1 **Sulfatase 2 Inhibition Sensitizes Triple-Negative Breast Cancer Cells to Chemotherapy Through**
2 **Augmentation of Extracellular ATP**

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19   **ABSTRACT**

20   **Background:** Breast cancer is the leading cause of cancer-related death among women worldwide.

21   Patients diagnosed with triple-negative breast cancer (TNBC) have limited therapeutic options that

22   produce durable responses. Hence, a diagnosis of TNBC is associated with a poor prognosis compared to

23   other types of breast cancer. As a result, there is a critical need for novel therapies that can deepen and

24   prolong responses.

25   We previously found that chemotherapy causes the release of extracellular adenosine triphosphate

26   (eATP). Augmenting eATP release can boost the response of TNBC cells to chemotherapy and cause

27   increased cell death. However, eATP concentrations are limited by several families of extracellular

28   ATPases, which complicates the design of compounds that attenuate eATP degradation.

29   In this study, we hypothesized that heparan sulfate (HS) would inhibit extracellular ATPases and

30   accentuate chemotherapy-induced cytotoxicity in TNBC by augmenting eATP. HS can be desulfated by

31   sulfatase 1 and 2; sulfatase 2 is consistently highly expressed in a variety of cancers including breast

32   cancer, whereas sulfatase 1 is not. We hypothesized that the sulfatase 2 inhibitor OKN-007 would

33   exacerbate chemotherapy-induced eATP release and TNBC cell death.

34   **Methods:** TNBC cell lines and nontumorigenic immortal mammary epithelial cells were treated with

35   paclitaxel in the presence of heparan sodium sulfate and/or OKN-007; eATP content and cell viability

36   were evaluated. In addition, protein and cell surface expression of sulfatases 1 and 2 were determined in

37   all examined cell lines via ELISA, Western blot, and flow cytometry analyses.

38   **Results:** Sulfatase 2 was highly expressed in TNBC cell lines and human breast cancer samples but not in

39   immortal mammary epithelial cells and much less so in normal human breast tissue and ductal carcinoma

40   in situ samples. OKN-007 exacerbated chemotherapy-induced eATP release and chemotherapy-induced

41   TNBC cell death. When combined with chemotherapy, OKN-007 attenuated cells with a cancer-initiating

42   cell phenotype.

43   **Conclusions:** These results suggest that sulfatase 2 inhibitors in combination with chemotherapy

44   attenuate the viability of TNBC cells more than chemotherapy alone by exacerbating eATP release. These

45   effects, as well as their capacity to attenuate the cancer-initiating cell fraction, may translate into

46   combination therapies for TNBC that induce deeper and more durable responses.

47   **Keywords**

48   Breast cancer, ATP, chemotherapy, sulfatases, heparan sodium sulfate, purinergic signaling

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58   **BACKGROUND**

59   Breast cancer diagnoses impacted 2,261,419 women in 2020 [1]. Among women, this form of cancer has

60   the highest mortality and incidence rates [2]. The mortality rate is largely due to the most aggressive form

61   of breast cancer, triple-negative breast cancer (TNBC). Due to a lack of effective targeted therapies,

62   chemotherapy is still the most efficacious treatment for TNBC, but a major downside is an inability to

63   fully eradicate metastatic disease despite transient responses [3]. Moreover, immunotherapy is only

64   effective in a small subset of patients. Hence, there is a critical need for innovative therapeutic strategies.

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65   The concentration of extracellular adenosine triphosphate (eATP) in tissues is between 0-10 nanomolar

66   (nM) under physiological conditions while the concentration of adenosine triphosphate (ATP)

67   intracellularly ranges from 3-10 millimolar (mM), a more than 10^6-fold difference [4]. However, this

68   minute eATP quantity fulfills a critical role as a signaling molecule through cell surface purinergic

69   receptors. Moreover, there is a marked increase in the concentration of eATP in the tumor

70   microenvironment (TME), which can reach the micromolar range [5-7]. Our published study showed that

71   eATP is toxic to TNBC cells in the high micromolar range but not in nontumorigenic immortal mammary

72   epithelial MCF-10A cells [8]. Thus, cancer cells "live" closer to the threshold for cytotoxicity.

73   Furthermore, our published data showed that chemotherapy treatment results in augmentation of eATP

74   concentration [8]. However, eATP concentration is limited by several families of ecto-nucleotidases,

75   including ecto-nucleoside triphosphate diphosphohydrolases (ENTPD), 5'-nucleotidases (5'-NTs), ecto-

76   nucleotide pyrophosphatases/phosphodiesterases (E-NPPase), and tissue nonspecific alkaline

77   phosphatases (TNAP), with E-NTPD considered to be the central enzyme responsible for ATP

78   degradation and extracellular 5'-NT responsible for the catalytic conversion of AMP to adenosine and

79   inorganic phosphate [9]. We previously showed that inhibitors of each of these families of ecto-ATPases

80   can augment chemotherapy-induced TNBC cell death and eATP release through P2RX4 and P2RX7 ion-

81   coupled purinergic receptors. This data suggests that all ecto-ATPases may need to be inhibited to

82   maximize eATP release and TNBC cell death. The existence of several families of structurally diverse

83   ecto-ATPases complicates the design of small-molecule inhibitors that can maximally suppress eATP

84   degradation.

85   We noted that sulfated polysaccharides have been reported to inhibit multiple families of extracellular

86   ATPases with nanomolar potency; the degree of sulfation, which imparts a negative charge to the

87   molecule, is critical for this inhibition [10]. We also noted that endogenous extracellular polysulfated

88   polysaccharide heparan sulfate (HS) inhibits eATP degradation [11]. Moreover, other publications show

89   that the ecto-ATPase ecto-nucleotide pyrophosphatases/phosphodiesterase 1 (E-NPP1) binds to

90   extracellular glycosaminoglycans, and its ATPase activity is competitively inhibited by these

91   glycosaminoglycans, including heparin and HS [12].

92   HS proteoglycans are ubiquitously expressed in the extracellular matrix and cell surface of animal cells.

93   They can be broadly divided into three groups: transmembrane syndecans; glycosylphosphatidylinositol-

94   linked glypicans; and extracellular matrix-associated perlecan, agrin, and collagen XVIII [13]. The

95   polysulfated polysaccharide HS is synthesized in the Golgi system and is composed of disaccharide units

96   that are negatively charged and unbranched with sulfation of the 3-O, 6-O, or N sites of glucosamine

97   along with the 6-O site of glucuronic/iduronic acid [14-17]. HS plays a tumor suppressor role: loss of

98   exostosin 1 or exostosin 2, proteins involved in the polymerization of HS, leads to hereditary multiple

99   exostoses, a hereditary cancer syndrome associated with an elevated risk of chondrosarcomas and

100   osteosarcomas [18-21]. Sulfatase 1 and sulfatase 2 are extracellular HS 6-O-endosulfatases that hydrolyze

101   the 6-O-sulfate groups on glucosamine residues in HS [14, 22-25]. Sulfatase 2 is highly expressed in a

102   variety of cancers including breast cancer, while sulfatase 1 is not [15, 22, 25-27]. Sulfatase 2 has been

103   shown to enhance tumor initiation and progression in a variety of cancers including breast (**Supplemental**

104   **Figure 1)** [24, 26, 28]. When sulfatase 2 was overexpressed in the breast cancer cell line MDA-MB 231,

105   there was an increase in breast cancer cell growth [24]. In contrast to sulfatase 2, sulfatase 1 is a tumor

106   suppressor, possibly due to its negative regulation of fibroblast growth factor signaling [22, 29]. Notably,

107   the expression of sulfotransferase 3-OST3A, an enzyme that adds sulfate groups to HS, is epigenetically

108   downregulated in estrogen receptor-positive (ER+) and TNBC cell lines as well as chondrosarcoma cell

109   lines [30, 31]. In addition, expression of sulfotransferase 3-OST3A is epigenetically silenced in human

110   breast, colon, lung, and pancreatic cancers [32, 33].

111   OKN-007 is a potent sulfatase inhibitor that has been demonstrated to decrease sulfatase 2 activity [34].

112   Moreover, OKN-007 is already in phase Ib clinical trials for glioblastoma multiforme (NCT03587038).

113   Hence, we hypothesized that sulfatase 2 inhibitors would accentuate the fully sulfated form of HS in the

114 TME, an endogenous inhibitor of ecto-ATPases, thus enhancing TNBC cell death by augmenting eATP

115 concentrations in the microenvironment of chemotherapy-treated cells **(Figure 1)**.

116 **METHODS**

117 *Cell culture, drugs, and chemicals*

118 Breast cancer cell lines MDA-MB 231 (ATCC HTB-26, RRID: CVCL_0062), MDA-MB 468 (ATCC

119 HTB-132, RRID: CVCL_0419), Hs 578t (ATCC HTB-126, RRID: CVCL_0332), HEK-293T ATCC

120 Cat# CRL-3216, RRID: CVCL_0063), and nontumorigenic immortal mammary epithelial MCF-10A

121 cells (ATCC Cat# CRL-10317, RRID: CVCL_0598) were authenticated and maintained as described

122 previously [8].

123 The following drugs and chemicals were used: ATP (Sigma), dimethyl sulfoxide/DMSO (Sigma),

124 paclitaxel (Calbiochem), OKN-007 (formally known as NXY-059) (Selleck Chemical), and heparan

125 sodium sulfate (Sigma). HS was dissolved in nuclease-free water (Invitrogen); paclitaxel and OKN-007

126 were dissolved in dimethyl sulfoxide (DMSO) (Sigma). **Table 1** shows the drugs' concentrations and

127 functions; we optimized the drug concentrations that were applied for the different assays by using

128 previously described drug concentrations as starting points [34-38]. Cells were treated at the designated

129 concentrations.

130 **Table 1. Drug concentrations and functions**

Drug	Concentration(s)	Function	Concentration reference
paclitaxel	50 and 100 µM	Chemotherapeutic agent	[1]
OKN-007	20 µM	Sulfatase inhibitor	[2]
A437809	20 µM	P2RX7 inhibitor	[3]
5-BDBD	20 µM	P2RX4 inhibitor	[4]
heparan sodium sulfate	50 µM	developmental processes, angiogenesis, and tumor metastasis	[5]

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132 *Measurement of ecto ATPase inhibition by heparan sulfate*

133 ATP was combined with recombinant proteins (enzymes): ENPP1, tissue non-specific alkaline

134 phosphatase (TNAP), and ecto-nucleoside triphosphate diphosphohydrolases 1 (CD39/ENTPD1) (R&D

135 Systems) in the presence or absence of heparan sodium sulfate. The amount of eATP was analyzed

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136   according to the protocol described by the manufacturer of ATPlite 1-step Luminescence Assay System

137   (PerkinElmer, Cat# 6016736) either at 0 hours (eATP concentrations were determined immediately) or

138   after 24 hours of incubation at 37°C. Luminescence readings were obtained from a Bioteck Synergy HT

139   plate reader. As a control, enzymes were boiled, and the subsequent experiments were carried out in the

140   same fashion. The student's t-test was applied to ascertain significance. **represents $p < 0.01$ for the

141   comparison of enzyme-treated ATP to enzyme-treated ATP and heparan sodium sulfate.

142

143   *Western blot analysis of expression of sulfatases*

144   An equal number of individual cell lines (TNBC MDA-MB 231, Hs 578t, MDA-MB 468 cells and

145   nontumorigenic immortal mammary epithelial MCF-10A cells) were seeded and cultured for 48 hours to

146   70%-80% confluency. Cell supernatants were collected. Total cell lysates were prepared; protein

147   quantification was performed; and proteins were denatured, separated, and transferred as previously

148   described [8]. For immunoblot of sulfatase 1 and sulfatase 2 on cell lysates, 100 µg of protein was loaded

149   onto gels. For immunoblot of sulfatase 1 and sulfatase 2 on cell supernatants, either 10 µL of cell

150   supernatants for the analysis of unnormalized expression levels or a volume that was normalized to the

151   total mass of protein measured in the corresponding lysate was loaded onto the gels. Unadjusted (with 10

152   µl supernatant) blots were stained with Ponceau S (Thermo Fisher Scientific) as a loading control. The

153   membranes were blocked with nonfat milk at room temperature for an hour and incubated overnight at

154   4°C with a primary antibody: sulfatase 1 (1:200 dilution; Novus Biologicals, Cat# NBP231584, RRID:

155   AB_2916043) and sulfatase 2 (1:500 dilution; Abcam, Cat# ab232835, RRID: AB_2916044) diluted in

156   5% nonfat milk. The membranes were washed and developed as described previously [8].

157   Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Cat #3683, RRID:

158   AB_1642205) was used as a loading control. Densitometry was performed on Licor Image Studio (RRID:

159   SCR_015795). The student's t-test was used for the applicable assays to ascertain significance. *

160   represents $p < 0.05$ and ** represents $p < 0.01$ relative to protein expression in MCF-10A cells.

161 *ELISAs*

162 TNBC cell lines, MDA-MB 231, Hs 578t, MDA-MB 468 cells, and nontumorigenic immortal mammary

163 epithelial MCF-10A cells were grown for 48 hours to 70-80% confluency, and supernatants were

164 collected. The concentrations of sulfatase 1 and 2 (Lifespan Biosciences, Cat# LS-F66757-1 and LS-

165 F35926-1) were measured in the supernatants of the examined cell lines at basal level via enzyme-linked

166 immunoassay (ELISA) analysis according to the manufacturer's directions.

167

168 *Flow cytometry analysis of sulfatases*

169 Cell surface expressions for sulfatase 1 and sulfatase 2 were measured in TNBC cell lines, MDA-MB

170 231, Hs 578t, MDA-MB 468 cells, nontumorigenic immortal mammary epithelial MCF-10A cells, and

171 HEK 293T cells. HEK 293T cells were transfected with either sulfatase 1 or sulfatase 2 expression

172 plasmids or empty vector control derived from pcDNA3.1 (RRID: Addgene_79663) using Lipofectamine

173 3000 (Thermo Fisher Scientific). Cells were detached with accutase (Thermo Fisher Scientific). One

174 million cells were washed in phosphate buffered saline (PBS) with 0.05% BSA, stained with sulfatase 1

175 (Novus Biologicals, Cat# NBP231584, RRID: AB_2916043) or sulfatase 2 (Abcam, Cat# ab232835,

176 RRID: AB_2916044) plus goat anti-rabbit IgG (H+L) secondary antibody-fluorescein isothiocyanate

177 (FITC) (Novus Biologicals, Cat# NB 7168, RRID: AB_524413) or stained with rabbit IgG Isotype

178 Control-FITC (Invitrogen, Cat# PA5-23092, RRID: AB_2540619) in flow cytometry staining buffer (2%

179 FBS, 0.02% sodium azide and PBS). Analysis was performed on BD fluorescence-activated cell sorting

180 (FACS) Fortessa using the FITC channel (530/30 nm) and Flowjo software (RRID: SCR_008520). The

181 student's t-test was applied to ascertain significance. * represents $p<0.05$ and ** represents $p<0.01$

182 relative to mean fluorescence intensity (MFI) in MCF-10A cells; + represents $p<0.05$ and ++ represents

183 $p<0.01$ relative to MFI in HEK293-empty vector transfected. O/E represents overexpressed.

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185 *Sulfatase activity assay*

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186   The sulfatase inhibitor OKN-007 (20 µM) or vehicle control was added to recombinant sulfatase enzyme

187   (0, 2, 4 µL) with sulfatase substrate (p-nitrocathecol sulfate) diluted in the sulfatase assay buffer, and

188   sulfatase activity was determined by comparison with the p-nitrocathecol standard dilution curve. The p-

189   nitrocathecol standard was prepared according to the manufacturer's protocol for the sulfatase activity

190   assay kit (Sigma, Cat# MAK276-1KT). Absorbance readings at 515 nm were obtained from a Bioteck

191   Synergy HT plate reader. The student's t-test was applied to ascertain significance. * represents $p<0.05$

192   and ** represents $p<0.01$, comparing the sulfatase activity between the recombinant sulfatase enzyme and

193   vehicle control addition and the combination of the sulfatase enzyme and OKN-007.

194

195   *Immunohistochemistry of sulfatase 2*

196   AMSBIO BR1202B breast cancer tissue array (120-core array) was sectioned at 5 µm and air-dried

197   overnight on Fisher Superfrost Plus slides. Also, normal breast tissue or ductal carcinoma in situ (DCIS)

198   was prepared on PERMAFLEX plus slides. All of the stainings were performed at Histowiz, Inc.

199   (Brooklyn, NY) using the Leica Bond RX automated stainer (Leica Microsystems) and using a standard

200   operating procedure with a fully automated workflow. Normal breast and DCIS samples were processed,

201   embedded in paraffin, and sectioned at 4 μm. The slides were dewaxed using xylene and alcohol-based

202   dewaxing solutions. Immunohistochemistry: Epitope retrieval was performed by heat-induced epitope

203   retrieval (HIER) of the formalin-fixed, paraffin-embedded tissue using citrate-based pH 6 solution (Leica

204   Microsystems, Cat#AR9961) for 10 minutes at 95°C. The tissues were first incubated with a peroxide

205   block buffer (Leica Microsystems, Cat# RE7101-CE), followed by incubation with the rabbit anti-

206   sulfatase 2 antibody (Abcam, Cat# ab232835, RRID: AB_2916044) at 1:50 dilution for 30 minutes,

207   followed by DAB rabbit secondary reagents: polymer, DAB refine, and hematoxylin (Bond Polymer

208   Refine Detection Kit, Leica Microsystems, Cat# DS9800) according to the manufacturer's protocol. The

209   slides were dried, coverslipped (TissueTek-Prisma Coverslipper), and visualized using a Leica Aperio

210   AT2 slide scanner (Leica Microsystems) at 40×.

211   Quantitation of protein expression: Automated analysis of protein expression was done at Histowiz, Inc.

212   For the analysis of the tissue microarray (TMA), the Halo TMA module was used to identify and extract

213   the individual TMA cores by means of constructing a grid over the TMA. All subsequent analysis steps

214   were the same for the 3 slides with DCIS tissue and 2 slides with normal breast tissue and the TMA slide.

215   In the first part of the analysis, the tumor area was identified by training a random forest classifier

216   algorithm to separate viable tumor tissue from any surrounding stroma and necrosis areas. Once the tumor

217   area was identified, the analysis then proceeded to identify positive and negative cells based on sulfatase

218   2 staining within the defined tumor area on each slide and each core from the TMA slide. Positive and

219   negative cells were identified using the Halo Multiplex IHC algorithm v3.4.1 by first defining the settings

220   for the hematoxylin counterstain, followed by setting thresholds to detect the sulfatase 2 stain positivity of

221   weak, moderate, and strong (Halo threshold settings 0.11, 0.35, 0.45). An H-score was then generated

222   following the convention below: weak positive (1+), moderate positive (2+), and strong positive (3+). For

223   the immunohistochemistry statistical analysis, group differences were calculated using Kruskal-Wallis

224   one-way analysis of variance (ANOVA). When shown to be statistically significant, a post hoc Dunn's

225   test was done to determine p values. P values were adjusted to account for multiple comparisons, and an

226   alpha level of 0.05 was used for all the tests. The software GraphPad Prism version 10.0.1 (RRID:

227   SCR_002798) was used for these tests.

228

229   *Cell viability and eATP assays*

230   TNBC MDA-MB 231, Hs 578t, MDA-MB 468 cells, and nontumorigenic immortal mammary epithelial

231   MCF-10A cells were plated as previously described and treated with paclitaxel (vehicle), heparan sodium

232   sulfate (50 µM), OKN-007 (20 µM), A438709 (20 µM), 5-BDBD (20 µM), or different combinations of

233   these drugs. Cells were treated with OKN-007 and heparan sodium sulfate for 48 hours and with

234   paclitaxel, A438709, or 5-BDBD for the final 6 hours of the 48-hour time course (we treated cells with

235   paclitaxel for 6 hours to replicate exposure times in patients); cell viability was assessed by applying the

236   PrestoBlue™ HS cell viability reagent (Invitrogen, Cat# P50201) following the manufacturer's

237   instructions [8]. ATP was assessed in supernatants as described above. Fluorescence readings (excitation

238   and emission ranges: 540–570 nm and 580–610 nm) were assessed using a Bioteck Synergy HT plate

239   reader. One-way ANOVA with Tukey's honestly significant difference (HSD) was calculated to ascertain

240   significance. * represents $p < 0.05$ and ** represents $p < 0.01$ when comparing vehicle addition to OKN-

241   007, to HS, and the combination of HS, OKN-007, and/or paclitaxel.

242

243   *Flow cytometry for cancer-initiating cells*

244   TNBC MDA-MB 231, Hs 578t, and MDA-MB 468 cells were collected and stained following the

245   protocol for the Aldeflour Kit (STEMCELL, Cat# 01700). Cells were washed and stained with CD24-PE

246   (eBioscience, Cat# 12-0247-42, RRID: AB_1548678), CD44-APC (eBioscience, Cat# 17-0441-82,

247   RRID: AB_469390), and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Cat# L10119)

248   for 30 minutes at 4°C. Cells were washed and resuspended in the Aldefluor Buffer provided in the

249   Aldefluor kit. Flow cytometry analysis was performed on BD FACS Fortessa using the FITC (ALDH),

250   PE (CD24), APC (CD44), and AF750 (Live/Dead) channels and applying the Flowjo software (RRID:

251   SCR_008520). One-way ANOVA with Tukey's HSD was calculated to ascertain significance. For

252   statistical analysis, * represents $p < 0.05$ and ** represents $p < 0.01$ when comparing paclitaxel to the

253   combination of paclitaxel and OKN-007.

254

255   *Tumorsphere formation efficiency assay*

256   TNBC MDA-MB 231, Hs 578t, and MDA-MB 468 cell lines were grown and treated with paclitaxel,

257   OKN-007, and/or heparan sodium sulfate as described above in the "Cell viability and eATP assay"

258   section. Cells were trypsinized, washed, resuspended in 3D Tumorsphere Medium XF (Sigma), and

259   plated at 10 viable cells per well after (45 µM) filtration on low-attachment, round-bottom 96-well plates.

260   Cells were grown for 7 days, and tumorspheres were counted for each different condition using the

261   Etaluma™ Lumascope 620. One-way ANOVA with Tukey's HSD was calculated to ascertain

262   significance. For statistical analysis, ** represents $p < 0.01$ when comparing paclitaxel to the combination

263   of paclitaxel and OKN-007.

264

265   **RESULTS**

266   **Effect of heparan sulfate on ecto-ATPase activity**

267   We first sought to determine if polysulfated HS prevents eATP degradation by different families of ecto-

268   ATPases: tissue non-specific alkaline phosphatase (TNAP), ecto-nucleoside triphosphate

269   diphosphohydrolases 1 (ENTPD1), and ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1). We

270   incubated ATP (500 µM) with vehicle or 100 units of one member of each of the families of ecto-ATPase

271   enzymes in the presence or absence of heparan sodium sulfate (50 µM) for 48 hours or 0 hours (ATP

272   concentrations determined immediately) at 37°C **(Figures 2A and B)**. In the absence of heparan sodium

273   sulfate, all three enzymes significantly decreased the concentration of ATP after 48 hours, but in the

274   presence of heparan sodium sulfate, eATP concentrations were not depressed. Additionally, as a control,

275   we used boiled enzymes added to ATP in the presence or absence of heparan sodium sulfate, and the

276   eATP concentrations did not change, demonstrating that the reduction in ATP observed with the addition

277   of enzymes is specifically due to their catalytic activity **(Figures 2C and D)**. We also did not observe any

278   change in ATP concentration when its level was measured at 0 hours (immediately) after the addition of

279   enzyme. This result indicates that HS does not positively or negatively interfere with the ATP assay.

280   These results demonstrate that HS inhibits the ATPase activity of each of the three major families of ecto-

281   ATPases.

282

283   **Analysis of expression of sulfatases in breast cancer**

284   *Expression of sulfatases in breast cancer cell lines and mammary epithelial cells by Western blot and*

285   *ELISA*

286   To compare the level of expression of sulfatase 2 and sulfatase 1 in TNBC cell lines to that in control

287   immortal mammary epithelial cells, we performed Western blot analysis on TNBC MDA-MB 231, Hs

288   578t, and MDA-MB 468 cell lines and nontumorigenic immortal epithelial mammary MCF-10A cells,

289   probing for sulfatase 2 and sulfatase 1 with GAPDH as the internal loading control **(Figures 3 and 4;**

290   **Supplemental Figures 2-7)**. TNBC cell lines expressed markedly higher levels of sulfatase 2 protein

291   intracellularly as well as extracellularly in supernatants when compared to MCF-10A cells, as assessed by

292   semi-quantitative densitometry. We separately probed for sulfatase 1 protein in the same cell lines and

293   determined that the TNBC cell lines express more sulfatase 1 extracellularly in comparison to the

294   immortal MCF-10A cells, but Hs578t was the only TNBC cell line to express more intracellular sulfatase

295   1 protein in comparison to immortal MCF-10A cells. We also checked the baseline expression levels of

296   sulfatases in the supernatants of TNBC cell lines and MCF-10A cells via ELISAs **(Supplemental Figure**

297   **8)**. We observed that TNBC cell lines expressed significantly more sulfatase 2 in their supernatants in

298   comparison to MCF-10A cells aligning with the Western blot analysis. TNBC cell lines expressed less

299   sulfatase 1 in their supernatants in comparison to control MCF-10A cells.

300   *Flow cytometry analysis of cell surface expression of sulfatases in breast cancer cell lines and immortal*

301   *mammary epithelial cells*

302   We examined the cell surface expressions of sulfatase 1 and sulfatase 2 in TNBC cell lines and immortal

303   mammary epithelial cells. We performed flow cytometry analysis on TNBCs, MCF-10A, and HEK 293T

304   cells transfected with sulfatase 2 as a positive control probing for cell surface expression of sulfatase 2

305   **(Figure 5A; Supplemental Figure 9)**. MDA-MB 231, Hs 578t, and MDA-MB 468 did not express

306   significantly more or less cell surface sulfatase 2 in comparison to control MCF-10A cells. We also

307   probed all the cell lines for sulfatase 1 and observed that immortal MCF-10A expressed the most cell

308   surface sulfatase 1 in comparison to the expressions in TNBC cell lines, which aligns with the results

309   from ELISAs.

Effect of OKN-007 on sulfatase activity

311   We wanted to confirm that the sulfatase 2 inhibitor OKN-007 inhibits sulfatase activity at the

312   concentrations planned for our experiments. We utilized a sulfatase activity kit that can measure the

313   desulfation of p-nitrocathecol sulfate by sulfatase to p-nitrocatechol. We added the sulfatase inhibitor

314   OKN-007 (20 µM) to recombinant sulfatase (0, 2, 4 µL) and sulfatase substrate diluted in the sulfatase

315   assay buffer, and sulfatase activity was determined through a calculated standard dilution of p-

316   nitrocathecol **(Figure 5B)**. We found that there was significantly decreased sulfatase activity when

317   sulfatase and sulfatase substrate were exposed to OKN-007. These results demonstrate that OKN-007

318   neutralizes sulfatase activity at the concentration utilized (20 µM).

319

Measurement of sulfatase 2 expression in human breast cancer samples by immunohistochemistry

321   An AMSBIO BR1202B breast cancer tissue array (120 cores with 82 TNBC cores) was stained with

322   sulfatase 2 **(Figure 5C; Supplemental Table 1; Supplemental Figure 10)**. We focused on sulfatase 2

323   because sulfatase 1 has been reported to be a tumor suppressor [24, 39]. Statistical analysis was

324   performed on the breast cancer tissue array stained with sulfatase 2 antibody. The expression of sulfatase

325   2 was compared between TNBCs, ER+/progesterone receptor-positive (PR+), human epidermal growth

326   factor receptor 2 positive (HER2+) breast cancer, normal breast tissue, and DCIS. We carried out the

327   Kruskal-Wallis test and found no significant difference when: comparing the average percentages of cells

328   expressing any level of sulfatase 2 between the different groups **(Figure 6A)**; the percentages of cells that

329   were weakly positive for sulfatase 2 **(Figure 6B)**; and the average percentages of cells that did not

330   express any sulfatase 2 **(Figure 6E)**. We also performed pairwise comparisons using Dunn's test and

331   found there was a significant difference between the percentages of cells that were moderately positive

332   for sulfatase 2 in tissue sections of TNBCs and ER+/PR+ breast cancers (p = 0.0497) **(Figure 6C)**. We

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333 found there was no significant difference when comparing the average percentages of cells that were

334 strongly positive for sulfatase 2 between the different groups **(Figure 6D)**. We found that there was a

335 significant difference between the percentages of cells that were positive for sulfatase 2 in tissue sections

336 of TNBC breast cancer stages 2A vs. 2B (p = 0.0003) **(Figure 6F)**. Additional statistical analysis was

337 carried out comparing the sulfatase 2 expression among normal breast tissue, DCIS, and various grades of

338 cancers **(Supplemental Figure 11)**. We saw that high sulfatase 2 expression was associated with lack of

339 expression of the PR **(Supplemental Figure 12)**. Furthermore, we observed that the higher percentage of

340 Ki67 was associated with a higher expression of sulfatase 2 **(Supplemental Figure 13)**.

341

342 **Effect of sulfatase inhibitor effect on cell viability and eATP**

343 We previously demonstrated that eATP augmentation by ecto-ATPase inhibitors increases chemotherapy-

344 induced TNBC cell death [8]. Given that polysulfated heparan is an ecto-ATPase inhibitor, we next

345 determined the effect of combining the sulfatase inhibitor (OKN-007) with chemotherapy (paclitaxel) to

346 ascertain its impact on the viability of TNBC MDA-MB 231, Hs 578t, and MDA-MB 468 cells in

347 comparison to nontumorigenic immortal mammary epithelial MCF-10A cells and its effects on eATP

348 release. For these experiments, all the cell lines were treated with OKN-007 and heparan sodium sulfate

349 for 48 hours and then paclitaxel or a corresponding vehicle was added to the medium for the final 6 hours.

350 The 6-hour duration of paclitaxel exposure was used to simulate the short duration of systemic exposure

351 in cancer patients **(Figure 7)** [40]. As the treatment with paclitaxel was for only 6 hours, we did not see

352 changes in the viability of cells treated with paclitaxel alone (vehicle addition). However, in all 3 TNBC

353 cell lines—MDA-MB 231, Hs 578t, and MDA-MB 468—when paclitaxel (100 µM) was combined with

354 the sulfatase inhibitor OKN-007 (20 µM), there was a significant loss of cell viability when compared to

355 paclitaxel alone (shown as mean percentage loss of viability compared to vehicle control). Additionally,

356 the loss of cell viability was further increased significantly when heparan sodium sulfate was added to the

357 combination of paclitaxel and OKN-007 when compared to paclitaxel alone. However, there was no

15

358   significant change in the viability of MCF-10A cells treated with paclitaxel, OKN-007, and heparan

359   sodium sulfate compared to paclitaxel alone, suggesting that this effect may be selective for transformed

360   cells **(Figure 7A)**.

361   Under the same conditions, we also measured the concentration of eATP in the supernatants of the

362   chemotherapy-treated (paclitaxel) cells **(Figure 8)**. Upon treatment with the combination of OKN-007

363   and paclitaxel, we saw significant increases in eATP levels when compared to vehicle addition in MCF-

364   10A cells as well as TNBC MDA-MB 231, Hs 578t, and MDA-MB 468. There was also a significant

365   increase in eATP with the addition of heparan sodium sulfate to the combination of paclitaxel and OKN-

366   007 when compared to the vehicle addition. Therefore, the sulfatase inhibitor OKN-007 significantly

367   increased eATP release upon chemotherapy treatment and sensitized TNBC cell lines to chemotherapy

368   treatment.

369   We also treated TNBC MDA-MB 231, Hs 578t and MDA-MB 468 cells and nontumorigenic

370   immortal mammary epithelial MCF-10A cells paclitaxel (100 µM) for 6 hours or ATP (500 µM)

371   for 48 hours and were probed for sulfatase 2 **(Supplemental Figures 14-16)**. We did not observe

372   any change in sulfatase 2 expression in the presence of paclitaxel or ATP. We also obtained dose

373   response graphs for treatments of increasing concentrations of paclitaxel and OKN-007

374   **(Supplemental Figures 17)**, There is some synergy (<0.1-1.0) for some dose combinations

375   (paclitaxel and OKN-007) for MDA-MB 231 cells while there were some drug dose

376   combinations (paclitaxel and OKN-007) that were additive (1-1.2) for Hs 578t and MDA-MB

377   468 cells.

378

379   **Role of purinergic signaling in the augmentation of chemotherapy-induced TNBC cell death by**

380   **sulfatase 2 inhibitors**

381   We had previously shown that eATP exerts its cytotoxic effects on TNBC cells through P2RX4 and

382   P2RX7 receptors [8]. We sought to confirm whether the exaggerated loss of cell viability in the presence

383   of OKN-007 is dependent on eATP-induced activation of P2RX4 or P2RX7 **(Figure 9)**. We chose to

384   examine Hs 578t cells because we observed the largest increase in eATP and loss of cell viability in this

385   cell type when exposed to the combination of paclitaxel, OKN-007, and heparan sodium sulfate. We

386   observed a reversal of the effects of OKN-007 on cell viability and eATP release upon exposure to both

387   the P2RX7 inhibitor A438079 **(Figures 8A and C)** and the P2RX4 inhibitor 5-BDBD **(Figures 9B and**

388   **D)**. We observed a significant decline in eATP ($p<0.0001$) and increased cell viability ($p<0.0001$) when

389   comparing the combination of paclitaxel with OKN-007 to that of paclitaxel with OKN-007 and A43709.

390   We observed a significant decline in eATP ($p<0.0001$) and increased cell viability ($p<0.0001$) when

391   comparing the combination of paclitaxel, heparan sodium sulfate and OKN-007 to paclitaxel, heparan

392   sodium sulfate, OKN-007 and 5-BDBD. This data reveals that the exaggerated loss of cell viability

393   observed when OKN-007 is combined with paclitaxel is dependent on the activation of P2RX4 and

394   P2RX7 by eATP.

395

396   **Purinergic signaling and cancer-initiating cells**

397   Additionally, we sought to analyze the impact of the sulfatase inhibitor OKN-007 in combination with the

398   chemotherapeutic agent paclitaxel on cancer-initiating cell properties of treated cells. Breast cancer-

399   initiating cells have previously been shown to express aldehyde dehydrogenase (ALDH) intracellularly

400   and CD44, but not CD24 at the cell surface [41-44]. Hence, flow cytometry analysis was performed on

401   TNBC cell lines MDA-MB 231, MDA-MB 468, and Hs 578t treated with OKN-007 for 48 hours and

402   paclitaxel (100 µM) for the final 6 hours to determine the fraction of residual cells with a cancer-initiating

403   cell phenotype (cells that express high levels of ALDH and CD44 but do not express CD24). Paclitaxel

404   alone increased the fraction of residual cancer-initiating cells while OKN-007 combined with paclitaxel

405   depressed the fraction of cancer-initiating cells **(Figures 10A-C; Supplemental Figures 18-22)**.

406   Furthermore, we applied an orthogonal approach to analyze cancer-initiating cells by conducting

407   tumorsphere formation efficiency assays. TNBC cell lines were treated with paclitaxel, OKN-007, and/or

408   heparan sodium sulfate and then cultured on low-attachment round-bottom plates in sphere-forming

409   medium in the absence of drug. After 7 days, we observed that the fraction of wells with tumorsphere

410   formation decreased in all TNBC cell lines—MDA-MB 231, MDA-MB 468, and Hs 578t—treated with

411   paclitaxel as compared to vehicle control but was lowest when treated with the combination of paclitaxel

412   and OKN-007. Spheroids were visualized using the Etaluma™ Lumascope 620 (**Figures 10D-F**). Images

413   were also recorded (**Supplemental Figures 23-25**). This data aligns with flow cytometry analysis in that

414   the sulfatase inhibitor OKN-007 depressed cancer-initiating cell formation.

415

416   **DISCUSSION**

417   Chemotherapy is still the most effective treatment for TNBC. A major drawback of chemotherapy is its

418   failure to eradicate metastatic disease, despite transient responses. Therapeutic strategies that deepen and

419   lengthen responses are urgently needed. eATP, in the high micromolar to millimolar range, is cytotoxic to

420   cancer cell lines. We previously showed that chemotherapy treatment augments eATP release from

421   TNBC cells [8]. We also showed that ecto-ATPase inhibitors exacerbate chemotherapy-induced eATP

422   release from TNBC cells and accentuate chemotherapy-induced cell death. However, one drawback of

423   attempting to develop therapeutic small-molecule ecto-ATPase inhibitors is the presence of multiple

424   families of ecto-ATPases in humans, each with multiple members.

425   Polysulfated polysaccharides have been shown to inhibit multiple classes of ecto-ATPases. The

426   endogenous polysulfated HS was shown to attenuate the degradation of eATP. Therefore, we

427   hypothesized that increasing fully sulfated HS in the microenvironment of TNBC cells using sulfatase 2

428   inhibitors would augment eATP concentrations in the pericellular environment of chemotherapy-treated

429   TNBC cells, and hence accentuate chemotherapy-induced cell death.

18

430 Furthermore, our immunoblot results show that sulfatase 2 is highly expressed intracellularly and

431 extracellularly in TNBC cells in comparison to the immortal mammary epithelial cells; this was

432 additionally confirmed through ELISAs. However, the cell surface expression of sulfatase 2 did not differ

433 among the TNBC cell lines and immortal mammary epithelial cells. Previous publications have suggested

434 that processed sulfatase 2 is primarily an extracellular secreted protein [24, 26, 39]. This could explain the

435 difference in the expression levels in cell supernatants as compared to cell surface expression. Staining

436 results also revealed that there was a difference in the expression of sulfatase 2 at different stages of

437 TNBC, with the most significant difference being between stages 2A and 2B.

438 Previously our lab showed that eATP in the high micromolar to millimolar range is toxic to TNBC cells

439 and that inhibitors of each of the major classes of ecto-ATPases exacerbate chemotherapy-induced

440 increases in eATP [8]. One defect of this approach as a potential therapeutic strategy is the need to use

441 different inhibitors for each ecto-ATPase class to maximally augment eATP levels. As polysulfated

442 polysaccharides such as HS inhibit all 3 families of ecto-ATPases, we sought to determine the effects of

443 sulfatase 2 inhibitors, which block the desulfation of HS, on chemotherapy-induced augmentation of

444 eATP and chemotherapy-induced TNBC cell death [8]. After verifying that the sulfatase 2 inhibitor

445 OKN-007 inhibits sulfatase activity at the concentration utilized, we showed that the combination of

446 OKN-007 and chemotherapy (paclitaxel) accentuated extracellular eATP concentrations and enhanced the

447 chemotherapeutic response in TNBCs, leading to a greater loss of viability. Additionally, the effects of

448 the sulfatase 2 inhibitor on eATP levels and TNBC cell death were reversed by specific inhibitors of

449 P2RX4 and P2RX7 eATP receptors, confirming that these effects are dependent on these purinergic

450 receptors; we have previously shown that these receptors are necessary for chemotherapy-induced eATP

451 release from TNBC cells and its cytotoxic effects [8].

452 We also examined the effects of combinations of sulfatase 2 inhibitors and chemotherapy on cancer-

453 initiating cells, as failure to eliminate these cells results in the failure of cytotoxic chemotherapy to

454 eradicate metastatic TNBC [41, 43-45]. We found that upon treatment with chemotherapy alone, there

19

455 was an enrichment of cancer-initiating cells, as assessed by flow cytometry, across all TNBC cell lines,

456 while combination with the sulfatase inhibitor prevented their enrichment in the surviving fraction of

457 cells.

458 As eATP is a known immune danger signal and its metabolite adenosine a potent immunosuppressant,

459 further work is necessary to determine the immune effects of ecto-ATPase inhibition by HS using

460 immunocompetent in vivo TNBC models. Moreover, further work is necessary to determine if the

461 cytotoxic effects on TNBC cells occur through non-specific permeability of P2RX7 ion-coupled channels

462 or through downstream activation of pyroptosis by P2RX7. Our future research will focus on this aspect

463 of the effects of HS in the TME. In addition, the precise reasons for the different properties of sulfatase 1

464 and sulfatase 2, one a tumor suppressor in many biological contexts and the other an established

465 oncogene, need to be determined. The basis for this may be complex and related to the precise identity of

466 the cell surface and extracellular matrix proteins targeted by each enzyme. Thus, additional work is

467 necessary in this area.

468 **CONCLUSION**

469 Sulfatase 2 inhibition sensitizes TNBC cell lines to chemotherapy by enhancing eATP concentrations in

470 the microenvironment of chemotherapy-treated cells. Combinations of sulfatase 2 inhibitors with

471 chemotherapy may attenuate the cancer-initiating cell fraction, unlike chemotherapy alone. Thus,

472 sulfatase 2 inhibitors may have the potential to induce deeper and more durable responses when combined

473 with chemotherapy. Moreover, as eATP is a known immune danger signal, it will be critical to evaluate

474 the immune effects of this strategy. Our future goals are to study the effects of sulfatase 2 inhibition in

475 vivo.

476 **DECLARATIONS**

477 **Ethics approval and consent to participate**

478 Not applicable.

479 **Consent for publication**

480 Not applicable.

481 **Availability of data and materials**

482 The datasets used and/or analyzed during the current study are available from the corresponding author

483 upon reasonable request.

484 **Competing interests**

485 All authors declare that they have no competing interests.

486 **Funding**

493 **Authors' contributions**

494 All authors contributed to review and analysis. JM performed a majority of the assays with JD carrying

495 out the Western blot analysis, and LM carrying out immunohistochemistry analysis. JM and MC

496 conceived of and designed the experiments, reviewed the data, and authored and edited the manuscript.

497 **Acknowledgments**

500 **References**

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505   oup%5B%5D=0&ages_group%5B%5D=17&group_cancer=1&include_nmsc=0&include_nmsc_ot
506   her=1]
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640   **ABBREVIATIONS**

641   ALDH: aldehyde dehydrogenase
642   ANOVA: analysis of variance
643   ATP: adenosine triphosphate
644   DCIS: ductal carcinoma in situ
645   DMSO: dimethyl sulfoxide
646   eATP: extracellular adenosine triphosphate
647   ELISA: enzyme-linked immunoassay
648   E-NPPase: ecto-nucleotide pyrophosphatases/phosphodiesterases
649   ENPP1: ecto-nucleotide pyrophosphatase/phosphodiesterase 1
650   ENTPD1: ecto-nucleoside triphosphate diphosphohydrolases 1
651   ER+: estrogen receptor-positive
652   FACS: fluorescence-activated cell sorting
653   FITC: fluorescein isothiocyanate
654   5'-NTs: 5'-nucleotidases
655   GAPDH: glyceraldehyde-3-phosphate dehydrogenase
656   HIER: heat-induced epitope retrieval
657   HER2+: human epidermal growth factor receptor 2-positive
658   HS: heparan sulfate
659   HSD: honestly significant difference
660   MFI: mean fluorescence intensity
661   mM: millimolar
662   nM: nanomolar
663   PAGE: polyacrylamide gel electrophoresis
664   PBS: phosphate buffered saline
665   PR+: progesterone receptor-positive
666   TMA: tissue microarray
667   TME: tumor microenvironment
668   TNAP: tissue nonspecific alkaline phosphatases
669   TNBC: triple-negative breast cancer
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671

672   **Figure Legends**

673   **Figure 1. Schematic displaying our proposed model for heparan sulfate's impact on eATP.** Our

674   proposed model suggests that accumulation of polysulfated polysaccharide HS, due to the presence of the

675   sulfatase inhibitor OKN-007, facilitates ATP accumulation in the extracellular environment of paclitaxel-

676   treated TNBC cells by preventing the breakdown of eATP by eATPases, which can lead to exacerbation

677   of chemotherapy-induced cell death.

678   **Figure 2. Heparan sulfate's influence on eATPases' activity.** ATP (500 µM) was incubated with

679   vehicle or 100 units of one member of each of the families of extracellular ATPases, tissue non-specific

25

680   alkaline phosphatase (TNAP), ENTPD1, and ENPP1, in the presence or absence of heparan sodium

681   sulfate (50 µM) for **(A)** 48 hours at 37°C or **(B)** 0 hours. For **(C)** and **(D)**, enzymes were boiled before

682   incubation, and eATP was measured after **(C)** 48 hours at 37°C or **(D)** 0 hours. ATP concentrations were

683   measured using a luciferase-based assay. HS blocked eATP degradation by all three families of enzymes.

684   The standard deviation was calculated from three independent experiments performed in triplicate. The

685   student's t-test was performed to determine significance, with ** indicating *p* value <0.01 for the

686   comparison of enzyme-treated ATP vs. enzyme and heparan sodium sulfate.

687   **Figure 3. Immunoblot analysis of sulfatase 2 expression in immortal mammary epithelial cells and**

688   **TNBC cell lines.** For the Western blot analysis of sulfatase 2, **(A)** 5 µl of cell supernatants of

689   nontumorigenic immortal mammary epithelial MCF-10A cells and TNBC cell lines—MDA-MB 231, Hs

690   578t, and MDA-MB 468 cells—were subjected to polyacrylamide gel electrophoresis (PAGE) and probed

691   with a human sulfatase 2-specific antibody. **(B)** Adjusted volume of cell supernatants inversely

692   proportionate to the protein concentration in the corresponding cell lysate were loaded for PAGE and

693   probed with a sulfatase 2-specific antibody to control for cell number and viability. **(C)** Equal amounts of

694   cell lysate from each cell line were probed with a sulfatase 2-specific antibody. All experiments were

695   repeated once with biological replicates. The densitometric analyses of the bands were performed using

696   Image Studio software (LI-COR Inc.). The student's t-test was performed to determine significance with

697   * representing *p*<0.05 and ** representing *p*<0.01 when comparing expression in MCF-10A cells to

698   expression in TNBC cell lines. Cropped blots are shown in the figure and full-length blots are presented

699   in **Supplementary Figures 3-5**.

700   **Figure 4. Immunoblot analysis of sulfatase 1 expression in immortal mammary epithelial cells and**

701   **TNBC cell lines.** For the Western blot analysis of sulfatase 1, **(A)** 5 µl of cell supernatants of

702   nontumorigenic immortal mammary epithelial MCF-10A cells and TNBC cell lines—MDA-MB 231, Hs

703   578t, and MDA-MB 468 cells—were subjected to PAGE and probed with a human sulfatase 1-specific

704   antibody. **(B)** Adjusted volume of cell supernatants inversely proportionate to the protein concentration in

26

705   the corresponding cell lysate were loaded for PAGE and probed with a sulfatase 1-specific antibody. **(C)**

706   Equal amounts of cell lysate from each cell line were probed with a sulfatase 1-specific antibody. All

707   experiments were repeated once with biological replicates. The densitometric analyses of the bands were

708   performed using Image Studio software (LI-COR Inc.). The student's t-test was performed to determine

709   significance with * representing $p<0.05$ and ** representing $p<0.01$ when comparing expression in MCF-

710   10A cells to expression in TNBC cell lines. Cropped blots are shown in the figure and full-length blots

711   are presented in **Supplementary Figures 6-8**.

712   **Figure 5. Cell surface expression of sulfatase 2, sulfatase activity, and immunohistochemistry. (A)**

713   Baseline sulfatase 2 cell surface expression levels were examined via flow cytometry analysis in

714   nontumorigenic immortal mammary epithelial MCF-10A cells and TNBC MDA-MB 231, Hs 578t, and

715   MDA-MB 468 cells by flow cytometry. The standard deviation (SD) was calculated from three

716   independent experiments performed in triplicate. The student's t-test was performed to determine

717   significance with * representing $p<0.05$ and ** representing $p<0.01$, comparing expression levels in

718   MCF-10A to those in the TNBC cell lines. **(B)** A sulfatase activity assay was carried out. OKN-007 (20

719   µM) was added to the vehicle (sulfatase; 0, 2, 4 µL) and sulfatase substrate; sulfatase activity (desulfation

720   of p-nitrocathecol sulfate to p-nitrocathecol) was determined using a standard dilution of p-nitrocathecol.

721   There was a decrease in sulfatase activity when sulfatase and sulfatase substrate were exposed to OKN-

722   007, confirming the antagonistic effect of this inhibitor on sulfatase. The SD was calculated from three

723   independent experiments performed in triplicate. The student's t-test was performed to determine

724   significance with * representing $p<0.05$ and ** representing $p<0.01$, comparing the sulfatase activity

725   between the vehicle (sulfatase) to OKN-007 and vehicle. **(C)** The AMSBIO BR1202B breast cancer

726   tissue array (120 cores with 82 TNBC cores; key can be found in **Supplemental Figure 10C**) was stained

727   with sulfatase 2.

728   **Figure 6. Statistical analysis for sulfatase 2 immunohistochemistry. (A)** The Kruskal-Wallis test

729   indicated that there was no significant difference in the average percentages of cells that stained positively

27

730   for sulfatase 2 in tissue sections of TNBC, ER+/PR+ breast cancer, HER2+ breast cancer, normal breast

731   tissue, and DCIS. **(B)** The Kruskal-Wallis test results showed that there was no significant difference

732   between the average percentages of cells that stained weakly positive for sulfatase 2 in tissue sections of

733   TNBC, ER+/PR+ breast cancer, HER2+ breast cancer, normal breast tissue, and DCIS. **(C)** Pairwise

734   comparisons using Dunn's test indicated that there was a significant difference between breast cancer sub-

735   types TNBC and ER+/PR+ ($p = 0.0497$) in the percentages of cells that stained moderately positive for

736   sulfatase 2. No other differences were statistically significantly between the other groups. **(D)** Pairwise

737   comparisons using Dunn's test showed that there was no significant difference between the percentages of

738   cells that stained positive for sulfatase 2 among TNBC, ER+/PR+ breast cancer, HER2+ breast cancer,

739   normal breast tissue, and DCIS. **(E)** The Kruskal-Wallis test indicated that there was no significant

740   difference in the percentages of cells that stained negatively for sulfatase 2 among TNBC, ER+/PR+

741   breast cancer, HER2+ breast cancer, normal breast tissue, and DCIS. **(F)** Pairwise comparisons using

742   Dunn's test showed that there was a significant difference between TNBC breast cancer stages 2A and 2B

743   ($p = 0.0003$) in the percentages of cells that stained positively for sulfatase 2. No other differences among

744   different TNBC stages were significant.

745   **Figure 7. Effects of sulfatase inhibitor OKN-007 combined with chemotherapeutic agent paclitaxel**

746   **on cell viability.** Percentage loss of cell viability was measured in treated **(A)** nontumorigenic immortal

747   mammary epithelial MCF-10A cells and TNBC cell lines **(B)** MDA-MB 231, **(C)** Hs 578t, and **(D)**

748   MDA-MB 468 cells. The treatments applied were vehicle addition (paclitaxel, purple), heparan sodium

749   sulfate (50 µM, teal), and OKN-007 (20 µM, light blue) or the combination (dark blue-green); heparan

750   sodium sulfate and OKN-007 were administered for 48 hours, and paclitaxel was added for the final 6

751   hours to replicate paclitaxel exposure times in patients. Standard deviation was calculated from three

752   independent experiments performed in triplicate. One-way ANOVA with Tukey's HSD was applied to

753   ascertain significance. * represents $p<0.05$ and ** represents $p<0.01$ when comparing vehicle addition to

754   heparan sodium sulfate, OKN-007, or the combination.

28

755   **Figure 8. Effect of sulfatase inhibitor OKN-007, chemotherapeutic agent paclitaxel and their**

756   **combination on extracellular ATP concentrations.** Extracellular ATP concentrations were measured in

757   the supernatants of treated **(A)** nontumorigenic immortal mammary epithelial MCF-10A cells and triple-

758   negative breast cancer cell lines **(B)** MDA-MB 231**, (C)** Hs 578t, and **(D)** MDA-MB 468 cells. The

759   treatments: vehicle addition (paclitaxel, purple), heparan sodium sulfate (50 µM, teal) and OKN-007 (20

760   µM, light blue), or the combination of all 3 (dark blue-green); heparan sodium sulfate and OKN-007 were

761   administered for 48 hours and paclitaxel was added for the final 6 hours to replicate exposure times to

762   paclitaxel in patients. Standard deviation was calculated from three independent experiments performed

763   in triplicate. One-way ANOVA with Tukey's HSD was applied to ascertain significance. * represents

764     $p<0.05$ and ** represents $p<0.01$ when comparing vehicle addition to heparan sodium sulfate, OKN-007,

765   or the combination regimen.

766   **Figure 9. Reversal of sulfatase inhibitor's effects by P2RX4 and P2RX7 inhibitors.** For experiments

767   corresponding to figures **(A)** and **(B),** Hs 578t cells were treated with OKN-007 (20 µM, 48 hours),

768   paclitaxel (100 µM, the final 6 hours of the 48-hour time course to replicate exposure times in patients),

769   heparan sodium sulfate (50 µM, 48 hours), the P2RX7 antagonist A437809 (20 µM, 6 hours), or a

770   combination of the different drug agents. Standard deviation was calculated from three independent

771   experiments performed in triplicate. One-way ANOVA with Tukey's HSD was applied to ascertain

772   significance. * represents $p<0.05$ and ** represents $p<0.01$ when comparing OKN-007 to the combination

773   of vehicle addition, OKN-007, and A437809; + represents $p<0.05$ and ++ represents $p<0.01$ when

774   comparing OKN-007 to the combination of vehicle addition, OKN-007, A437809, and heparan sodium

775   sulfate. For experiments corresponding to figures **(C)** and **(D),** Hs 578t cells were treated with OKN-007

776   (20 µM, 48 hours), paclitaxel (100 µM, final 6 hours of the 48-hour time course to replicate exposure

777   times in patients), heparan sodium sulfate (50 µM, 48 hours), the P2RX4 antagonist 5-BDBD (20 µM, 6

778   hours), or combinations. Standard deviation was calculated from three independent experiments

779   performed in triplicate. One-way ANOVA with Tukey's HSD was applied to ascertain significance. *

780   represents $p<0.05$ and ** represents $p<0.01$ when comparing OKN-007 to the combination of vehicle

781   addition, OKN-007, and 5-BDBD; + represents $p<0.05$ and ++ represents $p<0.01$ when comparing OKN-

782   007 to the combination of vehicle addition, OKN-007, 5-BDBD, and heparan sodium sulfate.

783   **Figure 10. Effect of OKN-007 and chemotherapy on the cancer-initiating cell fraction of TNBC**

784   **cells.** The three TNBC cell lines **(A)** MDA-MB 231, **(B)** MDA-MB 468, and **(C)** Hs 578t were treated

785   with OKN-007 (20 µM, 48 hours) and/or paclitaxel (100 µM, final 6 hours of the 48-hour time course to

786   replicate exposure times in patients). Paclitaxel alone increased the cancer-initiating cell fraction among

787   the surviving cells (cells that express high levels of ALDH and CD44 but do not express CD24), while the

788   combination of OKN-007 and paclitaxel decreased the cancer-initiating cell fraction. One-way ANOVA

789   with Tukey's HSD was applied to ascertain significance. * represents $p<0.05$ and ** represents $p<0.01$

790   when comparing paclitaxel to paclitaxel and OKN-007. Effects on cancer-initiating cells were also

791   determined through the tumorsphere formation efficiency assay in which TNBC cell lines **(D)** MDA-MB

792   231, **(E)** MDA-MB 468, and **(F)** Hs 578t cells were treated with vehicle (DMSO), paclitaxel (100 µM,

793   final 6 hours of the 48-hour time course to replicate exposure times in patients), heparan sodium sulfate

794   (50 µM, 48 hours), OKN-007 (20 µM, 48 hours), or the different combinations listed. Treated TNBC

795   cells were washed, passed through cell strainers, collected, and plated at approximately one cell per well

796   on round-bottom, low-attachment, 96-well plates; tumorspheres were allowed to form for 7 days. The

797   fraction of wells plated with at least one live cell positive for tumorspheres after 7 days were counted

798   using Etaluma™ Lumascope 620. The combination regimens showed a significant decrease in

799   tumorsphere formation when compared to the single-agent treatments of vehicle, paclitaxel, heparan

800   sodium sulfate, or OKN-007 treated cells. One-way ANOVA with Tukey's HSD was applied to ascertain

801   significance. ** represents $p<0.01$ when comparing paclitaxel to paclitaxel and OKN-007.

Figure 1
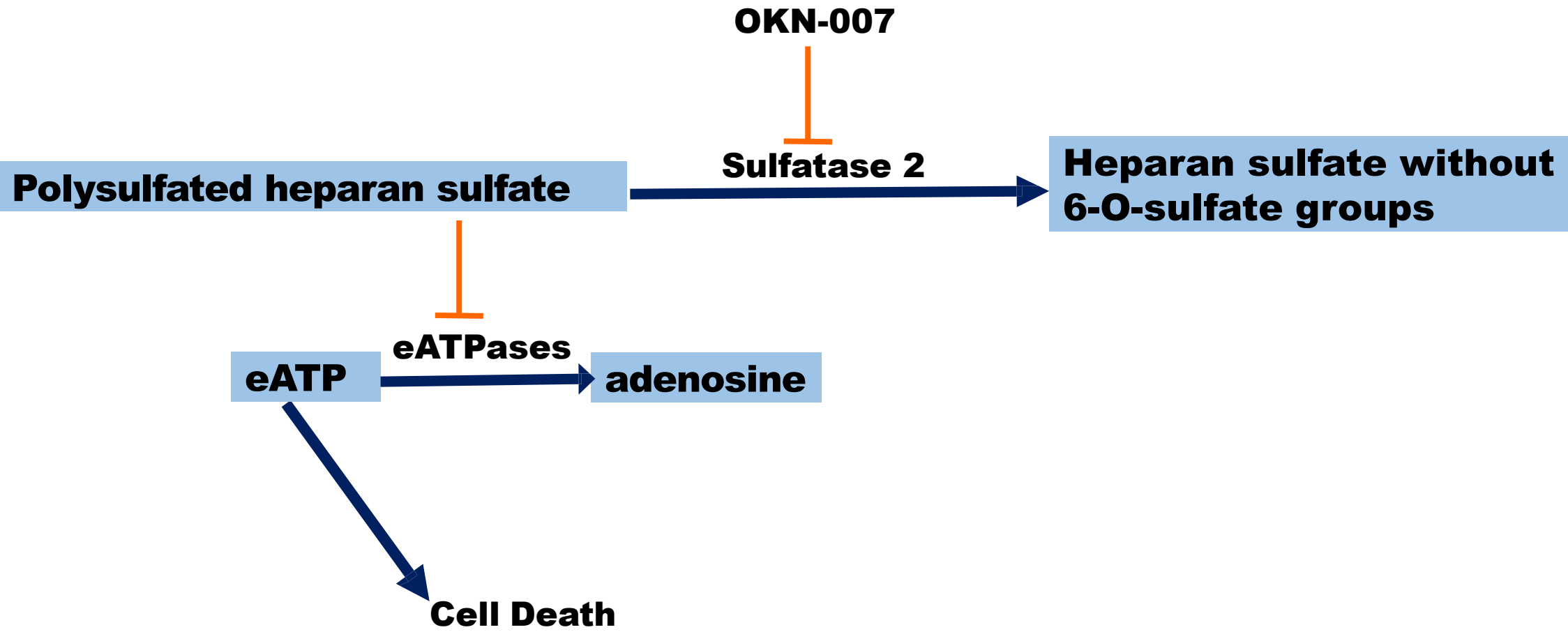
Figure 1

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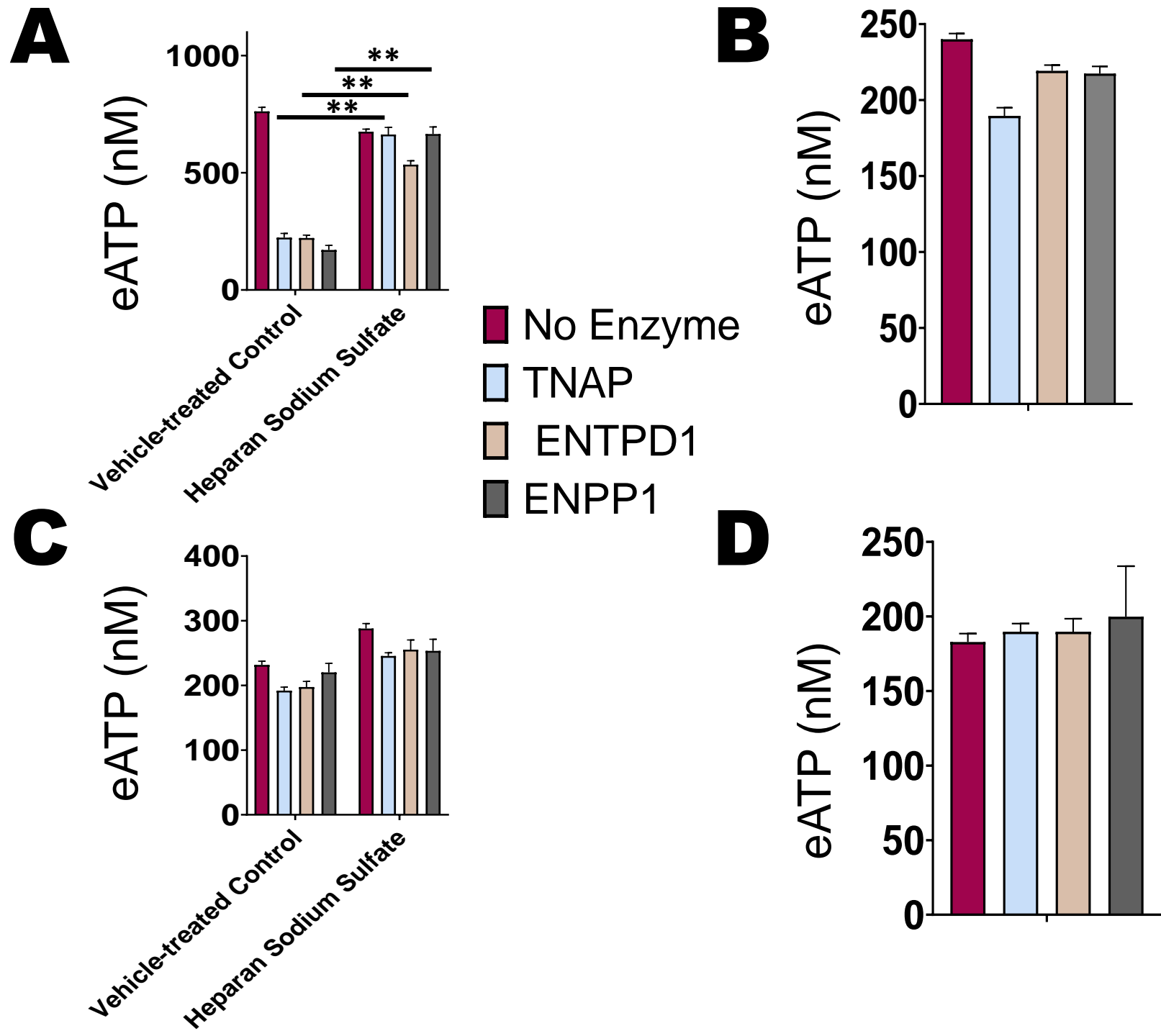
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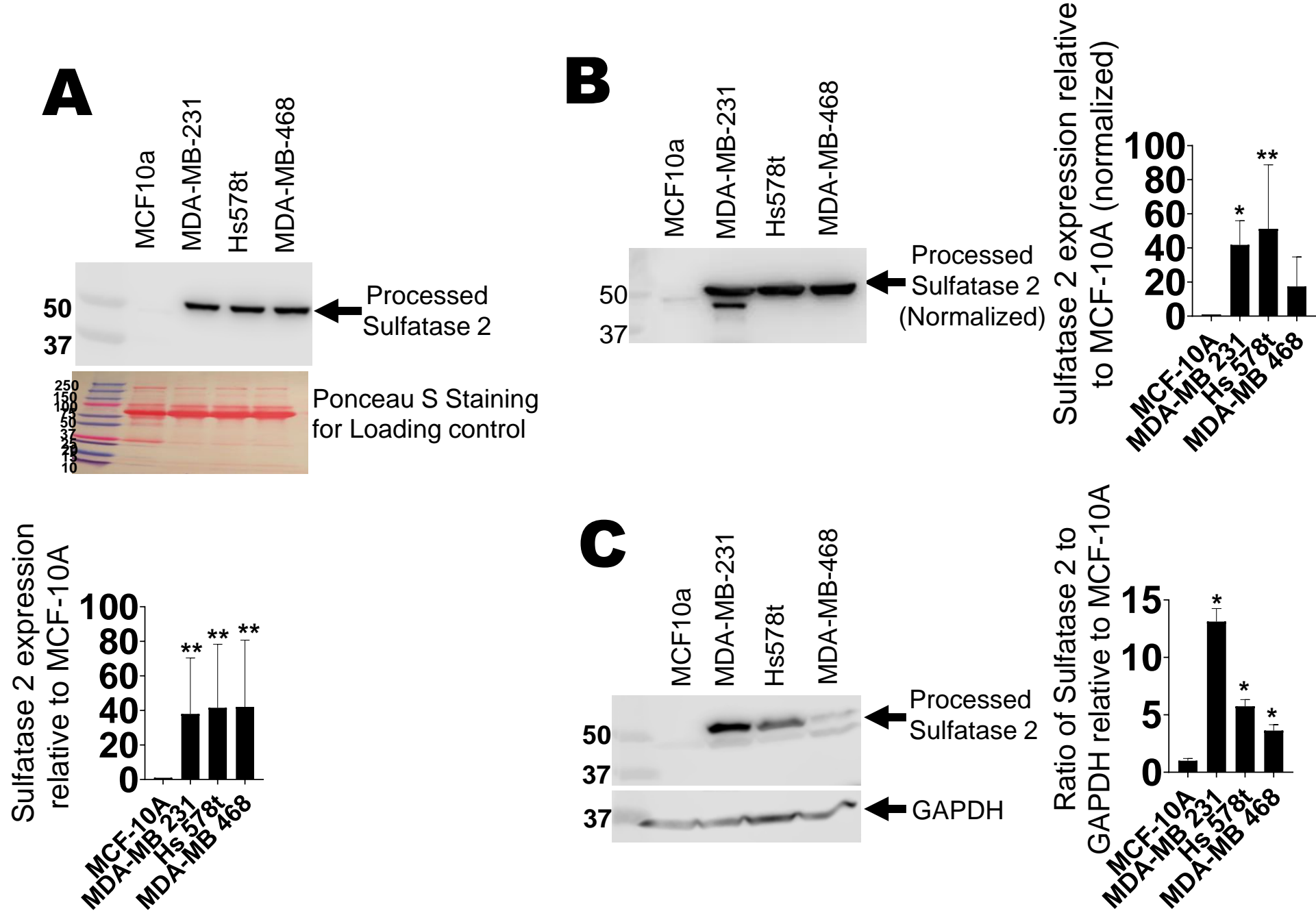
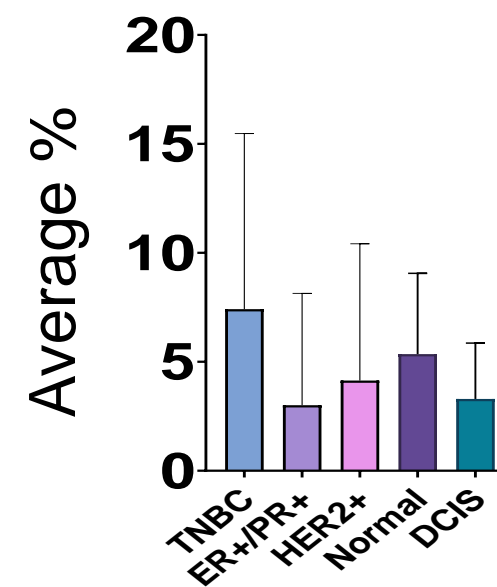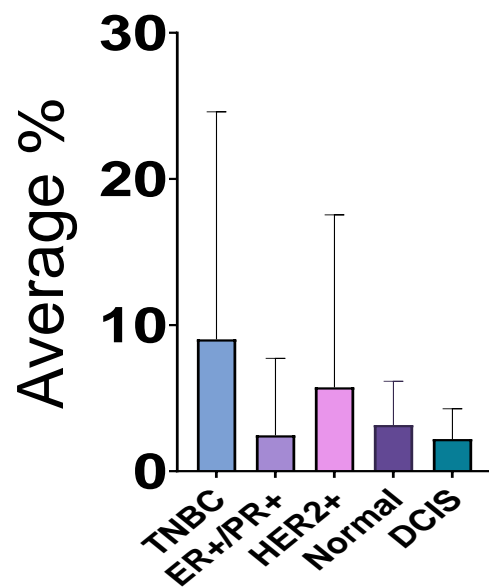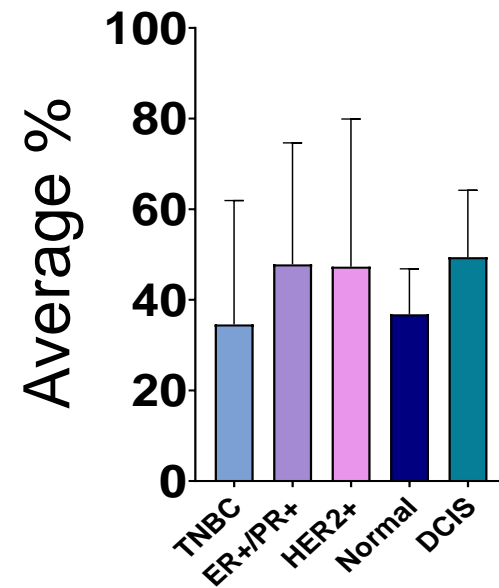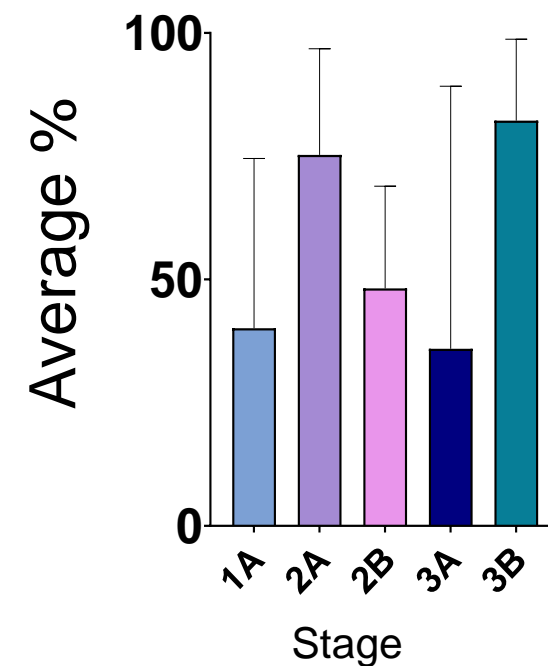**Figure 4**
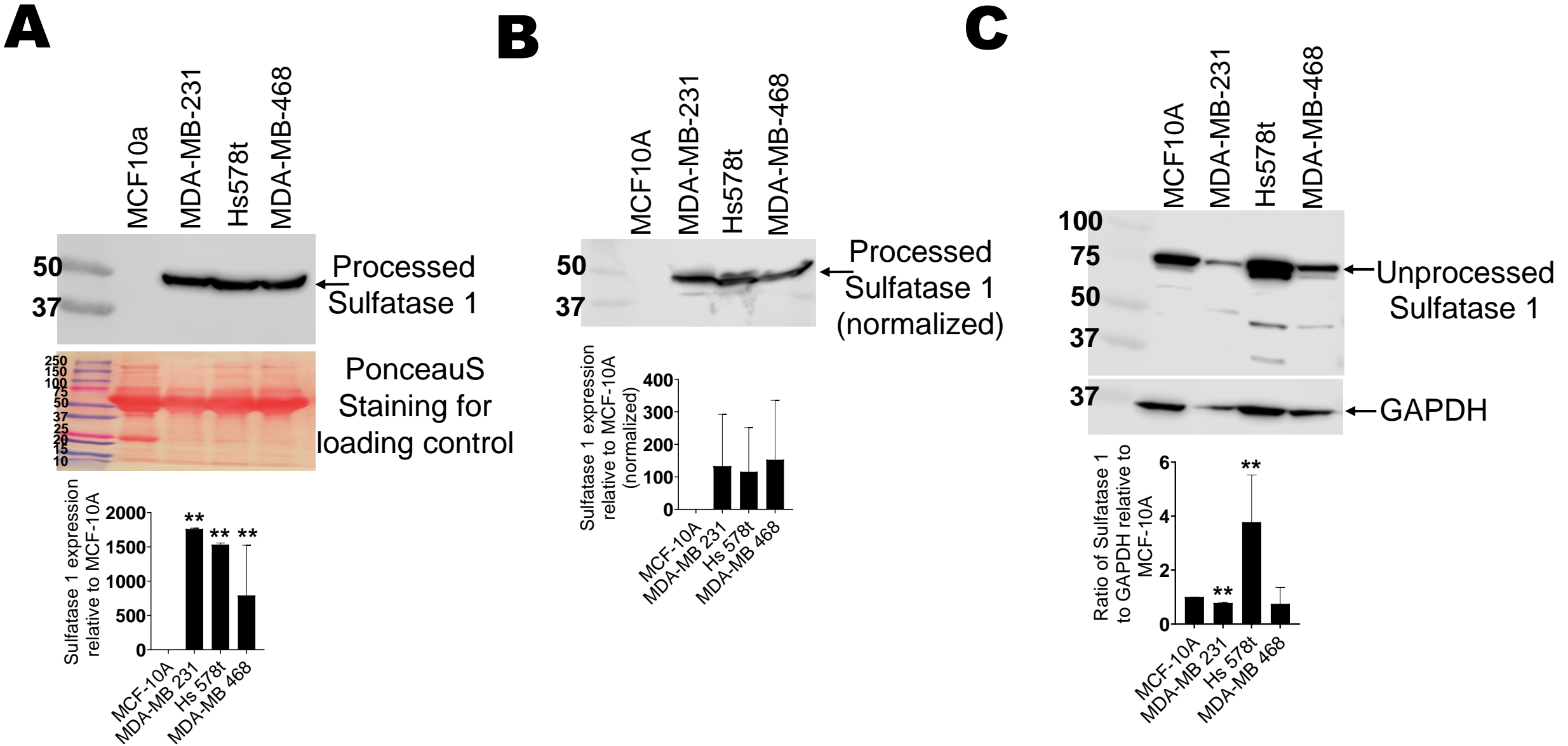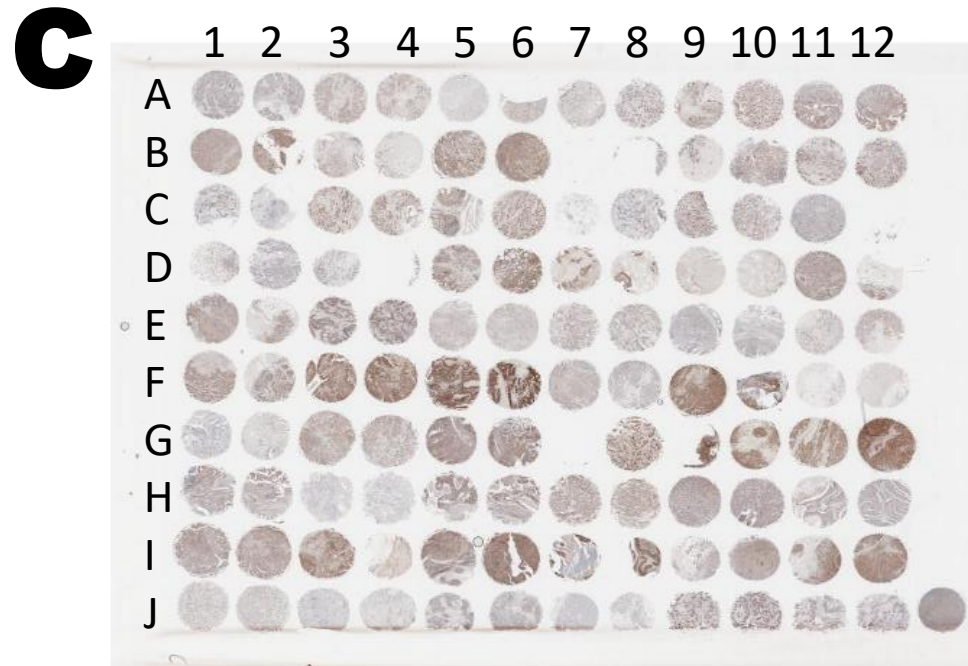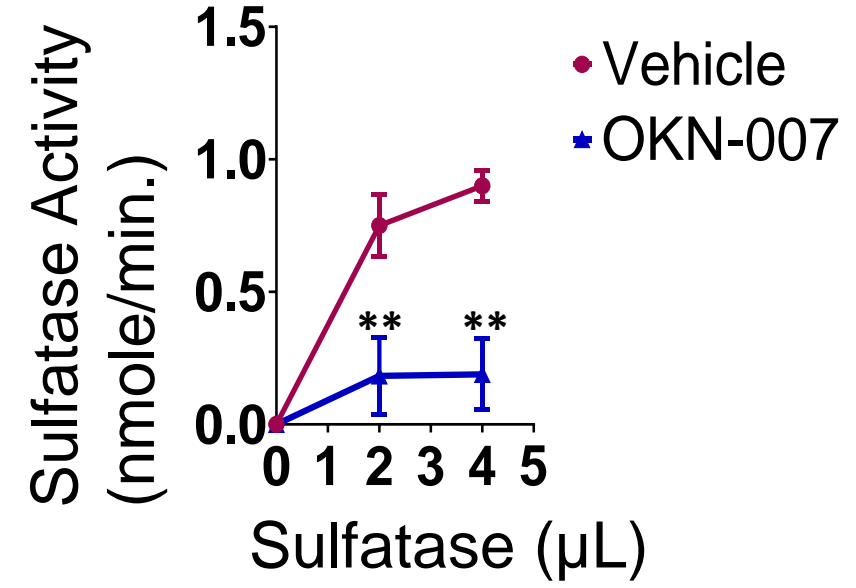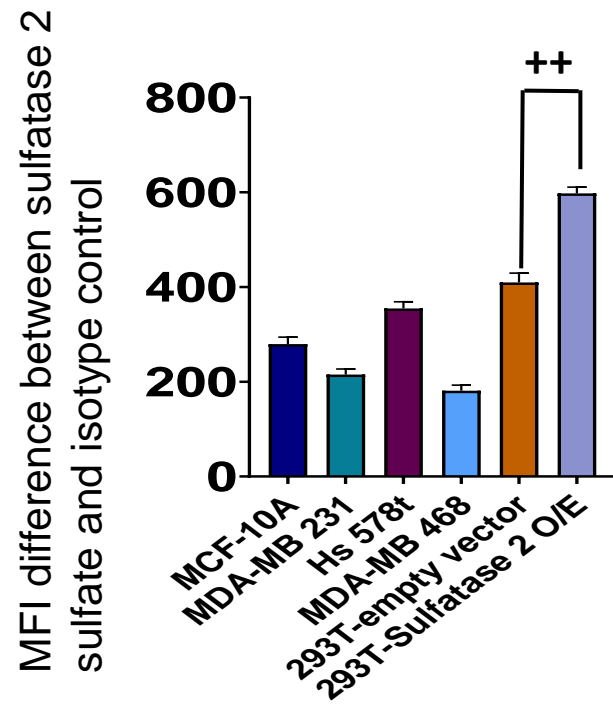
Figure 5

Figure 6

A Sulfatase 2 Positive Cells
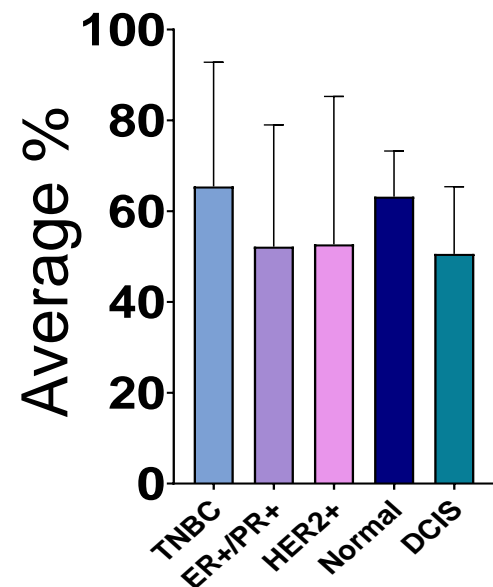
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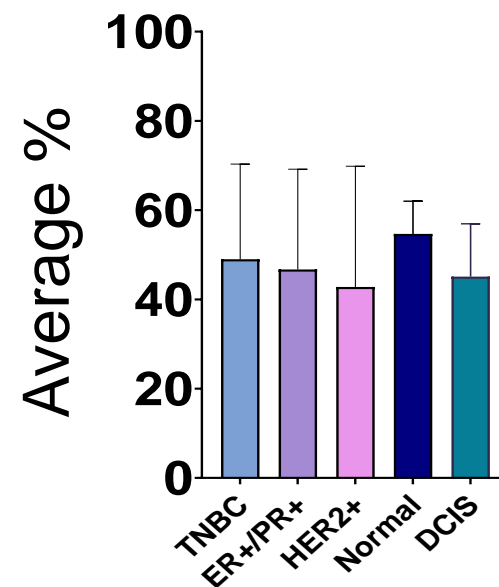
C Sulfatase 2 Moderate Positive Cells

D Sulfatase 2 Strong Positive Cells

E Sulfatase 2 Negative Cells

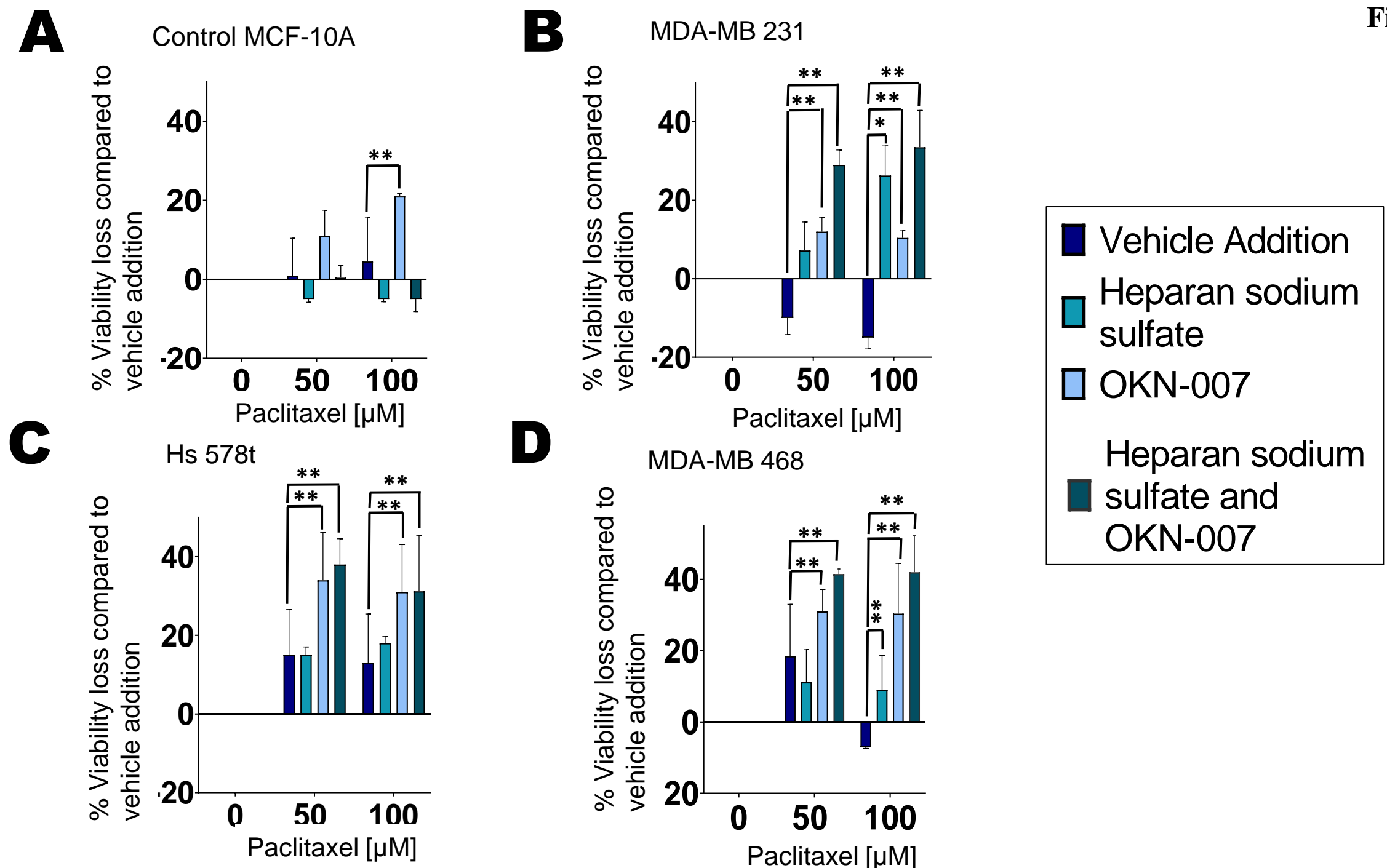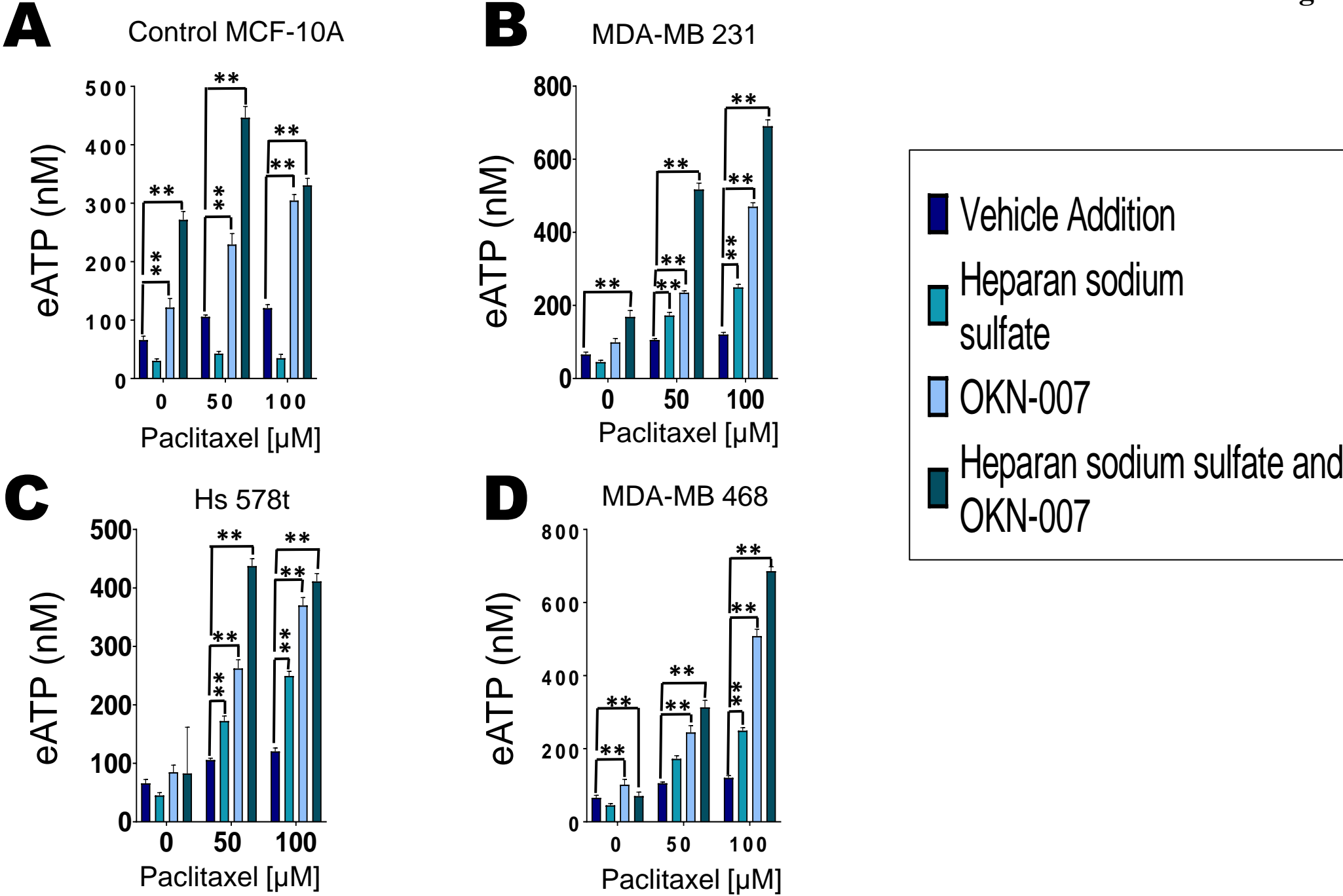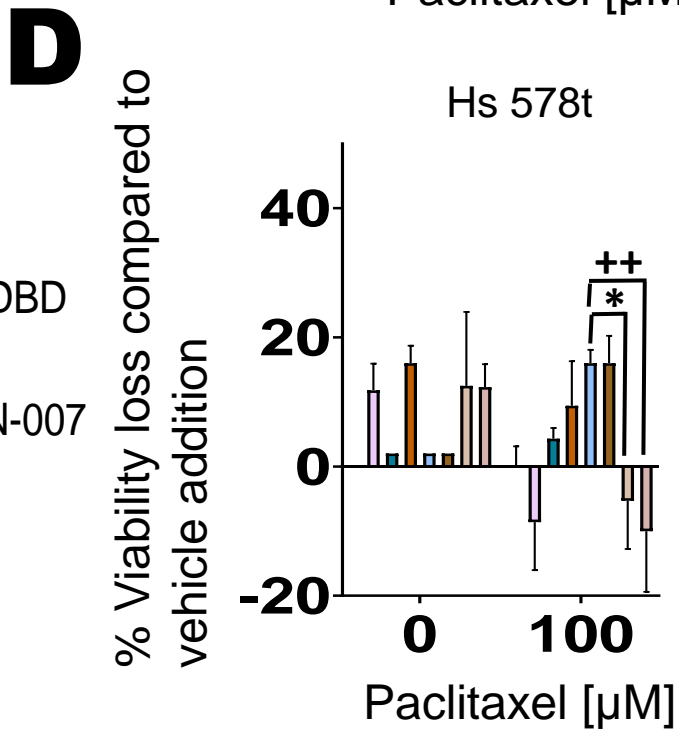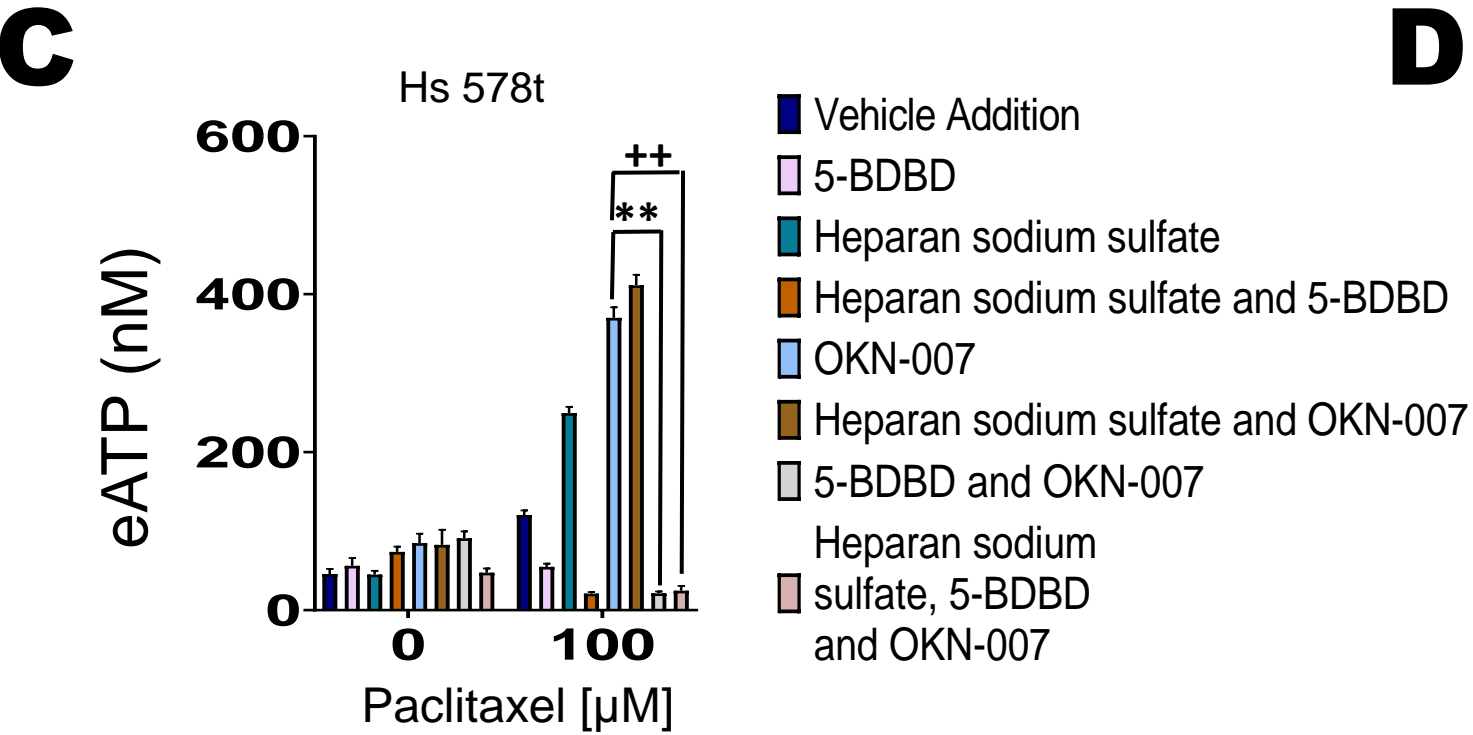**F** Sulfatase 2 Positive Cells in TNBC

Figure 7

Figure 8

Figure 9

Figure 10