wanted to investigate the

411 interaction between Sunitinib and MKK7, as they both interact via the ATP binding pocket.

412 The key question being if Sunitinib can bind as a ligand in the ATP binding pocket of MKK7

413 and if Sunitinib is able to block the MKK7/JNK pathway. Here we investigate the role of

414 cardiotoxic Sunitinib therapy on the ASK1/MKK7/JNK pathway phosphorylation and how the

415 interaction with the cardioprotective ASK1 specific agent NQDI-1 effects the

416 ASK1/MKK7/JNK pathway phosphorylation. Following Langendorff perfusion of Sunitinib and

15

417 NQDI-1, p-ASK1, p-MKK7 and p-JNK levels were measured in the left ventricular tissue of

418 the hearts by Western blot analysis.

419

420 Western blot analysis showed that Sunitinib treatment decreased p-ASK1, p-MKK7 and p-

421 JNK levels significantly when compared to control (density of phosphorylated protein

422 normalised to total protein: *p-ASK1*: Control: 389.43 ± 4.18 and Sunitinib: 16.47 ± 3.56,

423 p<0.001; *p-MKK7*: Control: 55.60 ± 4.86 and Sunitinib: 23.66 ± 4.53, p<0.001; *p-JNK*:

424 Control: 43.66 ± 2.82 and Sunitinib: 22.52 ± 2.74, p<0.001). Co-administration with NQDI-1

425 increased the p-ASK1, p-MKK7 and p-JNK levels, and these were statistically significantly

426 elevated when compared to heart treated with Sunitinib monotherapy Co-administration with

427 NQDI-1 restored the decrease in p-ASK1, p-MKK7 and p-JNK levels and increased them

428 back to control levels (density of phosphorylated protein normalised to total protein: *p-ASK1*:

429 Sunitinib + NQDI-1: 51.17 ± 3.66, p<0.001; *p-MKK7*: Sunitinib + NQDI-1: 38.11 ± 1.87,

430 p<0.05; *p-JNK*: 48.95 ± 2.76, p<0.001). The p-ASK1, p-MKK7 and p-JNK levels were

431 decreased in NQDI-1 treated hearts compared to control hearts (density of phosphorylated

432 protein normalised to total protein: *p-ASK1*: NQDI-1: 20.56 ±1.99, p<0.01; *p-MKK7*: NQDI-1:

433 18.40 ± 2.98, p<0.001; *p-JNK:* 28.78 ± 3.03, p<0.01) (Fig 4A-C).

434

435 These Western blot results show that Sunitinib decreased the phosphorylation of the

436 ASK1/MKK7/JNK pathway. The fact that Sunitinib was able to show a strong tendency of

437 decreasing the MKK7 mRNA highlights that the decreased MKK7 phosphorylation is

438 regulated at the transcriptional level, which is then affecting the post-transcriptional MKK7

439 phosphorylation levels. However, interestingly the cardiotoxic Sunitinib is having an inhibiting

440 effect throughout all three parts of the ASK1/MKK7/JNK pathway. Co-administration of

441 NQDI-1 is counteracting the Sunitinib inhibiting effect on the phosphorylation level

442 throughout the ASK1/MKK7/JNK pathway. These results show a clear indication of Sunitinib

443 interacting with ASK1, MKK7 and JNK at post-translational level and MKK7 gene expression

444 at pre-transcriptional level.

16

445

3.6. Cancer cell viability in response to Sunitinib with and without NQDI-1

447 The effect of Sunitinib on cell viability was examined in HL60 cells. The HL60 cells were

448 treated with Sunitinib for 24 hrs and then the level of mitochondrial metabolic-activity

449 inhibition was measured with the MTT assay. Sunitinib showed a pronounced decrease in

450 metabolic activity and a dose-dependent decrease in cell viability (Fig 5A). In particular,

451 Sunitinib significantly reduces cell viability at 1 nM (82.62 ± 6.55 %, $p<0.05$), 0.1 µM ($85.94 \pm$

452 3.80 %, $p<0.05$), 0.5 µM (83.94 ± 3.86 %, $p<0.05$), 1 µM (77.28 ± 6.58 %, $p<0.05$), 5 µM

453 (58.61 ± 4.44 % $p<0.001$) and 10 µM (47.14 ± 6.77 %, $p<0.001$) concentrations of Sunitinib.

454 The IC_{50} value was 6.16 µM. All the concentrations of Sunitinib in the absence and presence

455 of NQDI-1 used during this study produced significant reductions in HL60 cell viability

456 compared to vehicle treatment.

457

458 The co-administration of NQDI-1 (2.5 µM) to increasing concentrations of Sunitinib (1 nM -

459 10 µM) enhanced the inhibition of mitochondrial metabolism shown by Sunitinib (Fig 5A).

460 Specifically, co-treatment of Sunitinib with NQDI-1 reduced cell viability at 1 µM ($59.58 \pm$

461 6.30 %, $p<0.001$), 5 µM (36.62 ± 6.52 %, $p<0.001$) and 10 µM (15.10 ± 2.31 %, $p< 0.001$)

462 concentrations of Sunitinib compared to Sunitinib alone. The IC_{50} value for Sunitinib plus

463 NQDI-1 was 1.76 µM.

464

465 Interestingly, increasing concentrations of NQDI-1 alone (0.2-200 µM) only significantly

466 reduced cell viability at very high concentrations. Reductions in cell viability were significant

467 at 100 µM (55.44 ± 12.39 %, $p<0.05$), 200 µM (33.15 ± 9.67 %, $p<0.001$) (Fig 5B). The IC_{50}

468 value for NQDI-1 produced by the MTT assay was 130.8 µM.

469

4. Discussion

4.1. Involvement of ASK1/MKK7/JNK in Sunitinib-induced cardiotoxicity

472 The occurrence heart failure associated with anti-cancer treatment has been investigated

17

473 extensively (Khakoo et al. 2008), however, the underlying mechanism of cardiotoxicity is still

474 unclear. Determining cellular pathways involved in Sunitinib-induced cardiotoxicity could help

475 to develop therapies which could prevent the potential development of heart failure

476 associated with Sunitinib treatment.

477

478 Data presented in this study confirms that Sunitinib causes drug-induced myocardial injury

479 via an increase in infarct size (Fig 1). We observed that infarct size increased from ~ 8 % in

480 Control hearts to ~ 41 % in hearts perfused with Sunitinib. This observation is in accordance

481 with our previous study, where we investigated the involvement of A3 adenosine receptor

482 activation during Sunitinib induced cardiotoxicity in perfused rat hearts (Sandhu et al. 2017).

483 Also, the injury induced by Sunitinib administration is very similar to ischemia/reperfusion

484 injury rats investigated by the same Langendorff model (Gharanei et al. 2013). The Control

485 rat hearts do suffer from minor infarct injury during the brief period it takes from sacrificing

486 the animal and perfusing the heart with the Krebs buffer during the Langendorff model. This

487 insult accounts for the ~ 8 % infarct size we observe in Control hearts. A dose of 1 µM

488 Sunitinib increased the infarct to ~ 41 % in perfused heart, and this steady state blood

489 concentration of Sunitinib has been found in patients treated with Sunitinib (Henderson et al.

490 2013). As the rat hearts are physically much smaller than human hearts, the rat hearts may

491 have been more sensitive to the adverse effect of Sunitinib administration at the clinically

492 relevant dose of 1 µM.

493

494 Other animal studies investigating Sunitinib-induced cardiotoxicity were linked to a significant

495 decrease in left ventricular function (Henderson et al. 2013; Mooney et al. 2015), and

496 interestingly left ventricular dysfunction has also been identified in patients undergoing

497 Sunitinib chemotherapy (Di Lorenzo et al. 2009). Data presented in this study confirms that

498 Sunitinib causes drug-induced myocardial injury via an increase in infarct size (Fig 1). These

499 observations are in accordance with other studies investigating Sunitinib-induced

500 cardiotoxicity linked with a significant decrease in left ventricular function in animals

501 (Henderson et al. 2013; Mooney et al. 2015), and left ventricular dysfunction has also been

502 identified in patients undergoing Sunitinib chemotherapy (Di Lorenzo et al. 2009). Also,

503 sunitinib has also been associated with electrophysiological disturbances and an increased

504 pro-arrhythmic relative risk of developing QTc interval prolongation (Ghatalia et al. 2015;

505 Schmidinger et al. 2008). Several studies show that Sunitinib could potentially block human

506 ether-a-go-go-related gene (hERG) potassium channels and cause irregular contractions

507 (Doherty et al. 2013; Guo et al. 2013). The in vitro study by Thijs et al. 2015 investigated the

508 effect of Sunitinib on the contractile force measured during normal pacing or after simulated

509 ischemia on isolated human atrial trabeculae from patients awaiting coronary artery bypass

510 graft and/or aorta valve replacement. They showed that treatment with 81.3 nM Sunitinib did

511 not attenuate the recovery in contractile force of atrial cardiomyocytes after simulated

512 ischemia and reperfusion compared to vehicle treated atrial cardiomyocytes, and thus they

513 concluded that the development of heart failure in patients treated with Sunitinib could not be

514 explained by an acute cardiotoxic Sunitinib stimulation of cardiomyocytes. However, it

515 should be noted that they used a 12-times lower Sunitinib dose compared to what we have

516 used in this study, and thus they might have not used a dose of Sunitinib in the toxic range

517 (Thijs et al. 2015).

518

519 Modulation of ASK1 and its downstream targets MKK7 and JNK have been shown to play

520 important roles in regulating cardiomyocyte survival, apoptosis, hypertrophic remodeling and

521 intracellular signalling associated with heart failure (Mitchell et al. 2006). Therefore, we

522 hypothesised that successful modulation of the ASK1/MKK7/JNK pathway would produce an

523 effective cardioprotective treatment against Sunitinib induced cardiotoxicity.

524

525 As we were interested in the ASK1/MKK7/JNK signalling pathway we targeted ASK1 with

526 NQDI-1. Administration of the ASK1 specific inhibitor NQDI-1 resulted in abrogation of the

527 some cardiotoxic effects of Sunitinib. This study therefore, demonstrates the potentially

528 pivotal role that this kinase and the related pathway could play in protecting the heart from

529 Sunitinib induced cardiotoxicity. These observations are in accordance with other studies

530 that have also implicated the involvement of ASK1 in cardioprotection in other mammalian

531 models (Boehm 2015; Hao et al. 2016; He et al. 2003). Furthermore, inhibition of ASK1 with

532 thioredoxin results in reduction of infarct size compared to ischemic/reperfusion control

533 hearts (Gerczuk et al. 2012; Huang et al. 2015; Zhang et al. 2007).

534

535 ASK1 signalling pathway is facilitated through two main routes: either (i) MKK4/7 and JNK,

536 or (ii) MKK3/6 and p38 (Ichijo et al. 1997). As mentioned in the introduction the study by

537 Izumiya *et al.* 2003 showed the importance of ASK1 during angiotensin II induced

538 hypertension and cardiac hypertrophy in mice, as ASK1 knockout mice failed to develop

539 cardiac hypertrophy and remodelling through both JNK and p38 (Izumiya et al. 2003). In

540 another study by Yamaguchi *et al.* 2003 knockout of ASK1 in mice was also linked to

541 cardioprotection through the JNK pathway, as coronary artery ligation or thoracic transverse

542 aortic constriction in ASK1 deficient hearts showed no morphological or histological defects.

543 Both left ventricular end-diastolic and end-systolic ventricular dimensions were increased

544 less then wild-type mice, and the decreases in fractional shortening in both experimental

545 models wereas less when compared with wild-type mice (Yamaguchi et al. 2003). However,

546 in a study by Taniike *et al.* 2008 when ASK1 knockout mice were subjected to mechanical

547 stress it resulted in exaggerated hearth growth and hypertrophy development through p38

548 pathway (Taniike et al. 2008). It is worth noting that attenuation of ASK1 by NQDI-1 during

549 normoxic conditions and Sunitinib treatment could have affected the MKK3/6 and p38

550 signalling pathway, which would have led to the conflicting results that we observe during

551 our study: tThere was a significant reduction in infarct size when Sunitinib was administered

552 in the presence of the ASK-1 inhibitor NQDI-1, however administration of NQDI-1 alone also

553 increased the infarct size. The increase in infarct size with NQDI-1 was not as profound as

554 the increase observed with Sunitinib (Fig 1).

555

556 NQDI-1 is a recently discovered highly specific ASK1 inhibitor. In the presence of 25 µM

557 NQDI-1 the residual activity of ASK1 is reduced to 12.5 % using the ɣ-32P-ATP *in vitro*

558 kinase assay model. By using the same assay model Volynetys and colleagues determined

559 the residual activity of the tyrosine protein kinase fibroblast growth factor receptor 1 (FGFR1)

560 which was measured to be 44 % after 25 µM NQDI-1 exposure (Volynets et al. 2011).

561 FGFR1 as a key component involved in *in vivo* cardiomyocyte proliferation during early

562 stage heart development (Mima et al. 1995). Furthermore, FGFR1 is an essential regulator

563 of coronary vascular development through Hedgehog signalling activation, which in the adult

564 heart leads to increased coronary vessel density (Lavine et al. 2006). It is therefore possible

565 that NQDI-1 is blocking FGFR1 directly in the treated hearts of our study and thereby

566 exerting a slight increase in infarct size compared to the control.

567

568 In addition, NQDI-1's specificity towards other kinases has yet to be determined. In particular

569 its homologue apoptosis signal-regulating kinase 2 (ASK2) may potentially be inhibited by

570 NQDI-1 (Hattori et al. 2009). Both ASK1 and ASK2 is expressed in the heart (Iriyama et al.

571 2009). ASK2 is only stable and active when it forms a heteromeric complex with ASK1.

572 ASK2 mediate its stress response from the stable ASK1-ASK2 heteromeric complex

573 platform through both JNK and p38 and induce apoptosis. Furthermore, ASK1 and AS2 are

574 able to activate each other, and thus ASK1 assists ASK2 with ASK2 regulatory mechanisms

575 in addition to stabilising and activating ASK2 (Takeda et al. 2007). It is therefore very likely

576 that NQDI-1 will affect the ASK2 mediated signalling. Several studies by the team of Kataoka

577 have revealed the involvement of ASK2 during hypertension, cardiac hypertrophy and

578 remodelling development. They have shown that ASK2 deficient mice have a significantly

579 higher blood pressure and increased left ventricular weight then wild type mice, with an

580 underlying analysis revealing that perivascular and interstitial myocardial fibrosis was

581 increased (Kataoka 2008, 2009, 2010, 2011). Therefore, the relatively small adverse effect

582 of NQDI-1 administration on producing an increased infarct size could also be caused by the

583 NQDI-1 effect through attenuated ASK2 signalling. Therefore, the cardiac adverse effect of

584 NQDI-1 upon increased infarct size detected in this study could also be caused by the NQDI-

21

585 ~~1 effect through attenuated ASK2 signalling.~~

586

587 Further studies are required to unravel the underlying mechanisms associated with the

588 cardiac tissue injury and haemodynamic responses observed upon NQDI-1 stimuli to assess

589 if p38, FGFR1, and/or ASK2 are involved. Also, other concentration of NQDI-1 treatment of

590 hearts should be investigated, as lower concentrations of NQDI-1 most likely would cause

591 less cardiac adverse effects.

592

593 4.2. Profiling of cardiotoxicity linked microRNAs

594 MicroRNAs have been shown to have important roles in tissue formation and function in

595 response to injury and disease. The microRNAs miR-1, miR-27a, miR-133a and miR-133b

596 have been shown to produce differential expression patterns during the progression of heart

597 failure (Akat et al. 2014; Tijsen et al. 2012). Here we investigate the altered expression

598 profiles of microRNAs miR-1, miR-27a, miR-133a and miR-133b after Sunitinib-induced

599 cardiotoxicity with or without the ASK1 inhibitor NQDI-1 (Fig 2A-D). To the best of our

600 knowledge there are no other studies showing a significant altered expression of miR-1,

601 miR-27a, miR-133a and miR-133b after Sunitinib treatment. A reduction in hERG potassium

602 channels expression causes the delayed myocyte repolarisation attributed to a long QT

603 interval and interestingly the 3' untranslated region of hERG potassium channel transcripts

604 have a partial complimentary miR-133a target site (Xiao et al. 2007). The current study

605 shows an increase in miR-133a expression following Sunitinib treatment, which was

606 attenuated with NQDI-1 co-administration (Fig 2C). This could imply that miR-133a

607 overexpression inhibits the hERG potassium channel, which would have a negative impact

608 on the electrophysiological response (Xu et al. 2007). Over expression of miR-133a has also

609 been shown to have negative effects on cardiomyocyte proliferation and survival (Liu et al.

610 2008), which corroborates the results from the current study.

611

612 In the heart miR-1 and miR-133 maintain the heart beat rhythm by regulating the cardiac

613 conduction system (Kim 2013). Furthermore, miR-1 and miR-133 are downregulated during

614 cardiac hypertrophy in both mouse and human models. *In vitro* studies have shown that the

615 overexpression of miR-133 inhibits cardiac hypertrophy, whereas inhibition of miR-133

616 induces more pronounced hypertrophy (Care et al. 2007). In addition, a decreased in cardiac

617 expression of miR-133b is sufficient to induce hypertrophic gene expression (Sucharov et al.

618 2008). <u>In support of these studies our analysis shows that the miR-133b expression is</u>

619 <u>increased during NQDI-1 mono-therapy and co-administration of Sunitinib and NQDI-1 (Fig</u>

620 <u>2C),</u>~~In support of these studies our analysis shows that the miR-133b expression is~~

621 ~~increased during NQDI-1 mono-therapy and co-administration hearts (Fig 2C),~~ thus

622 protecting the heart against hypotrophy/cardiac damage development, which is the same

623 pattern as we observe in the infarct to risk analysis.

624

625 The miR-27a expression has been observed to downregulate FOXO-1 protein, a

626 transcription factor which regulates genes involved in the apoptotic response, cell cycle, and

627 cellular metabolism (Guttilla and White 2009). Moreover, miR-27a is downregulated in

628 coronary sinus samples of heart failure patients (Marques et al. 2016). <u>The increase in miR-</u>

629 <u>27a expression in our study during NQDI-1 mono-therapy and co-administration of Sunitinib</u>

630 <u>and NQDI-1</u> ~~The increase in miR-27a expression in our study during NQDI-1 mono-therapy~~

631 ~~and co-administration hearts~~ (Fig 2B) support the findings from these studies as the link

632 increased miR-27 expression to reduced apoptosis, which is what we observe in the infarct

633 to risk analysis.

634

635 **4.3. Sunitinib treatment supresses the ASK1/MKK7/JNK pathway**

636 **4.3.1. ASK1**

637 Western blot assessment of Sunitinib treated hearts showed a significant decrease in p-

638 ASK1 when compared to control perfused hearts, and this decrease in p-ASK1 levels was

639   abrogated with NQDI-1 co-administration. However, in p-ASK1 levels from hearts subjected

640   to Sunitinib and NQDI-1 co-administration were compared to control hearts we did observe a

641   significant increase in p-ASK1. . However, if p-ASK1 levels in Sunitinib and NQDI-1 co-

642   administration hearts were compared to control hearts we did observe a significant increase

643   in p-ASK1. Furthermore, NQDI-1 treated hearts had significantly decreased p-ASK1 levels

644   when compared to control hearts (Fig 4A).

645   ASK1 has multiple phosphorylation sites. The Akt/protein kinas B complex binds to and

646   phosphorylates Ser^{83} of ASK1, resulting in the inhibition of ASK1-mediated apoptosis (Kim et

647   al. 2001), the 14-3-3 interacts with phosphorylated Ser^{967} of ASK1 to block the function of

648   ASK1 (Zhang et al. 1999), protein phosphatase 5 dephosphorylates Thr^{845} within the

649   activation loop of ASK1 and thereby inhibits ASK1-mediated apoptosis (Morita et al. 2001),

650   while Ser^{1034} phosphorylation suppresses ASK1 proapoptotic function (Fujii et al. 2004).

651   ASK1 undergoes auto-phosphorylation at the Thr^{845} (Tobiume et al. 2002). It is possible that

652   auto-phosphorylation increased when Sunitinib was combined with NQDI-1, which led to the

653   increased levels of p-ASK1 identified by western blot analysis. In its inactive form, ASK1 is

654   complexed with thioredoxin (Saitoh et al. 1998). It has been proposed that auto-

655   phosphorylation at Thr^{845} is increased in response to H_2O_2 treatment, due to H_2O_2 preventing

656   thioredoxin from complexing with ASK1. This suggests that ASK1 activation is due to

657   oxidative stress (Tobiume et al. 2002). An increase in p-ASK1 could indicate increased

658   levels of oxidative stress, which potentially reduced cardiac function and generated a level of

659   infarct when NQDI-1 was administered alone compared to control.

660

661   Attenuation of ASK1 by the specific inhibitor NQDI-1 produces a protective role in the heart

662   compared to Sunitinib treatment alone. In this study we have focused on the ASK1 Thr^{845}

663   phosphorylation site, however, in future studies it would be interesting to investigate the

664   ASK1 Ser^{967} phosphorylation, as phosphorylation at this site has been linked to

665   cytoprotection (Kim et al. 2009). Another interesting aspect would be to identify if NQDI-1 is

666 able to inhibit the ASK1 homologue ASK2 as indicated by Nomura *et al.* 2013 (Nomura et al.

667 2013) and establish the K_i and IC_{50} values are.

668

669 ASK1 is also a key mediator of apoptotic signalling (Hattori et al. 2009; Ichijo et al. 1997) and

670 the team of Huynh et al. 2011 investigated the expression of ASK1 in tumours lysates from

671 mice bearing 06-0606 tumours treated with 40 mg/kg/day Sunitinib for 11 days (Huynh et al.

672 2011). Their study showed a significant increase in total ASK1 levels after Sunitinib

673 treatment compared to vehicle treated 06-0606 tumour expressing mice, however, in our

674 study the expression of total ASK1 levels in left ventricular coronary tissue did not differ in

675 hearts perfused with Sunitinib compared to vehicle treated hearts.

676

4.3.2. MKK7

678 We assessed MKK7 expression level at both transcriptional and post-translational levels in

679 hearts treated with Sunitinib with and without the upstream ASK1 inhibitor NQDI-1. There

680 was a strong tendency for MKK7 mRNA expression to be decreased in Sunitinib perfused

681 hearts compared to control, but without significance. ~~There was a strong tendency of~~

682 ~~decreased MKK7 mRNA expression in Sunitinib perfused hearts compared to control, but~~

683 ~~without significance.~~ The expression of MKK7 mRNA was increased significantly in Sunitinib

684 and NQDI-1 co-treated hearts compared to Sunitinib perfused hearts, and NQDI-1 solo

685 treatment did not alter the MKK7 mRNA expression compared to control hearts (Fig 3). The

686 p-MKK7 level was significantly decreased in Sunitinib treated hearts when compared to

687 control, and the p-MKK7 decrease is attenuated by NQDI-1 co-administration. The p-MKK7

688 levels are significantly decreased in Sunitinib and NQDI-1 co-treatment and NQDI-1 solo-

689 treatment hearts compared to control hearts (Fig 4B). We believe that this is the first study to

690 investigate the expression of MKK7 after Sunitinib therapy.

691

692 MKK7 has a vital role in protecting the heart from hypertrophic remodelling and

693 cardiomyocytes apoptosis during stress and therefore the transition into heart failure

25

694 (Mitchell et al. 2006). Studies by Liu *et al*. 2011 revealed the importance of MKK7 by

695 demonstrating that deprivation of MKK7 in cardiomyocytes provokes heart failure in mice

696 when exposed to pressure overload (Liu et al. 2011)**.** In addition to this, it has been shown

697 that inhibition and specific knockout of MKK7 increases the sensitivity of hepatocytes to

698 tumour necrosis factor alpha-induced apoptosis (Jia et al. 2015). Treatment with Sunitinib in

699 rat hearts in the current study down regulates both mRNA and phosphorylated protein levels

700 of MKK7. These studies suggest an important role of MKK7 in the maintenance of heart

701 homeostasis and expression of associated genes are important during cardiac hypertrophy

702 and heart failure.

703

704 MKK7 contains an ATP binding domain which could be inhibited by the ATP analogue,

705 Sunitinib (Roskoski 2007; Shukla et al. 2009; Song et al. 2013). It is therefore possible that

706 Sunitinib has an inhibitory effect on the MKK7/JNK signal transduction pathway. With

707 reduced MKK7 activity demonstrating both an incline towards cardiomyocyte damage and a

708 reversal of anti-tumour effects of chemotherapy, it would be interesting to assess both the

709 changes in expression levels and levels of phosphorylated MKK7 during Sunitinib treatment

710 affecting the heart in future studies. It may therefore be possible to identify a link between

711 MKK7 expression and tyrosine kinase inhibitor-induced cardiotoxicity.

712

713 Co-treatment of Sunitinib and NQDI-1 increases the level of p-MKK7 to levels compared to

714 Sunitinib treated hearts, restoring them almost back to the p-MKK7 levels observed in

715 control hearts. This could suggest that phosphorylated MKK7 is required to maintain at a

716 stable level to prevent damage to the heart. To illustrate this upregulation of transforming

717 growth factor beta has been found in compensatory hypertrophy, myocardial remodelling

718 and heart failure (Rosenkranz 2004). However, if MKK7 is removed entirely from the JNK

719 pathway cardiomyocyte damage ensues (Liu et al. 2011). Tang *et al*. 2012 demonstrated

720 that by causing an upregulation of MKK7 in hepatoma cells with the treatment with Alpinetin

721 it was possible to arrest cells in the G_0/G_1 phase of the cell cycle. However, by inhibiting

722 MKK7 the anti-tumour effect of the delta-opioid receptor agonist cis-diammined

723 dichloridoplatinum was reversed (Tang et al. 2012). This suggests that by agonising MKK7 it

724 may be possible to both enhance anti-cancer properties of chemotherapy agents, as well as

725 limiting apoptosis as the cell cycle is arrested.

726

4.3.3. JNK

728 Administration of Sunitinib decreased the p-JNK levels significantly when compared to

729 control hearts, and this decrease was abrogated with NQDI-1 co-treatment. Treatment with

730 NQDI-1 however also decreased the p-MKK7 levels when compared to control perfused

731 hearts (Fig 4C).

732

733 Many JNK knock out models have been used to determine the role of JNK in the

734 development of cardiac dysfunction. Kaiser *et al.* 2005 demonstrated the importance of JNK

735 in ischemia-reperfusion injury. They showed that a reduction in JNK activity in the heart

736 resulted in a reduced level of cardiac injury and cellular apoptosis. The same study

737 demonstrated an increase in JNK activity by using mouse models overexpressing MKK7 in

738 the heart, and this caused a significant protection against ischemia-reperfusion injury (Kaiser

739 et al. 2005). This highlights the complexity of JNK signalling. In this study, a significant

740 decrease in JNK phosphorylation was identified when hearts were treated with Sunitinib. In

741 addition, NQDI-1 treatment had a tendency to increase in JNK phosphorylation. It has been

742 established that a reduction in JNK activation is associated with cardiac hypertrophy and

743 cardiovascular dysfunction (Pan et al. 2014). The reduction in JNK activation caused by the

744 treatment of Sunitinib could also explain the increased infarct size and irregularities found in

745 the haemodynamic data.

746

747 Our study contradicts some previous studies, for example Wang *et al.* 1998 investigated the

748 role of MKK7 in cardiac hypertrophy in neonatal myocytes (Wang et al. 1998). Transgenic

749 neonatal rat cardiomyocytes expressing wild type MKK7 and a constitutively active mutant of

27

750 MKK7 were created. This study demonstrated JNK specific activation by MKK7 and showed

751 the key role of the JNK pathway in cardiac hypertrophy as cells infected with the

752 constitutively active form of MKK7 adopted characteristic features of myocardial stress

753 (Wang et al. 1998).

754

755 The study by Fenton et al 2010 looked at the expression of JNK in papillary cancer cells with

756 RET/PTC1 rearrangement treated with Sunitinib (Fenton et al. 2010). Sunitinib inhibited

757 proliferation of these RET/PTC1 subcloned papillary cancer cells, and furthermore inhibited

758 the JNK phosphorylation in the cytoplasm of the papillary cancer cells. In our study the

759 expression of p-JNK levels in left ventricular coronary tissue was also reduced in hearts

760 perfused with Sunitinib compared to vehicle treated hearts.

761

762 In summary, Sunitinib administration resulted in significant reduction in p-ASK1, p-MKK7 and

763 p-JNK levels, whilst NQDI-1 co-administration counteracted this increase. It is worth noting

764 that MKK7 is activated through phosphorylation at a special site at the C-terminal kinase

765 domain core called the "Domain for Versatile Docking" (DVD), which includes serine-

766 threonine sites (Wang et al. 2007). Sunitinib does not not discriminate between inhibition of

767 tyrosine kinases or serine-threonine kinases (Karaman et al. 2008). Therefore, Sunitinib

768 might potentially inhibiting serine-threonine kinases, however, it is much more likely that

769 Sunitinib inhibits tyrosine kinases as expected, resulting in the downstream inhibition of

770 ASK1, which then results in a downstream inhibition of MKK7 and JNK. However, more

771 detailed investigations into the pathway involvement are required to fully elucidate the

772 intracellular signalling pathways. In addition, the increase in p-ASK1, p-MKK7 and p-JNK

773 levels that we observe in the presence of both Sunitinib and NQDI-1, when compared to

774 Sunitinib perfused hearts, could be due to the fact that we only assessed the

775 phosphorylation of ASK1 at Thr^{845}, however, it is possible that NQDI-1 blocks both Ser^{83} and

776 Thr^{845} of ASK1, and further investigations of phosphorylation activity at both sides could

777 clarify this issue. This altered pattern in ASK1/MKK7/JNK pathway phosphorylation suggests

778 that Sunitinib has a direct effect on part of the ASK1/MKK7/JNK pathway. In summary,

779 Sunitinib administration resulted in significant reduction in p-ASK1, p-MKK7 and p-JNK

780 levels, whilst NQDI-1 co-administration counteracted this increase. It is possible that NQDI-1

781 blocks both Ser[83] and Thr[845], and that the increase in p-ASK1, p-MKK7 and p-JNK levels that

782 we observe in the presence of both Sunitinib and NQDI-1 when compared to Sunitinib

783 perfused hearts is due to fact only phosphorylation of ASK1 at Thr[845] was assessed. This

784 altered pattern in ASK1/MKK7/JNK pathway phosphorylation suggests that Sunitinib has a

785 direct effect on part of the ASK1/MKK7/JNK pathway.

786

787 **4.4. The anti-cancer properties of Sunitinib were enhanced by NQDI-1 treatment**

788 It is well established that Sunitinib achieves anti-tumour effects by inhibiting tyrosine kinases,

789 which have been over-expressed in cancer cells (Krause and Van Etten 2005). Sunitinib has

790 previously been shown to directly inhibit the survival and proliferation of a variety of cancer

791 cells, including leukaemia cells (Ilyas 2016).

792

793 We demonstrated a dose dependant decline in the cell viability of HL60 cells when treated

794 with Sunitinib (Fig 5A). This produced an IC_{50} value of 6.16 µM. Our results are in line with

795 existing data on the anti-proliferative effect of Sunitinib on HL60 cells. Sunitinib has

796 previously been shown to reduce the level of HL60 cell survival in a dose dependant manor

797 using a cell-titre blue reagent proliferation assay. This produced an IC_{50} value of 5.7 µM after

798 48 hrs of Sunitinib treatment (Ilyas 2016). Another group performed an MTT assay on a

799 variety of acute myelogenous leukaemia cell lines and found Sunitinib to have an IC_{50} values

800 between 0.007-13 µM (Hu et al. 2008).

801

802 To investigate the anti-proliferative effect of inhibition of the MKK7 pathway, HL60 cells were

803 treated with Sunitinib in co-treatment with NQDI-1 and NQDI-1 alone (Fig 5A-B). NQDI-1 is a

804 selective inhibitor for ASK1, the upstream regulator of MKK7. Previous studies have shown

805 ASK1 to have a crucial role in a variety of organ systems. However, ASK1 has also been

806 shown to promote tumorigenesis in gastric cancer and promote the proliferation of cancer

807 cells in skin cancer (Hayakawa et al. 2012; Iriyama et al. 2009).

808

809 Inhibition of ASK1 with compound K811 has been shown to prevent cell proliferation in

810 gastric cancer cell lines and reduce the size of xenograft tumours (Hayakawa et al. 2012).

811 Recently, Luo et al. 2016, investigated the involvement of ASK1 during proliferation in

812 pancreatic tumour cell line PANC-1 (Luo et al. 2016). The knock-down of ASK1 in mice with

813 pancreatic tumours reduced tumour growth, suggesting that ASK1 has an important role in

814 pancreatic tumorigenesis. The same group also demonstrated a dose-dependent inhibition

815 of the PANC-1 cell line when cells were treated with NQDI-1 at concentrations of 10 and 30

816 µM. However, the inhibition of ASK1 did not increase levels of apoptosis.

817

818 We have shown that increasing concentrations of NQDI-1 (0.2-200 µM) significantly reduce

819 the level of viable HL60 cells at 100 and 200 µM. This could suggest that ASK1 is expressed

820 at different levels in different cell types as a higher concentration was required in HL60 cells

821 compared to PANC-1 cells. Interestingly, the increasing concentrations of Sunitinib with 2.5

822 µM NQDI-1 enhanced the level of Sunitinib induced a reduction in HL60 cell proliferation.

823 The reason for this is not yet clear and further investigation into this is required.

824

825 As mentioned before NQDI-1 blocks FGFR-1 (Volynets et al. 2011). In cancer cells FGFR1

826 inhibitors have shown to elicit direct anti-tumour effects. The FGFR-1 inhibitors being

827 investigated in clinical trials for their anti-tumour qualities effecting various cancer types

828 include AZD4547, BGJ398, Debio-1347 and dovitinib (Katoh 2016). The apoptotic effect of

829 NQDI-1 we are observing in HL60 cells can therefore be a direct consequence of FGFR-1

830 inhibition.

831

832 In conclusion, our study demonstrates the potential of NQDI-1 as a valuable asset to cardiac

833 injury through the ASK1/MKK7/JNK transduction pathway, which could potentially lead to

834 development of cardioprotective adjunct therapy during drug-induced cardiac injury. NQDI-1

835 was observed to be cardioprotective as it reduced the Sunitinib-induced infarct size, and

836 addition it increased the apoptotic effect of Sunitinib in HL60 cells. This could indicate that

837 NQDI-1 - or an optimised derivative - could potentially be used as cardioprotective adjunct

838 therapy in e.g. Sunitinib treated leukaemia patients, which would not only protect the

839 patients' hearts but also boost the anti-cancer abilities of Sunitinib.

840

841

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Conflict of interest

All authors have no conflict of interest to declare.

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Figure legends

Figure 1: Infarct to whole heart ratio assessment. The hearts were drug perfused with Sunitinib and/or NQDI-1 for 125 min in an isolated Langendorff heart model. This establishes that Sunitinib-induced cardiotoxicity is reduced by ASK1 inhibitor NQDI-1. Groups: Control, Sunitinib (1 µM), Sunitinib (1 µM) and NQDI-1 (2.5 µM), and NQDI-1 (2.5 µM) (n=6 per group). Groups were assessed for statistical significance at each time point using one-way ANOVA. Control versus Sunitinib (***=$P<0.001$), Control versus Sunitinib+NQDI-1 (**=$P<0.01$), Control versus NQDI-1 (*=$P<0.05$), or Sunitinib vs Sunitinib+NQDI-1 (###=$P<0.001$).

Figure 2: Cardiotoxicity linked microRNAs expression. The effect of Sunitinib (1 µM) and the co-administration of ASK1 inhibitor, NQDI-1 (2.5 µM), on the expression of cardiotoxicity linked microRNAs following 125 minute drug perfusion in an isolated heart Langendorff model. The qRT-PCR results are shown as the ratio of target microRNA normalised to U6 with control group microRNA ratio set as 1 of microRNAs A) miR-1, B) miR-27a, C) miR-133a and D) miR-133b. The ratio of target microRNA normalised to U6 is presented on a log scale. Groups: Control (n=6 for miR-1, miR-27a and miR-133a; n=5 for miR-133b), Sunitinib

870 (1 µM) (n=6 for miR-1 and miR-27a; n=5 for miR-133a and miR-133b), Sunitinib (1 µM) and

871 NQDI-1 (2.5 µM) (n=6 for miR-1, miR-27a, miR-133a and miR-133b), and NQDI-1 (2.5 µM)

872 (n=4 for miR-1, miR-27a, miR-133a and miR-133b). Groups were assessed for statistical

873 significance at each time point using one-way ANOVA. Control versus Sunitinib

874 (***=P<0.001), Control versus Sunitinib+NQDI-1 (*=P<0.05, **=P<0.01), Control versus

875 NQDI-1 (**=P<0.01, ***=P<0.001), or Sunitinib vs Sunitinib+NQDI-1 (#=p<0.05,

876 ###=P<0.001).

877

878 **Figure 3:** MKK7 mRNA expression levels. The qRT-PCR assessment of MKK7 mRNA

879 expression levels in an isolated heart Langendorff model. The qRT-PCR results are shown

880 as the ratio of MKK7 mRNA normalised to GAPDH with control group ratio set as 1. Groups:

881 Control (n=5), Sunitinib (1 µM) (n=6), Sunitinib (1 µM) and NQDI-1 (2.5 µM) (n=3), and

882 NQDI-1 (2.5 µM) (n=3). Groups were assessed for statistical significance at each time point

883 using one-way ANOVA. Control versus Sunitinib, Control versus Sunitinib+NQDI-1, Control

884 versus NQDI-1, or Sunitinib vs Sunitinib+NQDI-1 (#=p<0.05).

885

886 **Figure 4:** ASK1/MKK7/JNK pathway western blot assessment. A) p-ASK1, B) p-MKK7, and

887 C) p-JNK phosphorylation levels in an isolated heart Langendorff model. Sorbitol was

888 included as a positive control in p-MKK7 Western blot analysis (n=4). Groups: Control (n=6

889 for p-ASK1; n=4 for p-MKK7 and p-JNK), Sunitinib (1 µM) (n=5 for p-ASK1; n=4 for p-MKK7

890 and p-JNK), Sunitinib (1 µM) and NQDI-1 (2.5 µM) (n=6 for p-ASK1; n=4 for p-MKK7 and p-

891 JNK) and NQDI-1 (2.5 µM) (n=5 for p-ASK1; n=4 for p-MKK7 and p-JNK). Groups were

892 assessed for statistical significance at each time point using one-way ANOVA. Control

893 versus Sunitinib (***=P<0.001), Control versus Sunitinib+NQDI-1 (*=p<0.05), Control versus

894 NQDI-1 (**=P<0.01; ***=P<0.001), or Sunitinib vs Sunitinib+NQDI-1 (#=p<0.05;

895 ###=P<0.001).

896

Figure 5: HL60 cell viability. HL60 cells (10^5 cells/ml) were incubated for 24 hours with control or with increasing concentrations of A) Sunitinib (0.1 – 10 μM) or Sunitinib (0.1 – 10 μM) + NQDI-1 (2.5 μM) or B) NQDI-1 (0.2 μM – 200 μM). Groups were assessed for statistical significance at each time point using one-way ANOVA. Control versus Sunitinib (*=$P<0.05$ and ***=$P<0.001$), Control versus NQDI-1 (*=$P<0.05$ and ***=$P<0.001$), or Sunitinib vs Sunitinib+NQDI-1 (###=$p<0.001$). **Figure 1:** Infarct to whole heart ratio assessment. The hearts were drug perfused with Sunitinib and/or NQDI-1 for 125 min in an isolated Langendorff heart model. This establishes that Sunitinib-induced cardiotoxicity is reduced by ASK1 inhibitor NQDI-1. Groups: Control, Sunitinib (1 μM), Sunitinib (1 μM) and NQDI-1 (2.5 μM), and NQDI-1 (2.5 μM) (n=6 per group). Groups were assessed for statistical significance at each time point using one-way ANOVA. Control versus Sunitinib (red ***=$P<0.001$), Control versus Sunitinib+NQDI-1 (green **=$P<0.01$), Control versus NQDI-1 (blue *=$P<0.05$), or Sunitinib vs Sunitinib+NQDI-1 (purple $$$=$P<0.001$).

Figure 2: Cardiotoxicity linked microRNAs expression. The effect of Sunitinib (1 μM) and the co-administration of ASK1 inhibitor, NQDI-1 (2.5 μM), on the expression of cardiotoxicity linked microRNAs following 125 minute drug perfusion in an isolated heart Langendorff model. The qRT-PCR results are shown as the ratio of target microRNA normalised to U6 with control group microRNA ratio set as 1 of microRNAs A) miR-1, B) miR-27a, C) miR-133a and D) miR-133b. The ratio of target microRNA normalised to U6 is presented on a log scale. Groups: Control (n=6 for miR-1, miR-27a and miR-133a; n=5 for miR-133b), Sunitinib (1 μM) (n=6 for miR-1 and miR-27a; n=5 for miR-133a and miR-133b), Sunitinib (1 μM) and NQDI-1 (2.5 μM) (n=6 for miR-1, miR-27a, miR-133a and miR-133b), and NQDI-1 (2.5 μM) (n=4 for miR-1, miR-27a, miR-133a and miR-133b). Groups were assessed for statistical significance at each time point using one-way ANOVA. Control versus Sunitinib (red ***=$P<0.001$), Control versus Sunitinib+NQDI-1 (green *=$P<0.05$, **=$P<0.01$), Control versus NQDI-1 (blue **=$P<0.01$, ***=$P<0.001$), or Sunitinib vs Sunitinib+NQDI-1 (purple $=$p<0.05$, $$$=$P<0.001$).

Figure 3: MKK7 mRNA expression levels. The qRT-PCR assessment of MKK7 mRNA expression levels in an isolated heart Langendorff model. The qRT-PCR results are shown as the ratio of MKK7 mRNA normalised to GAPDH with control group ratio set as 1. Groups: Control (n=5), Sunitinib (1 µM) (n=6), Sunitinib (1 µM) and NQDI-1 (2.5 µM) (n=3), and NQDI-1 (2.5 µM) (n=3). Groups were assessed for statistical significance at each time point using one-way ANOVA. Control versus Sunitinib, Control versus Sunitinib+NQDI-1, Control versus NQDI-1, or Sunitinib vs Sunitinib+NQDI-1 (purple $=p<0.05).

Figure 4: ASK1/MKK7/JNK pathway western blot assessment. A) p-ASK1, B) p-MKK7, and C) p-JNK phosphorylation levels in an isolated heart Langendorff model. Sorbitol was included as a positive control in p-MKK7 Western blot analysis (n=4). Groups: Control (n=6 for p-ASK1; n=4 for p-MKK7 and p-JNK), Sunitinib (1 µM) (n=5 for p-ASK1; n=4 for p-MKK7 and p-JNK), Sunitinib (1 µM) and NQDI-1 (2.5 µM) (n=6 for p-ASK1; n=4 for p-MKK7 and p-JNK) and NQDI-1 (2.5 µM) (n=5 for p-ASK1; n=4 for p-MKK7 and p-JNK). Groups were assessed for statistical significance at each time point using one-way ANOVA. Control versus Sunitinib (red ***=P<0.001), Control versus Sunitinib+NQDI-1 (green *=p<0.05), Control versus NQDI-1 (blue **=P<0.01; ***=P<0.001), or Sunitinib vs Sunitinib+NQDI-1 (purple $=p<0.05; $$$=P<0.001).

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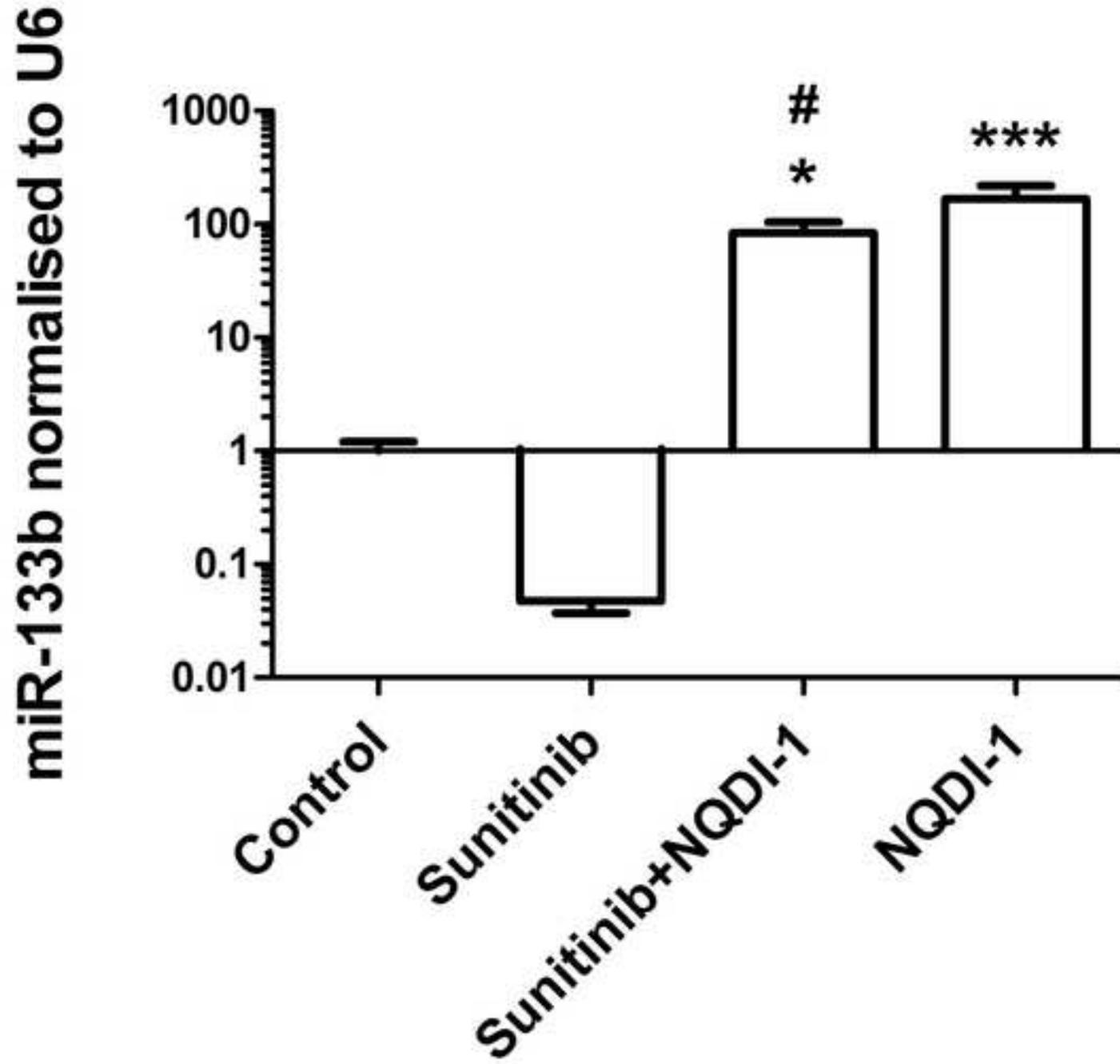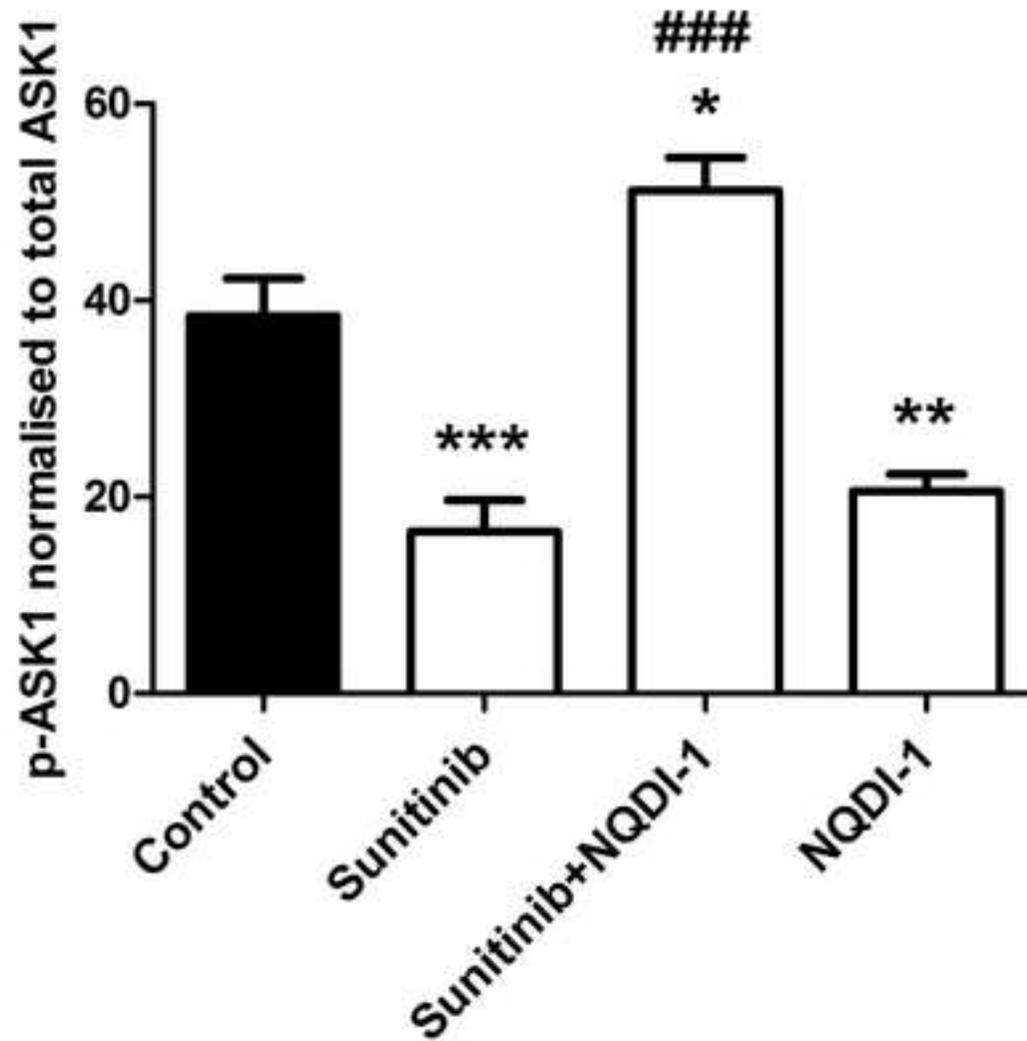1249 induced cell death by 14-3-3 proteins. Proc Natl Acad Sci U S A 96, 8511-8515.

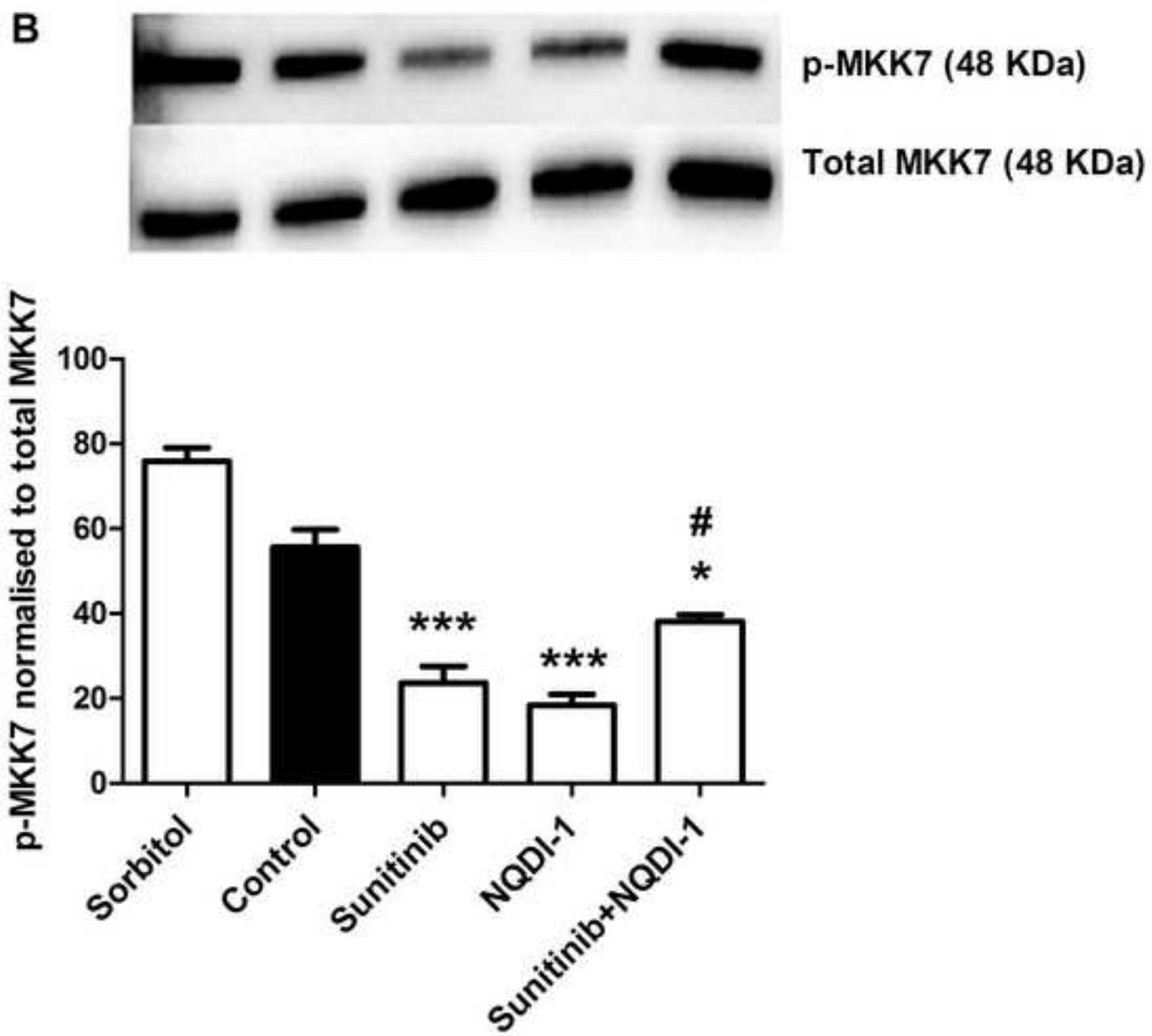1250

Figure 2B

Figure 3
Click here to download high resolution image

A   p-ASK1 (155 KDa)
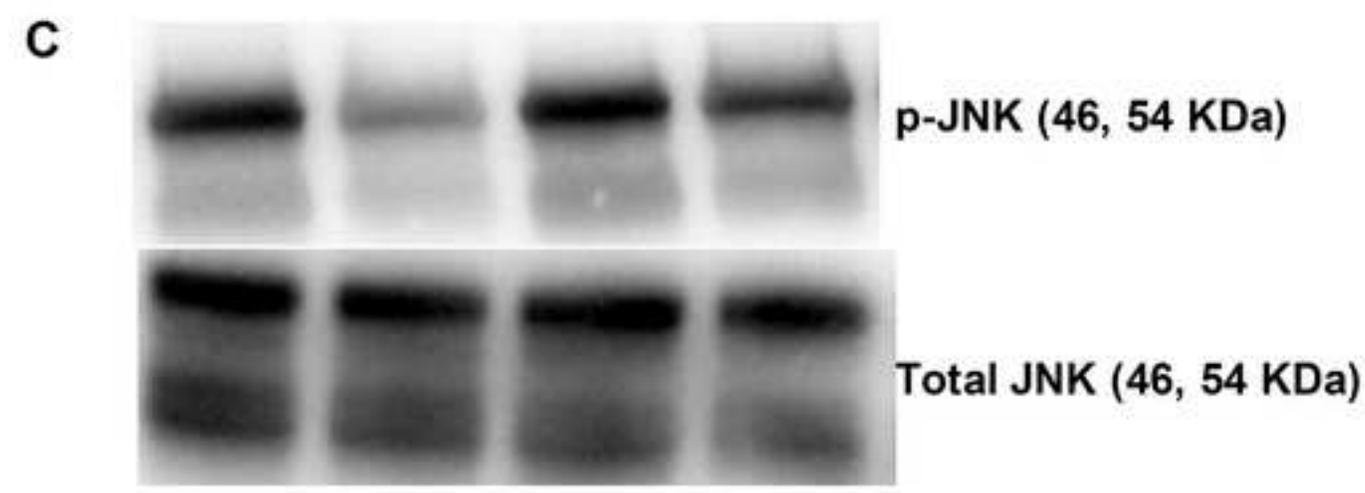
Total ASK1 (155 KDa)

B

p-MKK7 (48 KDa)

Total MKK7 (48 KDa)

C

p-JNK (46, 54 KDa)

Total JNK (46, 54 KDa)

A
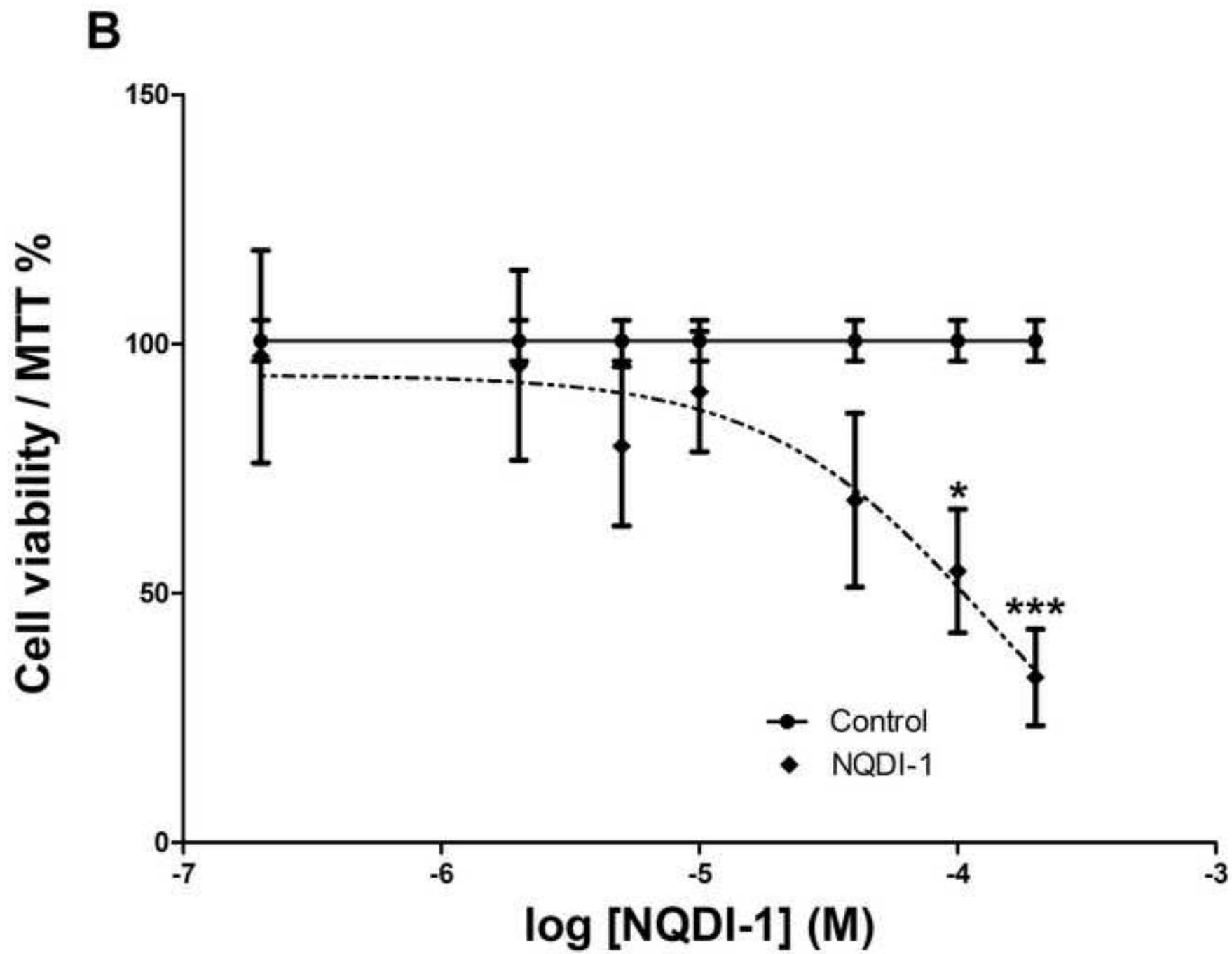
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