

1 **Specific Inhibition of GSK-3 β by Tideglusib: Potential Therapeutic Target for** 2 **Neuroblastoma Cancer Stem Cells**

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58
59 **Running title:** GSK-3 β inhibition in human neuroblastoma cancer stem cells

60 **Abstract:**

61

62 Neuroblastoma is an embryonic tumor that represents the most common extracranial
63 solid tumor in children. Resistance to therapy is attributed, in part, to the persistence of a
64 subpopulation of slowly dividing cancer stem cells (CSCs) within those tumors. Glycogen
65 synthase kinase (GSK)-3 β is an active proline-directed serine/threonine kinase, well-known to be
66 involved in different signaling pathways entangled in the pathophysiology of neuroblastoma.
67 This study aims to assess the potency of an irreversible GSK-3 β inhibitor drug, Tideglusib
68 (TDG), in suppressing proliferation, viability, and migration of human neuroblastoma cell lines,
69 as well as its effects on their CSCs subpopulation *in vitro* and *in vivo*. Our results showed that
70 treatment with TDG significantly reduced cell proliferation, viability, and migration of SK-N-SH
71 and SH-SY5Y cells. TDG also significantly inhibited neurospheres formation capability in both
72 cell lines, eradicating the self-renewal ability of highly resistant CSCs. Importantly, TDG
73 potently inhibited neuroblastoma tumor growth and progression *in vivo*. In conclusion, TDG
74 proved to be an effective *in vitro* and *in vivo* treatment for neuroblastoma cell lines and may
75 hence serve as a potential adjuvant therapeutic agent for this aggressive nervous system tumor.

76

77 **Key Words:** neuroblastoma; GSK-3 β ; Tideglusib; cancer stem cells; targeted therapy.

78 **1. Introduction:**

79

80 Neuroblastoma (NB) is a common childhood tumor that originates from embryonic
81 neural crest cells that serve as precursor cells of the sympathetic nervous system. It represents the
82 most common extracranial solid tumor among pediatric patients [1], accounting for 6% of all
83 cancer diagnoses of children (0-14 years) in the US [2]. This disease is remarkable for its varied
84 clinical outcomes, whereas some tumors spontaneously regress into mature non-malignant tissue,
85 while others progress and metastasize regardless of intense treatment measures [1, 3]. Current
86 treatment regimens include an induction radiotherapy, surgical excision of the tumor, and high-
87 dose chemotherapy regimen [4]. Even though the overall 5-year survival rate of the NB patients
88 in the US remains around 80% [2], patients with high-risk disease (stage 4, amplified MYCN) –
89 accounting for the majority of the diagnoses – suffer from a long-term survival rate below 50%
90 [5], with cure relapse and tumor recurrence seen in almost 50% of the cases [6].

91

92 The malignant recurrence of NB after complete clinical remission, as well as other solid
93 tumors, is notably attributed to the failure in the complete eradication of cancer stem cells (CSC),
94 a subpopulation of cells within the tumor bulk that possess an indefinite self-renewal ability, and
95 play an integral role in tumor initiation, progression, and recurrence [7]. One of the main
96 characteristics of CSC is their potential to resist conventional treatment regimens through the
97 development of multi-drug resistance as seen NB patients and neuroblastoma cell lines *in vitro*
98 [8, 9], which highlights the need to develop effective targeted therapy able to target this
99 subpopulation.

100

101 Glycogen Synthase Kinase 3 Beta (GSK-3 β) is an active proline-directed serine/threonine
102 kinase that plays a regulatory role in glucose metabolism, as well as other signaling pathways,
103 including cell-fate determination, cellular differentiation and cell division [10]. GSK3- β plays a
104 controversial role in cancer pathophysiology: while it plays a tumor suppressor protein by
105 activating the adenomatous polyposis coli (APC)- β -Catenin destruction complex in colon
106 cancers, recent studies investigated its potential role as a target protein in other cancers, such as
107 pancreatic adenocarcinoma and acute myeloid leukemias [11-14], suggesting non-conventional
108 mechanisms by which GSK-3 β regulates carcinogenesis.

109

110 In this study, we tested the effect of GSK-3 β inhibition by Tideglusib (TDG) on NB cell
111 lines *in vitro* and *in vivo*. Tideglusib is a well-tolerated, irreversible non-ATP competitive GSK-
112 3 β inhibitor that was clinically tested for its effect on different neurological disorders, including
113 Alzheimer's disease and progressive supranuclear palsy [15-17]. Here, we show that treating
114 human NB cell lines with TDG hinders cellular proliferation, decreases cellular survival, and
115 blocks cellular migration. Furthermore, we further focused on its effects on the CSC
116 subpopulation in NB cell lines, using a 3D-sphere formation and propagation model [18-20] over
117 4 sequential generations of neurospheres.

118

119 **2. Materials and Methods:**

120

121 **2.1. Cell Culture**

122

123 Two neuroblastoma cell lines SK-N-SH (ATCC[®] HTB-11[™], USA) [21] and SH-SY5Y

124 (ATCC[®] CRL-2266[™], USA) [22, 23], were cultured and maintained in Dulbecco's Modified
125 Eagle Media (DMEM) Ham's F12 (Sigma-Aldrich; cat #D8437), supplemented with 10% of
126 heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich; cat #F9665), 1%
127 Penicillin/Streptomycin (Biowest; cat #L0022-100) and Plasmocin[™] prophylactic (Invivogen;
128 cat #ant-mpp). Cell lines were checked using the ICLAC Database of Cross-contaminated or
129 Misidentified Cell Lines confirming they are not misidentified or contaminated. Cells were
130 incubated at 37°C in a humidified incubator containing 5% CO₂. Tideglusib (TDG) was
131 purchased from Sigma-Aldrich (Cat. # SML0339-10MG; Lot # 123M4615V and 016M4605V)
132 and reconstituted in dimethyl sulfoxide (DMSO; Amresco; cat #0231-500ML), per
133 manufacturer's instructions.

134

135 **2.2. MTT/Cell Proliferation Assay**

136

137 The anti-proliferative effects of Tideglusib (TDG) on the used cell lines were measured
138 *in vitro* using MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) (Sigma-
139 Aldrich; cat #M5655-1G) assay according to the manufacturer's instructions [24-26]. In brief,
140 cells (6×10^3 cells in 100µL full media, per well) were seeded in 3 different 96-well culture plates
141 (one for every time point: 24h, 48h and 72h) and incubated overnight at 37°C and 5% CO₂. Wells
142 were randomly distributed across different treatment conditions – 3 wells/condition (Control:
143 media only, Vehicle: Media + 0.1% DMSO, treatment groups: 5µM, 25µM, and 50µM TDG in
144 full media). At every time point, media was removed and replaced with fresh media along with
145 10µL of MTT yellow dye (5mg/mL in DMSO) per well. Afterwards, the cells were incubated for
146 4 hours, after which 100µL of the solubilizing agent was added to each well. The plates were

147 incubated overnight at room temperature, and the absorbance intensity of every well was
148 measured by the microplate ELISA reader (Multiscan EX) at 595nm. The percentage of cell
149 proliferation was presented as an optical density (OD) ratio of the treated to the untreated cells
150 (control).

151

152 **2.3. Trypan Blue/Cell Viability Assay**

153

154 The effects of TDG on cell viability was measured *in vitro* using the trypan blue assay
155 [27]. In brief, SH-SY5Y and SK-N-SH cells (60×10^3 cells/well in 500 μ L full media) were
156 seeded in 3 different 12-well culture plates (one for each time point: 24h, 48h, and 72h). Cells
157 were incubated overnight at 37 \square and 5% CO₂. Wells were randomly distributed, in duplicate,
158 across different treatment conditions (Control: media only, Vehicle: Media + 0.1% DMSO,
159 treatment groups: 5 μ M, 25 μ M, and 50 μ M TDG in full media). At every time point, cells from
160 each well were treated with Trypsin and viable cells were counted on a hemocytometer under an
161 inverted light microscope after staining cell suspension with Trypan blue. The percentage of cell
162 viability was determined as a ratio of viable cells counted in treated to untreated conditions.

163

164 **2.4. Wound Healing Assay:**

165

166 Wound healing assay was used to assess the effects of TDG on cell migration. SH-SY5Y
167 and SK-N-SH cells (5×10^5 cells/well) were seeded in 6-well culture plates and incubated at 37 \square
168 and 5% CO₂ until they reached 90% confluence. Cells were then treated with 5mg/mL of
169 Mitomycin C (Sigma-Aldrich; cat #M0503-5x2MG) for 1 hour to block cellular proliferation. A

170 sterile 200 μ L pipet was used to create two scratches per well in each monolayer. Cells were then
171 washed twice with dulbecco's phosphate buffered saline (D-PBS) (Sigma-Aldrich; cat #D8537-
172 500ML) to remove cell debris. The remaining cells were distributed into three conditions:
173 Control (Full media), Vehicle (full media + 0.1% DMSO), and treatment (25 μ M TDG in full
174 media). Pictures of the scratches were taken using an inverted light microscope at the following
175 time points: 0h, 6h, 12h, 24h, and 48h. The distance travelled by the cells was measured using
176 Zen Microscope Software (Zen 2.3).

177

178 **2.5. 3D culture and Sphere-Formation Assay**

179

180 The sphere formation assay was used as previously reported by our lab [19, 20]. In brief,
181 single SH-SY5Y and SK-N-SH cell suspensions (10×10^3 cells/well) were seeded in
182 MatrigelTM/serum free DMEM Ham's F-12 (1:1). The solution was then plated gently around the
183 rim of individual wells of 24-well culture plate (50 μ L per well). The MatrigelTM (Corning Life
184 Sciences; cat #354230) was allowed to solidify for 1 hour at 37°C in a humidified incubator.
185 Wells were randomly assigned to control and treatment conditions (control, 0.1 μ M, 1 μ M, and
186 5 μ M TDG). 500 μ L/well of complete media (+5% FBS) was gently added to the center of each
187 well and changed regularly every 3 days. At day 9 after plating, spheres were pictured and
188 counted. SH-SY5Y spheres were further harvested for propagation.

189

190 For spheres propagation, the medium was aspirated from the center of the wells. The
191 MatrigelTM was digested with 0.5mL of 1mg/mL Dispase II solution (ThermoFisher; cat #17105-
192 041) dissolved in serum-free DMEM Ham's F-12 for 60 minutes at 37°C in a humidified

193 incubator. SH-SY5Y and SK-N-SH spheres were collected and incubated in warm trypsin at
194 37°C for 5 minutes; trypsin was used to dissociate spheres into single cell suspensions. Cells
195 were counted and re-seeded at 2×10^3 cells/well. The propagation of the spheres was repeated
196 over 4 generations. The sphere forming unit (SFU) was calculated as the ratio of the number of
197 spheres counted at day 9 to the number of cells originally seeded. Bright field images of the
198 spheres were obtained using Axiovert microscope from Zeiss at 5× magnification.

199

200 **2.6. Western Blotting Analysis**

201

202 For 2D, SH-SY5Y cells were cultured in 6-well plates (5×10^5 cells/well) until they
203 reached 70% confluency. Three wells were then treated with 25µM TDG for 48 hours, while the
204 remaining wells were taken as control. The plates were then washed with ice-cold D-PBS to
205 remove any residual media. Similarly, for 3D, SH-SY5Y spheres (G1) were treated with 5µM
206 TDG while others were taken as control. G1 spheres were collected and washed with ice-cold D-
207 PBS. Cells/spheres were treated and lysed using radioimmunoprecipitation (RIPA) buffer (0.1%
208 sodium dodecyl sulfate (SDS) (v/v), 0.5% sodium deoxyolate (v/v), 150mM sodium chloride
209 (NaCl), 100mM EDTA, 50mM Tris-HCl (pH=8), 1% Tergitol (NP40) (v/v), 1mM PMSF, and
210 protease and phosphatase inhibitors (one tablet of each in 10mL buffer, Roche, Germany)),
211 scraped off the plates, transferred into micro-centrifuge tubes and incubated on ice for 30
212 minutes. Sonication was used to maximize the protein yield. Lysates were then centrifuged at
213 13,600 rpm for 15 minutes at 4°C, to pellet the cell debris.

214

215 Protein concentrations of the collected supernatants were quantified using DC™ Protein

216 Assay (Bio-Rad). For immunoblotting, 50µg of proteins were electrophoresed in 8% or 12%
217 polyacrylamide gel and then transferred to PVDF membranes (Bio-Rad Laboratory, CA, USA)
218 overnight. Membranes were blocked with 5% bovine serum albumin (BSA) (v/v) (Amresco; cat
219 #0332-100G) for 2 hours and blotted at 4°C overnight with primary antibodies as follows: rabbit
220 anti-phospho-GSK-3β (Ser9) (5B3) (1/500 dilution; Cell Signaling; cat #9323), rabbit anti-GSK-
221 3β (1/1000 dilution; Cell Signaling; cat #27C10), and mouse anti-GAPDH (1/5000 dilution;
222 Novus Biologicals; cat #NB300-221). The next day, membranes were washed and incubated at
223 room temperature for 2 hours with the appropriate HRP-conjugated secondary antibodies as
224 follows: mouse anti-rabbit (1/1000 dilution; Santa Cruz; cat #sc-2357) and mouse IgGκ BP
225 (1/1000 dilution; Santa Cruz; cat #sc-516102). Finally, bands were detected using Lumi-Light
226 Western Blotting Substrate (Roche; cat #12015200001) and visualized using autoradiography.
227 Band intensities were digitized and analyzed using ImageJ software.

228

229 **2.7. Mouse Neuroblastoma Xenografts**

230

231 This study was approved by the Institutional Animal Care and Utilization Committee
232 (IACUC) of the American University of Beirut. Neuroblastoma xenografts were generated using
233 mouse SH-SY5Y cells. Cells were injected at a concentration of 1.2×10^6 cells in 100µL total
234 volume of cells and Matrigel™ (1:1) using a 27 G needle subcutaneously, into the flanks of
235 NOD-SCID male mice (6-8 weeks old) [28]. Once palpable tumor (approximate size 1mm^3) was
236 detected, mice were intraperitoneally injected 3 times a week for 2 weeks with 20mg/kg TDG or
237 vehicle only (Lipofundin/DMSO) and tumor volumes were measured every 3 days by direct
238 physical measurements using a digital caliper (Model DC150-S) to determine tumor size and

239 expansion. Mice weight was monitored at the initiation of the experiment and at the time of
240 sacrifice. The following formula for volume assessment was applied: $V = (3.14/6) \times L \times W \times H$;
241 where V is the tumor volume in mm^3 , L is the tumor length in mm, W is the tumor width in mm,
242 and H is the tumor height in mm. Measurements were carried out until the termination of the
243 experiment. Data represent an average of n=3 mice. The data are reported as mean \pm SEM.

244

245 **2.8. Data Analyses**

246

247 Statistical analysis was performed using GraphPad Prism 7 software. The significance of
248 the data was determined using proper statistical tests, including the student *t*-test and the two-
249 way ANOVA statistical test, followed by multiple comparisons using Bonferroni post-hoc
250 analysis. P-values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were labeled significant, highly
251 significant and very highly significant, respectively.

252

253 **3. Results:**

254

255 **3.1. GSK-3 β mRNA Expression Patterns in Human Neuroblastoma Tissues**

256

257 In our study, we first aimed to assess the expression pattern of *GSK-3 β* gene in human
258 neuroblastoma tumor tissues as compared to other body cancer tissues. For this, we surveyed a
259 publicly available dataset (Neale Multi-cancer Statistics, 60 samples; data retrieved from
260 Oncomine.org) encompassing human tumor tissues from different organs. mRNA expression
261 analysis revealed high expression of *GSK-3 β* gene among neuroblastoma tissues relative to other

262 organ specific tumor tissues in three out of four probes of the dataset (**Fold change = 1.639; $p =$**
263 **$4.06E-4$**) (**Fig 1 and Supp Fig 1**).

264

265 **3.2. Tideglusib decreases cell viability and cell proliferation of human NB cell lines**

266

267 The effect of TDG on the cellular proliferation and cellular viability of human
268 neuroblastoma cell lines, SK-N-SH and SH-SY5Y, was assessed *in vitro* using the MTT Assay
269 (**Fig 2A and 2B**) and the Trypan Blue assay, respectively (**Fig 2C and 2D**). TDG significantly
270 inhibited the proliferative ability of SH-SY5Y and SK-N-SH cell lines in a dose-dependent
271 manner (two-way ANOVA showed significant effect for treatment $p < 0.0001$, for both cell lines).
272 TDG treatment of 25 μ M achieved nearly a 50% inhibitory effect on both cell lines, after 72h
273 (**Fig 2A and 2B**). In addition, for further validation, we saw a significant effect of TDG
274 treatment on cell viability using the trypan blue exclusion assay. At 72h of treatment with 25 μ M
275 of TDG, there was a significant decrease in the number of viable cells in culture, for both SH-
276 SY5Y and SK-N-SH cell lines (**Fig 2C and 2D**).

277

278 **3.3. Tideglusib inhibits cell migration of human NB cells *in vitro***

279

280 Following that, we assessed the effect of TDG on cellular migration, the main feature that
281 underlie cancer spread and metastasis. This was done using a wound healing assay on both cell
282 lines. Mitomycin C was used to block cell proliferation. In untreated conditions, both cell lines
283 were able to migrate through and close the wounds within 48 hours. Under 25 μ M treatment with
284 TDG, the wound made in SH-SY5Y and SK-N-SH monolayers remained patent by 60% and

285 70% respectively (**Fig 3**). This shows that TDG treatment is effective in impeding the migrative
286 ability of neuroblastoma cell lines in culture.

287

288 **3.4. Tideglusib reduces the sphere-forming ability of SH-SY5Y and SK-N-SH cells**

289

290 Single cell suspensions of SH-SY5Y were cultured under non-adherent conditions in
291 Matrigel™ for 14 days. Sphere forming ability was monitored daily using an inverted light
292 microscope, and pictures were taken to keep track of the size and shape of neurospheres. The
293 sphere formation assay was used as a functional assay to study the stem/progenitor cells
294 subpopulation within SH-SY5Y cell line. Treating cells with TDG after seeding the cells in
295 Matrigel™ significantly decreased the percentage of SFUs in a dose dependent manner (one-way
296 ANOVA, $p=0.0037$) (**Fig 4A and 4B**), as well as the average sphere volume (one-way ANOVA,
297 $p<0.001$) (**Fig 4C**). Notably, inhibitory effects of TDG were achieved at lower concentration in
298 3D assay compared to functional assays on cellular monolayers. To further validate our results,
299 we performed the spheres formation assay on SK-N-SH cell lines. Effect of TDG was consistent
300 with that observed with SH-SY5Y where a decrease in the percentage of SFUs at G1 spheres of
301 SK-N-SH was observed in a dose dependent manner (one-way ANOVA, $p<0.001$) (**Supp Fig**
302 **2A**).

303

304 **3.5. Tideglusib inhibits the sphere self-renewal ability by targeting an enriched population** 305 **of SH-SY5Y and SK-N-SH cancer stem/progenitor cells**

306

307 One of the main characteristics of CSCs is their self-renewal ability, largely responsible

308 of cancer recurrence. To study the effect of TDG on this characteristic, we propagated SH-SY5Y
309 and SK-N-SH spheres over multiple generations, wherein the cells taken from one generation of
310 spheres were isolated into single cell suspensions and seeded again under non-adherent
311 conditions. Consecutive generational propagations of those spheres are thought to enrich the
312 cancer stem/progenitor cells subpopulation, by emphasis on their ability of anchorage-
313 independent growth [29]. The experimental design and results of three independent experiments
314 are shown in **Fig 5** for SH-SY5Y and in **Supp Fig 2B** for SK-N-SH. Noteworthy, treating the G4
315 spheres, which acquired an enriched stem/progenitor subpopulation of cells, with 5 μ M of TDG
316 significantly decreased the percentage of SFUs by around 95% for SH-SY5Y cells (student
317 independent *t*-test, $p < 0.001$, **Supp Fig 3A**) and 80% for SK-N-SH cells (student independent *t*-
318 test, $p < 0.001$, **Supp Fig 3B**).

319
320 For SH-SY5Y cells, we decided to test the self-renewal ability of the cells by propagating
321 the same spheres, into two new conditions: control and treated. We noticed that after a single
322 exposure to treated conditions at G1, the SFU significantly dropped to 1.13% compared to 6.21%
323 in control conditions (student independent *t*-test, $p < 0.0001$) (**Fig 5**). However, once propagated
324 into normal conditions again, the cells successfully regain their self-renewal ability (SFU =
325 4.73% at G2). According to our data, it takes two treatment regimens in two generations to
326 completely abolish the self-renewal ability of the spheres, i.e. single cell suspensions from
327 spheres previously treated in two generations fail to form any more spheres after propagation
328 (**Fig 5**).

329

330 **3.6. Tideglusib inhibits GSK-3 β at protein levels**

331

332 To validate the direct effect of TDG on its respective target GSK-3 β , we used western
333 blotting in order to detect differences in protein expression between the cellular lysates of treated
334 (25 μ M of TDG) and non-treated SH-SY5Y cells and G1 spheres. GSK-3 β inhibition by TDG
335 was established by monitoring the levels of expression of the inhibited form of GSK-3 β ,
336 phosphorylated at Serine 9 (p-GSK-3 β Ser 9). Treating cells with TDG significantly increased
337 the expression of p-GSK-3 β (Ser 9) in SH-SY5Y cell lines by around 2.62 times ($p=0.0102$) as
338 compared to the control group (**Supp Fig 4A**), signifying GSK-3 β inhibition. Besides, treating
339 SH-SY5Y G1 spheres with TDG significantly increased the expression of p-GSK-3 β (Ser 9) by
340 around 1.15 times ($p=0.0445$) as compared to the control group (**Supp Fig 4B**).

341

342 **3.7. Tideglusib inhibits neuroblastoma growth *in vivo***

343

344 Lastly, we assessed the potential effect of TDG on neuroblastoma tumor growth *in vivo*.
345 We injected NOD \times SCID mice with SH-SY5Y cells subcutaneously generating neuroblastoma
346 xenografts. The average weight of the mice was monitored throughout the experiment and was
347 maintained within a normal range during the study, signifying that TDG treatment was well
348 tolerated (**Fig 6A**). Treatment with TDG (20mg/kg TDG) resulted in significant inhibition of
349 tumor growth in SH-SY5Y injected mice which was reflected by the reduction in the volume of
350 tumors after 15 days of treatment (**Fig 6B**). These results indicate that TDG significantly reduces
351 neuroblastoma tumor cell growth in xenograft mouse models.

352

353 **4. Discussion:**

354

355 Neuroblastoma is a solid tumor of the peripheral nervous system that arises from neural
356 crest cells and is typically localized within the medulla of suprarenal glands or within the
357 sympathetic nerve ganglia [3]. The standard care of treatment for most nervous system tumors
358 comprises of maximal surgical resection, radiation therapy, and chemotherapy; yet, tumors
359 eventually recur in the majority of patients despite multimodal treatment. The main reason
360 behind the failure of conventional chemotherapy is hypothesized to be the presence of dormant
361 slowly dividing CSCs within the tumor bulk that develop multi-drug resistance and drive tumor
362 recurrence [30]. Thus, there is ultimate need to come up with novel effective therapies that
363 uniquely target the CSCs population and its related molecular pathways [31]. Several nervous
364 system cancers have been reported to harbor CSCs, such as medulloblastomas [32],
365 glioblastomas [33], and neuroblastomas [20, 34].

366

367 Molecular alteration in different signaling pathways of CSCs have been linked to
368 abnormal proliferation, self-renewal and differentiation of these cells, and accordingly many of
369 those pathways have served as potential therapeutic targets and prognostic factors in human
370 oncology [33]. In neuroblastoma tumors [35], some of the oncogenic signaling pathways
371 implicated include PI3K/Akt/mTOR/S6K1, Ras/MAPK, VEGF, EGFR, and p53 [36-38].
372 Interestingly, GSK-3 β represents a signaling node at the crossroads of many of those pathways
373 [12, 20, 39]. This molecule has been associated with several pathological processes in the human
374 body such as bipolar depression, Alzheimer's disease, Parkinson's disease and non-insulin-
375 dependent diabetes mellitus (NIDDM) [40].

376

377 In oncology, GSK-3 β has shown to express opposite actions in different tumors; it has
378 been an oncogenic molecule in some tumors, but a tumor suppressor in others [41]. The exact
379 underlying mechanism of action, at cellular and molecular levels, of GSK-3 β in the context of
380 boosting tumor progression is not fully understood; yet, it has been related to blockade of GSK-
381 3 β -mediated upregulation of NF- κ B-mediated gene transcription [14]. Hence, we hypothesized
382 that targeting this molecule with selective inhibitors, such as TDG - which is now under Phase II
383 Clinical Trials for Alzheimer's Disease and patients with progressive supranuclear palsy, with
384 minimal adverse effects being reported among patients under study - might carry hope as a novel
385 potential CSCs-targeted therapy to patients suffering from neuroblastoma tumor [15, 42].

386

387 First, we surveyed an online publicly available dataset (Neale Multi-cancer Statistics) to
388 determine and compare between mRNA expression patterns of *GSK-3 β* in human neuroblastoma
389 tissues and other body tumor tissues. Interestingly, *GSK-3 β* was significantly overexpressed in
390 neuroblastoma tissues relative to other tumor tissues, with a fold change of 1.639 ($p = 4.06E-4$).

391

392 Next, we assessed the anti-tumor properties and mechanism of action of TDG - an in-
393 clinical-trial drug that specifically inhibits GSK-3 β - on two human neuroblastoma cells, SH-
394 SY5Y and SK-N-SH, respectively, and investigated its effect on cell proliferation, viability, and
395 migration *in vitro*, all hallmarks of tumorigenesis. Our results revealed that TDG significantly
396 inhibited the proliferation and survival of both cell lines, in a dose- and time- dependent
397 manners. TDG also significantly reduced migratory ability of both cells. Our results are
398 consistent with those of Mathuram *et al.*, where they have shown that TDG, at molecular level,
399 induces apoptosis in human neuroblastoma IMR32 cells, provoking sub-G0/G1 accumulation

400 and ROS generation [43].

401

402 Eventually, we also sought to determine the ability of TDG to target the sub-population
403 of cancer stem/progenitor cells in SH-SY5Y cells using a 3D neurospheres formation assay in
404 Matrigel™ *in vitro* [20, 44]. Treatment with TDG at a concentration as low as 0.1μM
405 significantly inhibited SFU as well as sphere size of SH-SY5Y cells. Notably, significant
406 progressive decrease in the number and size of cultured G1 neurospheres followed a dose-
407 dependent manner. Furthermore, consecutive treatment of SH-SY5Y neurospheres at G1 and G2
408 with the same concentrations of TDG, caused prominent reduction in SFU where neurospheres
409 formation was completely abolished at G3. Sphere formation assay was performed on SK-N-SH
410 cells as well to validate the effect of TDG showing consistent results. Thus, we concluded that
411 TDG is effective in targeting the self-renewal ability of CSCs, a hall mark of cancer progression.
412 When compared to 2D culture, TDG treatment was more potent when used in a 3D culture,
413 whereby lower drug dosages were sufficient to exert an even stronger cytotoxic effect on
414 neuroblastoma cells. Consistent with the *in vitro* data, SH-SY5Y cells treated with TDG *in vivo*,
415 drastically reduced the tumorigenic potential of tumor cells.

416

417 Lastly, we believe that our study has some limitations related to the methodology and
418 experimental design. First, we assessed in our work the inhibitory effect of TDG on two human
419 neuroblastoma cell lines as models for this nervous system tumor. Future experiments will
420 follow after acquiring more human cell line models for neuroblastoma to assess the inhibitory
421 effect of TDG using different cell lines. Second, in our study, we mainly relied on experimental
422 assays that serve as functional reporters of the progenitor activity of neuroblastoma cell lines, as

423 well as the differentiation and self-renewal ability of the stem/progenitor cell population. Future
424 studies will aim at evaluating the inhibitory effect of TDG, at a molecular level, on different
425 GSK-3 β -related signaling pathways that are entangled in the pathophysiology of neuroblastoma
426 and its CSCs. Based on future experiments and knowing that TDG has made it into clinical trials
427 for Alzheimer's disease, it is thus worthy of consideration in nervous system tumors as well such
428 as neuroblastoma, especially that we proved in our current study its efficiency in targeting the
429 CSC population in this tumor type.

430

431 **5. Conclusions:**

432

433 In conclusion, TDG proved to be an effective *in vitro* and *in vivo* treatment for
434 neuroblastoma cell lines and may hence serve as a potential adjuvant therapeutic agent for this
435 aggressive nervous system tumor. Henceforth, our study supports the notion that targeting GSK-
436 3 β , causing decrease in CSCs viability, may be crucial to halt neuroblastoma tumor progression.

437 **6. Acknowledgments**

438

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441 Building at the American University of Beirut (AUB) for their help and support.

442

443 **7. Financial Disclosure**

444

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447 Medicine, Lebanese University (LU) (to HH). Funders had no role in the study design; in the
448 collection, analysis and interpretation of data; in the writing of the report; and in the decision to
449 submit the article for publication.

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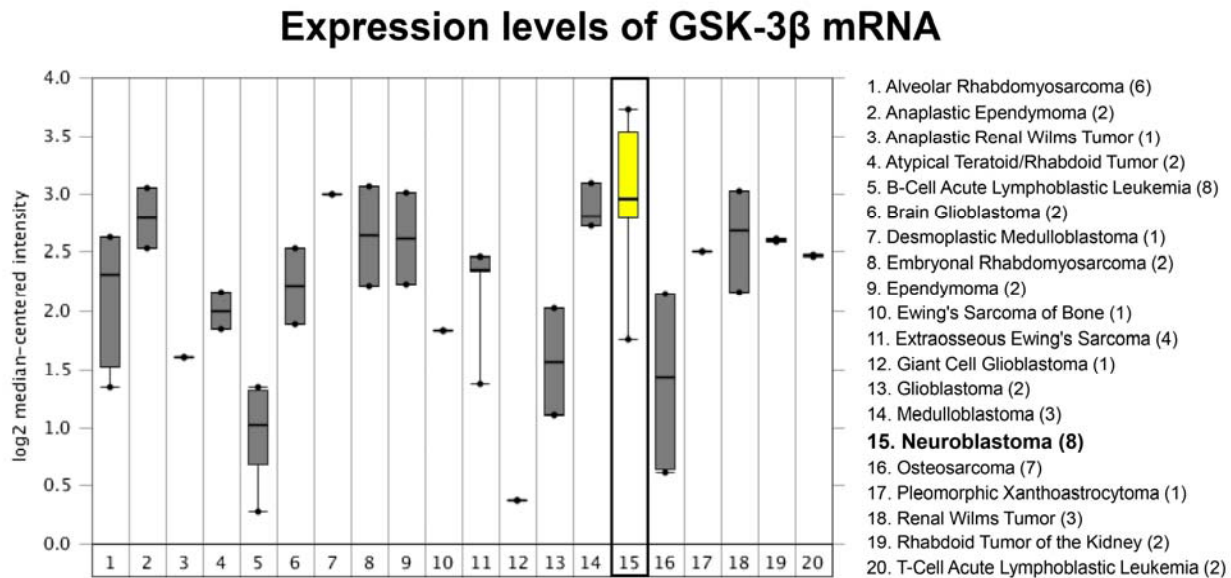
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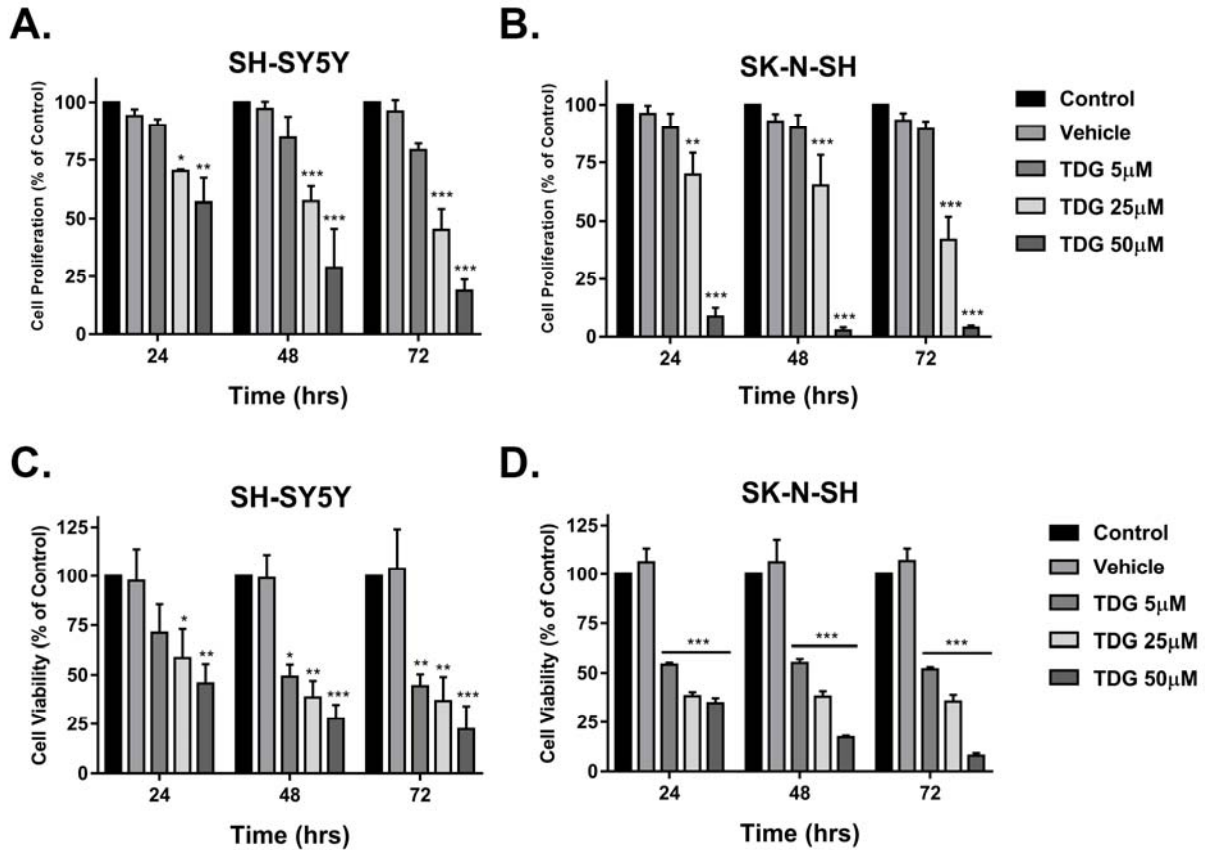
574 **Figures:**

575

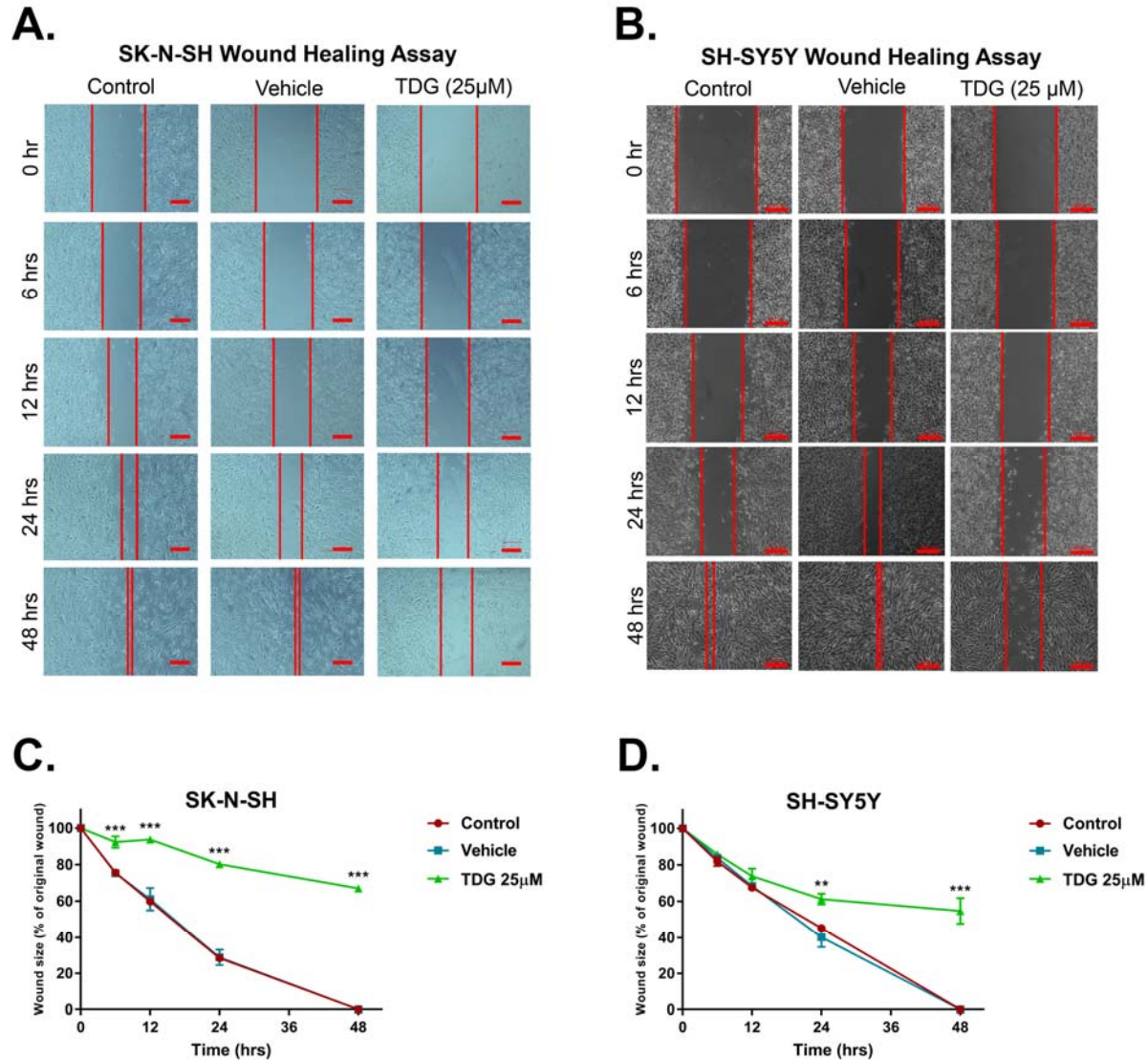


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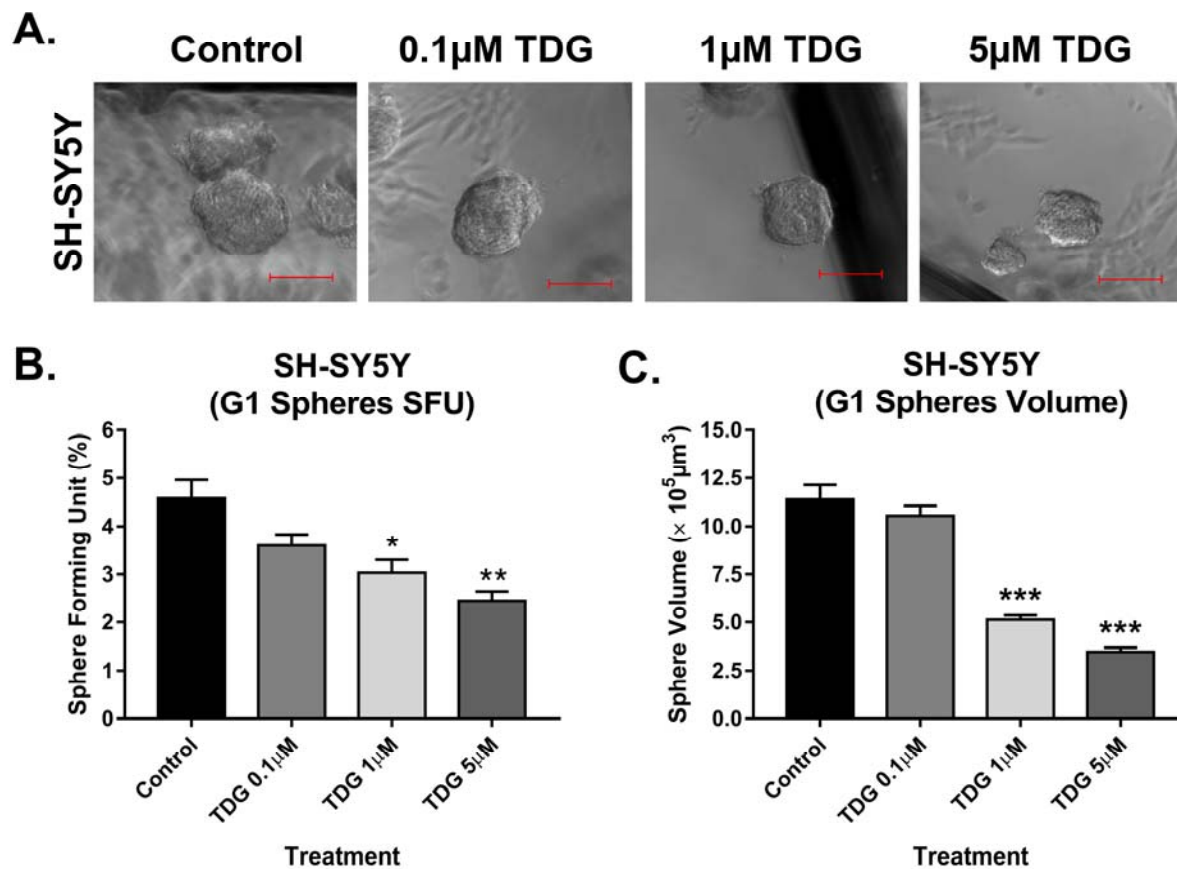
577 **Fig 1. Expression levels of *GSK-3 β* mRNA were assessed in an array set comprised of**
578 **human pan-tumor samples (Neale Multi-cancer Statistics, Reporter 226183_at is presented;**
579 **the remaining probes of the dataset are presented in Supp Fig 1: Reporters 209945_s_at,**
580 **226191_at, and 242336_at). Expression within tumor tissues was presented by log (base 2)**
581 **median-centered expression of *GSK-3 β* . Box and whiskers plots indicate median and**
582 **interquartile range. *p* values were obtained using *t*-tests (Neale Multi-cancer Statistics, 60**
583 **samples; data retrieved from Oncomine.org). Analysis revealed that mRNA expression of *GSK-***
584 ***3 β* gene was the highest among neuroblastoma tissues relative to other organ specific tumor**
585 **tissues (Fold change = 1.639; *p* = 4.06E-4).**



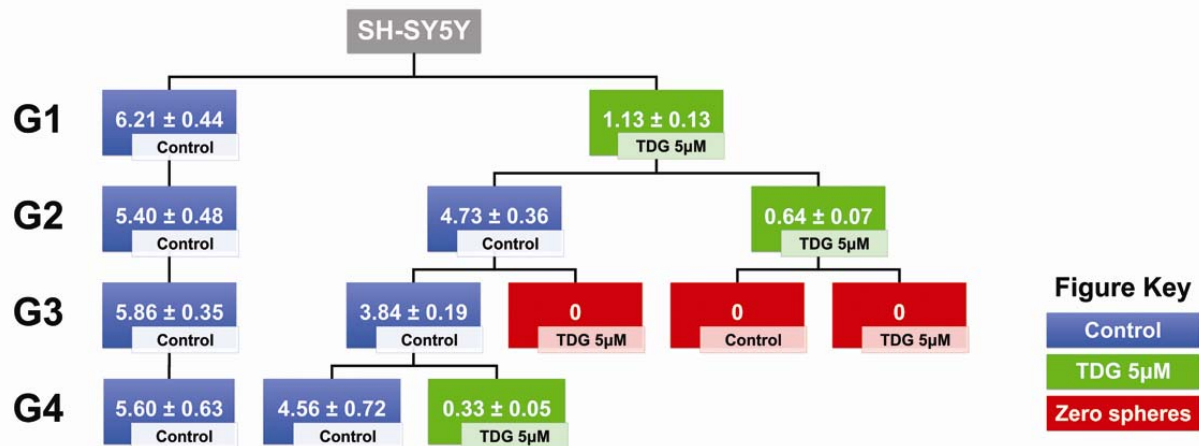
586
 587 **Fig 2. Tideglusib significantly decreases cell proliferation and cell viability of human**
 588 **neuroblastoma cells.** (A) The effect of TDG on cell proliferation was determined using the
 589 MTT assay. Tideglusib significantly decreases cell proliferation of SK-N-SH (two-way
 590 ANOVA; treatment $F_{(4, 30)} = 143, p < 0.001$; time $F_{(2, 30)} = 2.02, p = 0.15$; interaction $F_{(8, 30)} = 1.29,$
 591 $p = 0.2858$) and SH-SY5Y (two-way ANOVA; treatment $F_{(4, 30)} = 50.78, p < 0.001$; time $F_{(2, 30)} =$
 592 $5.801, p = 0.0074$; interaction $F_{(8, 30)} = 1.738, p = 0.1303$) cells in dose-dependent manner, as
 593 determined by MTT. (B) The effect of TDG on cell viability was determined using the trypan
 594 blue assay. Tideglusib significantly decreases the percentage of viable cells in SK-N-SH (two-
 595 way ANOVA; treatment $F_{(4, 30)} = 248.5, p < 0.001$; time $F_{(2, 30)} = 2.791, p = 0.0773$; interaction $F_{(8,$
 596 $30)} = 2.002, p = 0.0808$) and SH-SY5Y (two-way ANOVA; treatment $F_{(4, 30)} = 25.22, p < 0.001$;
 597 time $F_{(2, 30)} = 2.16, p = 0.1329$; interaction $F_{(8, 30)} = 0.524, p = 0.8289$) cells in dose-dependent
 598 manner, as determined by MTT. The data are reported as mean \pm SEM of three independent
 599 experiments. Bonferroni post-hoc analysis was done to determine simple factor effects.
 600 (** $p < 0.01$, *** $p < 0.001$).



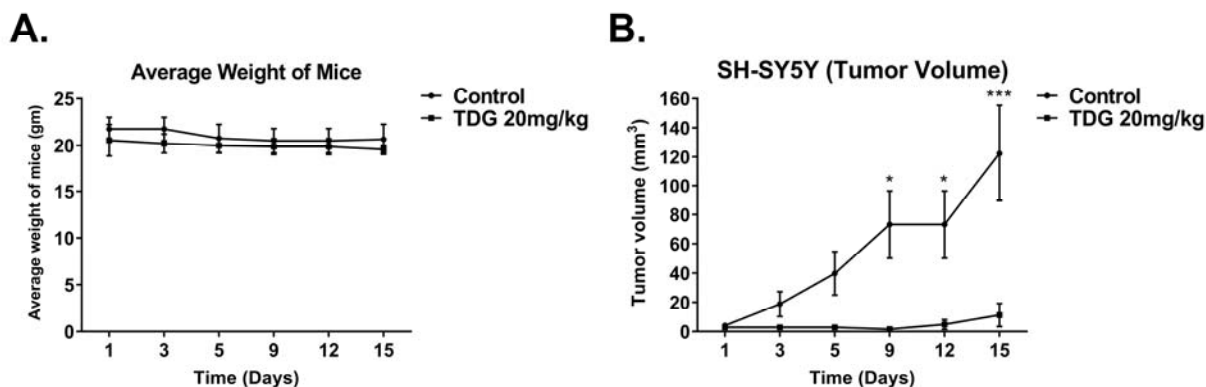
601
602 **Fig 3. Tideglusib inhibits cell migration of SK-N-SH and SH-SY5Y human neuroblastoma**
603 **cells.** Representative figures showing the scratch made in SK-N-SH (A) and SH-SY5Y (B) cell
604 lines at five different timepoints: 0h, 6h, 12h, 24h and 48h. These figures show closure of the
605 wound after 48 hours in control and vehicle treated conditions as opposed to the TDG (25 µM)
606 treated conditions. Scale = 200µm in (A) and 100µm in (B). A scratch was made to cells seeded
607 in 6-well plates at T=0h using a 200µL pipet tip; distances between cells were assessed at the
608 different timepoints to determine the drug's effects on cellular migration. The data are reported
609 as percentages of the distance between cells relative to original wound size at T=0h. Tideglusib
610 (25 µM) significantly inhibited cell migration of SK-N-SH (C) (two-way ANOVA with repeated
611 measures: treatment $F_{(2, 6)} = 1659, p < 0.001$; time $F_{(4, 24)} = 454.3, p < 0.001$; interaction $F_{(8, 24)} =$
612 $38.83, p < 0.001$) and SH-SY5Y (D) (two-way ANOVA with repeated measures: treatment $F_{(2, 6)}$
613 $= 80.98, p < 0.001$; time $F_{(4, 24)} = 340.8, p < 0.001$; interaction $F_{(8, 24)} = 19.29, p < 0.001$) cell lines,
614 in a dose- and time- dependent manners. The data are reported as mean \pm SEM of three
615 independent experiments. Bonferroni post-hoc analysis was done to determine simple factor
616 effects. (** $p < 0.01$, *** $p < 0.001$ when compared to control).



617
618 **Fig 4. Tideglusib effectively decreases the percentage of self-forming units and volume of**
619 **spheres in the sphere formation assay on SH-SY5Y cells. (A)** Representative images taken of
620 SH-SY5Y spheres under different conditions (control, 0.1 μM TDG, 1 μM TDG and 5 μM TDG)
621 using inverted light microscopy showing the gradual decrease in size of spheres in treatment
622 dose-dependent manner. **(B)** Tideglusib decreases the percentage of SFUs in SH-SY5Y cell
623 suspensions in a dose-dependent manner. (One-way ANOVA followed by Bonferroni multiple
624 comparisons: treatment $F_{(3, 8)} = 10.6, p=0.0037$) **(C)** TDG treatment decreases the volume of the
625 formed spheres in a dose-dependent manner (one-way ANOVA followed by Bonferroni multiple
626 comparisons: treatment $F_{(3, 356)} = 66.27, p<0.001$). *The data are reported as mean ± SEM of*
627 *three independent experiments. Bonferroni post-hoc analysis was done to determine simple*
628 *factor effects. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ when compared to control).*

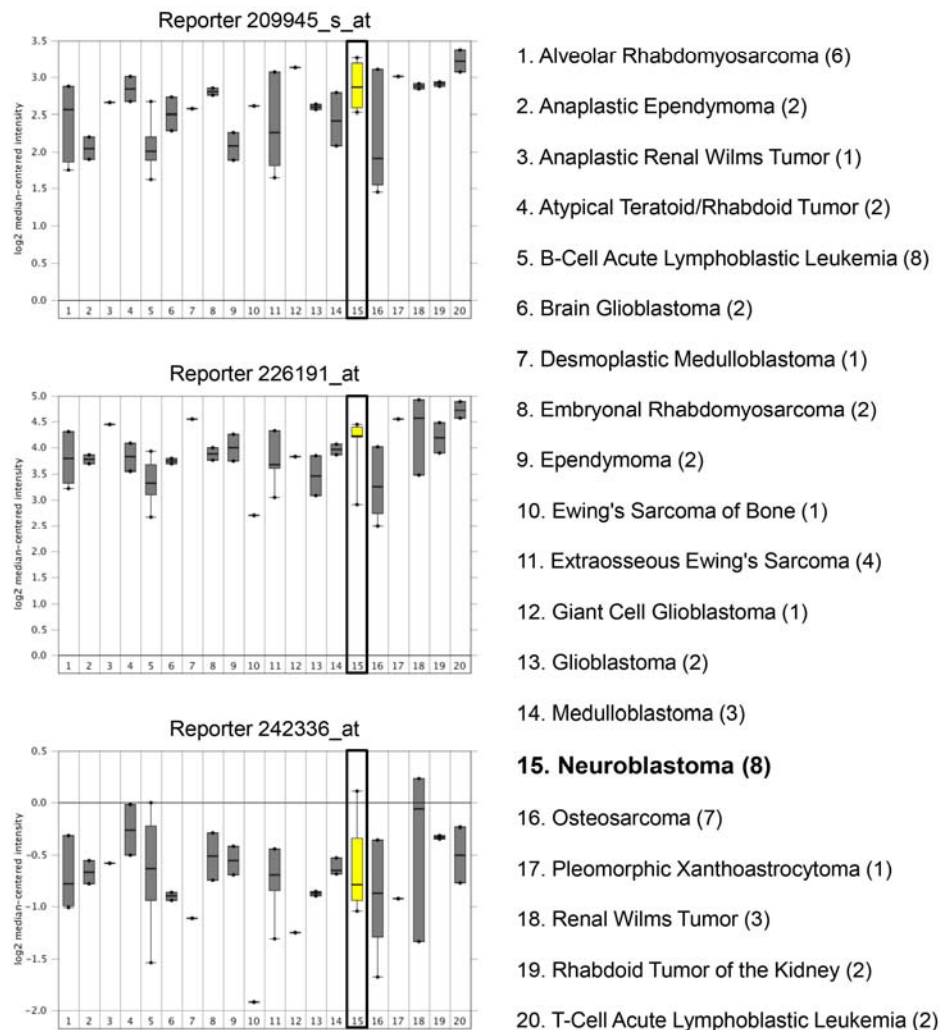


629 **Fig 5. Tideglusib targets an enriched cancer stem/progenitor subpopulation within SH-**
 630 **SY5Y cell line, decreasing SFU across multiple generations.** Schematic summarizing the
 631 experimental design and results of serial propagation of spheres across 4 generations. Spheres
 632 from control and treated conditions were isolated, dissociated into single cell suspensions and
 633 seeded under non-adherent conditions. Wells were then randomly distributed into treated and
 634 non-treated conditions to assess the effect of treatment across generations. The numbers shown
 635 represent the average percentage of SFUs as obtained from three independent experiments. The
 636 data was analyzed using multiple independent *t*-tests across each generation.

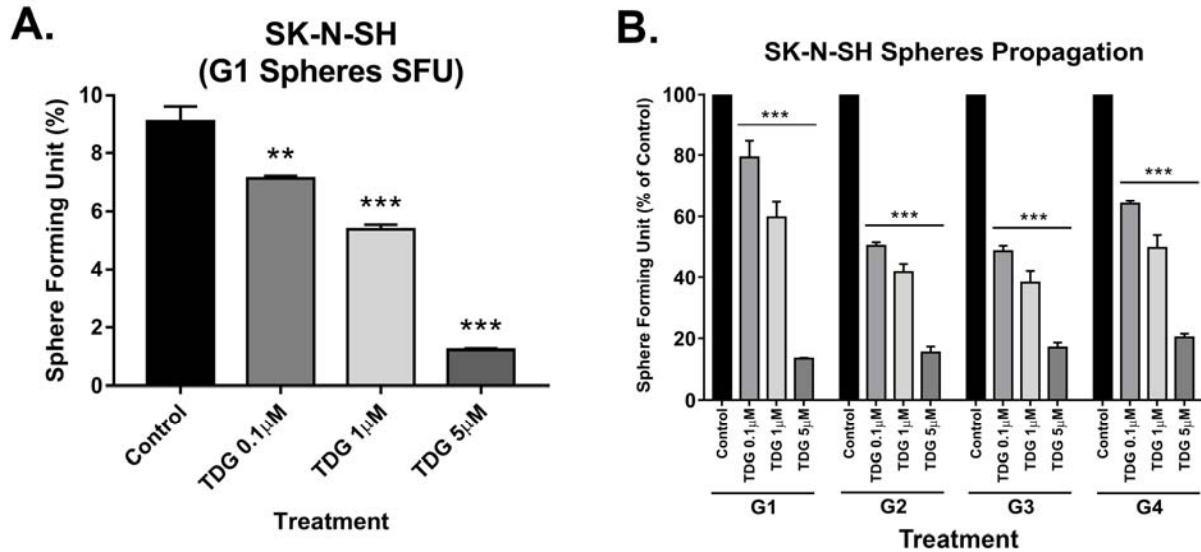


637
638 **Fig 6. TDG treatment drastically reduces neuroblastoma tumor burden in xenograft mouse**
639 **models.** 1.2×10^6 SH-SY5Y cells were subcutaneously transplanted in 6 to 8 weeks old
640 NOD-SCID mice. (A) Average weight of mice throughout the experiment was recorded. (B)
641 Tumor size measurements were initiated upon the detection of a palpable tumor post-cell
642 injection. Tumor volume was assessed by direct physical measurements of the tumors at the
643 primary site of injection, every 3 days, until the termination of the experiment. The following
644 general formula was applied: $V = (3.14/6) \times L \times W \times H$; where V is the tumor volume in mm³, L is
645 the tumor length in mm, W is the tumor width in mm, and H is the tumor height in mm. (two-
646 way ANOVA; treatment $F_{(1, 24)} = 37.12, p < 0.001$; time $F_{(5, 24)} = 5.053, p = 0.0026$; interaction $F_{(5,$
647 $24)} = 3.953, p = 0.0093$). Data represent an average of n=3 mice. The data are reported as
648 mean \pm SEM. (* $P < 0.05$; *** $P < 0.001$).

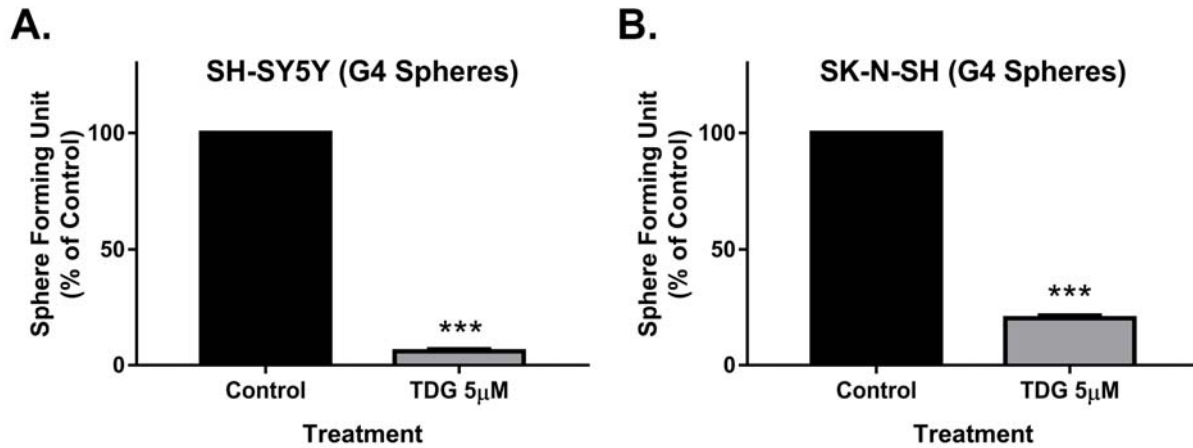
649 **Supplementary Figures:**



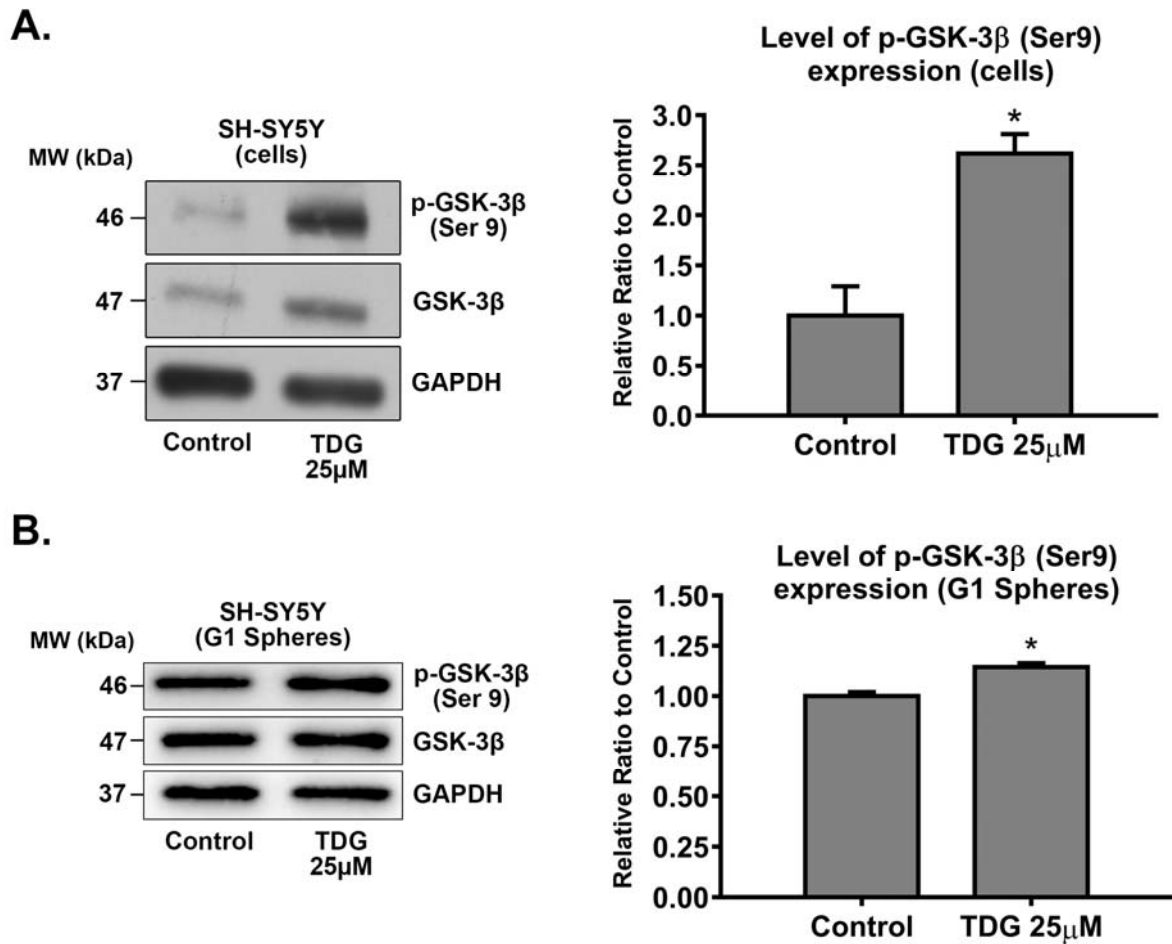
650 **Supp Fig 1. Assessment of the expression levels of *GSK-3β* mRNA in three out of four**
 651 **probes of the Neale Multi-cancer Statistics array set comprised of human pan-tumor**
 652 **samples.** Data from different probes of the same dataset are shown: Reporters 209945_s_at
 653 (upper panel), 226191_at (middle panel), and 242336_at (lower panel). Expression within tumor
 654 tissues was presented by log (base 2) median-centered expression of *GSK-3β*. Box and whiskers
 655 plots indicate median and interquartile range. *p*-values were obtained using *t*-tests (Neale Multi-
 656 cancer Statistics, 60 samples; data retrieved from Oncomine.org). Analysis revealed that mRNA
 657 expression of *GSK-3β* gene was amongst the highest in neuroblastoma tissues relative to other
 658 organ specific tumor tissues in reporters 209945_s_at and 226191_at.



659
 660 **Supp Fig 2. Tideglusib effectively decreases the percentage of SFUs of spheres in the sphere**
 661 **formation assay on SK-N-SH cells and targets an enriched cancer stem/progenitor**
 662 **subpopulation within those cells, decreasing SFU across multiple generations.** (A)
 663 Tideglusib decreases the percentage of SFUs in SK-N-SH cell suspensions in a dose-dependent
 664 manner. (One-way ANOVA followed by Bonferroni multiple comparisons: treatment $F_{(3, 8)} =$
 665 $147.6, p < 0.001$). (B) SFU obtained from serially passaged SK-N-SH spheres over 4 generations
 666 (G1–G4) is shown under untreated condition (control) and with increasing concentration of
 667 TDG: 0.1, 1, and 5 μM (treated at each generation from G1 to G4) (two-way ANOVA with
 668 repeated measures: treatment $F_{(3, 32)} = 18.92, p < 0.001$; generation $F_{(3, 32)} = 688.6, p < 0.001$;
 669 interaction $F_{(9, 32)} = 8.084, p < 0.001$). The data are reported as mean \pm SEM of three independent
 670 experiments. Bonferroni post-hoc analysis was done to determine simple factor effects.
 671 (** $P < 0.01$ and *** $p < 0.001$ when compared to control).



672
673 **Supp Fig 3. Treating an enriched cancer stem/progenitor subpopulation within SH-SY5Y**
674 **and SK-N-SH cells significantly decreased SFU at G4.** Treating an enriched cancer
675 stem/progenitor subpopulation in SH-SY5Y (A) and SK-N-SH (B) cells leads to a significant
676 decrease in the percentage of SFUs at G4. (***) $P < 0.001$; treatment compared to control, student
677 independent *t*-test).



678
679 **Supp Fig 4. Tideglusib selectively inhibits GSK-3β by increasing expression of its inhibited**
680 **form, phosphorylated at Serine 9 (p-GSK-3β Ser 9).** After treating SH-SY5Y cells with 25μM
681 TDG (for 48 hours) (A) and G1 spheres with 5μM TDG (B), proteins were extracted using RIPA
682 buffer, and used to detect differences in expression of the phosphorylated form of GSK-3β (Ser
683 9). Bands were detected by enhanced chemiluminescence (ECL) using ChemiDoc MP Imaging
684 System. Protein expression was quantified using Image Lab software, relative to the expression
685 of GAPDH, a housekeeping gene equally expressed in treated and non-treated cells/spheres.
686 Results are expressed as relative ratio to control. Analysis of p-GSK-3β (Ser 9) protein level was
687 done after normalization with total GSK-3β protein levels. Data represent an average of three
688 independent experiments. The data are reported as mean ± SEM. (* $P < 0.05$; treatment compared
689 to control, student independent t-test).