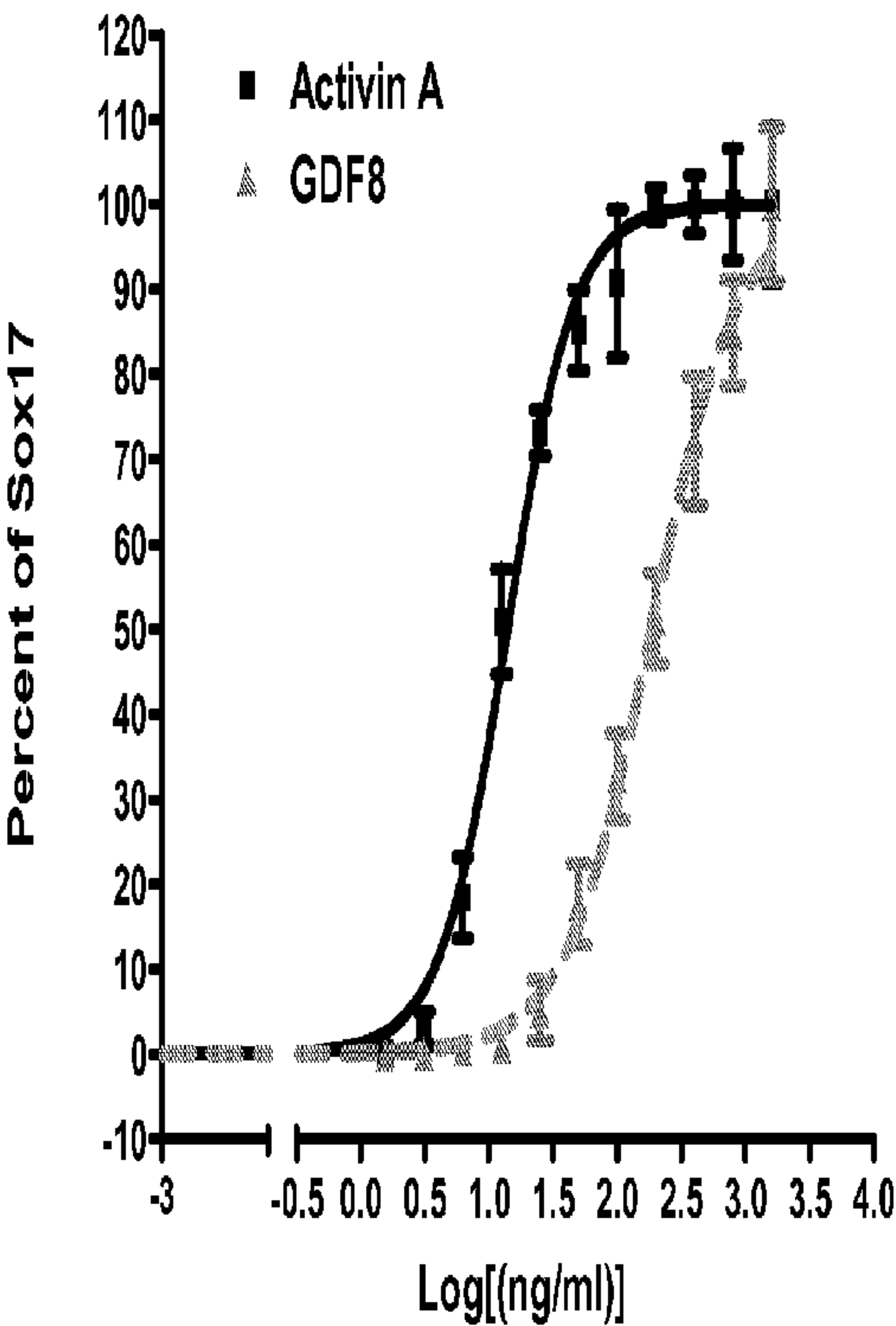




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(54) Titre : DIFFERENCIATION DE CELLULES SOUCHES PLURIPOTENTES
(54) Title: DIFFERENTIATION OF PLURIPOTENT STEM CELLS



(57) **Abrégé/Abstract:**
The present invention is directed to methods to differentiate pluripotent stem cells. In particular, the present invention is directed to methods and compositions to differentiate pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage comprising culturing the pluripotent stem cells in medium comprising a sufficient amount of GDF-8 to cause the differentiation of the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage.

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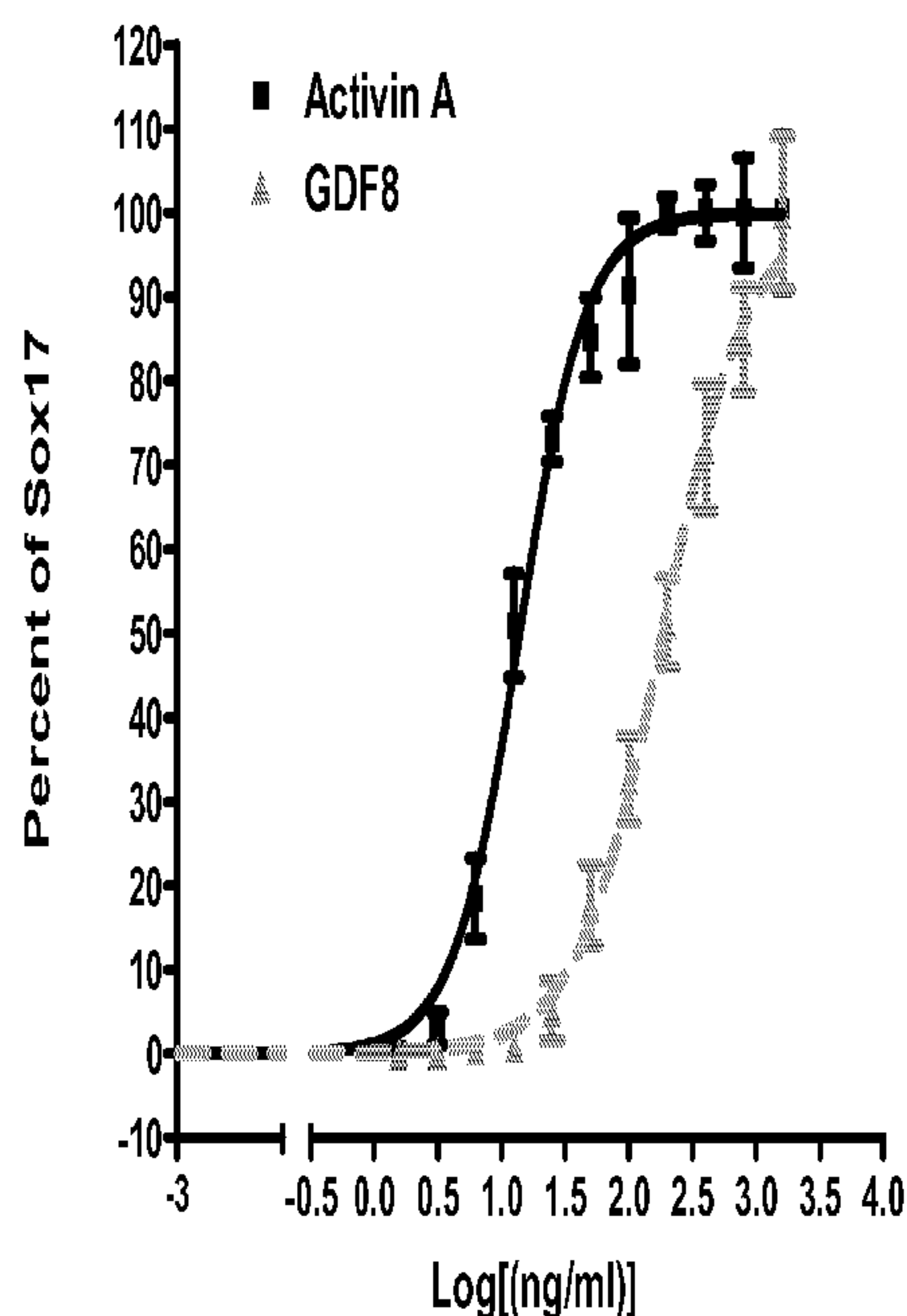
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[Continued on next page]

(54) Title: DIFFERENTIATION OF PLURIPOTENT STEM CELLS

Figure 2

(57) Abstract: The present invention is directed to methods to differenti-
ate pluripotent stem cells. In particular, the present invention is directed to
methods and compositions to differentiate pluripotent stem cells into cells
expressing markers characteristic of the definitive endoderm lineage com-
prising culturing the pluripotent stem cells in medium comprising a suffi-
cient amount of GDF-8 to cause the differentiation of the pluripotent stem
cells into cells expressing markers characteristic of the definitive endo-
derm lineage.

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DIFFERENTIATION OF PLURIPOTENT STEM CELLS

- [0001] The present invention claims priority to application serial number 61/076,900, filed June 30, 2008, application serial number 61/076,908, filed June 30, 2008, and application serial number 61/076,915, filed June 30, 2008.

FIELD OF THE INVENTION

- [0002] The present invention is directed to methods to differentiate pluripotent stem cells. In particular, the present invention is directed to methods and compositions to differentiate pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage comprising culturing the pluripotent stem cells in medium comprising a sufficient amount of GDF-8 to cause the differentiation of the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage.

BACKGROUND

- [0003] Advances in cell-replacement therapy for Type I diabetes mellitus and a shortage of transplantable islets of Langerhans have focused interest on developing sources of insulin-producing cells, or β cells, appropriate for engraftment. One approach is the generation of functional β cells from pluripotent stem cells, such as, for example, embryonic stem cells.
- [0004] In vertebrate embryonic development, a pluripotent cell gives rise to a group of cells comprising three germ layers (ectoderm, mesoderm, and endoderm) in a process known as gastrulation. Tissues such as, for example, thyroid, thymus, pancreas, gut, and liver, will develop from the endoderm, via an intermediate stage. The intermediate stage in this process is the formation of definitive endoderm. Definitive endoderm cells express a number of markers, such as, for example, HNF-3 β , GATA4, MIXL1, CXCR4 and SOX17.
- [0005] Formation of the pancreas arises from the differentiation of definitive endoderm into pancreatic endoderm. Cells of the pancreatic endoderm express the pancreatic-duodenal homeobox gene, PDX1. In the absence of PDX1, the pancreas fails to develop beyond the formation of ventral and dorsal buds. Thus, PDX1 expression

marks a critical step in pancreatic organogenesis. The mature pancreas contains, among other cell types, exocrine tissue and endocrine tissue. Exocrine and endocrine tissues arise from the differentiation of pancreatic endoderm.

- [0006] Cells bearing the features of islet cells have reportedly been derived from embryonic cells of the mouse. For example, Lumelsky *et al.* (Science 292:1389, 2001) report differentiation of mouse embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Soria *et al.* (Diabetes 49:157, 2000) report that insulin-secreting cells derived from mouse embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice.
- [0007] In one example, Hori *et al.* (PNAS 99: 16105, 2002) discloses that treatment of mouse embryonic stem cells with inhibitors of phosphoinositide 3-kinase (LY294002) produced cells that resembled β cells.
- [0008] In another example, Blyszczuk *et al.* (PNAS 100:998, 2003) reports the generation of insulin-producing cells from mouse embryonic stem cells constitutively expressing Pax4.
- [0009] Micallef *et al.* reports that retinoic acid can regulate the commitment of embryonic stem cells to form Pdx1 positive pancreatic endoderm. Retinoic acid is most effective at inducing Pdx1 expression when added to cultures at day 4 of embryonic stem cell differentiation during a period corresponding to the end of gastrulation in the embryo (Diabetes 54:301, 2005).
- [0010] Miyazaki *et al.* reports a mouse embryonic stem cell line over-expressing Pdx1. Their results show that exogenous Pdx1 expression clearly enhanced the expression of insulin, somatostatin, glucokinase, neurogenin3, p48, Pax6, and HNF6 genes in the resulting differentiated cells (Diabetes 53: 1030, 2004).
- [0011] Skoudy *et al.* reports that activin A (a member of the TGF- β superfamily) up-regulates the expression of exocrine pancreatic genes (p48 and amylase) and endocrine genes (Pdx1, insulin, and glucagon) in mouse embryonic stem cells.
- [0012] The maximal effect was observed using 1 nM activin A. They also observed that the expression level of insulin and Pdx1 mRNA was not affected by retinoic acid;

however, 3 nM FGF7 treatment resulted in an increased level of the transcript for Pdx1 (Biochem. J. 379: 749, 2004).

- [0013] Shiraki *et al.* studied the effects of growth factors that specifically enhance differentiation of embryonic stem cells into Pdx1 positive cells. They observed that TGF β 2 reproducibly yielded a higher proportion of Pdx1 positive cells (Genes Cells. 2005 June; 10(6): 503-16).
- [0014] Gordon *et al.* demonstrated the induction of brachyury [positive]/ HNF-3beta [positive] endoderm cells from mouse embryonic stem cells in the absence of serum and in the presence of activin along with an inhibitor of Wnt signaling (US 2006/0003446A 1).
- [0015] Gordon *et al.* (PNAS, Vol 103, page 16806, 2006) states: "Wnt and TGF-beta/ nodal/ activin signaling simultaneously were required for the generation of the anterior primitive streak."
- [0016] However, the mouse model of embryonic stem cell development may not exactly mimic the developmental program in higher mammals, such as, for example, humans.
- [0017] Thomson *et al.* isolated embryonic stem cells from human blastocysts (Science 282:114, 1998). Concurrently, Gearhart and coworkers derived human embryonic germ (hEG) cell lines from fetal gonadal tissue (Shamblott *et al.*, Proc. Natl. Acad. Sci. USA 95:13726, 1998). Unlike mouse embryonic stem cells, which can be prevented from differentiating simply by culturing with Leukemia Inhibitory Factor (LIF), human embryonic stem cells must be maintained under very special conditions (U.S. Pat. No. 6,200,806; WO 99/20741; WO 01/51616).
- [0018] D'Amour *et al.* describes the production of enriched cultures of human embryonic stem cell-derived definitive endoderm in the presence of a high concentration of activin and low serum (D'Amour K A *et al.* 2005). Transplanting these cells under the kidney capsule of mice resulted in differentiation into more mature cells with characteristics of some endodermal organs. Human embryonic stem cell-derived definitive endoderm cells can be further differentiated into PDX1 positive cells after addition of FGF-10 (US 2005/0266554A1).

- [0019] D'Amour *et al.* (Nature Biotechnology--24, 1392-1401 (2006)) states: "We have developed a differentiation process that converts human embryonic stem (hES) cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin. This process mimics *in vivo* pancreatic organogenesis by directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor en route to cells that express endocrine hormones."
- [0020] In another example, Fisk *et al.* reports a system for producing pancreatic islet cells from human embryonic stem cells (US2006/0040387A1). In this case, the differentiation pathway was divided into three stages. Human embryonic stem cells were first differentiated to endoderm using a combination of n-butyrate and activin A. The cells were then cultured with TGF- β antagonists such as Noggin in combination with EGF or betacellulin to generate PDX1 positive cells. The terminal differentiation was induced by nicotinamide.
- [0021] In one example, Benvenistry *et al.* states: "We conclude that over-expression of PDX1 enhanced expression of pancreatic enriched genes, induction of insulin expression may require additional signals that are only present *in vivo*" (Benvenistry *et al.*, Stem Cells 2006; 24:1923-1930).
- [0022] Activin A is a TGF-beta family member that exhibits a wide range of biological activities including regulation of cellular proliferation and differentiation, and promotion of neuronal survival. Isolation and purification of activin A is often complex and can often result in poor yields. For example, Pangas, S.A. and Woodruff, T.K states: "Inhibin and activin are protein hormones with diverse physiological roles including the regulation of pituitary FSH secretion. Like other members of the transforming growth factor- β gene family, they undergo processing from larger precursor molecules as well as assembly into functional dimers. Isolation of inhibin and activin from natural sources can only produce limited quantities of bioactive protein." (J. Endocrinol. 172 (2002) 199-210).
- [0023] In another example, Arai, K. Y. *et al* states: "Activins are multifunctional growth factors belonging to the transforming growth factor- β superfamily. Isolation of activins from natural sources requires many steps and only produces limited

quantities. Even though recombinant preparations have been used in recent studies, purification of recombinant activins still requires multiple steps.” (Protein Expression and Purification 49 (2006) 78-82).

[0024] Therefore, there still remains a significant need for alternatives for activin A to facilitate the differentiation of pluripotent stem cells.

SUMMARY

[0025] In one embodiment, the present invention provides a method to differentiate pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage, comprising culturing the pluripotent stem cells in medium comprising a sufficient amount of GDF-8 to cause the differentiation of the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage.

[0026] In one embodiment, the medium comprising a sufficient amount of GDF-8 also contains at least one other compound. In one embodiment, the at least one other compound is an aniline-pyridinotriazine. In an alternate embodiment, the at least one other compound is a cyclic aniline-pyridinotriazine.

[0026A] In one embodiment, there is provided a method of differentiating pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage comprising culturing the pluripotent stem cells in a medium lacking activin A, and supplemented with (i) GDF-8 and (ii) one or more compound selected from the group consisting of: an aniline-pyridinotriazine, a cyclic aniline-pyridinotriazine, EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D, VEGF, muscimol, PD98059, LY294002, U0124, U0126, 6-[(2-{[4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl]amino}ethyl)amino]pyridine-3-carbonitrile and sodium butyrate, for a period of time sufficient for the pluripotent stem cells to differentiate into cells expressing markers characteristic of the definitive endoderm lineage.

BRIEF DESCRIPTION OF THE FIGURES

- [0027] **Figure 1** shows the differentiation of H1 human embryonic stem cells into cells expressing markers characteristic of the definitive endoderm lineage. Differentiation was determined by measuring cell number (Panel A) and SOX17 intensity (Panel B) using an IN Cell Analyzer 1000 (GE Healthcare). Human embryonic stem cells were treated for a total of four days with medium containing 20 ng/ml Wnt3a plus activin A at the concentrations indicated (black bars) or medium lacking Wnt3a but with activin A at the concentrations indicated (white bars).
- [0028] **Figure 2** shows the dose response relationship of activin A and GDF8 used to differentiate cells of the human embryonic stem cell line H1 toward cells expressing markers characteristic of the definitive endoderm lineage. Cells were treated for a total of three days with activin A or GDF8 at the concentrations shown in combination with 20ng/ml Wnt3a on the first day of assay. Differentiation was

determined by measuring SOX17 intensity using a fluorescent antibody probe and high content analysis on a GE Healthcare IN Cell Analyzer.

- [0029] **Figure 3** shows the expression of CXCR4 in cells following the first step of differentiation, according to the methods described in Example 12. H1 cells were treated with 100ng/ml activin A or 200ng/ml GDF-8 for a total of three days in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 34 or 2.5 μ M Compound 56 for all three days. CXCR4 expression was measured using a fluorescent antibody probe and flow cytometry, yielding the percentages of positive cells shown.
- [0030] **Figure 4** shows the expression of SOX17 in cells after three days differentiation to definitive endoderm according to the methods described in Example 12. H1 cells were treated for a total of three days with 100ng/ml activin A or 200ng/ml GDF-8 in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 34 or 2.5 μ M Compound 56 for all three days. Differentiation was determined by measuring SOX17 intensity (black bars) and resulting cell number (white bars) with fluorescent antibody probes and high content analysis on a GE Healthcare IN Cell Analyzer.
- [0031] **Figure 5** shows the expression of PDX1 and CDX2 protein in cells following the third step of differentiation, according to the methods described in Example 12. H1 cells were treated for a total of three days with 100ng/ml activin A or 200ng/ml GDF-8 in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 34 or 2.5 μ M Compound 56 for all three days followed by subsequent differentiation through the second and third steps of differentiation. Protein expression and cell numbers, as determined with fluorescent antibody probes and high content analysis, are depicted for each treatment group. For comparative purposes, values are normalized relative to treatment with activin A/Wnt3a.
- [0032] **Figure 6** shows the expression of PDX1 protein (white bars) and cell number (black bars) in cells following the fourth step of differentiation, according to the methods described in Example 12. H1 cells were treated for a total of three days with 100ng/ml activin A or 200ng/ml GDF-8 in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 34 or 2.5 μ M Compound 56 for all three days followed by subsequent differentiation through the second, third, and fourth steps of

differentiation. Protein expression and cell numbers, as determined with fluorescent antibody probes and high content analysis, are depicted for each treatment group. For comparative purposes, values are normalized relative to treatment with activin A/Wnt3a.

[0033] **Figure 7** shows the protein expression for insulin and glucagon and cell number in cells differentiated according to the methods described in Example 12. H1 cells were treated for a total of three days with 100ng/ml activin A or 200ng/ml GDF-8 in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 34 or 2.5 μ M Compound 56 for all three days followed by subsequent differentiation through the second, third, fourth, and fifth steps of differentiation. Protein expression and cell numbers, as determined with fluorescent antibody probes and high content analysis, are depicted for each treatment group. For comparative purposes, values are normalized relative to treatment with activin A/Wnt3a.

[0034] **Figure 8** shows SOX17 protein expression and cell number in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 13. H1 cells were treated for a total of four days with 100ng/ml of activin A or 100ng/ml of a GDF- growth factor in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 34 or 2.5 μ M Compound 56 for the first two days of assay. SOX17 protein expression (black bars) and cell numbers (white bars), as determined with fluorescent antibody probes and high content analysis, are depicted for each treatment group. For comparative purposes, values are normalized relative to treatment with activin A/Wnt3a. Panel 8A shows a series of control conditions for differentiation in the absence of any growth factors (NONE), or with activin A/Wnt3a treatment (AA/Wnt3a) or with individual reagents alone. Panel 8B shows differentiation with GDF-3, alone or in multiple combinations with Wnt3a, Compound 34, or Compound 56. Panel 8C shows differentiation with GDF-5, alone or in multiple combinations with Wnt3a, Compound 34, or Compound 56. Panel 8D shows differentiation with GDF-8, alone or in multiple combinations with Wnt3a, Compound 34, or Compound 56. Panel 8E shows differentiation with GDF-10, alone or in multiple combinations with Wnt3a, Compound 34, or Compound 56. Panel 8F shows differentiation with GDF-11, alone or in multiple combinations with Wnt3a,

Compound 34, or Compound 56. Panel 8G shows differentiation with GDF-15, alone or in multiple combinations with Wnt3a, Compound 34, or Compound 56.

[0035] **Figure 9** shows SOX17 protein expression in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 14. H1 cells were treated for a total of three days with 100ng/ml of activin A or various growth factors at the concentrations shown in combination with 20ng/ml Wnt3a or 2.5 μ M Compound 34 for the first day of assay. SOX17 protein expression (black bars) and cell numbers (white bars), as determined with fluorescent antibody probes and high content analysis, are depicted for each treatment group. For comparative purposes, values are normalized relative to treatment with activin A/Wnt3a. Panel 9A shows a series of control conditions for differentiation with Wnt3a alone or in the absence of any growth factors (None) or with activin A/Wnt3a treatment (AA/Wnt3a). Panel 9B shows differentiation with GDF-8 (Vendor PeproTech), at the concentrations shown, in combination with 20ng/ml Wnt3a. Panel 9C shows differentiation with GDF-8 (Vendor Shenendoah), at the concentrations shown, in combination with 20ng/ml Wnt3a. Panel 9D shows differentiation with TGF β 1, at the concentrations shown, in multiple combinations with Wnt3a or Compound 34. Panel 9E shows differentiation with BMP2, at the concentrations shown, in multiple combinations with Wnt3a or Compound 34. Panel 9F shows differentiation with BMP3, at the concentrations shown, in multiple combinations with Wnt3a or Compound 34. Panel 9G shows differentiation with BMP4, at the concentrations shown, in multiple combinations with Wnt3a or Compound 34.

[0036] **Figure 10** shows SOX17 protein expression in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of activin A or 100ng/ml GDF-8 in combination with 20ng/ml Wnt3a. SOX17 protein expression, as determined with fluorescent antibody probes and high content analysis, is shown as total intensity values for each treatment group, testing control conditions for differentiation with no growth factors added (no treatment), with Wnt3a alone, with activin A or GDF-8 alone, or with activin A/Wnt3a treatment or GDF-8/Wnt3a treatment, where Wnt3a was added only for the first day of assay or for all three days of assay as shown.

- [0037] **Figure 11** shows SOX17 protein expression in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of activin A in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added only on the first day of assay. Protein expression for SOX17, as determined with fluorescent antibody probes and high content analysis, is depicted by total intensity values.
- [0038] **Figure 12** shows SOX17 protein expression in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of activin A in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added for all three days of assay. Protein expression for SOX17, as determined with fluorescent antibody probes and high content analysis, is depicted by total intensity values.
- [0039] **Figure 13** shows SOX17 protein expression in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of GDF-8 in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added only on the first day of assay. Protein expression for SOX17, as determined with fluorescent antibody probes and high content analysis, is depicted by total intensity values.
- [0040] **Figure 14** shows SOX17 protein expression in human embryonic stem cells after differentiation to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of GDF-8 in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D),

Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added for all three days of assay. Protein expression for SOX17, as determined with fluorescent antibody probes and high content analysis, is depicted by total intensity values.

[0041] **Figure 15** shows cell number yields after differentiation of human embryonic stem cells to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of activin A or 100ng/ml GDF-8 in combination with 20ng/ml Wnt3a. Cell numbers, as determined with a fluorescent nuclear probe and high content analysis, are shown for each treatment group, testing control conditions for differentiation with no growth factors added (no treatment), with Wnt3a alone, with activin A or GDF-8 alone, or with activin A/Wnt3a treatment or GDF-8/Wnt3a treatment, where Wnt3a was added only for the first day of assay or for all three days of assay as shown.

[0042] **Figure 16** shows cell number yields after differentiation of human embryonic stem cells to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of activin A in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added only on the first day of assay. Cell number yields, as determined with a fluorescent nuclear probe and high content analysis, are shown.

[0043] **Figure 17** shows cell number yields after differentiation of human embryonic stem cells to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of activin A in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added for all three days of assay. Cell number yields, as determined with a fluorescent nuclear probe and high content analysis, are shown.

- [0044] **Figure 18** shows cell number yields after differentiation of human embryonic stem cells to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of GDF-8 in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added only on the first day of assay. Cell number yields, as determined with a fluorescent nuclear probe and high content analysis, are shown.
- [0045] **Figure 19** shows cell number yields after differentiation of human embryonic stem cells to definitive endoderm, according to the methods described in Example 15. H1 cells were treated for a total of three days in various timed exposures with 100ng/ml of GDF-8 in combination with test compound (Compound 181 (Panel A), Compound 180 (Panel B), Compound 19 (Panel C), Compound 202 (Panel D), Compound 40 (Panel E), Compound 34 (Panel F), or GSK3 inhibitor BIO (Panel G)) at the concentrations shown, where test compound was added for all three days of assay. Cell number yields, as determined with a fluorescent nuclear probe and high content analysis, are shown.
- [0046] **Figure 20** shows the expression of various protein markers in cells throughout multiple steps of differentiation according to the methods described in Example 16. H1 cells were treated with 100ng/ml activin A or 100ng/ml GDF-8 for a total of three days in combination with 20ng/ml Wnt3a for the first day or 2.5μM various compounds (Compound 19, Compound 202, Compound 40, or GSK3 inhibitor BIO) added only on the first day. **Figure 20, panel A** shows FACS analysis for the definitive endoderm marker, CXCR4, in cells after the first step of differentiation. CXCR4 expression was measured using a fluorescent antibody probe and flow cytometry, yielding the percentages of positive cells as shown. **Figure 20, panel B** shows high content image analysis for normalized SOX17 protein expression (black bars) and recovered cell numbers (white bars) resulting from the first step of differentiation, testing the corresponding treatments shown. **Figure 20, panel C** shows high content image analysis for relative cell numbers recovered from cultures treated through differentiation step 5. **Figure 20, panel D** shows high content image

analysis for glucagon protein expression from cultures treated through differentiation step 5. **Figure 20, panel E** shows high content image analysis for insulin protein expression from cultures treated through differentiation step 5. **Figure 20, panel F** shows the ratio of glucagon to insulin expression in cells from cultures treated through differentiation step 5. For comparison purposes, expression values in panels B, C, D, E, and F are normalized to the control treatment with activin A and Wnt3a during step 1.

[0047] **Figure 21** shows the expression of various protein and RT-PCR markers in cells throughout multiple steps of differentiation according to the methods described in Example 17. H1 cells were treated with 100ng/ml activin A or 100ng/ml GDF-8 for a total of three days in combination with 20ng/ml Wnt3a for the first day or various compounds at the following concentrations (Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, Compound 56, or GSK3 inhibitor BIO) added only on the first day. FACS analysis for the definitive endoderm marker, CXCR4, is shown in cells after the first step of differentiation where treatment combined activin A (**Panel A**) or GDF-8 (**Panel B**) with Wnt3a or various compounds. CXCR4 expression was measured using a fluorescent antibody probe and flow cytometry, yielding the percentages of positive cells as shown. In subsequent panels of Figure 21, normalized RT-PCR values for various differentiation markers are shown with respective treatments using activin A or GDF-8 during the first step of differentiation as follows: markers at the end of step one of differentiation for treatments combining activin A (**Panel C**) or GDF-8 (**Panel D**); markers at the end of step three of differentiation for treatments combining activin A (**Panel E**) or GDF-8 (**Panel F**); markers at the end of step four of differentiation for treatments combining activin A (**Panel G**) or GDF-8 (**Panel H**); markers at the end of step five of differentiation for treatments combining activin A (**Panel I**) or GDF-8 (**Panel J**). At the conclusion of step five of differentiation, high content analysis was performed to measure recovered cell numbers for corresponding treatments during the first step of differentiation using activin A (**Panel K**) or GDF-8 (**Panel M**). High content analysis was also used to measure glucagon and insulin intensity in recovered cell populations at the end of step five of differentiation, corresponding to treatment with activin A (**Panel L**) or GDF-8 (**Panel N**) during the first step of differentiation.

- [0048] **Figure 22** shows the expression of various protein and RT-PCR markers in cells treated according to the methods described in Example 18. H1 cells were treated with 100ng/ml activin A or 100ng/ml GDF-8 for a total of three days in combination with 20ng/ml Wnt3a for the first day or 2.5 μ M Compound 40 or 2.5 μ M Compound 202 only on the first day. **Figure 22, panel A** shows FACS analysis for the definitive endoderm marker, CXCR4, in cells after the first step of differentiation. CXCR4 expression was measured using a fluorescent antibody probe and flow cytometry, yielding the percentages of positive cells as shown. In **Figure 22, panel B**, normalized RT-PCR values for various differentiation markers in cells recovered after the fourth step of differentiation are shown corresponding to respective treatments using activin A/Wnt3a or GDF-8/Compound 40 or GDF-8/Compound 202 during the first step of differentiation.
- [0049] **Figure 23** shows the level of C-peptide detected in SCID-beige mice that received cells at the end of step four of the differentiation protocol as described in Example 18.
- [0050] **Figure 24** panel A shows the expression of CXCR4, as determined by FACS in cells at the end of step one of the differentiation protocol described in Example 19. Panel B shows the expression of various genes, as determined by RT-PCR in cells at the end of step four of the differentiation protocol described in Example 19. Two different experimental replicates are shown (Rep-1 and Rep-2), each subjected to identical treatment protocols. Panel C shows the level of C-peptide detected in SCID-beige mice that received cells at the end of step four of the differentiation protocol as treated with GDF-8 and Wnt3a during the first step of *in vitro* differentiation. Panel D shows the level of C-peptide detected in SCID-beige mice that received cells at the end of step four of the differentiation protocol as treated with GDF-8 and Compound 28 during the first step of *in vitro* differentiation.
- [0051] **Figure 25** shows the cell number (panel A) and expression of CXCR4 (panel B) from cells grown on microcarrier beads, treated according to the methods of the present invention as described in Example 22. Cells were grown on Cytodex3 beads without treatment (undifferentiated) or with treatment combining 100ng/ml activin A with 20ng/ml Wnt3a (AA/Wnt3a) or with various treatments combining GDF-8 as shown: 50ng/ml GDF-8 with 2.5 μ M Compound 34 (Cmp 34+8); or 50ng/ml GDF-8 with 2.5 μ M Compound 34 and 50ng/ml PDGF (Cmp 34+8+D); or 50ng/ml GDF-8 with

2.5 μ M Compound 34 and 50ng/ml PDGF and 50ng/ml VEGF (Cmp 34+8+D+V); or 50ng/ml GDF-8 with 2.5 μ M Compound 34 and 50ng/ml PDGF and 50ng/ml VEGF and 20ng/ml muscimol (Cmp 34+8+D+V+M).

- [0052] **Figure 26** shows the proliferation of cells following treatment of the compounds of the present invention as described in Example 23. **Panels B** through **I** show assay results for treatment using a compound in combination with GDF-8 and measuring MTS OD readings at 1 day, 2 days, and 3 days after initiating the differentiation assay.
- [0053] **Figure 27** shows the expression of various proteins and genes from cells grown on microcarrier beads, treated according to the methods of the present invention. **Panel A** shows the percent positive expression of CXCR4, CD99, and CD9 as determined by FACS in cells at the end of step one of the differentiation protocol described in Example 24. **Panel B** shows cells recovered from treatments as shown differentiated through step three of the differentiation protocol. **Panel C** shows ddCT values for various gene markers expressed in cells treated as shown in step and differentiated through step three of the protocol.

DETAILED DESCRIPTION

- [0054] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments, or applications of the present invention.

Definitions

- [0055] Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

[0056] Stem cells are classified by their developmental potential as: (1) totipotent, meaning able to give rise to all embryonic and extraembryonic cell types; (2) pluripotent, meaning able to give rise to all embryonic cell types; (3) multipotent, meaning able to give rise to a subset of cell lineages but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self-renewal), blood cell restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood); (4) oligopotent, meaning able to give rise to a more restricted subset of cell lineages than multipotent stem cells; and (5) unipotent, meaning able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

[0057] Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a nerve cell or a muscle cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. De-differentiation refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

[0058] "β-cell lineage" refers to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, HNF-3 beta, MAFA, PAX4, or PAX6. Cells expressing markers characteristic of the β cell lineage include β cells.

[0059] "Cells expressing markers characteristic of the definitive endoderm lineage", or "Stage 1 cells", or "Stage 1", as used herein, refers to cells expressing at least one of

the following markers: SOX17, GATA4, HNF-3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

[0060] “Cells expressing markers characteristic of the pancreatic endoderm lineage”, as used herein, refers to cells expressing at least one of the following markers: PDX1, HNF-1 beta, PTF1 alpha, HNF6, or HB9. Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

[0061] “Cells expressing markers characteristic of the pancreatic endocrine lineage”, or “Stage 5 cells”, or “Stage 5”, as used herein, refers to cells expressing at least one of the following markers: NGN3, NEUROD, ISL1, PDX1, NKX6.1, PAX4, or PTF-1 alpha. Cells expressing markers characteristic of the pancreatic endocrine lineage include pancreatic endocrine cells, pancreatic hormone expressing cells, and pancreatic hormone secreting cells, and cells of the β -cell lineage.

[0062] “Definitive endoderm”, as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express the following markers: HNF-3 beta, GATA4, SOX-17, Cerberus, OTX2, goosecoid, C-Kit, CD99, or MIXL1.

[0063] “Extraembryonic endoderm”, as used herein, refers to a population of cells expressing at least one of the following markers: SOX7, AFP, or SPARC.

[0064] “Markers”, as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

- [0065] “Mesendoderm cell”, as used herein, refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX17, DKK4, HNF-3 beta, GSC, FGF17, or GATA-6.
- [0066] “Pancreatic endocrine cell”, or “pancreatic hormone expressing cell”, as used herein, refers to a cell capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.
- [0067] “Pancreatic endoderm cell”, or “Stage 4 cells”, or “Stage 4”, as used herein, refers to a cell capable of expressing at least one of the following markers: NGN3, NEUROD, ISL1, PDX1, PAX4, or NKX2.2.
- [0068] “Pancreatic hormone producing cell”, as used herein, refers to a cell capable of producing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.
- [0069] “Pancreatic hormone secreting cell”, as used herein, refers to a cell capable of secreting at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.
- [0070] “Posterior foregut cell” or “Stage 3 cells”, or “Stage 3”, as used herein, refers to a cell capable of secreting at least one of the following markers: PDX1, HNF1, PTF-1 alpha, HNF6, HB-9, or PROX-1.
- [0071] “Pre-primitive streak cell”, as used herein, refers to a cell expressing at least one of the following markers: Nodal, or FGF8.
- [0072] “Primitive gut tube cell” or “Stage 2 cells”, or “Stage2”, as used herein, refers to a cell capable of secreting at least one of the following markers: HNF1, HNF-4 alpha.
- [0073] “Primitive streak cell”, as used herein, refers to a cell expressing at least one of the following markers: Brachyury, Mix-like homeobox protein, or FGF4.

Isolation, expansion, and culture of pluripotent stem cells*Characterization of pluripotent stem cells*

- [0074] The pluripotency of pluripotent stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.
- [0075] Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

Sources of pluripotent stem cells

- [0076] The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10 to 12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example, the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, GA).
- [0077] In one embodiment, human embryonic stem cells are prepared as described by Thomson *et al.* (U.S. Pat. No. 5,843,780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995).

- [0078] In one embodiment, pluripotent stem cells are prepared as described by Takahashi *et al.* (Cell 131: 1-12, 2007).

Culture of pluripotent stem cells

- [0079] In one embodiment, pluripotent stem cells are typically cultured on a layer of feeder cells that support the pluripotent stem cells in various ways. Alternatively, pluripotent stem cells are cultured in a culture system that is essentially free of feeder cells but nonetheless supports proliferation of pluripotent stem cells without undergoing substantial differentiation. The growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type. Alternatively, the growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a chemically defined medium.
- [0080] The pluripotent stem cells may be plated onto a suitable culture substrate. In one embodiment, the suitable culture substrate is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL[®] (Becton Dickenson). MATRIGEL[®] is a soluble preparation from Engelbreth-Holm-Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.
- [0081] Other extracellular matrix components and component mixtures are suitable as an alternative. Depending on the cell type being proliferated, this may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations.
- [0082] The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium that promotes cell survival, propagation, and retention of the desirable characteristics. All these characteristics benefit from careful attention to the seeding distribution and can readily be determined by one of skill in the art.
- [0083] Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092;

Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco # 15039-027; non-essential amino acid solution, Gibco 11140-050; β -mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

Formation of pancreatic hormone producing cells from pluripotent stem cells

[0084] In one embodiment, the present invention provides a method for producing pancreatic hormone producing cells from pluripotent stem cells, comprising the steps of:

- a. Culturing pluripotent stem cells,
- b. Differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,
- c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, and
- d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

[0085] In one aspect of the present invention, the pancreatic endocrine cell is a pancreatic hormone producing cell. In an alternate aspect, the pancreatic endocrine cell is a cell expressing markers characteristic of the β -cell lineage. A cell expressing markers characteristic of the β -cell lineage expresses PDX1 and at least one of the following transcription factors: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, HNF-3 beta, MAFA, PAX4, or Pax6. In one aspect of the present invention, a cell expressing markers characteristic of the β -cell lineage is a β -cell.

[0086] Pluripotent stem cells suitable for use in the present invention include, for example, the human embryonic stem cell line H9 (NIH code: WA09), the human embryonic stem cell line H1 (NIH code: WA01), the human embryonic stem cell line H7 (NIH code: WA07), and the human embryonic stem cell line SA002 (Cellartis, Sweden). Also suitable for use in the present invention are cells that express at least one of the

following markers characteristic of pluripotent cells: ABCG2, cripto, CD9, FOXD3, Connexin43, Connexin45, OCT4, SOX2, Nanog, hTERT, UTF-1, ZFP42, SSEA-3, SSEA-4, Tra1-60, or Tra1-81.

- [0087] The pluripotent stem cells may be cultured on a feeder cell layer. Alternatively, the pluripotent stem cells may be cultured on an extracellular matrix. The extracellular matrix may be a solubilized basement membrane preparation extracted from mouse sarcoma cells (as sold by BD Biosciences under the trade name MATRIGELTM). Alternatively, the extracellular matrix may be growth factor-reduced MATRIGELTM. Alternatively, the extracellular matrix may be fibronectin. In an alternate embodiment, the pluripotent stem cells are cultured and differentiated on tissue culture substrate coated with human serum.
- [0088] The extracellular matrix may be diluted prior to coating the tissue culture substrate. Examples of suitable methods for diluting the extracellular matrix and for coating the tissue culture substrate may be found in Kleinman, H.K., *et al.*, Biochemistry 25:312 (1986), and Hadley, M.A., *et al.*, J.Cell.Biol. 101:1511 (1985).
- [0089] In one embodiment, the extracellular matrix is MATRIGELTM. In one embodiment, the tissue culture substrate is coated with MATRIGELTM at a 1:10 dilution. In an alternate embodiment, the tissue culture substrate is coated with MATRIGELTM at a 1:15 dilution. In an alternate embodiment, the tissue culture substrate is coated with MATRIGELTM at a 1:30 dilution. In an alternate embodiment, the tissue culture substrate is coated with MATRIGELTM at a 1:60 dilution.
- [0090] In one embodiment, the extracellular matrix is growth factor-reduced MATRIGELTM. In one embodiment, the tissue culture substrate is coated with growth factor-reduced MATRIGELTM at a 1:10 dilution. In an alternate embodiment, the tissue culture substrate is coated with growth factor-reduced MATRIGELTM at a 1:15 dilution. In an alternate embodiment, the tissue culture substrate is coated with growth factor-reduced MATRIGELTM at a 1:30 dilution. In an alternate embodiment, the tissue culture substrate is coated with growth factor-reduced MATRIGELTM at a 1:60 dilution.
- [0091] Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX17, GATA4, HNF-3 beta, GSC, CER1, Nodal, FGF8, Brachyury,

Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, and OTX2. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the definitive endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

[0092] Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of PDX1, HNF-1 beta, PTF1 alpha, HNF6, HB9 and PROX1. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell.

[0093] Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NGN3, NEUROD, ISL1, PDX1, NKX6.1, PAX4, and PTF-1 alpha. In one embodiment, a pancreatic endocrine cell is capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endocrine lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endocrine lineage is a pancreatic endocrine cell. The pancreatic endocrine cell may be a pancreatic hormone expressing cell. Alternatively, the pancreatic endocrine cell may be a pancreatic hormone secreting cell.

Formation of cells expressing markers characteristic of the definitive endoderm lineage

[0094] In one aspect of the present invention, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium comprising a sufficient amount of

GDF-8 to cause the differentiation of the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage.

- [0095] The pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day to about seven days. Alternatively, the pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day to about six days. Alternatively, the pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day to about five days. Alternatively, the pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day to about four days. Alternatively, the pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day to about three days. Alternatively, the pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day to about two days. Alternatively, the pluripotent stem cells may be cultured in the medium containing a sufficient amount of GDF-8 for about one day.
- [0096] In one embodiment, the GDF-8 is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the GDF-8 is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the GDF-8 is used at a concentration from about 5 ng/ml to about 25 ng/ml. In an alternate embodiment, the GDF-8 is used at a concentration of about 25 ng/ml.
- [0097] In one embodiment, the medium comprising a sufficient amount of GDF-8 also contains at least one other factor. In one embodiment, the at least one other factor is selected from the group consisting of: EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D, VEGF, muscimol, PD98059, LY294002, U0124, U0126, and sodium butyrate.
- [0098] In one embodiment, the EGF is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the EGF is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the EGF is used at a concentration of about 50 ng/ml.
- [0099] In one embodiment, the FGF4 is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the FGF4 is used at a concentration from

about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the FGF4 is used at a concentration of about 50 ng/ml.

[0100] In one embodiment, the PDGF-A is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the PDGF-A is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the PDGF-A is used at a concentration of about 50 ng/ml.

[0101] In one embodiment, the PDGF-B is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the PDGF-B is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the PDGF-B is used at a concentration of about 50 ng/ml.

[0102] In one embodiment, the PDGF-C is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the PDGF-C is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the PDGF-C is used at a concentration of about 50 ng/ml.

[0103] In one embodiment, the PDGF-D is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the PDGF-D is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the PDGF-D is used at a concentration of about 50 ng/ml.

[0104] In one embodiment, the VEGF is used at a concentration from about 5 ng/ml to about 500 ng/ml. In an alternate embodiment, the VEGF is used at a concentration from about 5 ng/ml to about 50 ng/ml. In an alternate embodiment, the VEGF is used at a concentration of about 50 ng/ml.

[0105] In one embodiment, the muscimol is used at a concentration from about 1 μ M to about 200 μ M. In an alternate embodiment, the muscimol is used at a concentration from about 1 μ M to about 20 μ M. In an alternate embodiment, the muscimol is used at a concentration of about 20 μ M.

[0106] In one embodiment, the PD98059 is used at a concentration from about 0.1 μ M to about 10 μ M. In an alternate embodiment, the PD98059 is used at a concentration

from about 0.1 μM to about 1 μM . In an alternate embodiment, the PD98059 is used at a concentration of about 1 μM .

[0107] In one embodiment, the LY294002 is used at a concentration from about 0.25 μM to about 25 μM . In an alternate embodiment, the LY294002 is used at a concentration from about 0.25 μM to about 2.5 μM . In an alternate embodiment, the LY294002 is used at a concentration of about 2.5 μM .

[0108] In one embodiment, the U0124 is used at a concentration from about 0.1 μM to about 10 μM . In an alternate embodiment, the U0124 is used at a concentration from about 0.1 μM to about 1 μM . In an alternate embodiment, the U0124 is used at a concentration of about 1 μM .

[0109] In one embodiment, the U0126 is used at a concentration from about 0.1 μM to about 10 μM . In an alternate embodiment, the U0126 is used at a concentration from about 0.1 μM to about 1 μM . In an alternate embodiment, the U0126 is used at a concentration of about 1 μM .

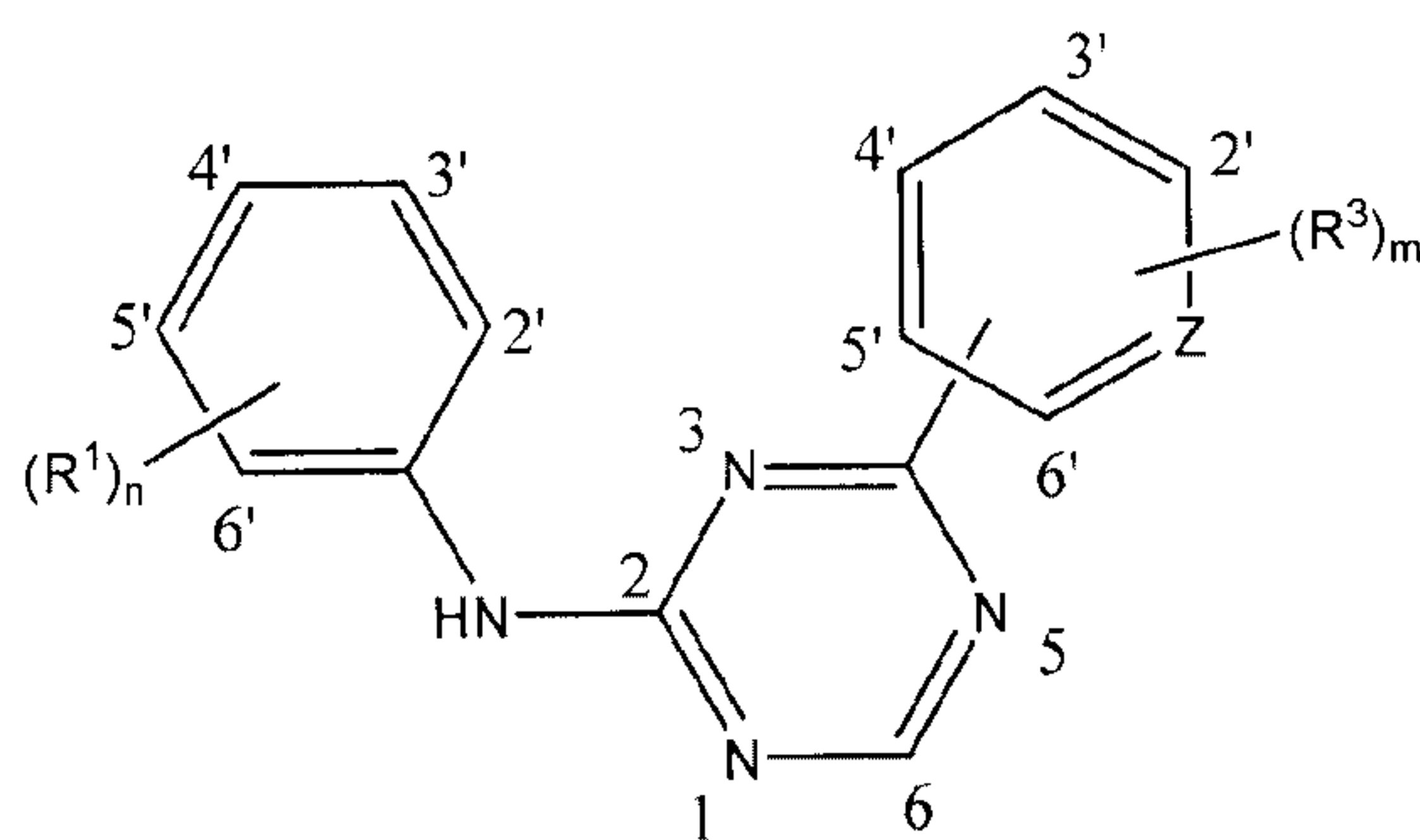
[0110] In one embodiment, the sodium butyrate is used at a concentration from about 0.05 μM to about 5 μM . In an alternate embodiment, the sodium butyrate is used at a concentration from about 0.05 μM to about 0.5 μM . In an alternate embodiment, the sodium butyrate is used at a concentration of about 0.5 μM .

[0111] In an alternate embodiment, the at least one other factor is selected from the group consisting of: an aniline-pyridinotriazine, a cyclic aniline-pyridinotriazine, N- $\{[1-(\text{Phenylmethyl})\text{azepan-4-yl}]\text{methyl}\}$ -2-pyridin-3-ylacetamide, 4- $\{[4-(4-\{[2-(\text{Pyridin-2-ylamino})\text{ethyl}]\text{amino}\}-1,3,5\text{-triazin-2-yl})\text{pyridin-2-yl}]\text{oxy}\}$ butan-1-ol, 3- $\{[3-[4-(\{2-[\text{Methyl}(\text{pyridin-2-yl})\text{amino}]\text{ethyl}\}\text{amino})-1,3,5\text{-triazin-2-yl}]\text{pyridin-2-yl}\}\text{amino}\}$ propan-1-ol, N $\sim 4\sim$ - $[2-(3\text{-Fluorophenyl})\text{ethyl}]\text{-N}\sim 2\sim$ - $[3-(4\text{-methylpiperazin-1-yl})\text{propyl}]\text{pyrido}[2,3\text{-d}]\text{pyrimidine-2,4-diamine}$, 1-Methyl-N- $[(4\text{-pyridin-3-yl-2-}\{[3-(\text{trifluoromethyl})\text{phenyl}]\text{amino}\}-1,3\text{-thiazol-5-yl})\text{methyl}]\text{piperidine-4-carboxamide}$, 1,1-Dimethylethyl $\{2-[4-(\{5-[3-(3\text{-hydroxypropyl})\text{phenyl}]\text{-4H-1,2,4-triazol-3-yl}\}\text{amino})\text{phenyl}]\text{ethyl}\}$ carbamate, 1,1-Dimethylethyl $\{[3-(\{5-[5-(3\text{-hydroxypropyl})\text{-2-(methyloxy})\text{phenyl}]\text{-1,3-oxazol-2-yl}\}\text{amino})\text{phenyl}]\text{methyl}\}$ carbamate, 1- $\{[5-[6-(\{4-[(4\text{-Methylpiperazin-1-yl})\text{sulfonyl}]\text{phenyl}\}\text{amino})\text{pyrazin-2-yl}]\text{thiophen-2-}$

yl}methyl)piperidin-4-ol, 1-({4-[6-({4-[(4-Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidine-4-carboxamide, and 2-{{4-(1-Methylethyl)phenyl}amino}-N-(2-thiophen-2-ylethyl)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxamide.

The compounds of the present invention

- [0112] The present invention provides compounds that are capable of differentiating pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage.
- [0113] In one embodiment, the compound that is capable of differentiating pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage is an aniline-pyridinotriazine of the Formula (1):



Formula (1).

- [0114] The N-oxide forms, the pharmaceutically acceptable addition salts and the stereochemically isomeric forms thereof, wherein:
- [0115] m represents an integer from 1 to 4; n represents an integer from 1 to 4; Z represents N or C;
- [0116] R¹ and R⁸ each independently represent hydrogen, Het¹⁴, cyano, halo, hydroxy, C₁₋₆alkoxy-, C₁₋₆alkyl-, mono- or di(C₁₋₄alkyl)amino-carbonyl-, mono- or di(C₁₋₄alkyl)amino-sulfonyl, C₁₋₆alkoxy-substituted with halo or R¹ represents C₁₋₆alkyl substituted with one or where possible two or more substituents selected from hydroxy or halo;
- [0117] R² and R⁹ each independently represents hydrogen, C₁₋₄alkyl, C₂₋₄alkenyl, Het³, Het⁴-C₁₋₄alkyl-, Het⁵-C₁₋₄alkylcarbonyl-, mono- or di(C₁₋₄alkyl)amino-C₁₋₄alkyl-

carbonyl-or phenyl optionally substituted with one or where possible two or more substituents selected from hydrogen, hydroxy, amino or C₁₋₄alkyloxy-;

- [0118] R³ and R⁷ each independently represent hydrogen, C₁₋₄alkyl, Het⁶, Het⁷-C₁₋₄alkyl-, C₂₋₄alkenylcarbonyl-optionally substituted with Het⁸-C₁₋₄alkylaminocarbonyl-, C₂₋₄alkenylsulfonyl-, C₁₋₄alkyloxyC₁₋₄alkyl-or phenyl optionally substituted with one or where possible two or more substituents selected from hydrogen, hydroxy, amino or C₁₋₄alkyloxy-;
- [0119] R₄, R₅, R₆ and R¹⁰ each independently represent hydrogen or C₁₋₄alkyl optionally substituted with hydroxy, Het⁹ or C₁₋₄alkyloxy;
- [0120] Het¹ and Het² each independently represent a heterocycle selected from pyrrolidinyl, piperidinyl, piperazinyl, pyridinyl, pyrimidinyl, pyrazinyl, imidazolidinyl or pyrazolidinyl wherein said Het¹ and Het² are optionally substituted with amino, hydroxy, C₁₋₄alkyl, hydroxy-C₁₋₄alkyl-, phenyl, phenyl-C₁₋₄alkyl-, C₁₋₄alkyl-oxy-C₁₋₄alkyl-mono-or di(C₁₋₄alkyl) amino-or amino-carbonyl-;
- [0121] Het³ and Het⁶ each independently represent, heterocycle selected from pyrrolidinyl or piperidinyl wherein said Het³ and Het⁶ are optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-;
- [0122] Het⁴, Het⁷ and Het⁹ each independently represent a heterocycle selected from morpholinyl, pyrrolidinyl, piperazinyl or piperidinyl wherein said Het⁴, Het⁷ and Het⁹ are optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-;
- [0123] Het⁵ represents a heterocycle selected from morpholinyl, pyrrolidinyl, piperazinyl or piperidinyl wherein said Het⁵ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-;
- [0124] Het¹⁰, Het¹¹ and Het¹³ each independently represent a heterocycle selected from pyrrolidinyl, piperidinyl, piperazinyl, pyridinyl, pyrimidinyl, pyrazinyl,

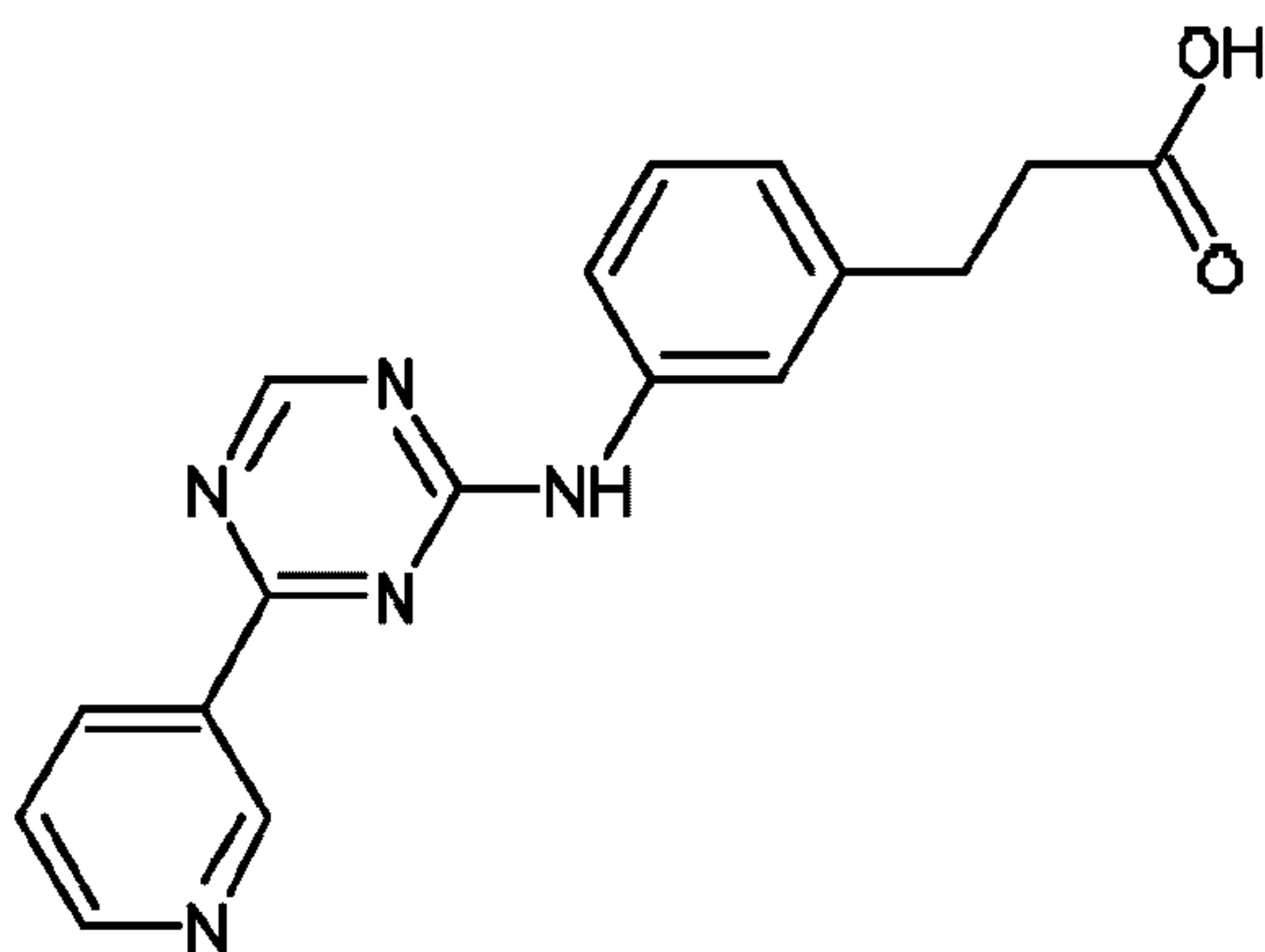
imidazolidinyl or pyrazolidinyl wherein said Het¹⁰, Het¹¹ and Het¹³ are optionally substituted with amino, hydroxy, C₁₋₄alkyl, hydroxy-C₁₋₄alkyl-, phenyl, phenyl-C₁₋₄alkyl-, C₁₋₄alkyl-oxy-C₁₋₄alkyl-, amino-carbonyl-or mono-or di(C₁₋₄alkyl)amino-;

[0125] Het¹² represents a heterocycle selected from pyrrolidinyl, piperidinyl, piperazinyl, pyridinyl, pyrimidinyl, pyrazinyl, imidazolidinyl or pyrazolidinyl wherein said Het¹² is optionally substituted with amino, hydroxy, C₁₋₄alkyl, hydroxy-C₁₋₄alkyl-, phenyl, phenyl-C₁₋₄alkyl-, C₁₋₄alkyl-oxy-C₁₋₄alkyl-; mono-or di(C₁₋₄alkyl)amino-or amino-carbonyl-;

[0126] Het¹⁴ represents a heterocycle selected from morpholinyl; pyrrolidinyl; piperazinyl; imidazolyl; pyrrolyl; 2,3,4-triazapyrrolyl; 1,2,3-triazolyl; pyrazolyl; or piperidinyl wherein said Het¹⁴ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-; in particular Het¹⁴ represents a heterocycle selected from morpholinyl; pyrrolidinyl; pyrrolyl; 2,3,4-triazapyrrolyl; piperazinyl or piperidinyl wherein said Het¹⁴ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-; more particular Het¹⁴ represents a heterocycle selected from morpholinyl; pyrrolidinyl; piperazinyl or piperidinyl wherein said Het¹⁴ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-.

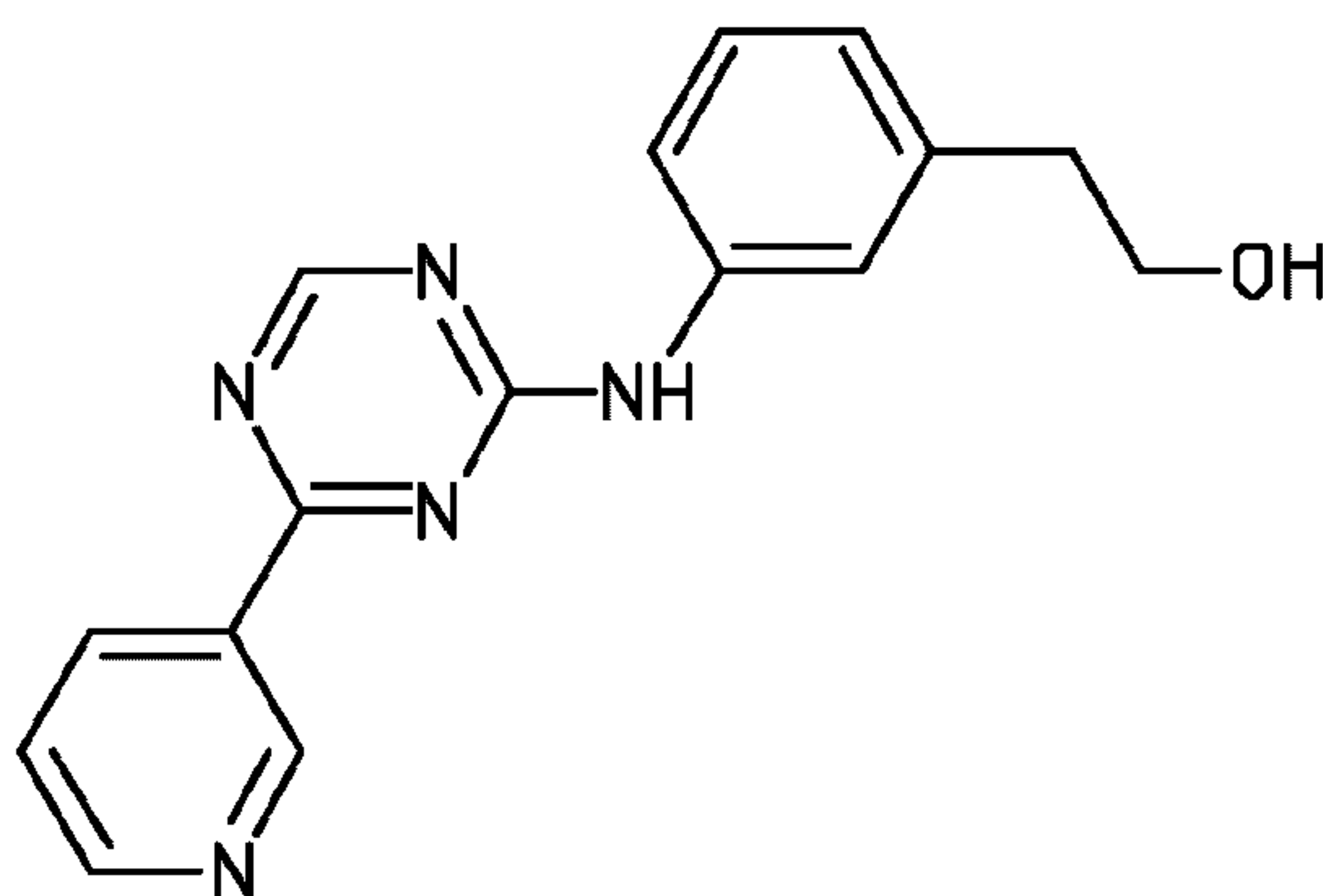
[0127] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (1).

[0128] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (2).



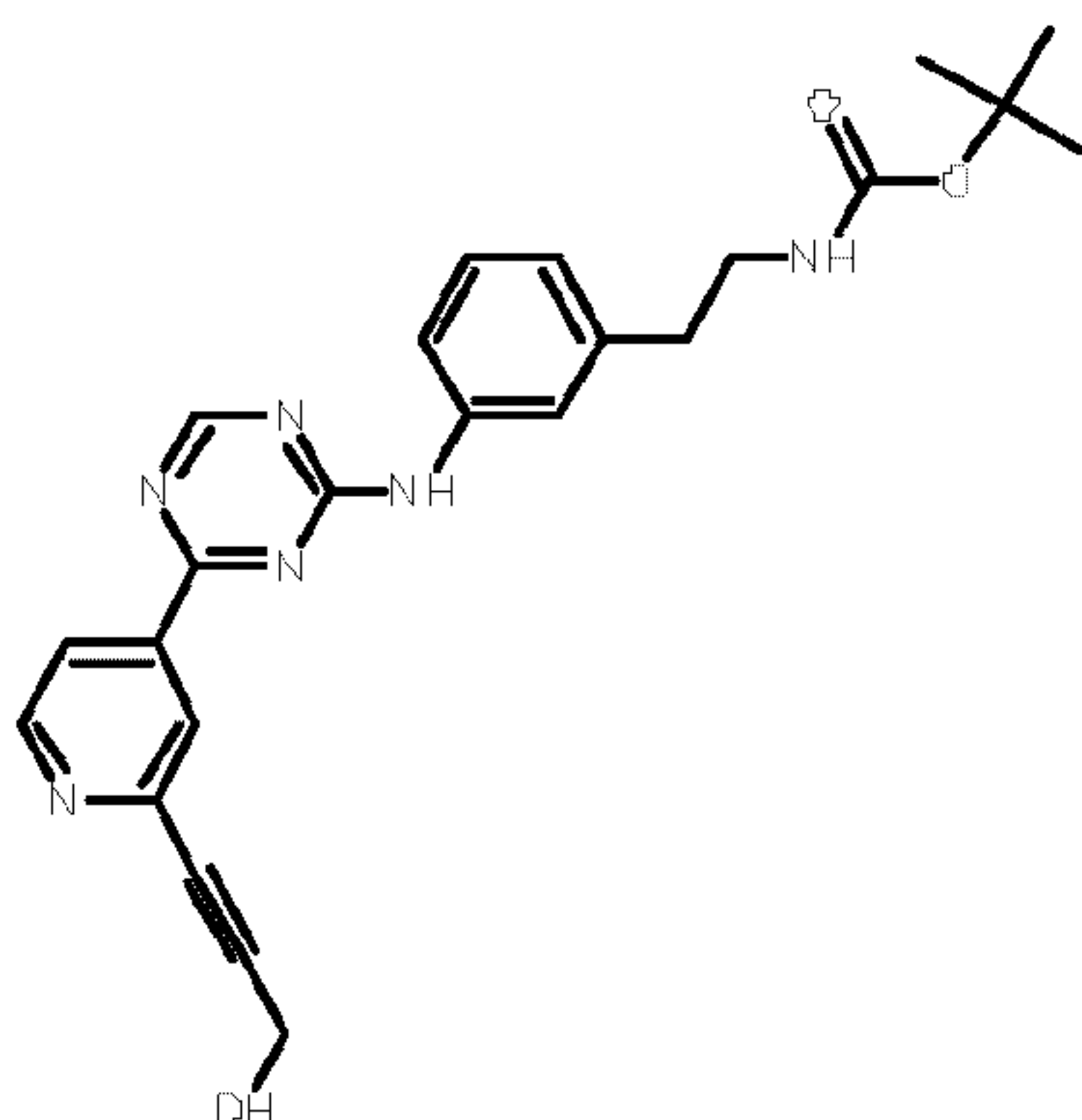
Formula (2): 3-{3-[(4-Pyridin-3-yl-1,3,5-triazin-2-yl)amino]phenyl}propanoic acid. Referred to herein as “Compound 1”.

[0129] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (3).



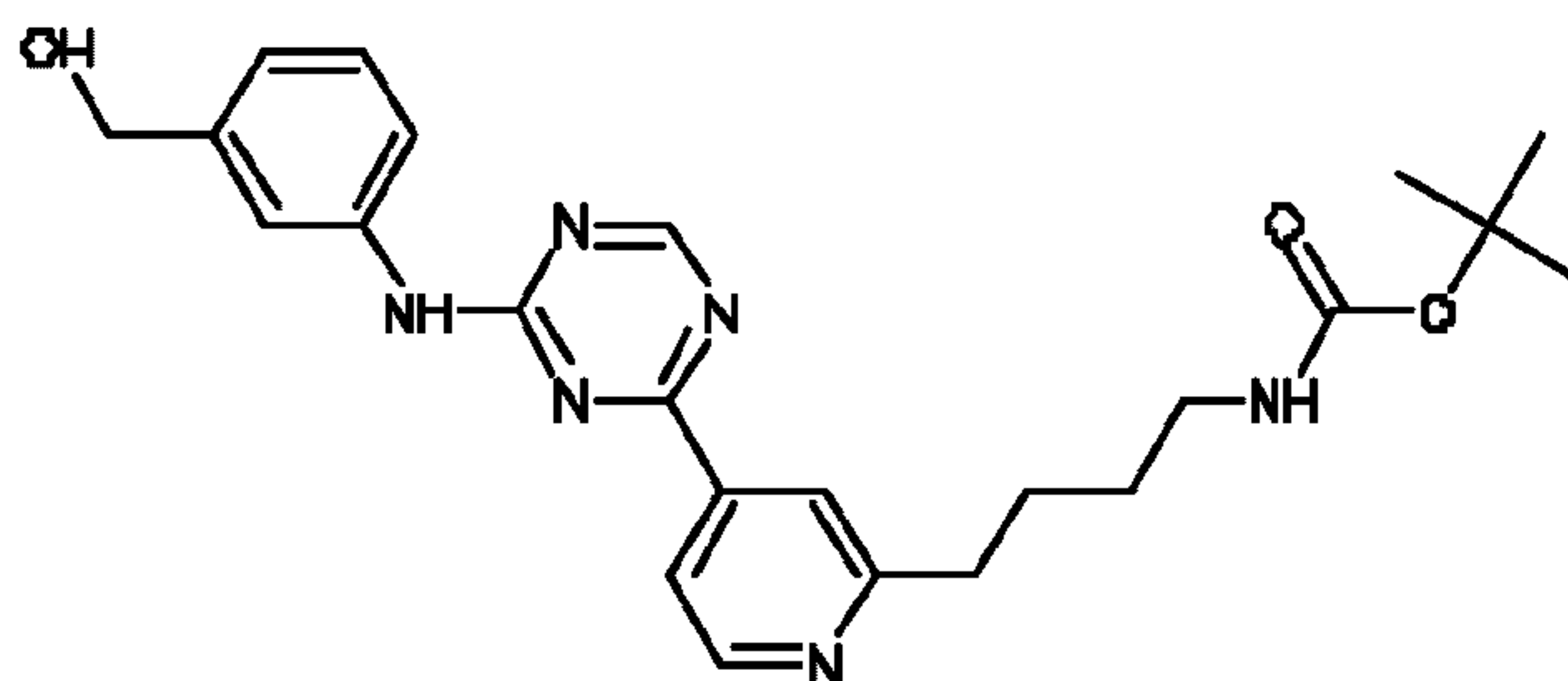
Formula (3): 2-{3-[(4-Pyridin-3-yl-1,3,5-triazin-2-yl)amino]phenyl}ethanol. Referred to herein as “Compound 2”.

[0130] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (4).



Formula (4): 1,1-Dimethylethyl {2-[3-({4-[2-(3-hydroxyprop-1-yn-1-yl)pyridin-4-yl]-1,3,5-triazin-2-yl}amino)phenyl]ethyl}carbamate. Referred to herein as “Compound 3”.

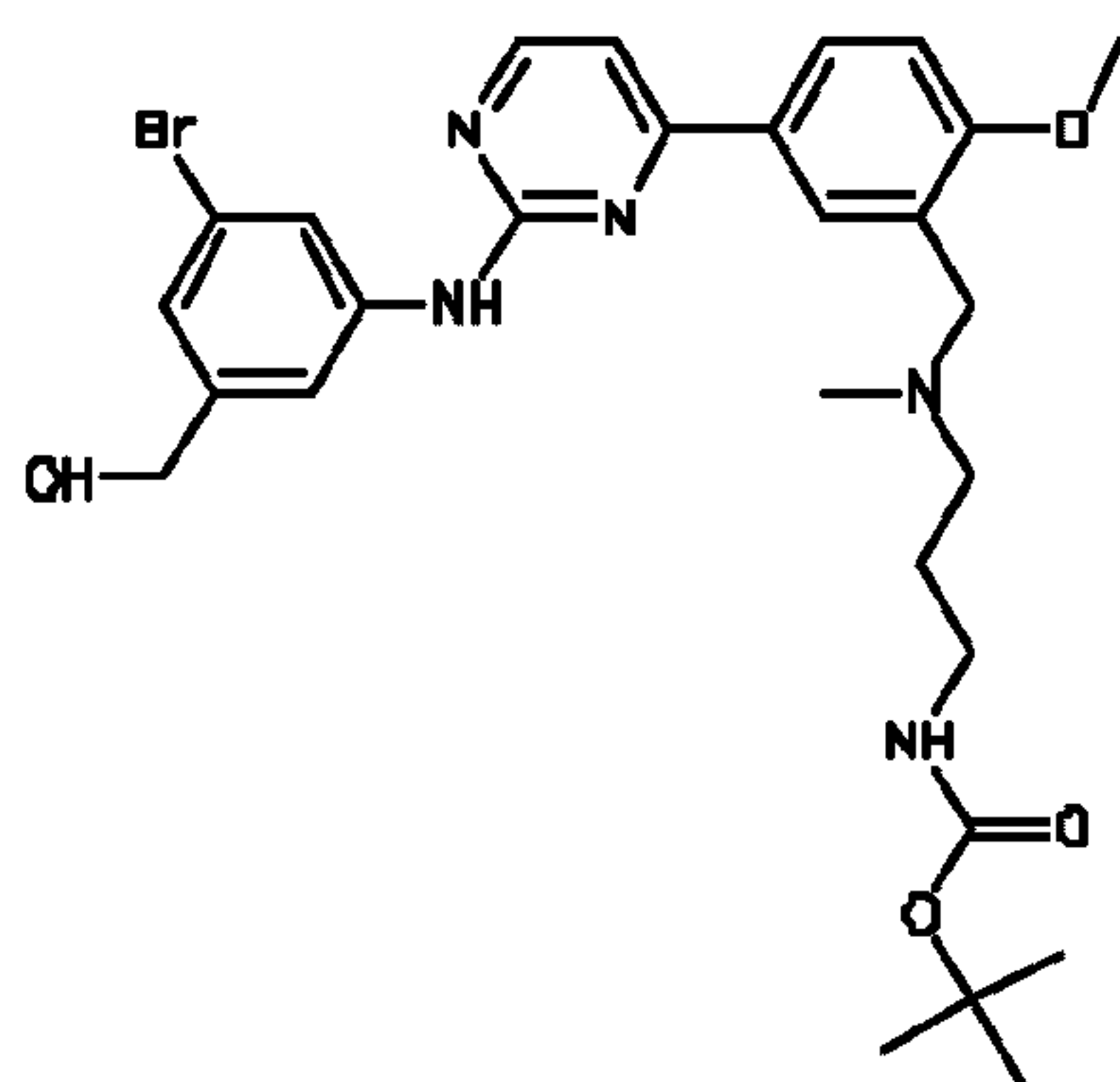
[0131] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (5).



Formula (5): 1,1-Dimethylethyl

{4-[4-(4-{[3-(hydroxymethyl)phenyl]amino}-1,3,5-triazin-2-yl)pyridin-2-yl]butyl} carbamate. Referred to herein as “Compound 4”.

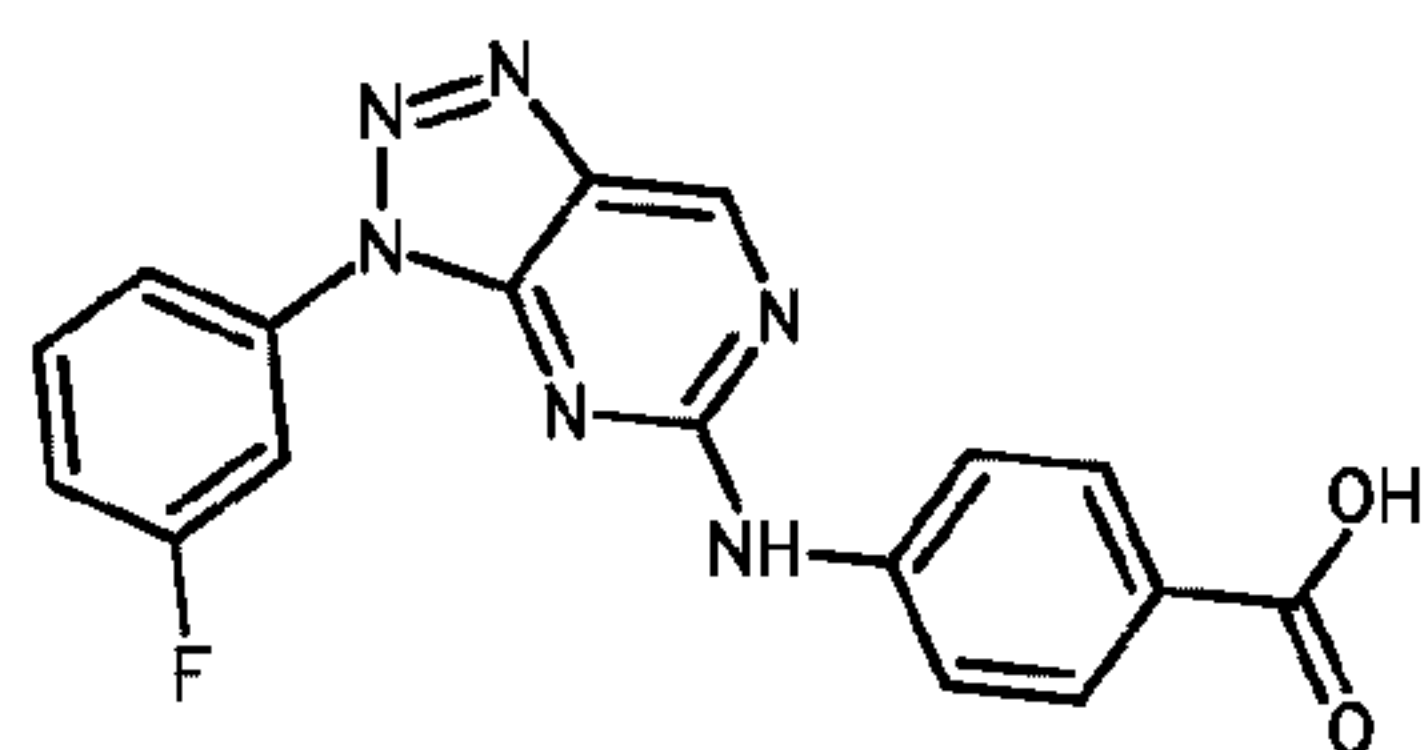
[0132] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (6).



Formula (6): 1,1-Dimethylethyl

{3-[[5-(2-{[3-bromo-5-(hydroxymethyl)phenyl]amino}pyrimidin-4-yl)-2-(methoxy)phenyl]methyl}(methyl)amino]propyl} carbamate. Referred to herein as “Compound 5”.

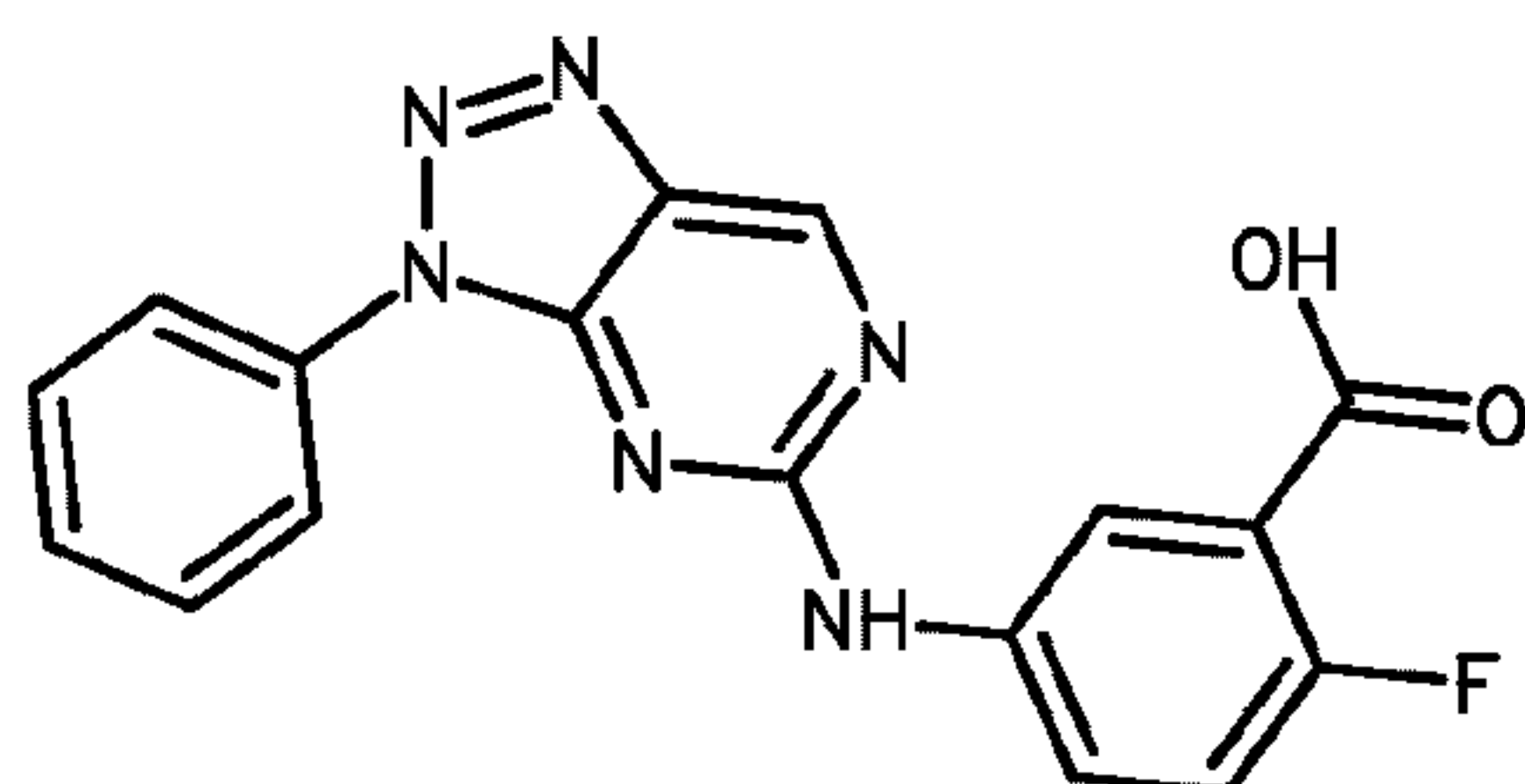
[0133] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (7).



Formula (7): 4-{[3-(3-Fluorophenyl)-3H-

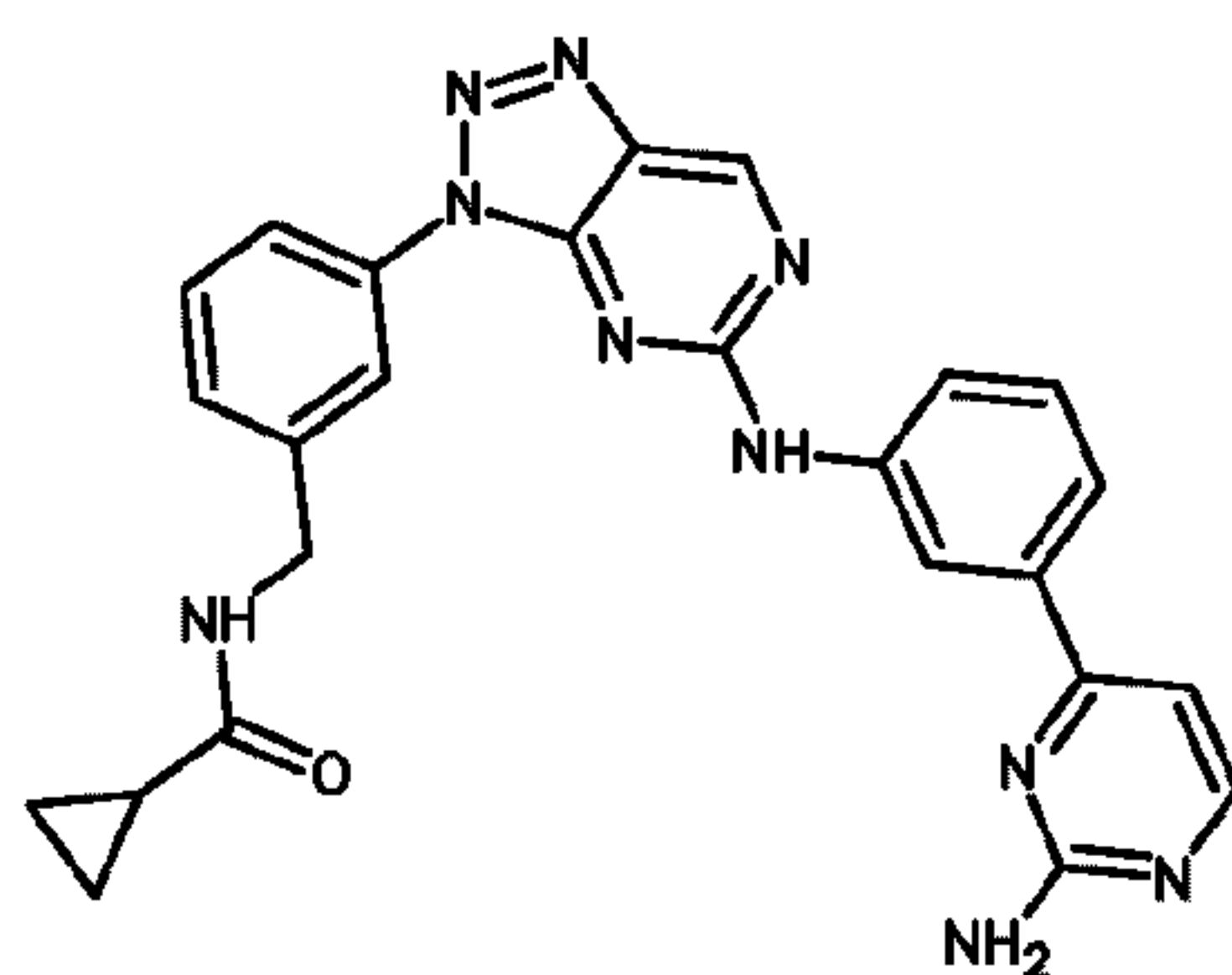
[1,2,3]triazolo[4,5-d]pyrimidin-5-yl]amino}benzoic acid. Referred to herein as “Compound 6”.

[0134] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (8).



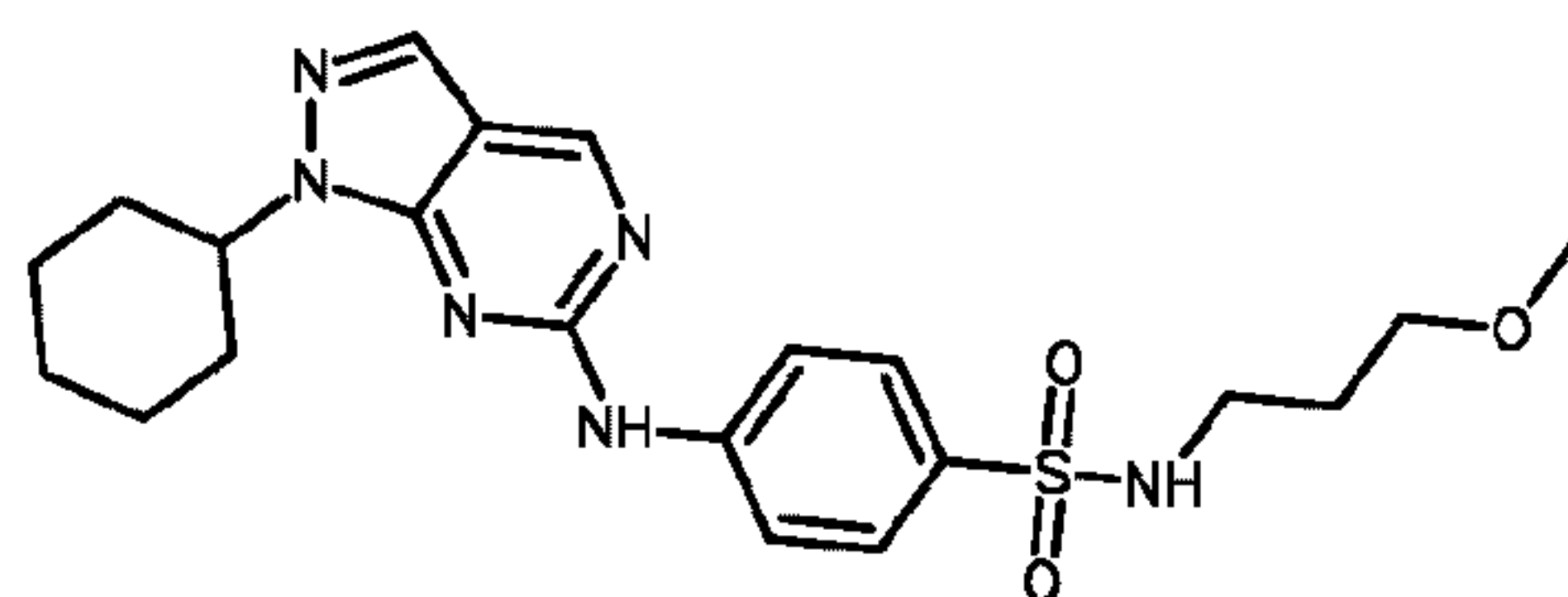
Formula (8): 2-Fluoro-5-[(3-phenyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-5-yl)amino]benzoic acid. Referred to herein as “Compound 7”.

[0135] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (9).



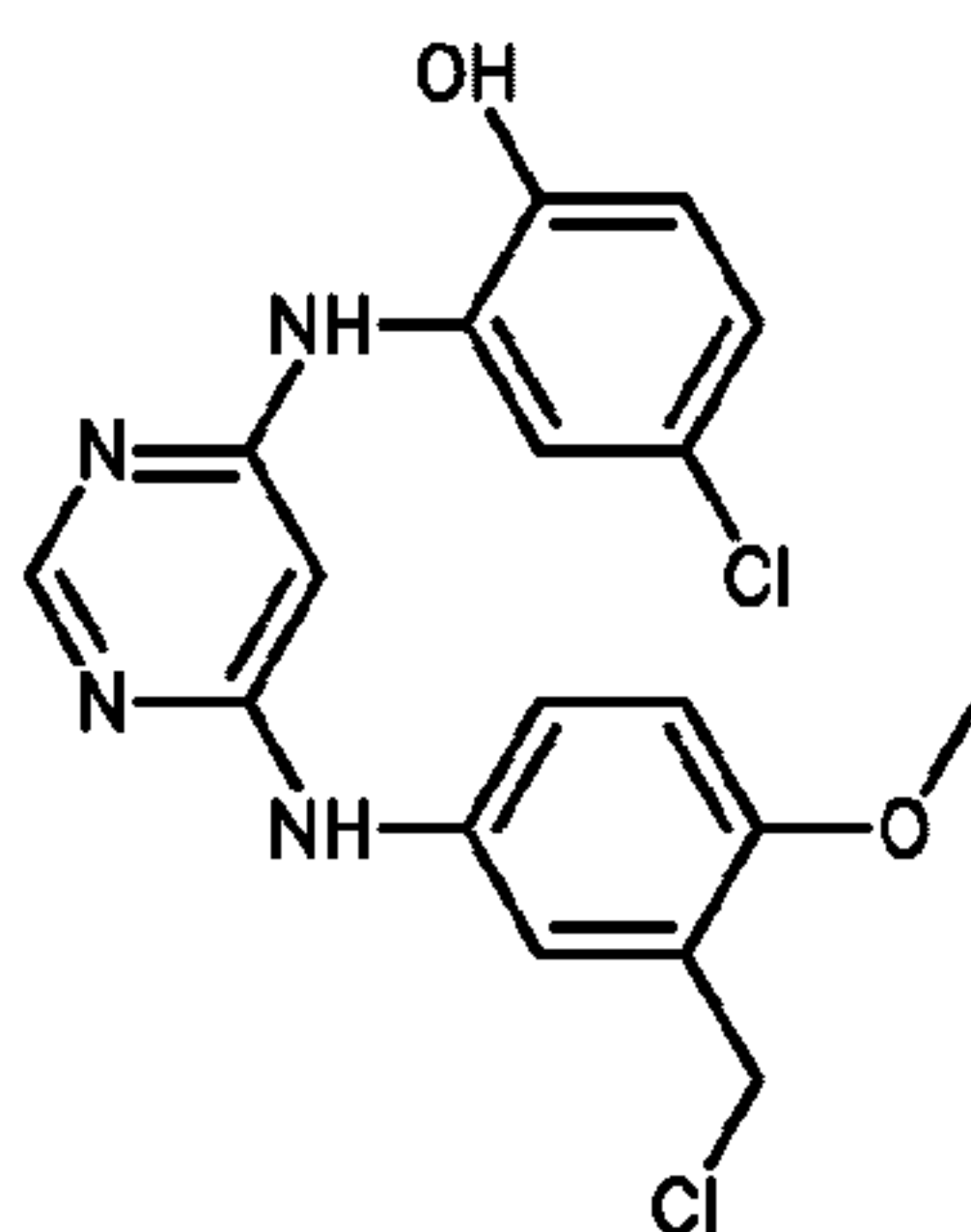
Formula (9): N-{[3-(5-{[3-(2-Aminopyrimidin-4-yl)phenyl]amino}-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)phenyl]methyl}cyclopropanecarboxamide. Referred to herein as “Compound 8”.

[0136] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (10).



Formula (10): 4-[(1-Cyclohexyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)amino]-N-[3-(methyloxy)propyl]benzenesulfonamide. Referred to herein as “Compound 9”.

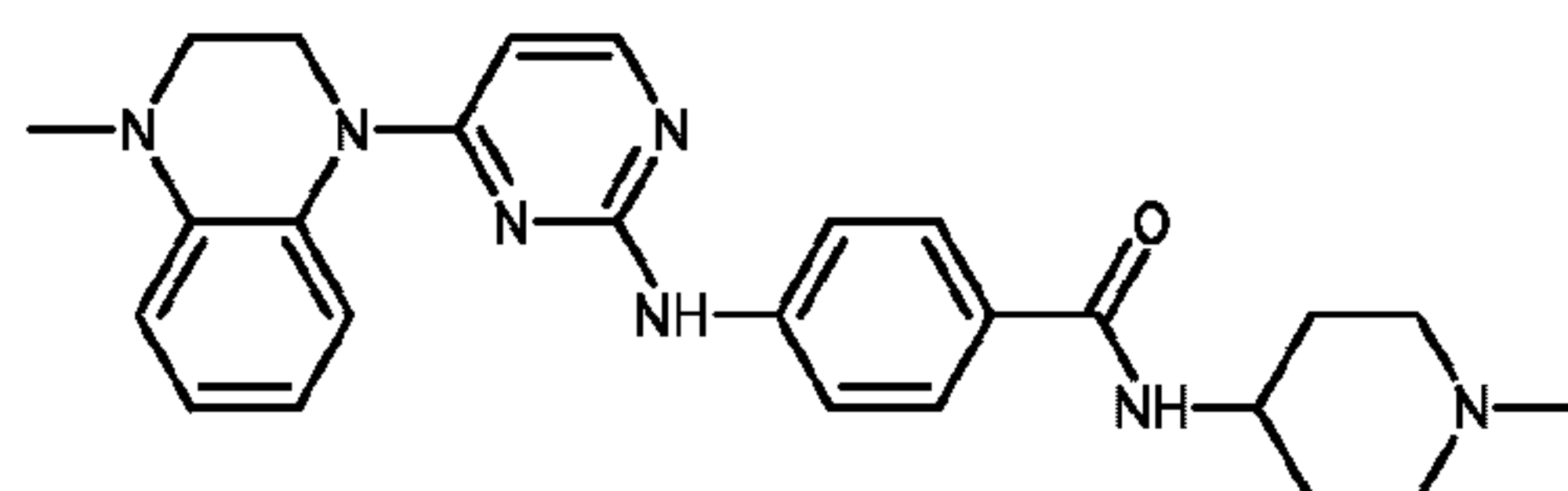
[0137] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (11).



Formula (11): 4-Chloro-2-[(6-{[3-(chloromethyl)-4-methoxyphenyl]amino}pyrimidin-4-yl)amino]phenol.

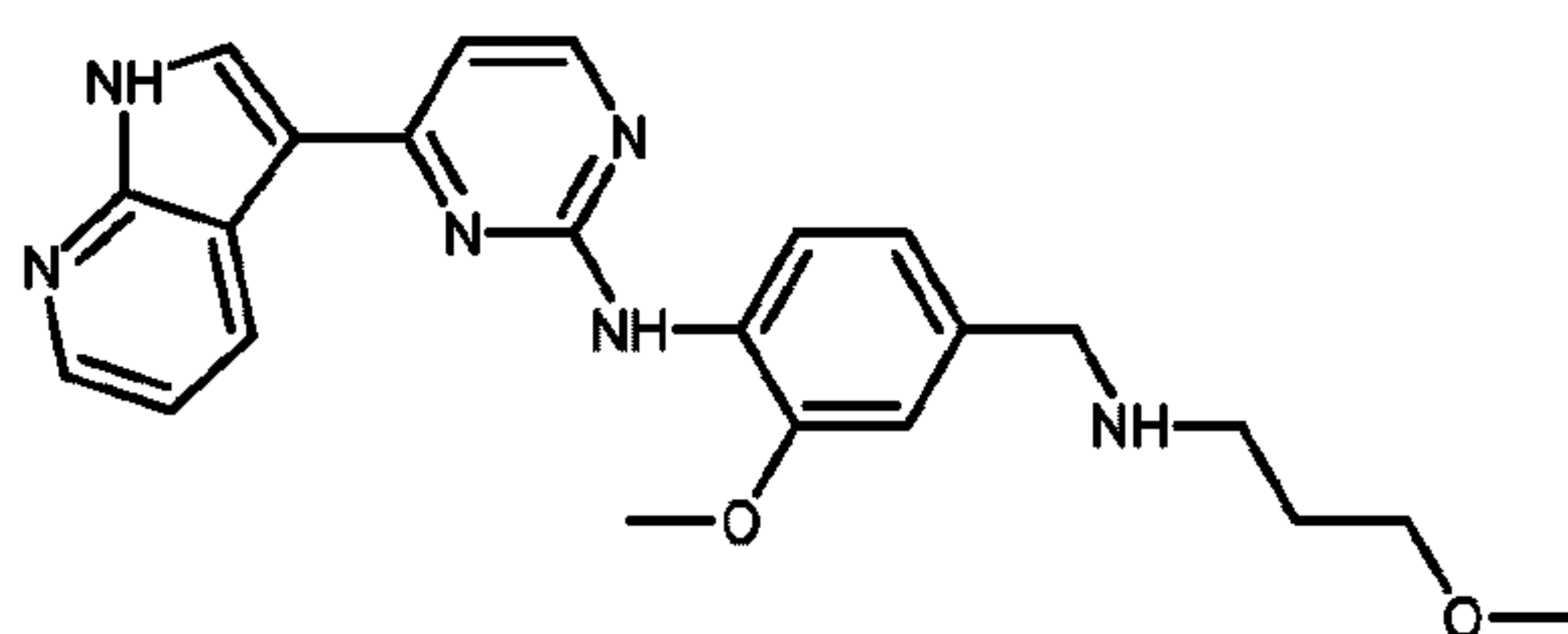
Referred to herein as “Compound 10”.

[0138] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (12).



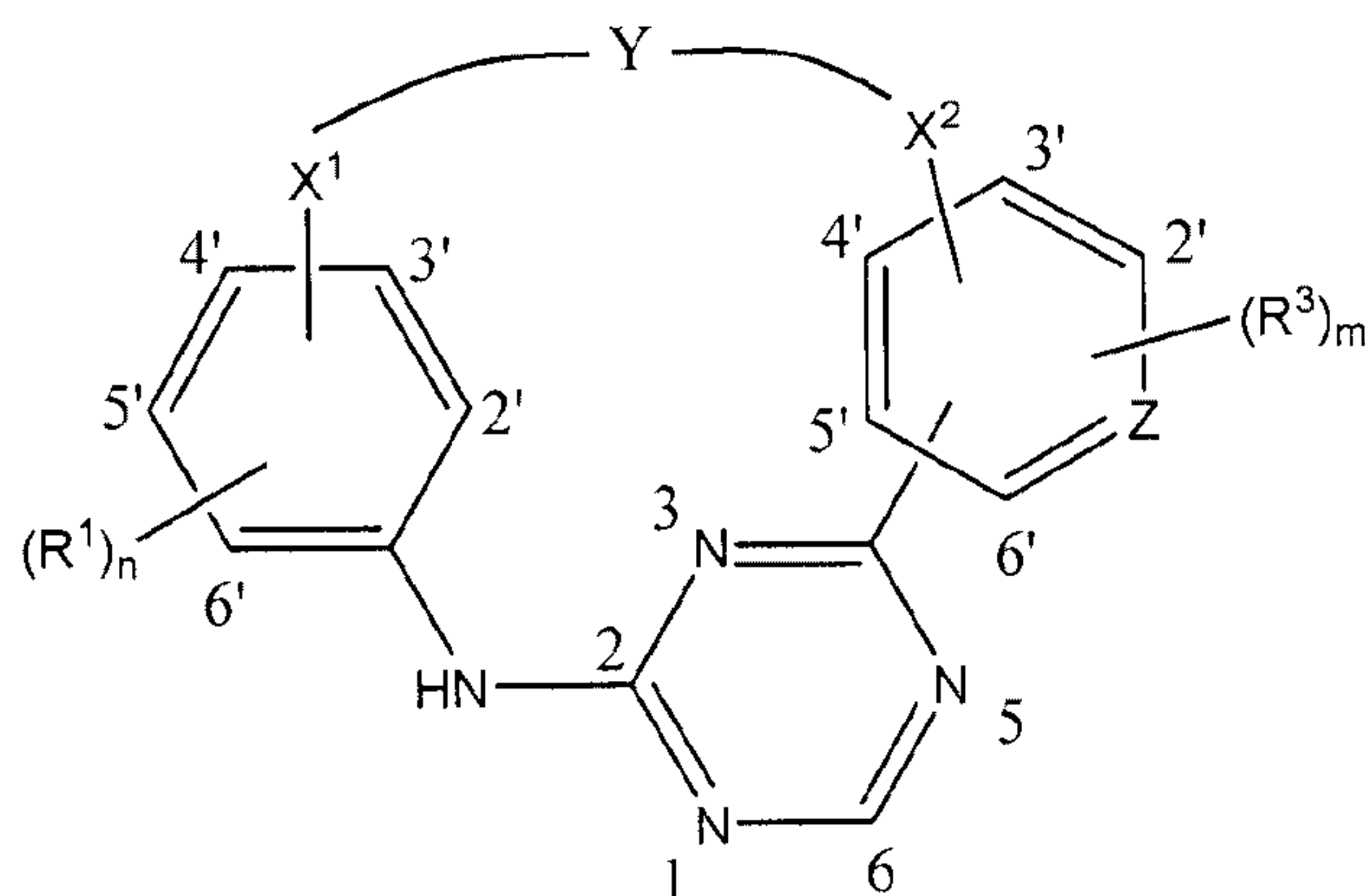
Formula (12): 4-{[4-(4-Methyl-3,4-dihydroquinoxalin-1(2H)-yl)pyrimidin-2-yl]amino}-N-(1-methylpiperidin-4-yl)benzamide. Referred to herein as “Compound 11”.

[0139] In one embodiment, the aniline-pyridinotriazine is a compound of the Formula (13).



Formula (13): N-(2-Methoxy-4-{[(3-methoxypropyl)amino]methyl}phenyl)-4-(1H-pyrrolo[2,3-b]pyridin-3-yl)pyrimidin-2-amine. Referred to herein as “Compound 12”.

[0140] In one embodiment, the compound that is capable of differentiating pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage is a cyclic aniline-pyridinotriazine of the Formula (14):



Formula (14).

- [0141] The N-oxide forms, the pharmaceutically acceptable addition salts and the stereochemically isomeric forms thereof, wherein:
- [0142] m represents an integer from 1 to 4; n represents an integer from 1 to 4; Z represents N or C;
- [0143] Y represents $\text{-NR}^2\text{-C}_{1-6}\text{alkyl-CO-NR}^4\text{-}$, $\text{-C}_{1-4}\text{alkyl-NR}^9\text{-C}_{1-4}\text{alkyl-}$, $\text{C}_{1-6}\text{alkyl-CO-Het}^{10}\text{-}$, $\text{-Het}^{11}\text{-CO-C}_{1-6}\text{alkyl-}$, $\text{-Het}^{12}\text{-C}_{1-6}\text{alkyl-}$, $\text{-CO-Het}^{13}\text{-C}_{1-6}\text{alkyl-}$, $\text{-CO-NR}^{10}\text{-C}_{1-6}\text{alkyl-}$, $\text{-Het}^1\text{-C}_{1-6}\text{alkyl-CO-NR}^5\text{-}$, or $\text{-Het}^2\text{-CO-NR}^6\text{-}$ wherein the $\text{-C}_{1-6}\text{alkyl-}$ linker in $\text{-NR}^2\text{-C}_{1-6}\text{alkyl-CO-NR}^4\text{-}$ or $\text{-Het}^1\text{-C}_{1-6}\text{alkyl-CO-NR}^5\text{-}$ is optionally substituted with one or where possible two or more substituents selected from hydroxy, methoxy, aminocarbonyl, halo, phenyl, indolyl, methylsulfide, thiol, hydroxyphenyl, cyanophenyl, amino and hydroxycarbonyl;
- [0144] X^1 represents a direct bond, $\text{C}_{1-4}\text{alkyl}$, $\text{C}_{1-4}\text{alkyloxy-}$, $\text{C}_{1-4}\text{alkyl-CO-}$, $\text{C}_{2-4}\text{alkenyl}$, $\text{C}_{2-4}\text{alkynyl}$, or $\text{C}_{1-4}\text{alkyl-NR}^3\text{-}$, wherein said $\text{C}_{1-4}\text{alkyl}$ or $\text{C}_{2-4}\text{alkenyl}$ is optionally substituted with one or where possible two or more halo substituents;
- [0145] X^2 represents a direct bond, $\text{C}_{1-4}\text{alkyl}$, $\text{C}_{1-4}\text{alkyloxy-}$, $\text{C}_{1-4}\text{alkyl-CO-}$, $\text{C}_{2-4}\text{alkenyl}$, $\text{C}_{2-4}\text{alkynyl}$, or $\text{C}_{1-4}\text{alkyl-NR}^7\text{-}$, wherein said $\text{C}_{1-4}\text{alkyl}$ or $\text{C}_{2-4}\text{alkenyl}$ is optionally substituted with one or where possible two or more halo substituents;
- [0146] R^1 and R^8 each independently represent hydrogen, Het^{14} , cyano, halo, hydroxy, $\text{C}_{1-6}\text{alkoxy-}$, $\text{C}_{1-6}\text{alkyl-}$, mono- or di($\text{C}_{1-4}\text{alkyl}$)amino-carbonyl-, mono- or di($\text{C}_{1-4}\text{alkyl}$)amino-sulfonyl, $\text{C}_{1-6}\text{alkoxy-}$ substituted with halo or R^1 represents $\text{C}_{1-6}\text{alkyl}$

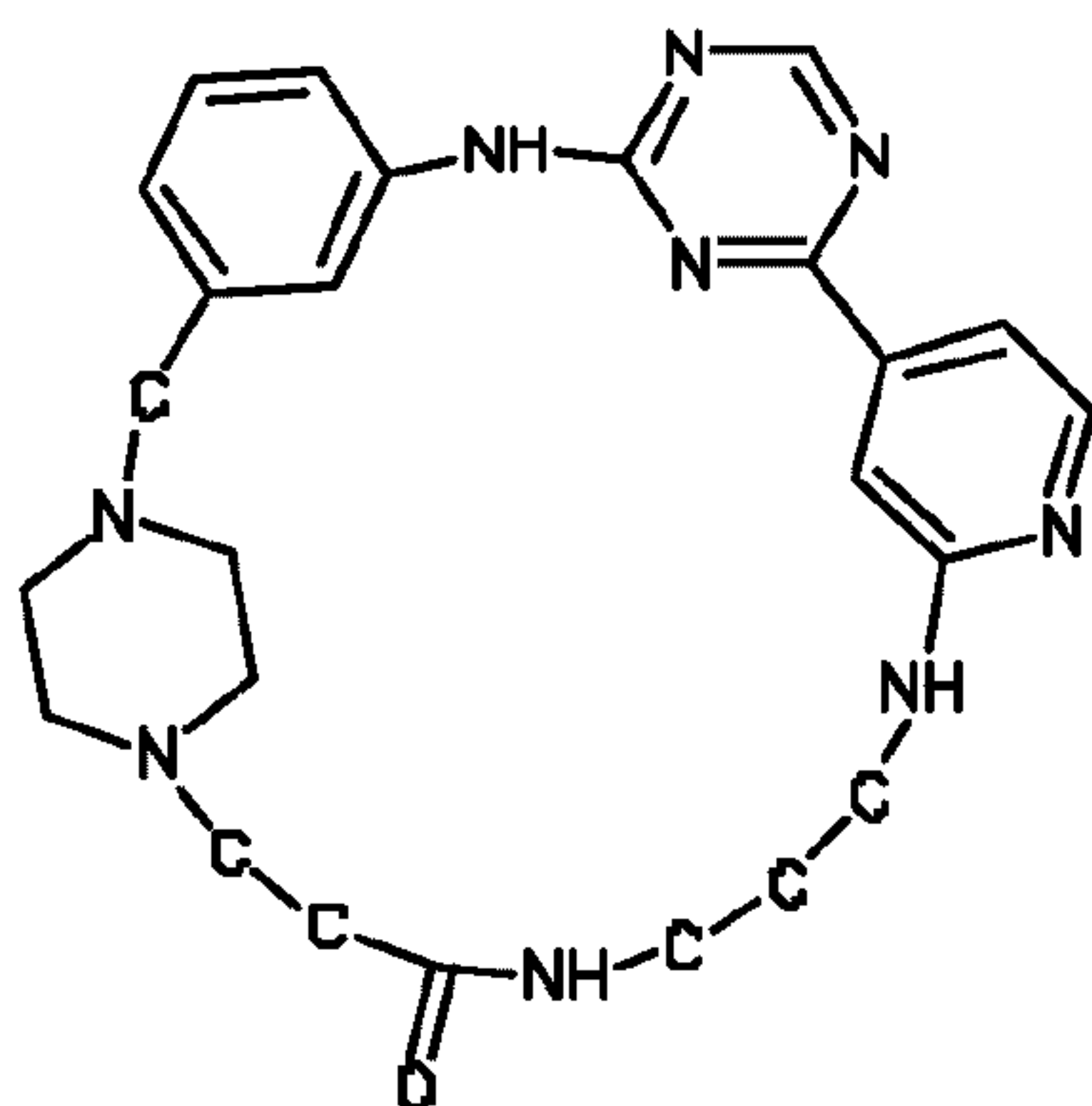
substituted with one or where possible two or more substituents selected from hydroxy or halo;

- [0147] R^2 and R^9 each independently represents hydrogen, C_{1-4} alkyl, C_{2-4} alkenyl, Het^3 , Het^4 - C_{1-4} alkyl-, Het^5 - C_{1-4} alkylcarbonyl-, mono-or di(C_{1-4} alkyl)amino- C_{1-4} alkylcarbonyl-or phenyl optionally substituted with one or where possible two or more substituents selected from hydrogen, hydroxy, amino or C_{1-4} alkyloxy-;
- [0148] R^3 and R^7 each independently represent hydrogen, C_{1-4} alkyl, Het^6 , Het^7 - C_{1-4} alkyl-, C_{2-4} alkenylcarbonyl- optionally substituted with Het^8 - C_{1-4} alkylaminocarbonyl-, C_{2-4} alkenylsulfonyl-, C_{1-4} alkyloxy C_{1-4} alkyl-or phenyl optionally substituted with one or where possible two or more substituents selected from hydrogen, hydroxy, amino or C_{1-4} alkyloxy-;
- [0149] R_4 , R_5 , R_6 and R^{10} each independently represent hydrogen or C_{1-4} alkyl optionally substituted with hydroxy, Het^9 or C_{1-4} alkyloxy;
- [0150] Het^1 and Het^2 each independently represent a heterocycle selected from pyrrolidinyl, piperidinyl, piperazinyl, pyridinyl, pyrimidinyl, pyrazinyl, imidazolidinyl or pyrazolidinyl wherein said Het^1 and Het^2 are optionally substituted with amino, hydroxy, C_{1-4} alkyl, hydroxy- C_{1-4} alkyl-, phenyl, phenyl- C_{1-4} alkyl-, C_{1-4} alkyl-oxy- C_{1-4} alkyl-mono-or di(C_{1-4} alkyl) amino-or amino-carbonyl-;
- [0151] Het^3 and Het^6 each independently represent, heterocycle selected from pyrrolidinyl or piperidinyl wherein said Het^3 and Het^6 are optionally substituted with one or where possible two or more substituents selected from C_{1-4} alkyl, C_{3-6} cycloalkyl, hydroxy- C_{1-4} alkyl-, C_{1-4} alkyloxy C_{1-4} alkyl or polyhydroxy- C_{1-4} alkyl-;
- [0152] Het^4 , Het^7 and Het^9 each independently represent a heterocycle selected from morpholinyl, pyrrolidinyl, piperazinyl or piperidinyl wherein said Het^4 , Het^7 and Het^9 are optionally substituted with one or where possible two or more substituents selected from C_{1-4} alkyl, C_{3-6} cycloalkyl, hydroxy- C_{1-4} alkyl-, C_{1-4} alkyloxy C_{1-4} alkyl or polyhydroxy- C_{1-4} alkyl-;
- [0153] Het^5 represents a heterocycle selected from morpholinyl, pyrrolidinyl, piperazinyl or piperidinyl wherein said Het^5 is optionally substituted with one or where possible two

or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-;

- [0154] Het¹⁰, Het¹¹ and Het¹³ each independently represent a heterocycle selected from pyrrolidinyl, piperidinyl, piperazinyl, pyridinyl, pyrimidinyl, pyrazinyl, imidazolidinyl or pyrazolidinyl wherein said Het¹⁰, Het¹¹ and Het¹³ are optionally substituted with amino, hydroxy, C₁₋₄alkyl, hydroxy-C₁₋₄alkyl-, phenyl, phenyl-C₁₋₄alkyl-, C₁₋₄alkyl-oxy-C₁₋₄alkyl-, amino-carbonyl- or mono- or di(C₁₋₄alkyl)amino-;
- [0155] Het¹² represents a heterocycle selected from pyrrolidinyl, piperidinyl, piperazinyl, pyridinyl, pyrimidinyl, pyrazinyl, imidazolidinyl or pyrazolidinyl wherein said Het¹² is optionally substituted with amino, hydroxy, C₁₋₄alkyl, hydroxy-C₁₋₄alkyl-, phenyl, phenyl-C₁₋₄alkyl-, C₁₋₄alkyl-oxy-C₁₋₄alkyl-; mono- or di(C₁₋₄alkyl)amino- or amino-carbonyl-;
- [0156] Het¹⁴ represents a heterocycle selected from morpholinyl; pyrrolidinyl; piperazinyl; imidazolyl; pyrrolyl; 2,3,4-triazapyrrolyl; 1,2,3-triazolyl; pyrazolyl; or piperidinyl wherein said Het¹⁴ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-; in particular Het¹⁴ represents a heterocycle selected from morpholinyl; pyrrolidinyl; pyrrolyl; 2,3,4-triazapyrrolyl; piperazinyl or piperidinyl wherein said Het¹⁴ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-; more particular Het¹⁴ represents a heterocycle selected from morpholinyl; pyrrolidinyl; piperazinyl or piperidinyl wherein said Het¹⁴ is optionally substituted with one or where possible two or more substituents selected from C₁₋₄alkyl, C₃₋₆cycloalkyl, hydroxy-C₁₋₄alkyl-, C₁₋₄alkyloxyC₁₋₄alkyl or polyhydroxy-C₁₋₄alkyl-.
- [0157] Compounds of Formula (7) are disclosed in WO2007/003525, assigned to Janssen Pharmaceutica N.V.
- [0158] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (14).

[0159] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (15).



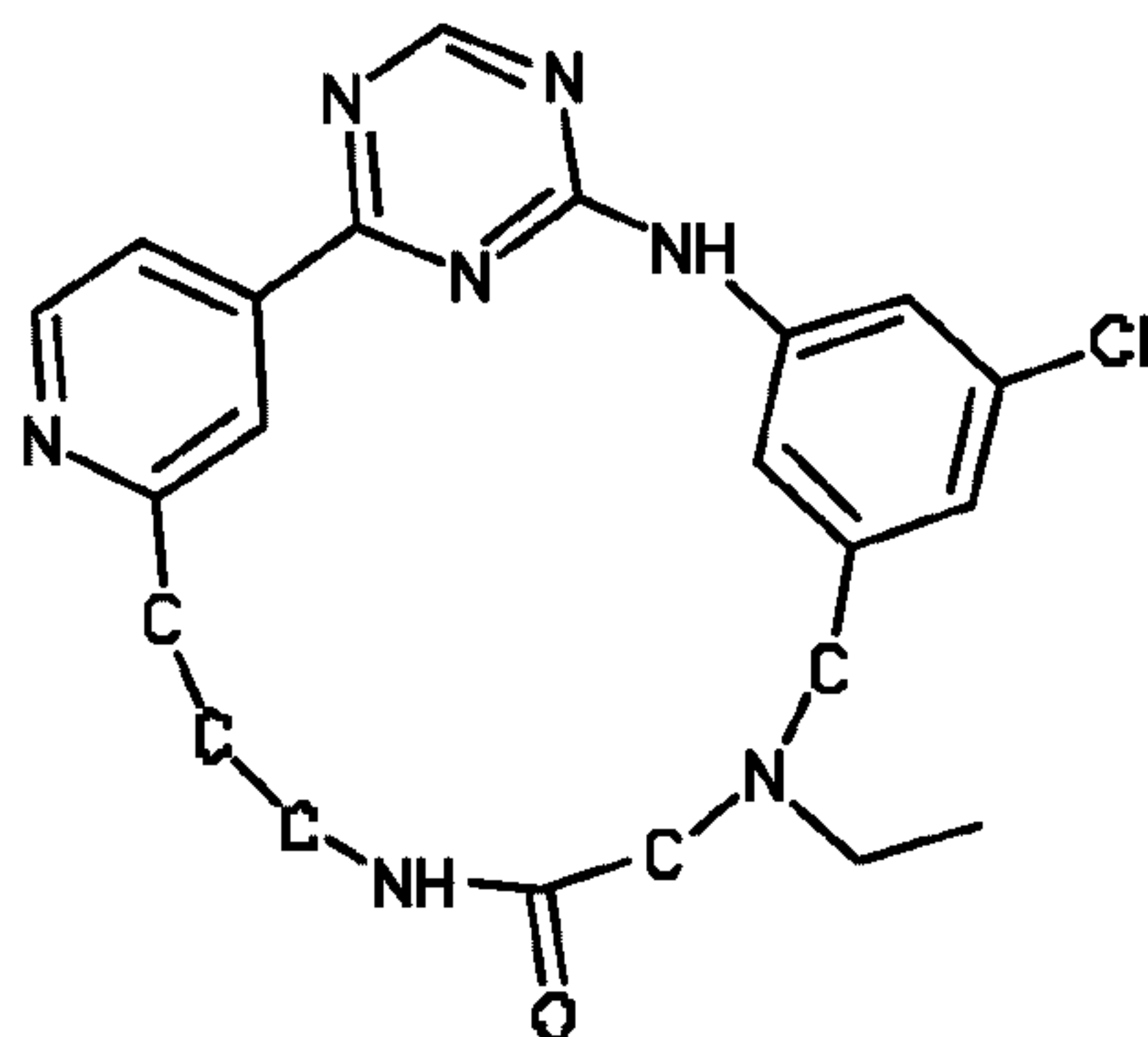
Formula (15):

1,8,10,12,17,19,23,27,33-

Nonaazapentacyclo[25.2.2.1~3,7~.1~9,13~.1~14,18~]tetratriaconta-

3(34),4,6,9(33),10,12,14(32),15,17-nonaen-24-one. Referred to herein as "Compound 13".

[0160] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (16).

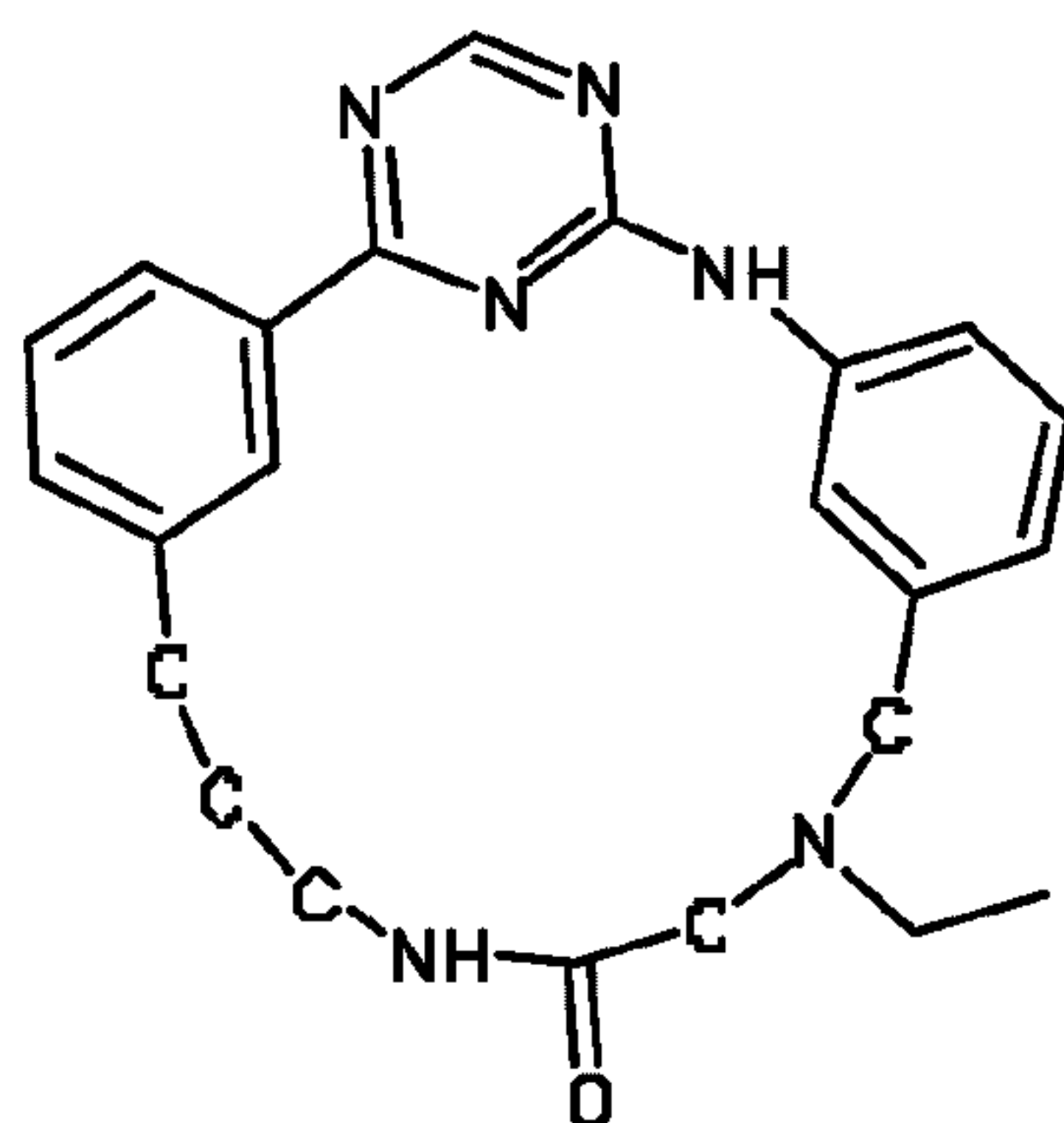


Formula (16): 10-Chloro-14-ethyl-

3,5,7,14,17,22,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacos-

1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one. Referred to herein as "Compound 14".

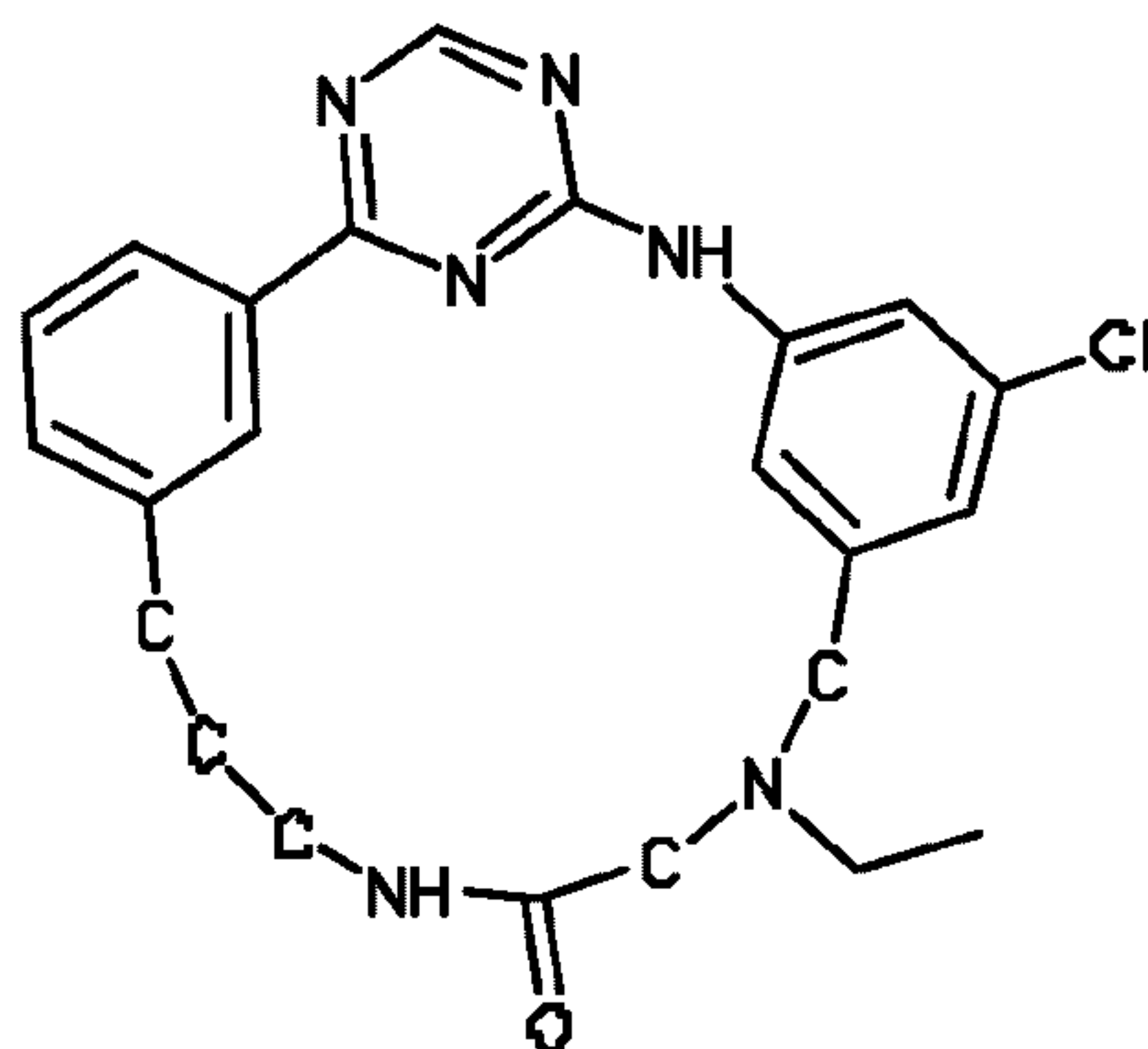
[0161] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (17).



Formula (17): 14-Ethyl-

3,5,7,14,17,27-hexaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosan-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one. Referred to herein as “Compound 15”.

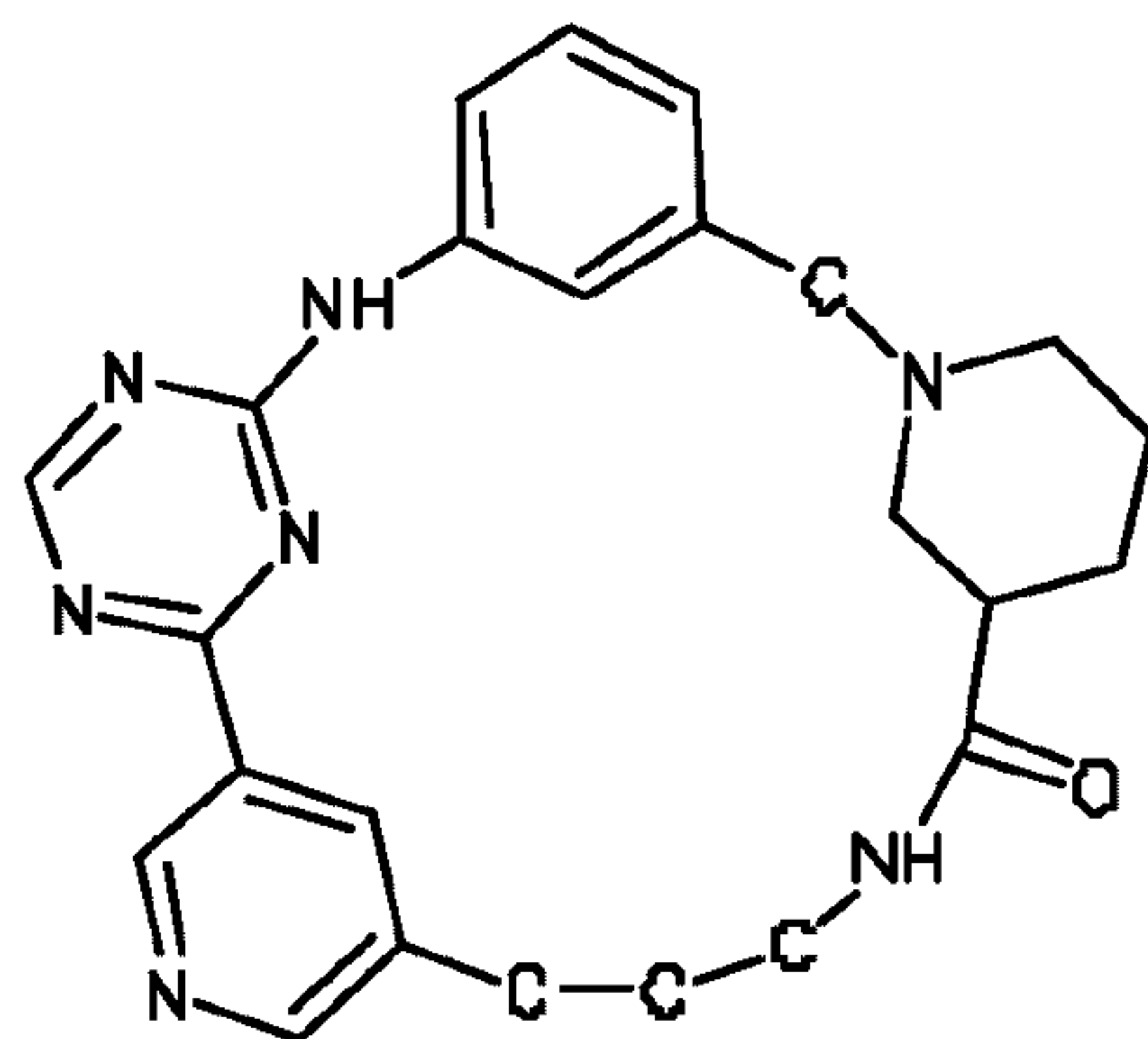
[0162] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (18).



Formula (18): 10-Chloro-14-

ethyl-3,5,7,14,17,27-hexaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosan-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one. Referred to herein as “Compound 16”.

[0163] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (19).

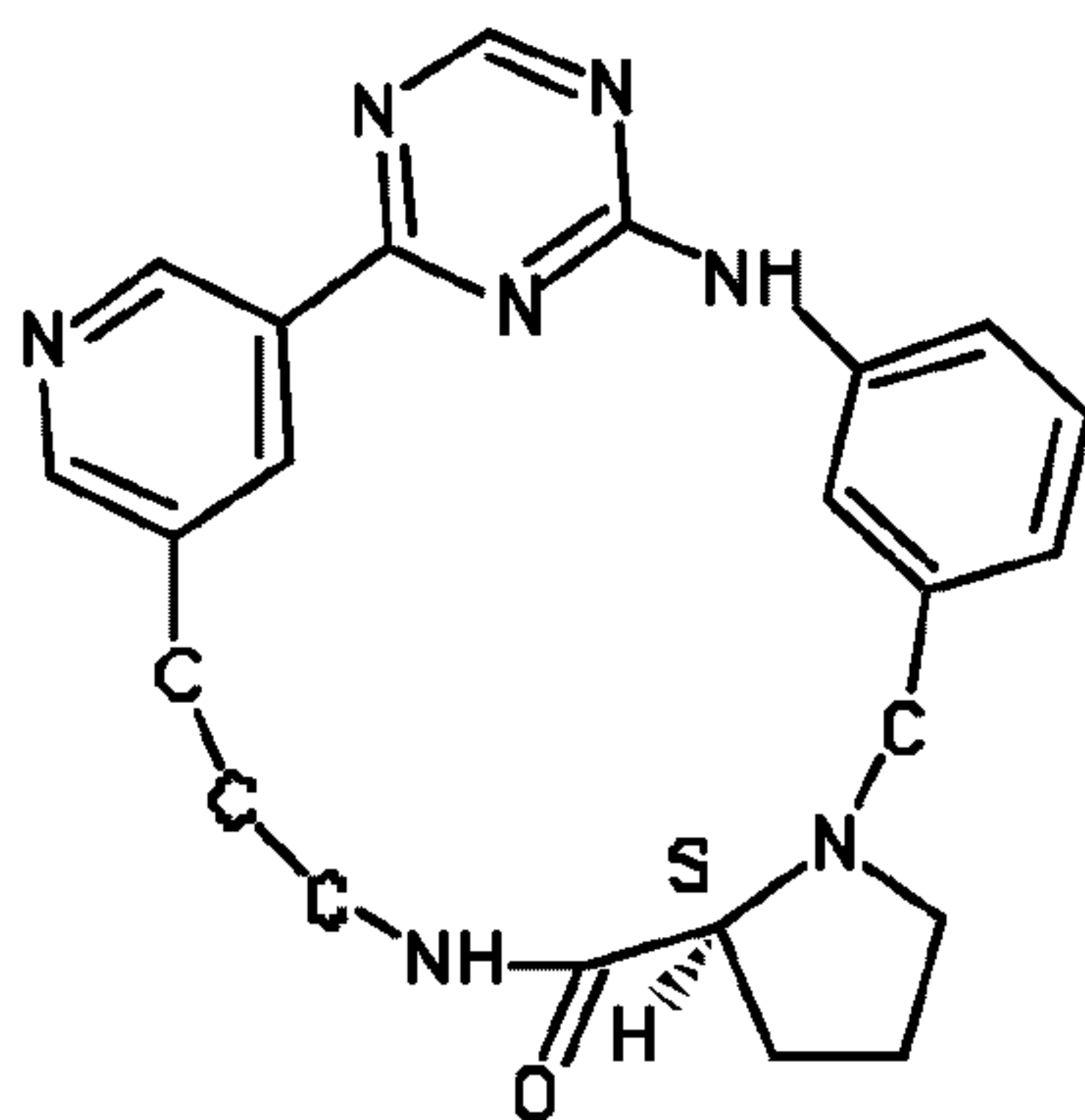


Formula (19): 3,5,7,14,20,26,31-

Heptaazapentacyclo[22.3.1.1~2,6~.1~8,12~.1~14,18~]hentriaconta-

1(28),2(31),3,5,8(30),9,11,24,26-nonaen-19-one. Referred to herein as “Compound 17”.

[0164] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (20).

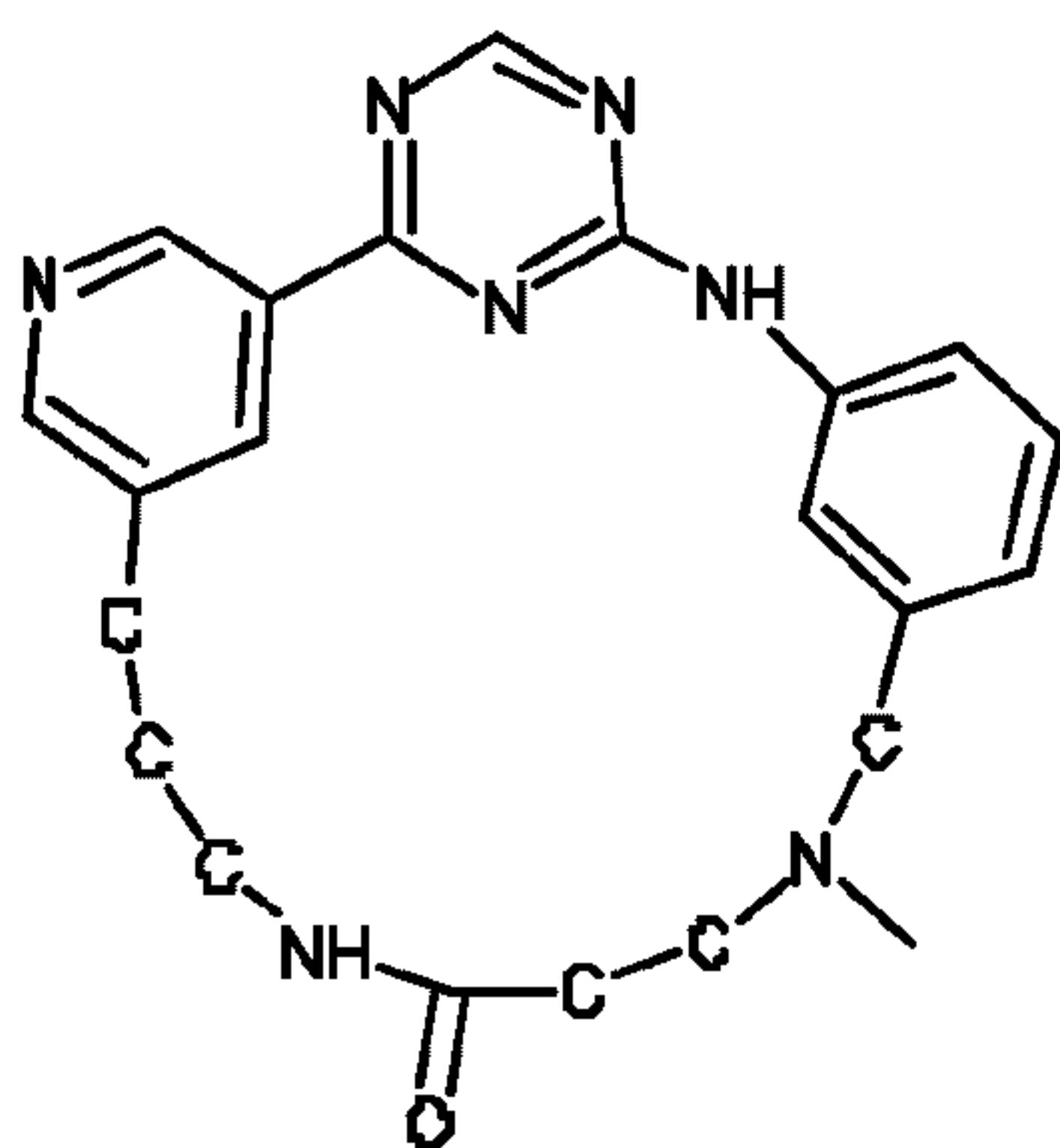


Formula (20): (18S)-

3,5,7,14,20,26,30-Heptaazapentacyclo[22.3.1.1~2,6~.1~8,12~.0~14,18~]triaconta-

1(28),2(30),3,5,8(29),9,11,24,26-nonaen-19-one. Referred to herein as “Compound 18”.

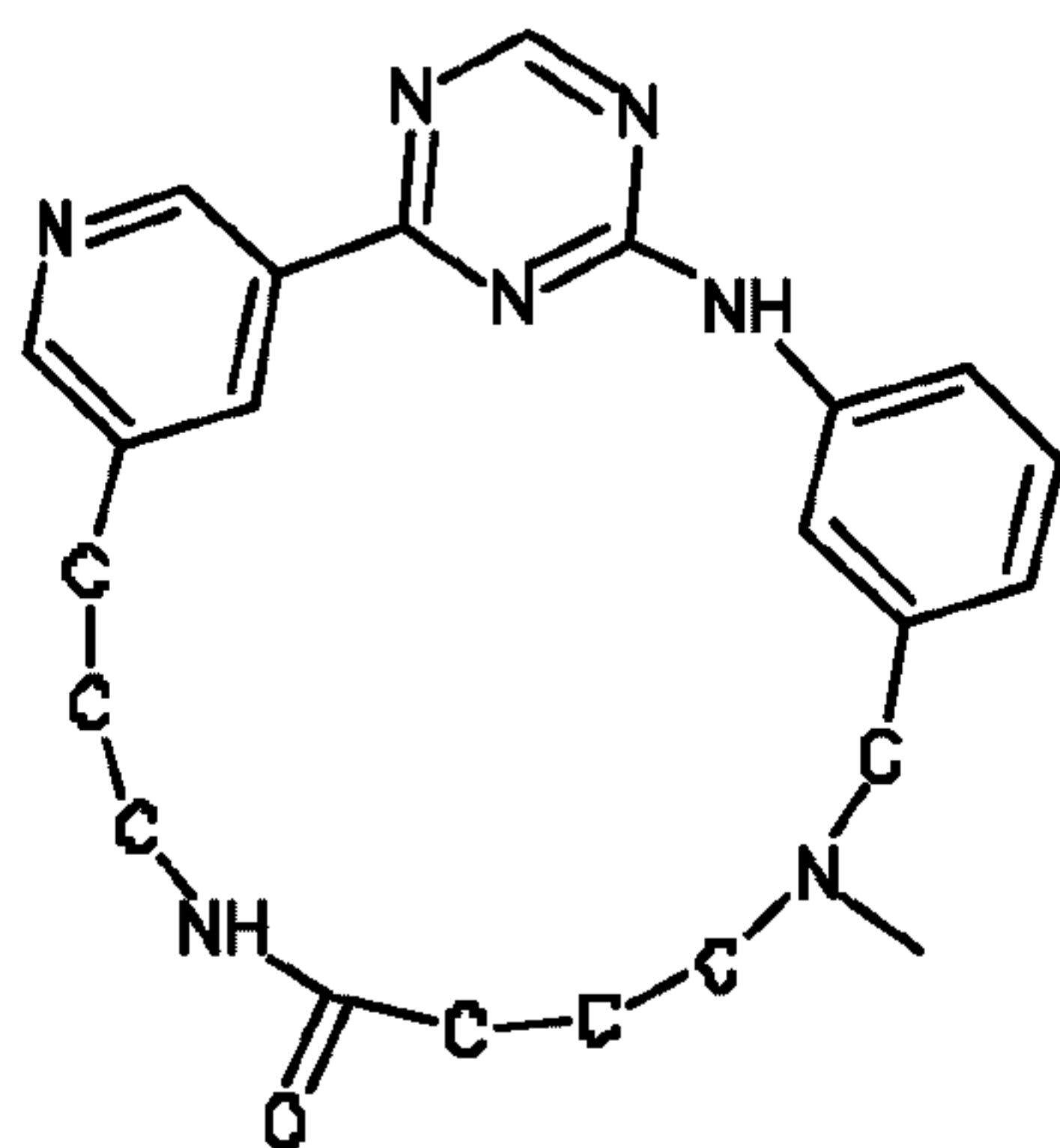
[0165] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (21).



Formula (21): 14-Methyl-

3,5,7,14,18,24,28-heptaazatetracyclo[20.3.1.1~2,6~.1~8,12~]octacosa-1(26),2(28),3,5,8(27),9,11,22,24-nonaen-17-one. Referred to herein as “Compound 19”.

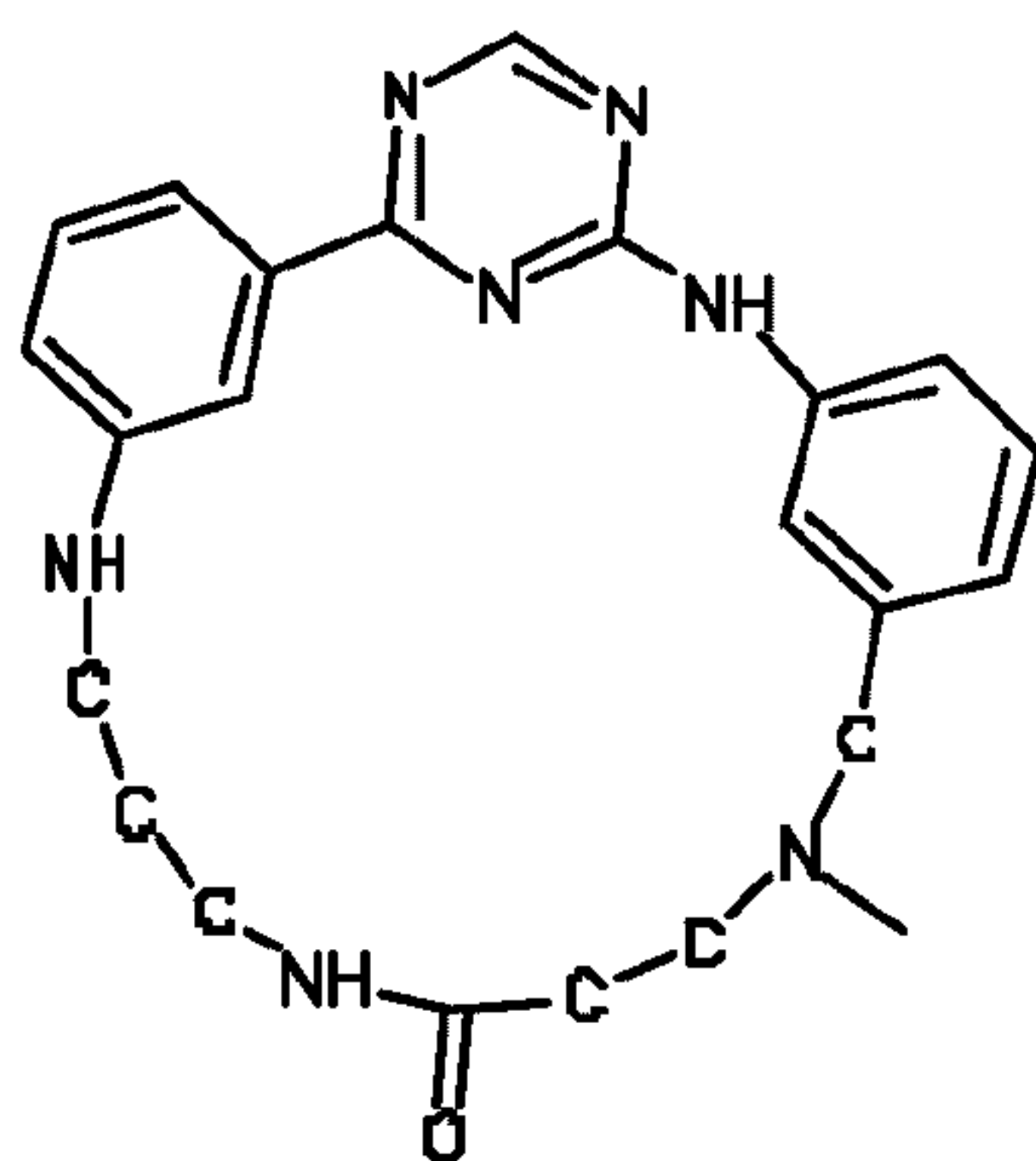
[0166] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (22).



Formula (22): 14-Methyl-

3,5,7,14,19,25,29-heptaazatetracyclo[21.3.1.1~2,6~.1~8,12~]nonacosa-1(27),2(29),3,5,8(28),9,11,23,25-nonaen-18-one. Referred to herein as “Compound 20”.

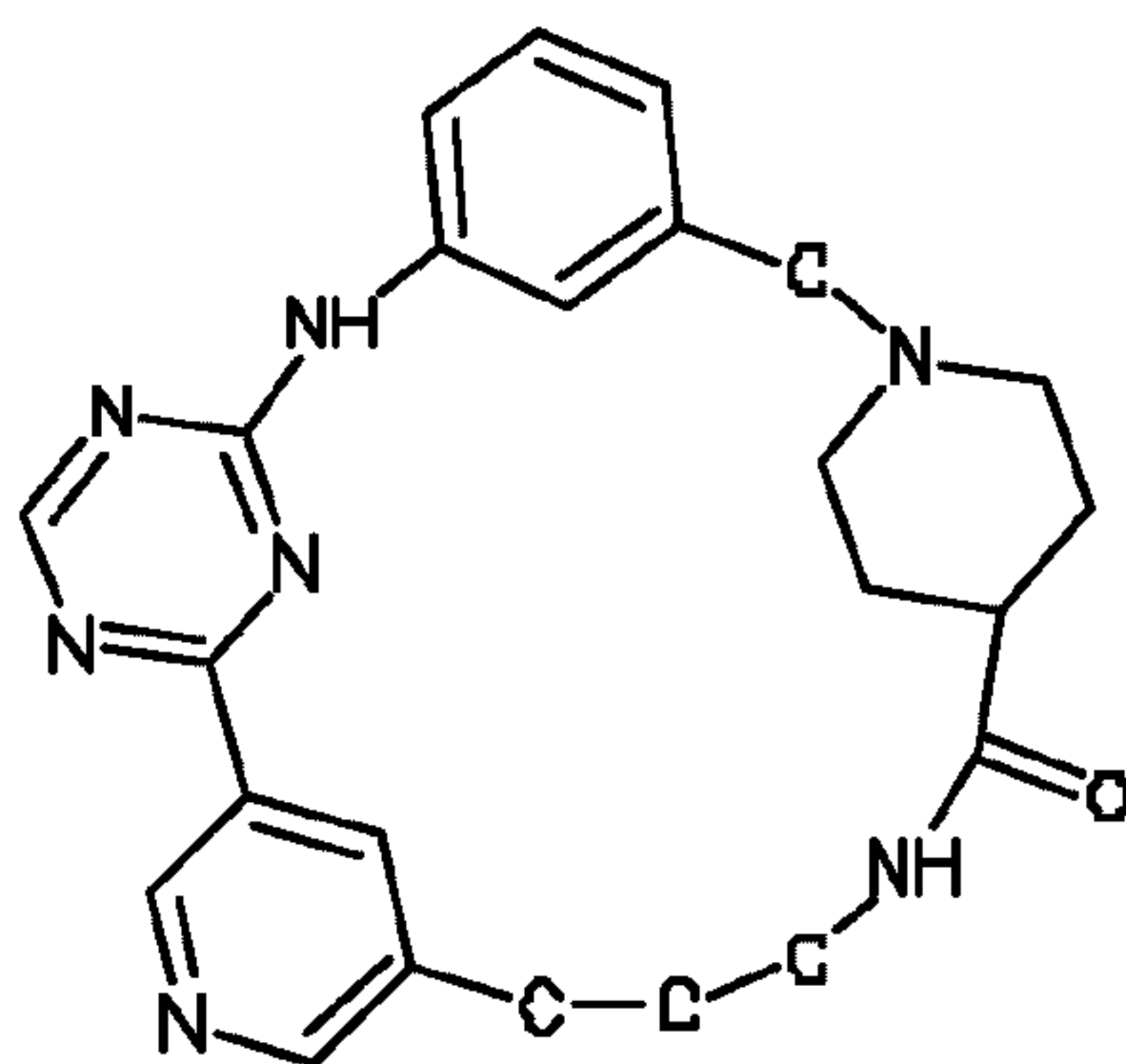
[0167] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (23).



Formula (23): 14-Methyl-

3,5,7,14,18,22,29-heptaazatetracyclo[21.3.1.1~2,6~.1~8,12~]nonacosa-1(27),2(29),3,5,8(28),9,11,23,25-nonaen-17-one. Referred to herein as “Compound 21”.

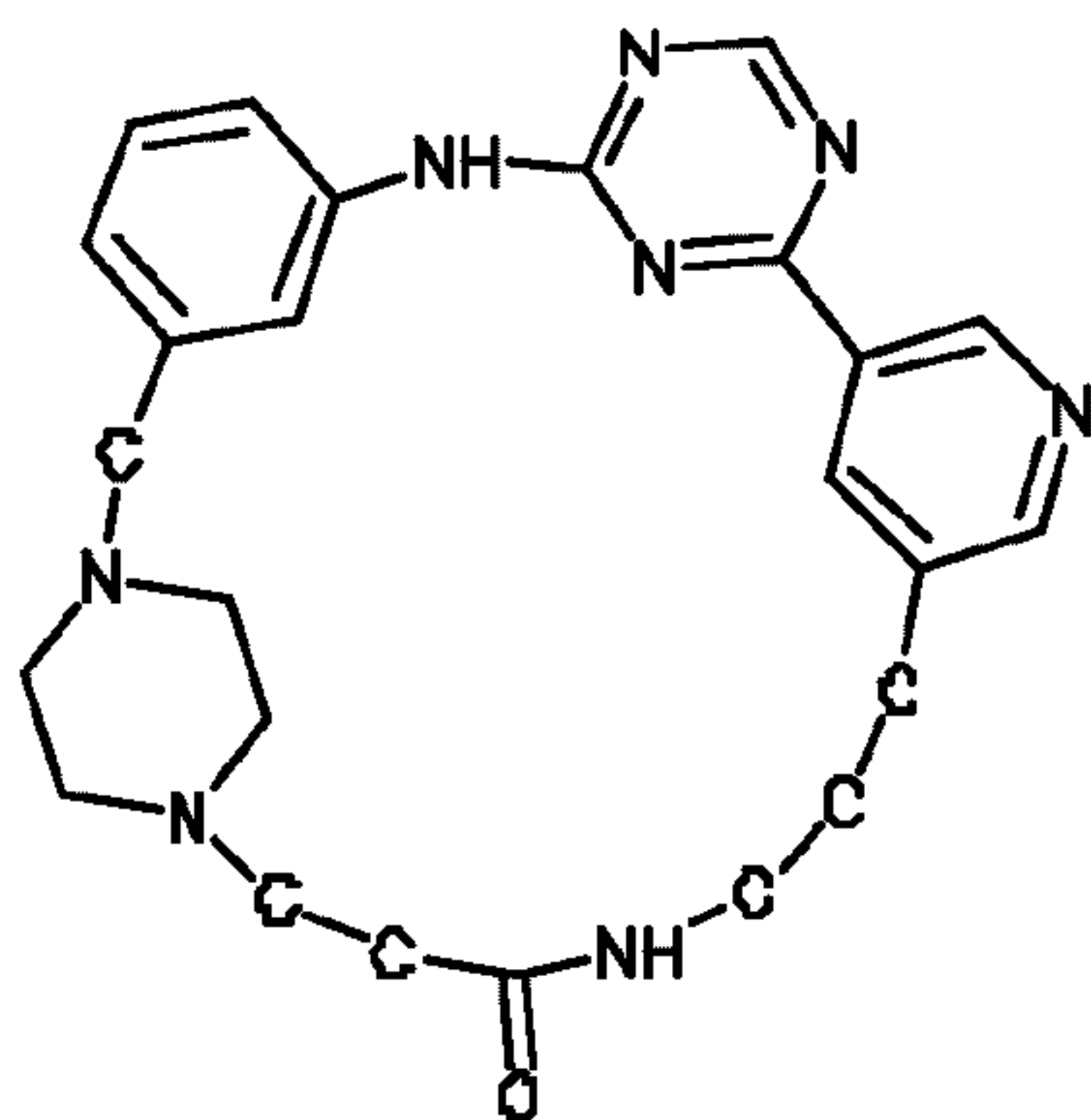
[0168] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (24).



Formula (24): 1,8,10,12,16,22,30-

Heptaazapentacyclo[22.2.2.1~3,7~.1~9,13~.1~14,18~]hentriaconta-3(31),4,6,9(30),10,12,14(29),15,17-nonaen-23-one. Referred to herein as “Compound 22”.

[0169] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (25).



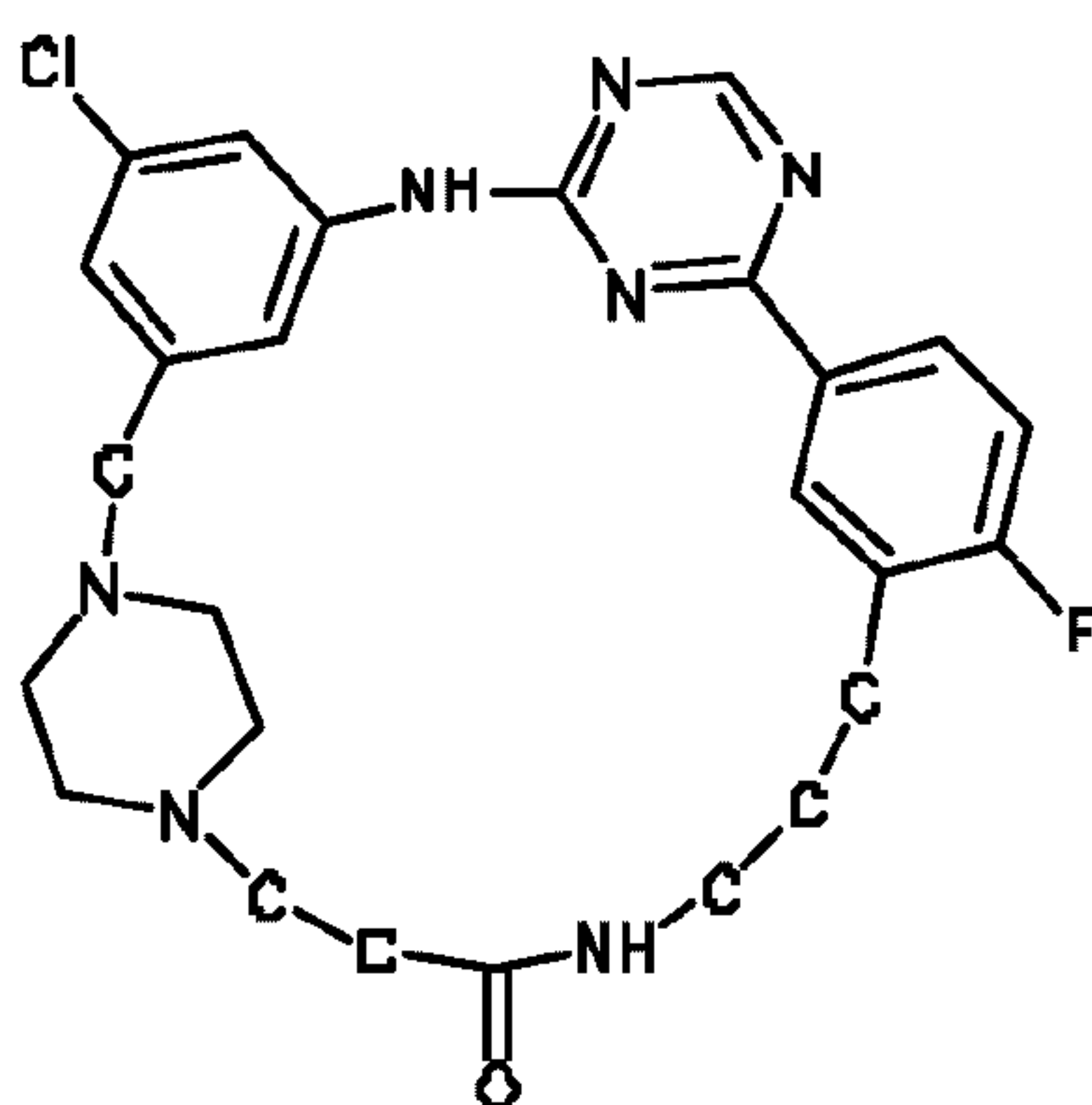
Formula (25):

1,8,10,12,16,22,26,32-

Octaazapentacyclo[24.2.2.1~3,7~.1~9,13~.1~14,18~]tritiaconta-

3(33),4,6,9(32),10,12,14(31),15,17-nonaen-23-one. Referred to herein as “Compound 23”.

[0170] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (26).

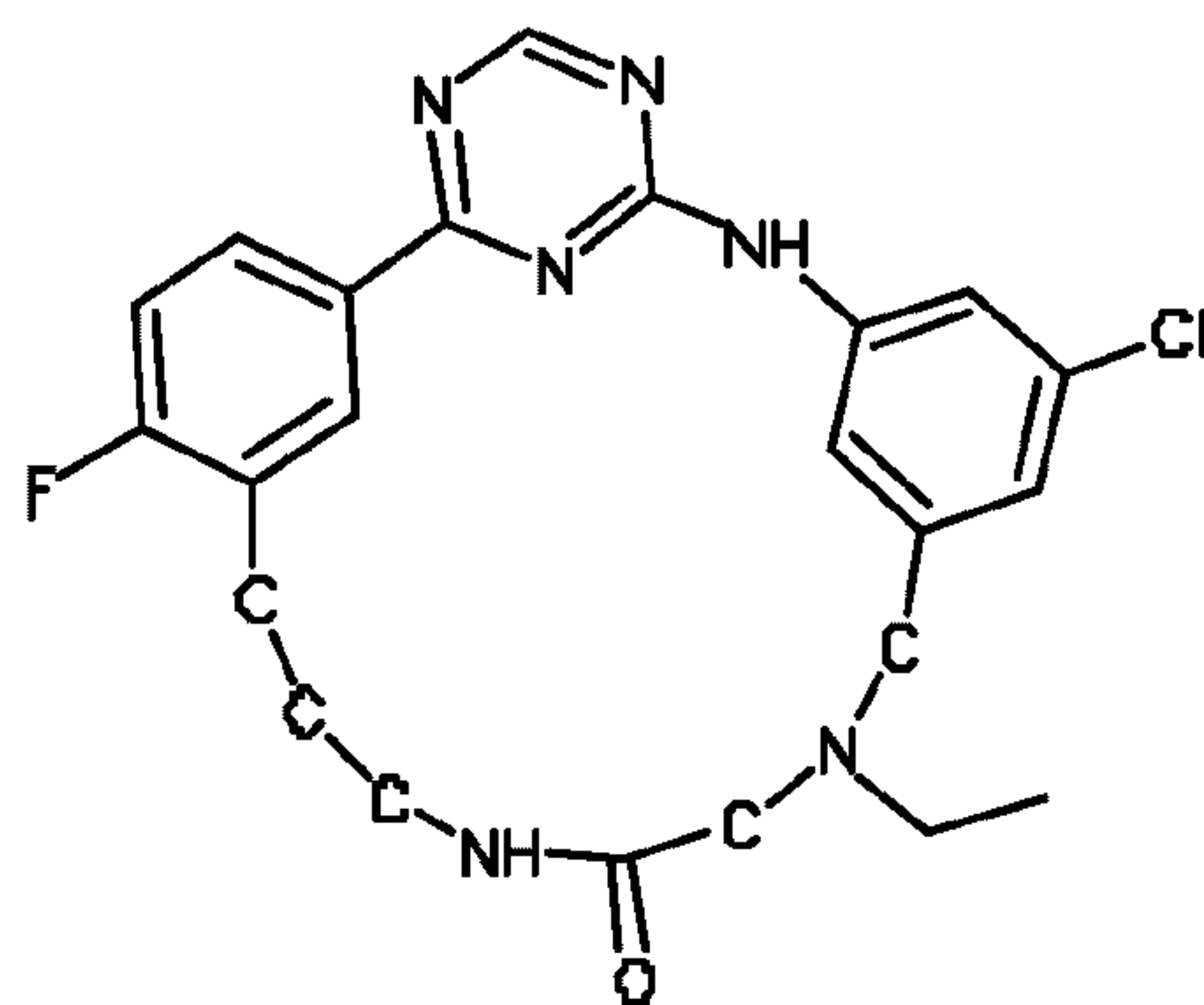


Formula (26): 5-Chloro-17-fluoro-

1,8,10,12,22,26,32-heptaazapentacyclo[24.2.2.1~3,7~.1~9,13~.1~14,18~]tritiaconta-

3(33),4,6,9(32),10,12,14(31),15,17-nonaen-23-one. Referred to herein as “Compound 24”.

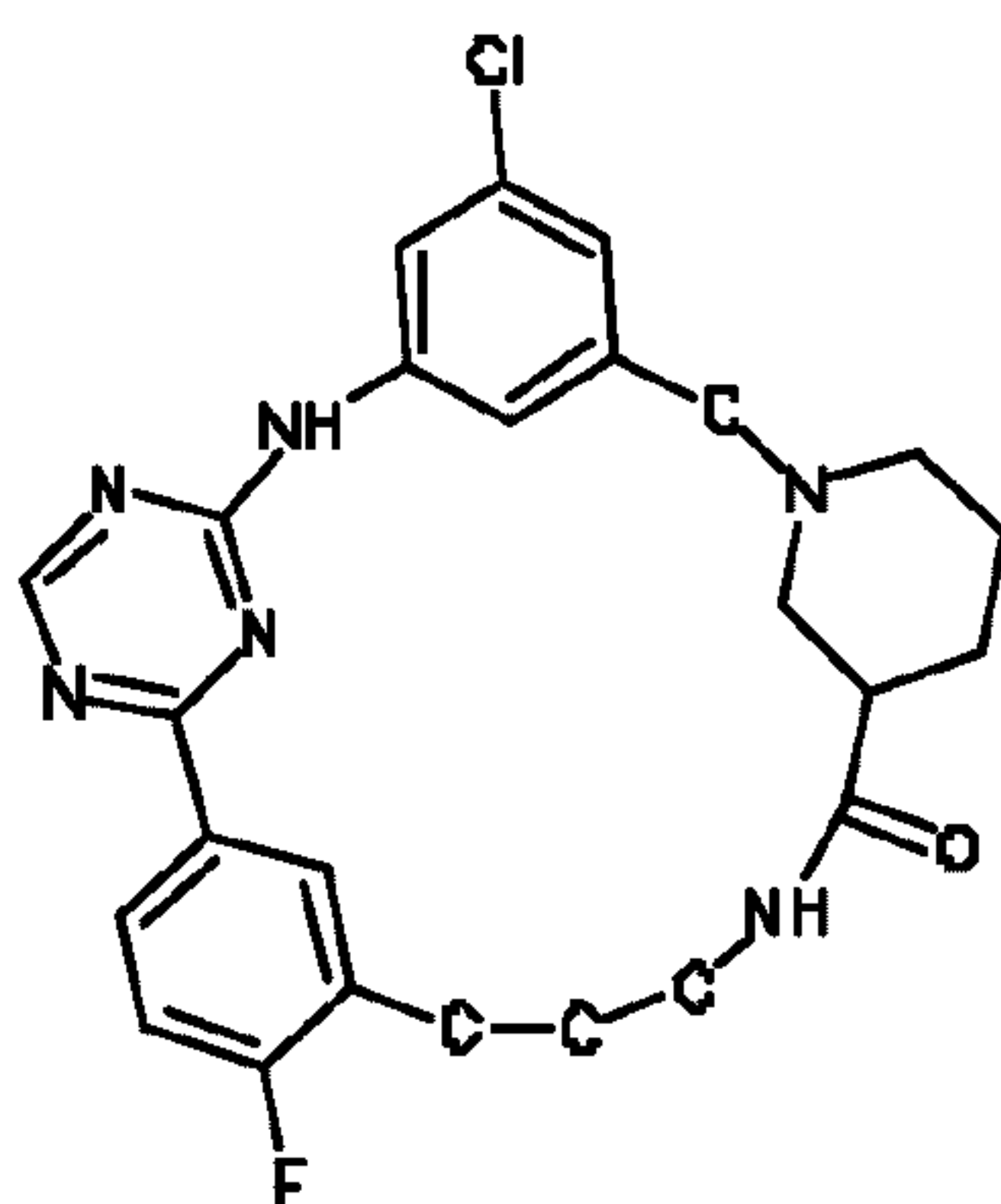
[0171] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (27).



Formula (27): 10-Chloro-14-ethyl-

22-fluoro-3,5,7,14,17,27-hexaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacos-
1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one. Referred to herein as “Compound
25”.

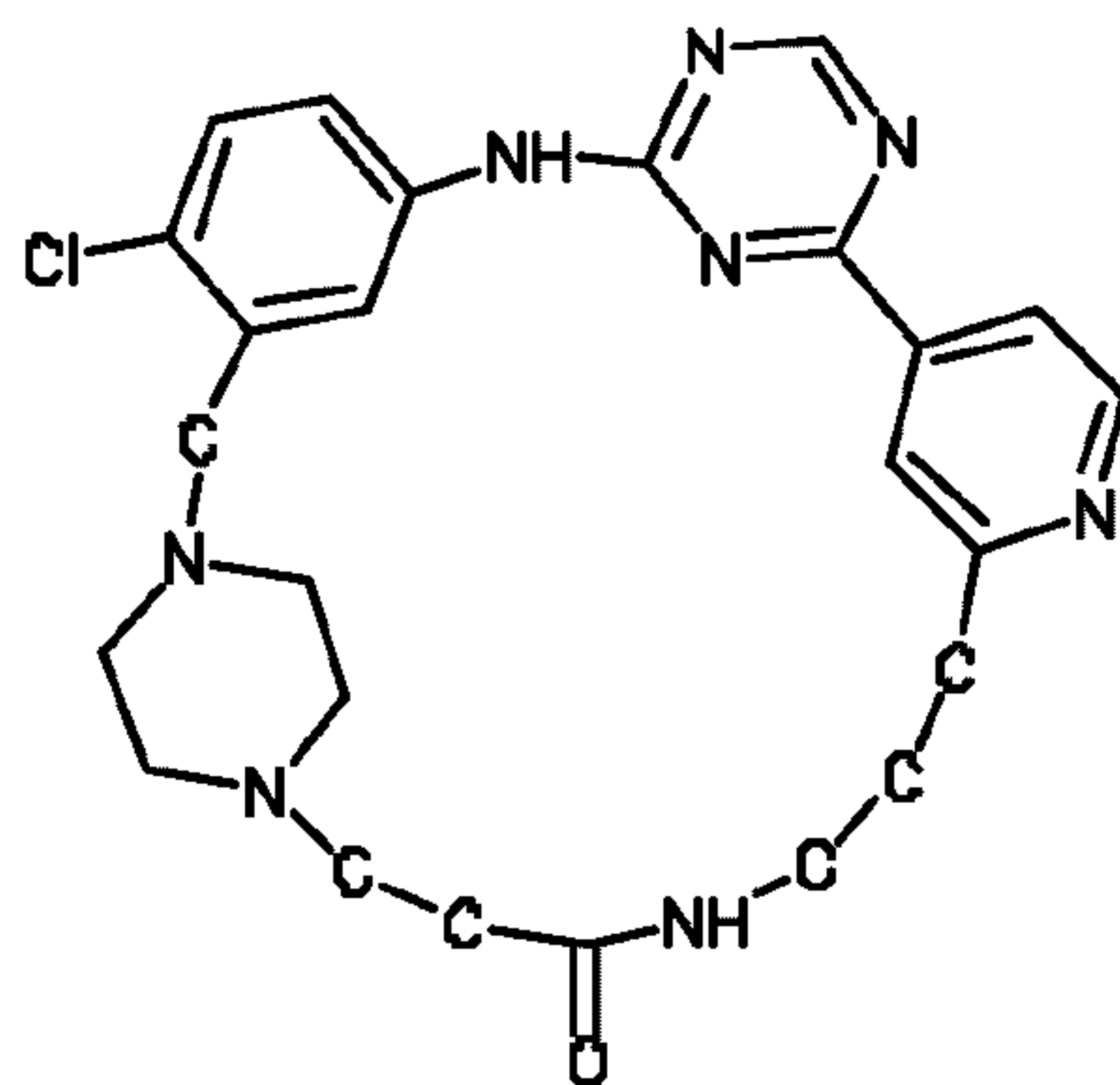
[0172] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (28).



Formula (28): 10-Chloro-25-

fluoro-3,5,7,14,20,31-
hexaazapentacyclo[22.3.1.1~2,6~.1~8,12~.1~14,18~]hentriaconta-
1(28),2(31),3,5,8(30),9,11,24,26-nonaen-19-one. Referred to herein as “Compound
26”.

[0173] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (29).



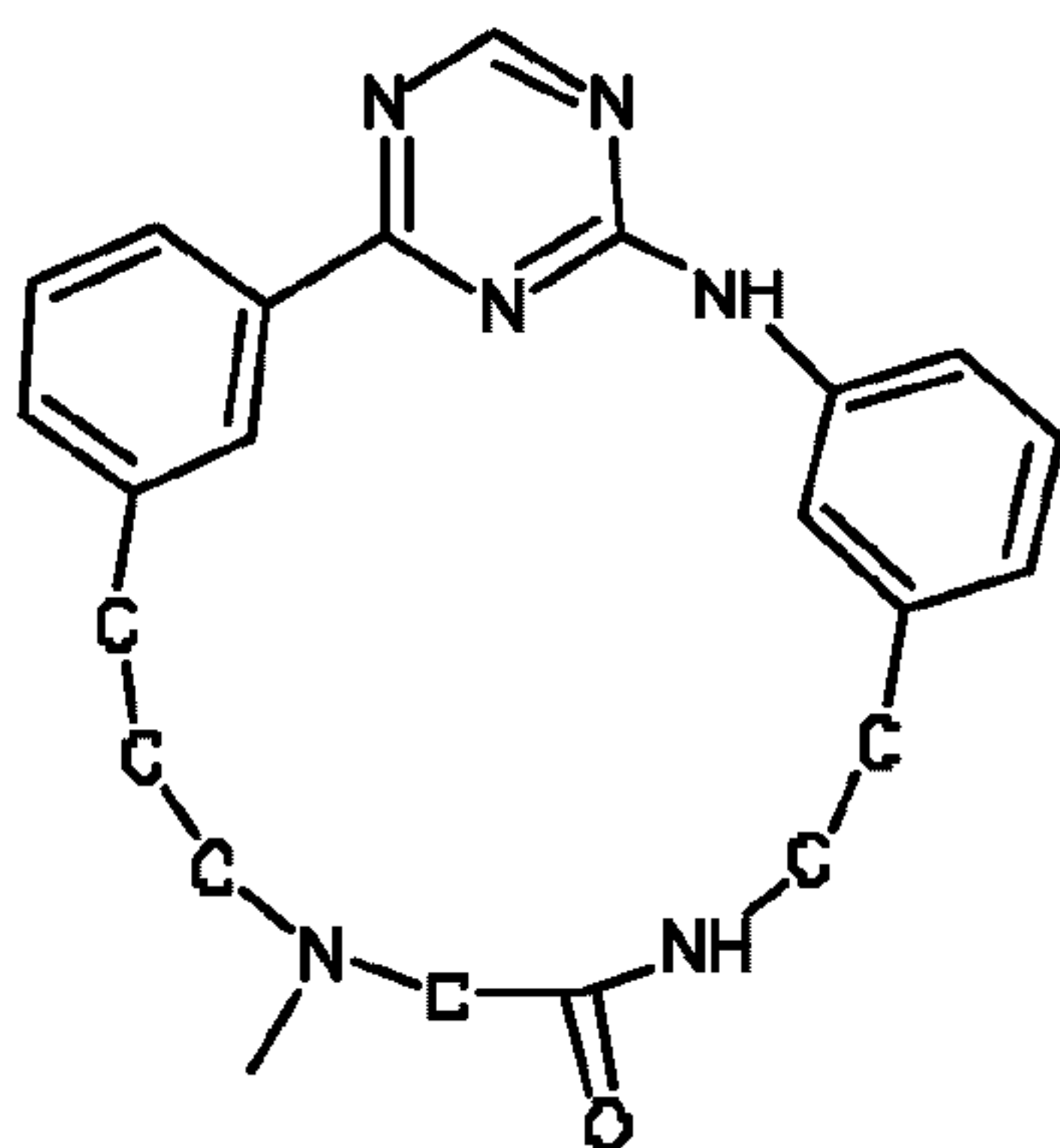
Formula (29): 4-Chloro-

1,8,10,12,17,22,26,32-

octaazapentacyclo[24.2.2.1~3,7~.1~9,13~.1~14,18~]tritriaconta-

3(33),4,6,9(32),10,12,14(31),15,17-nonaen-23-one. Referred to herein as "Compound 27".

[0174] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (30).

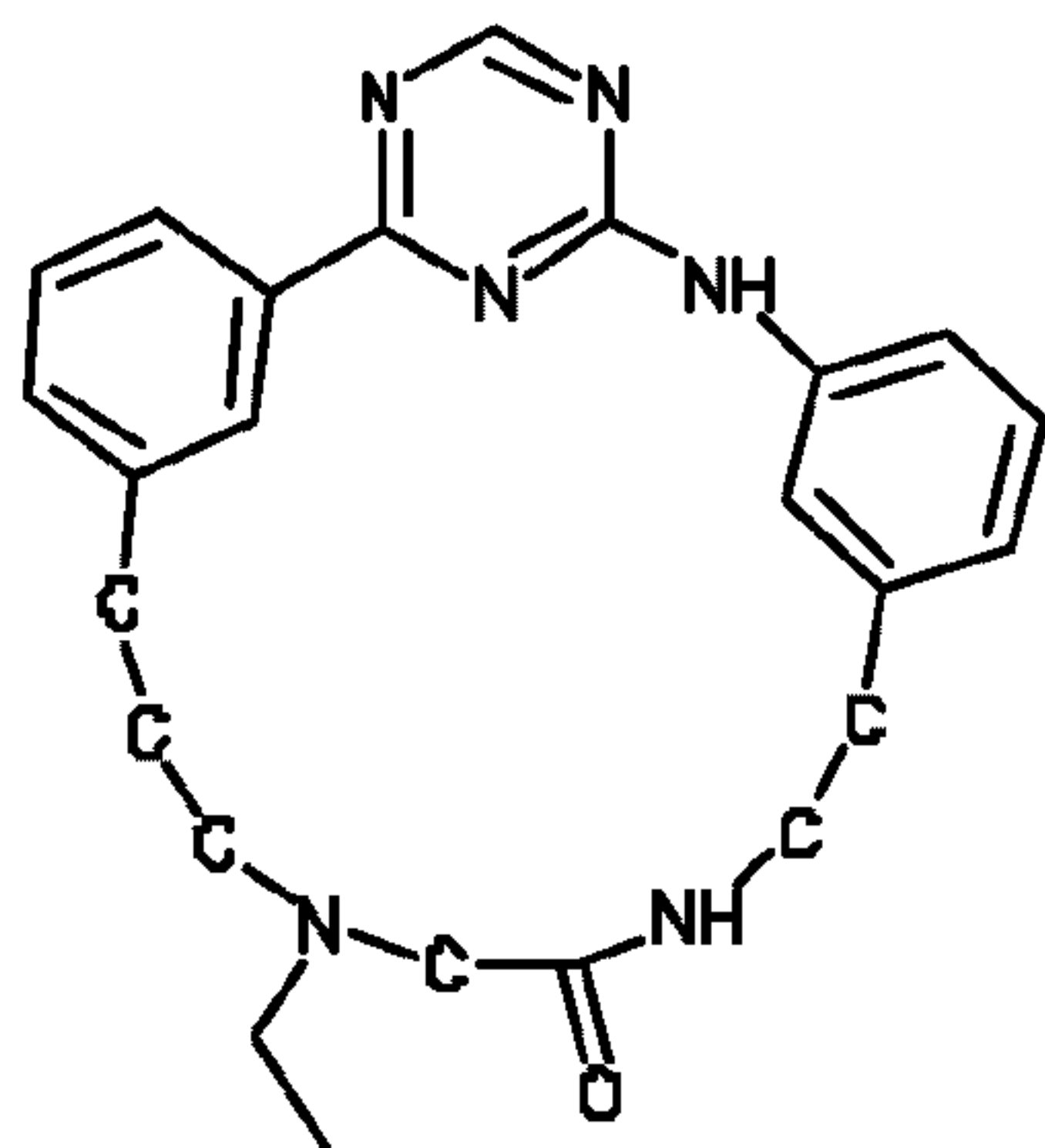


Formula (30): 18-Methyl-

3,5,7,15,18,28-hexaazatetracyclo[20.3.1.1~2,6~.1~8,12~]octacosa-

1(26),2(28),3,5,8(27),9,11,22,24-nonaen-16-one. Referred to herein as "Compound 28".

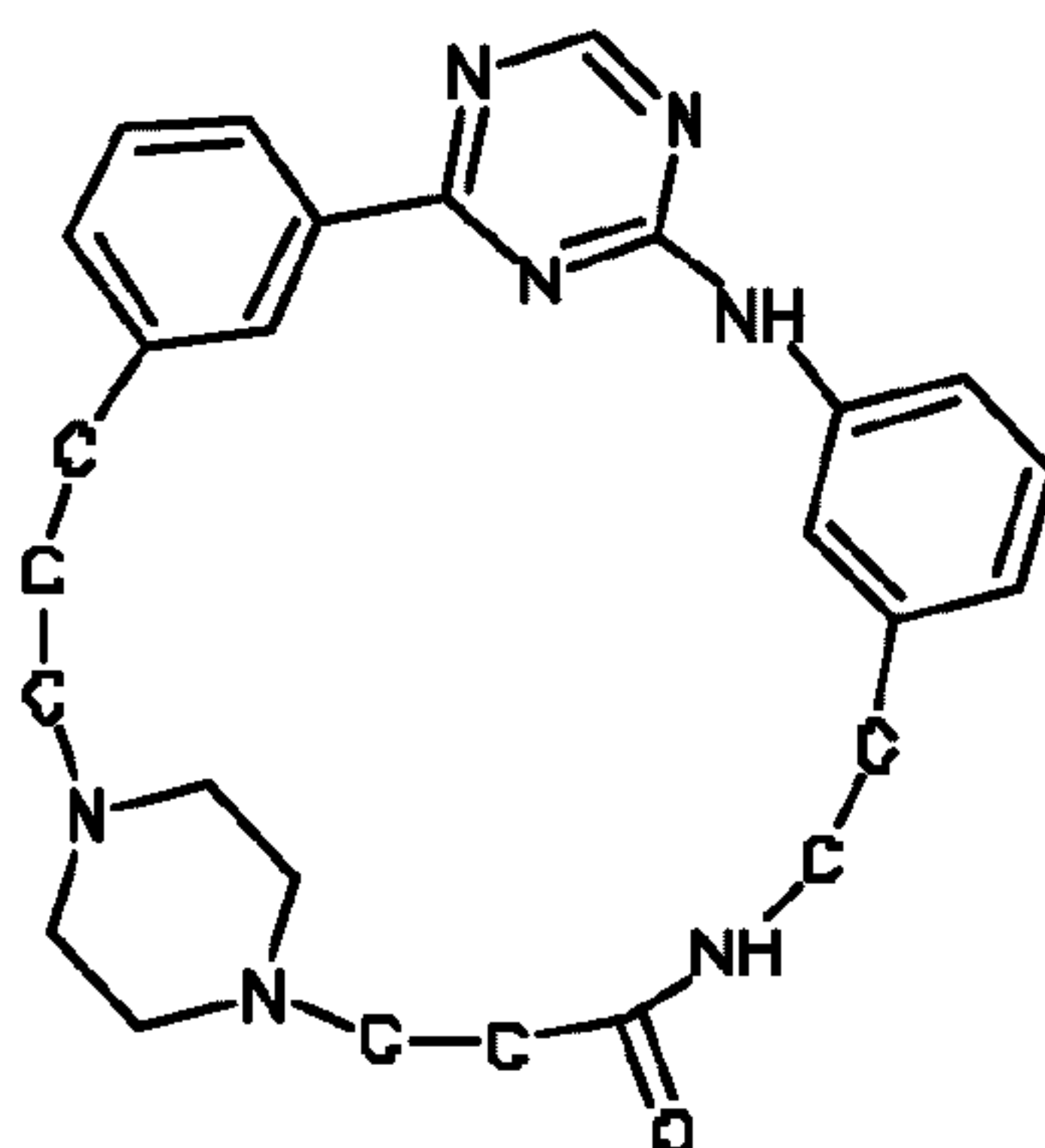
[0175] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (31).



Formula (31): 18-Ethyl-

3,5,7,15,18,28-hexaazatetracyclo[20.3.1.1.1~2,6~.1~8,12~]octacosan-1(26),2(28),3,5,8(27),9,11,22,24-nonaen-16-one. Referred to herein as “Compound 29”.

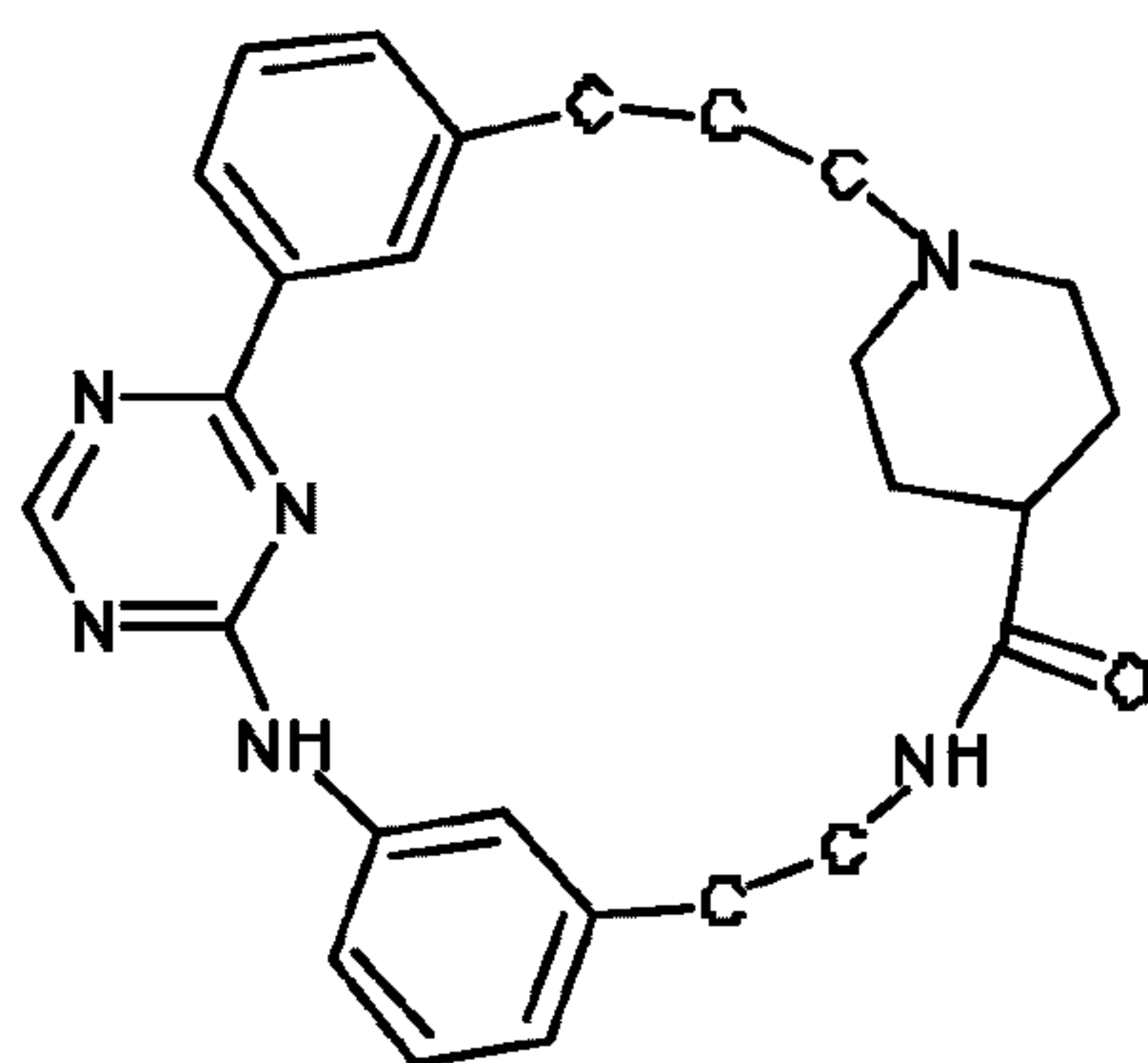
[0176] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (32).



Formula (32):

1,8,10,12,17,19,23,27,33-Nonaazapentacyclo[25.2.2.1~3,7~.1~9,13~.1~14,18~]tetratriacontan-3(34),4,6,9(33),10,12,14(32),15,17-nonaen-24-one. Referred to herein as “Compound 30”.

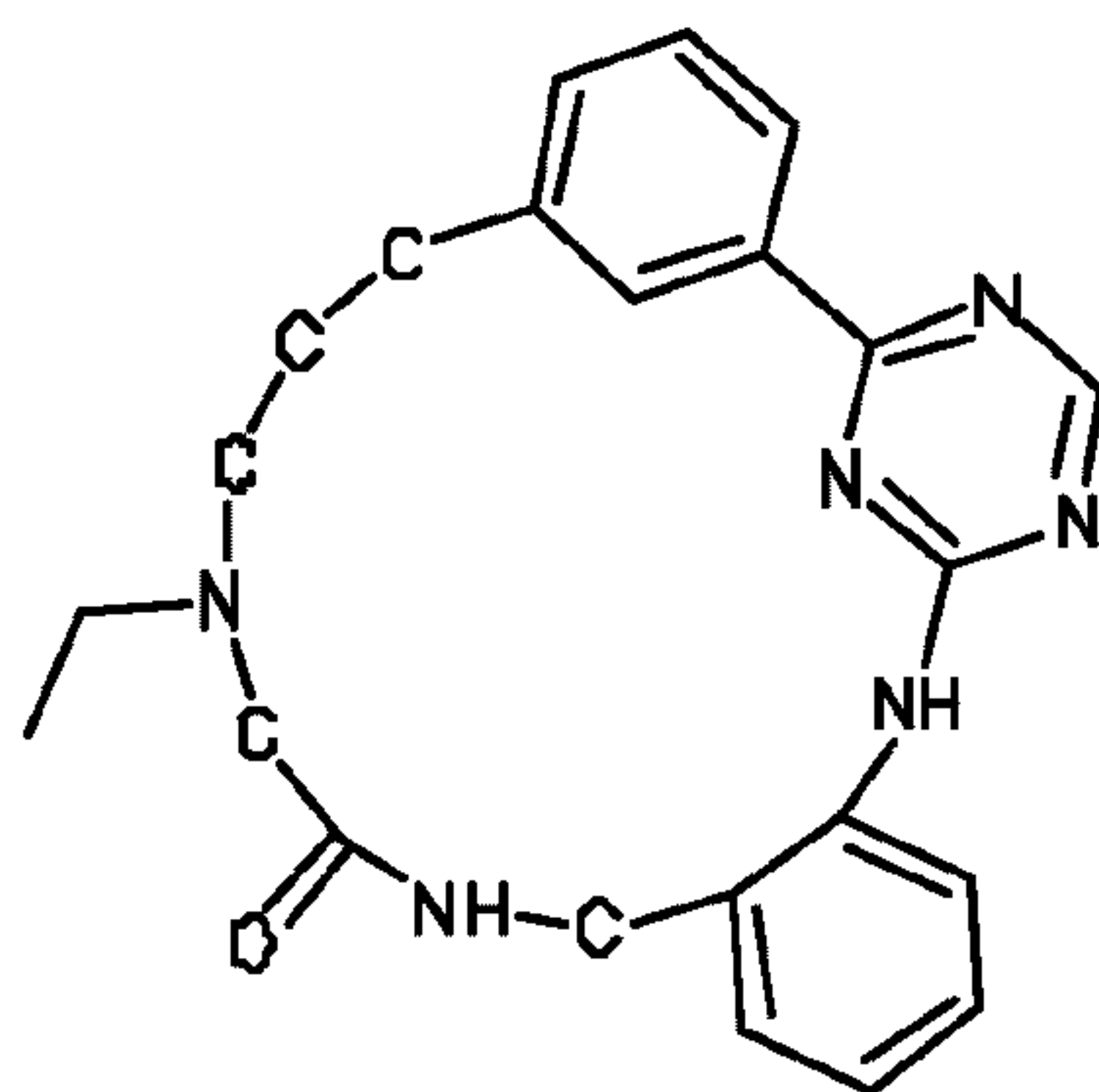
[0177] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (33).



Formula (33): 1,11,13,15,23,31-

Hexaazapentacyclo[23.2.2.1~5,9~.1~10,14~.1~16,20~]dotriaconta-5(32),6,8,10(31),11,13,16(30),17,19-nonaen-24-one. Referred to herein as “Compound 31”.

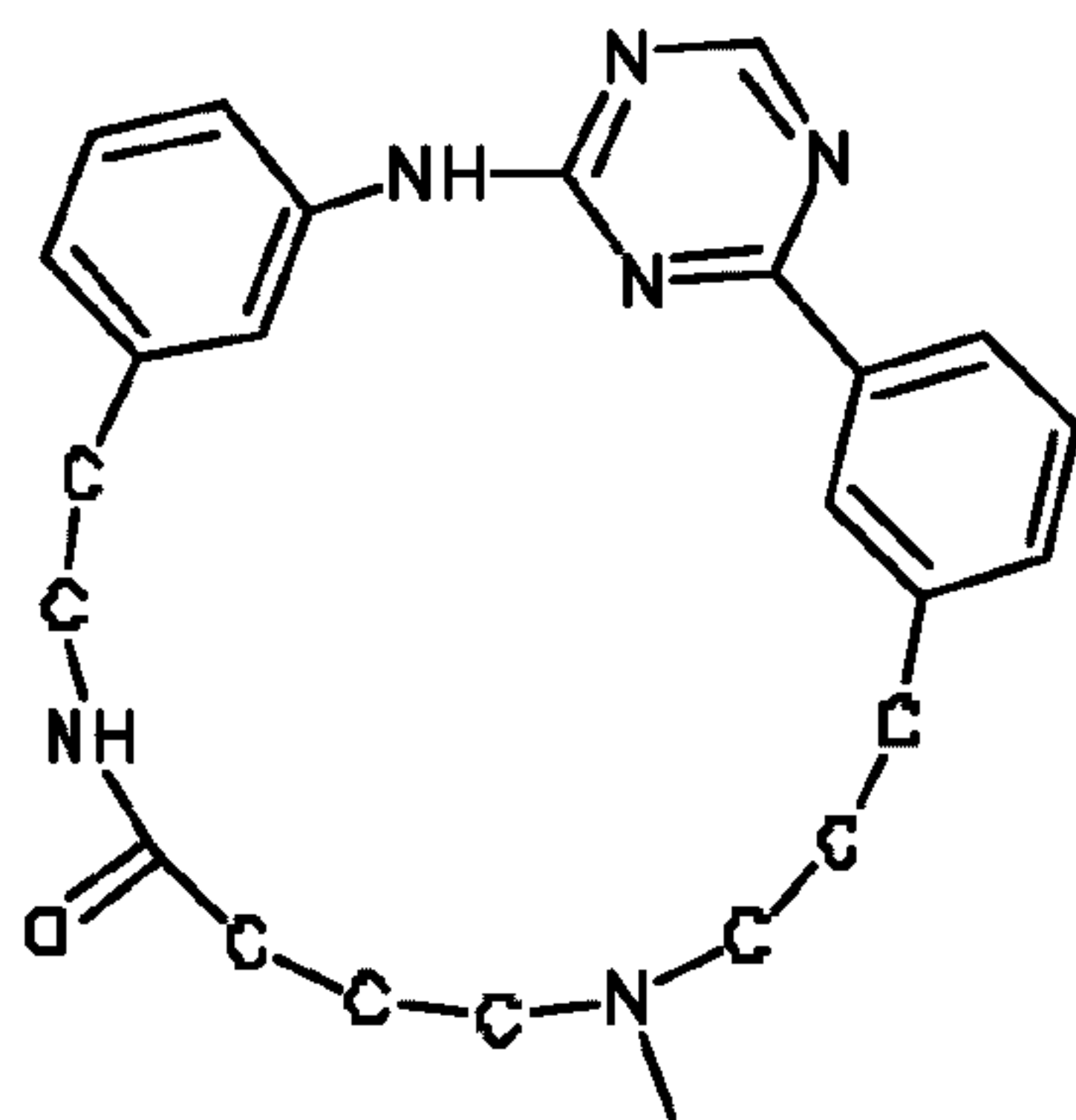
[0178] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (34).



Formula (34): 15-Ethyl-

13,14,15,16,18,19-hexahydro-1H-6,2-(azeno)-7,11-(metheno)-1,3,5,15,18-benzopentaazacyclohenicosin-17(12H)-one. Referred to herein as “Compound 32”.

[0179] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (35).

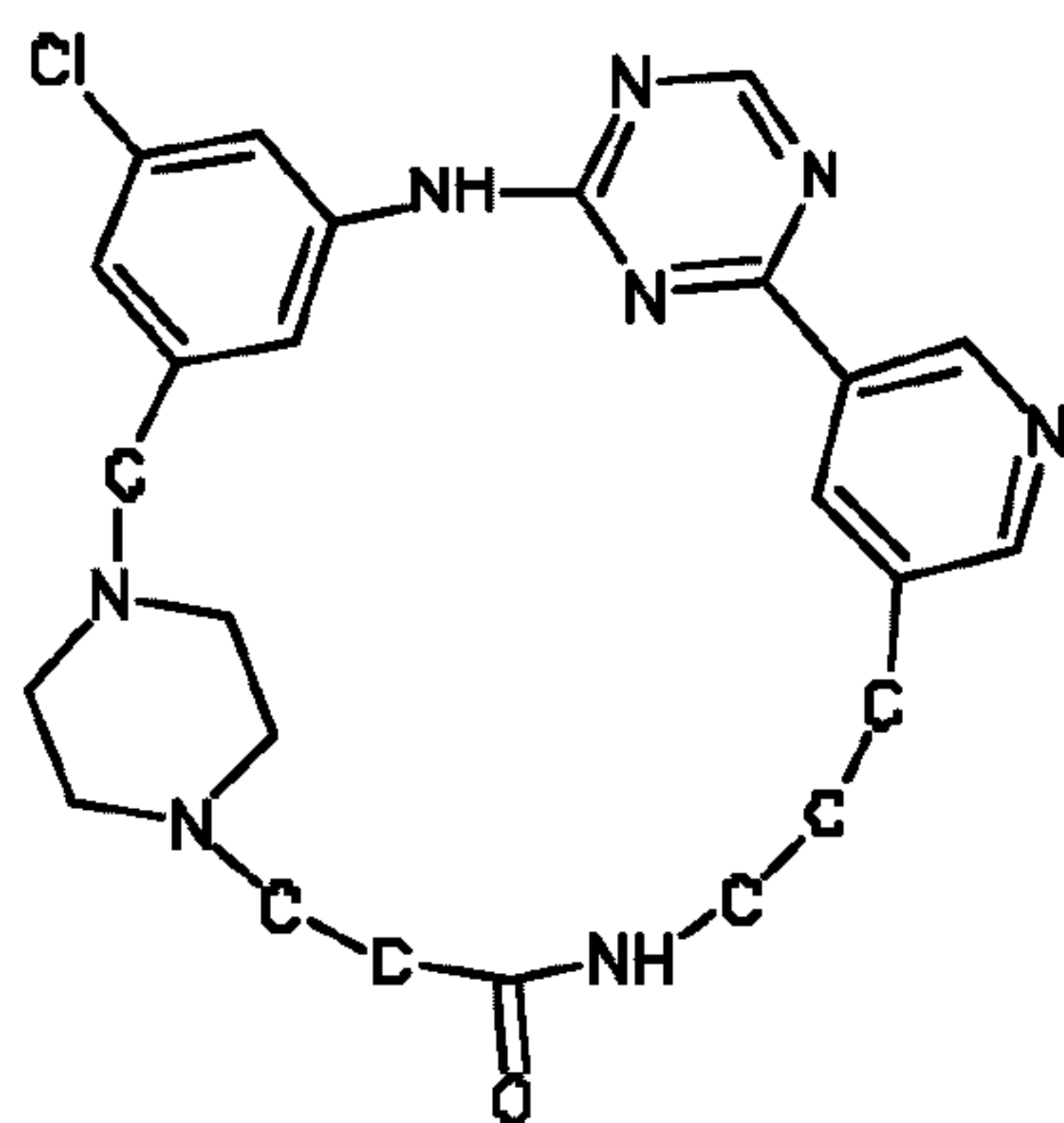


Formula (35): 20-Methyl-

3,5,7,15,20,30-hexaazatetracyclo[22.3.1.1~2,6~.1~8,12~]triaconta-

1(28),2(30),3,5,8(29),9,11,24,26-nonaen-16-one. Referred to herein as “Compound 33”.

[0180] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (36).



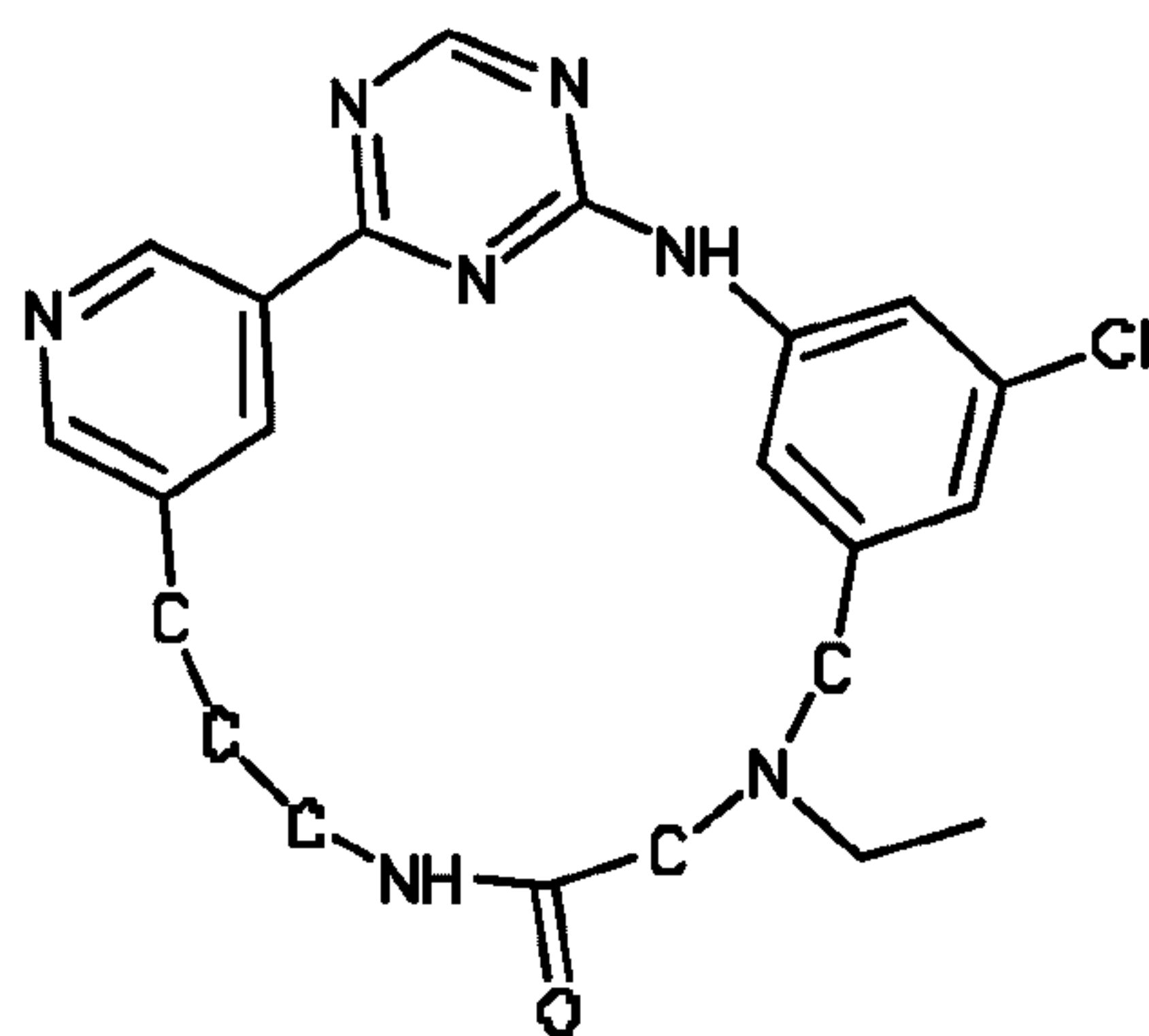
Formula (36): 5-Chloro-

1,8,10,12,16,22,26,32-

octaazapentacyclo[24.2.2.1~3,7~.1~9,13~.1~14,18~]tritriaconta-

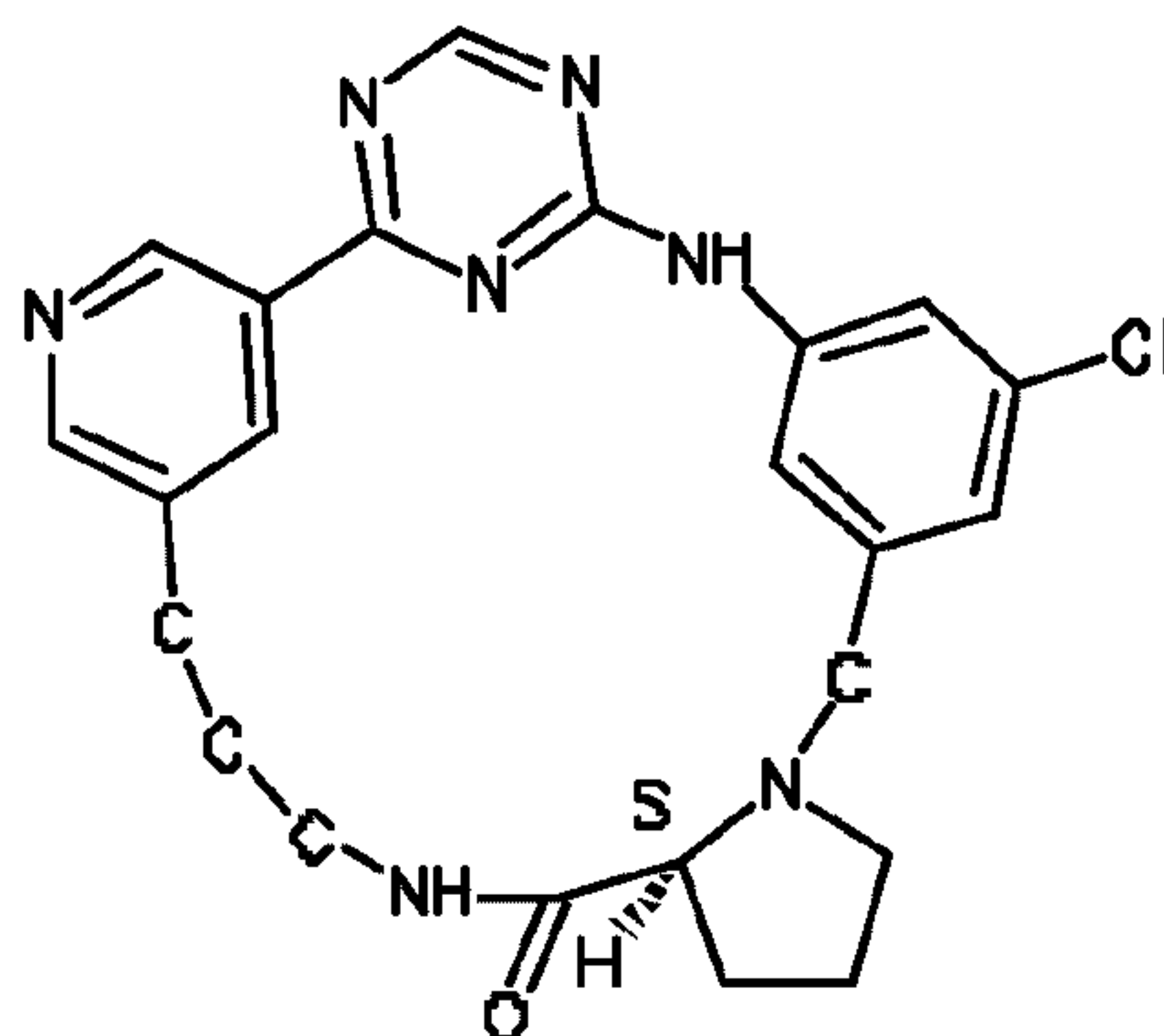
3(33),4,6,9(32),10,12,14(31),15,17-nonaen-23-one. Referred to herein as “Compound 34”.

[0181] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (37).



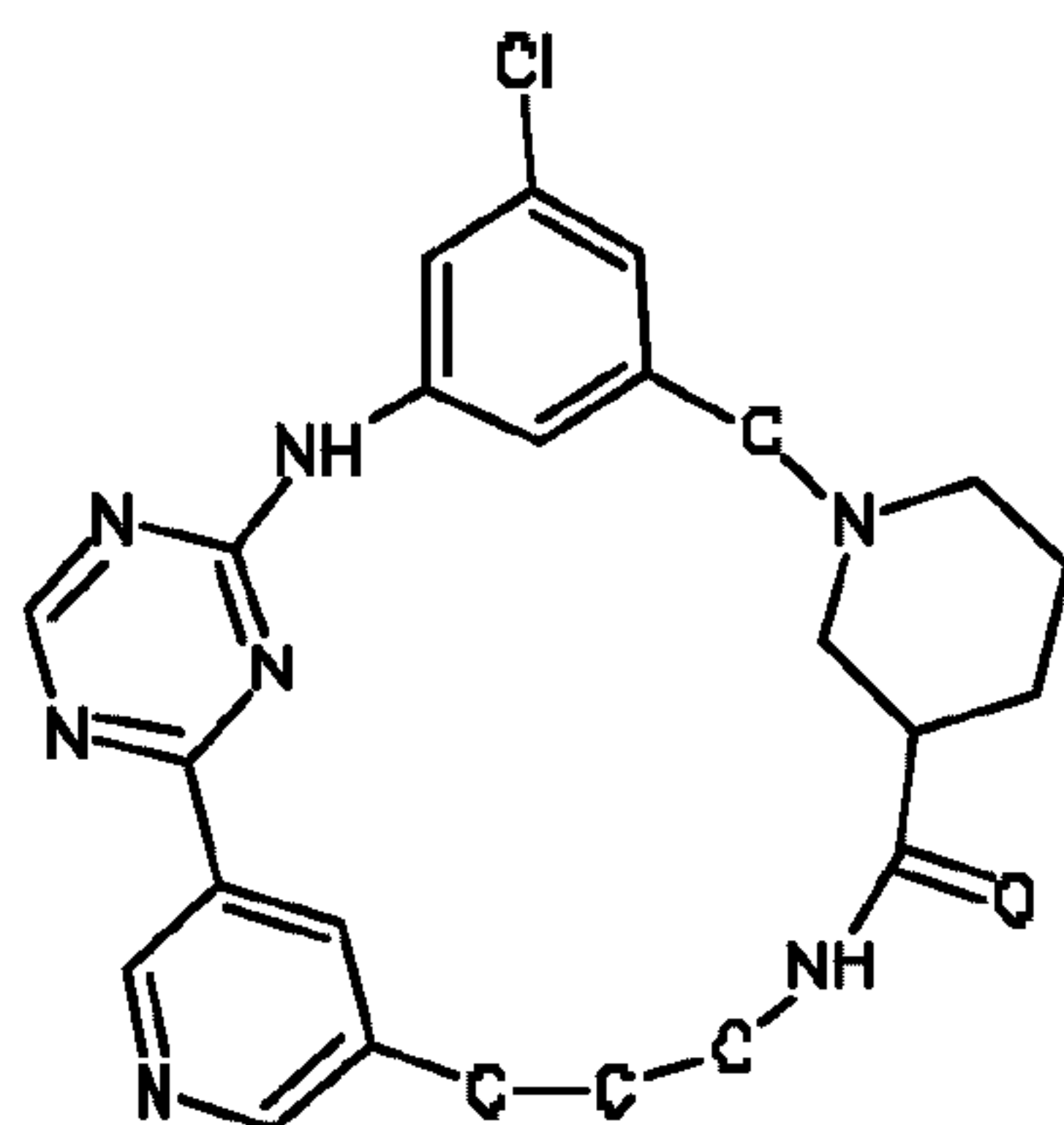
Formula (37): 10-Chloro-14-ethyl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacos-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one. Referred to herein as “Compound 35”.

[0182] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (38).



Formula (38): (18S)-10-Chloro-3,5,7,14,20,26,30-heptaazapentacyclo[22.3.1.1~2,6~.1~8,12~.0~14,18~]triacont-1(28),2(30),3,5,8(29),9,11,24,26-nonaen-19-one. Referred to herein as “Compound 36”.

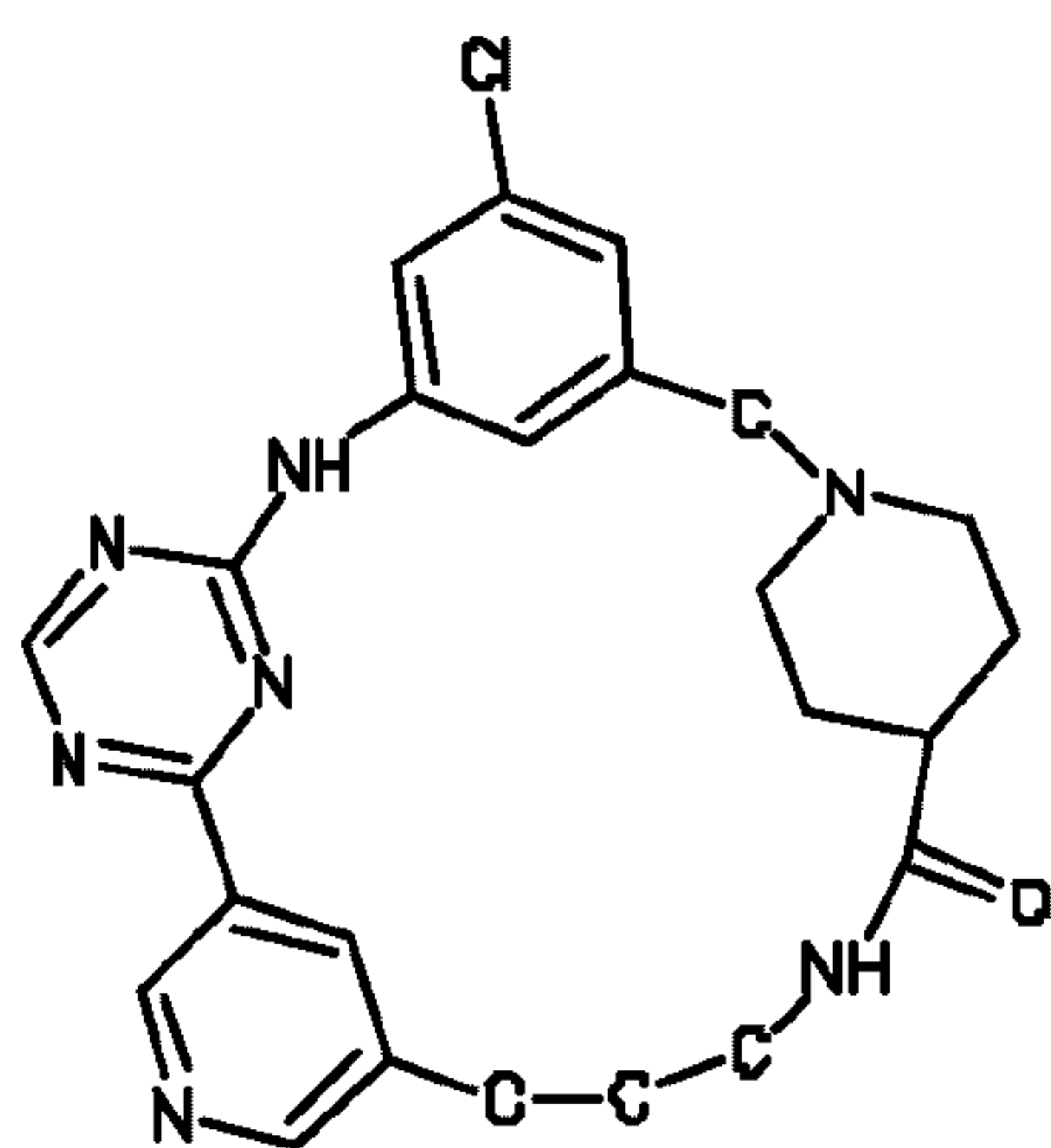
[0183] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (39).



Formula (39): 10-Chloro-

3,5,7,14,20,26,31-heptaazapentacyclo[22.3.1.1~2,6~.1~8,12~.1~14,18~]hentriaconta-1(28),2(31),3,5,8(30),9,11,24,26-nonaen-19-one. Referred to herein as “Compound 37”.

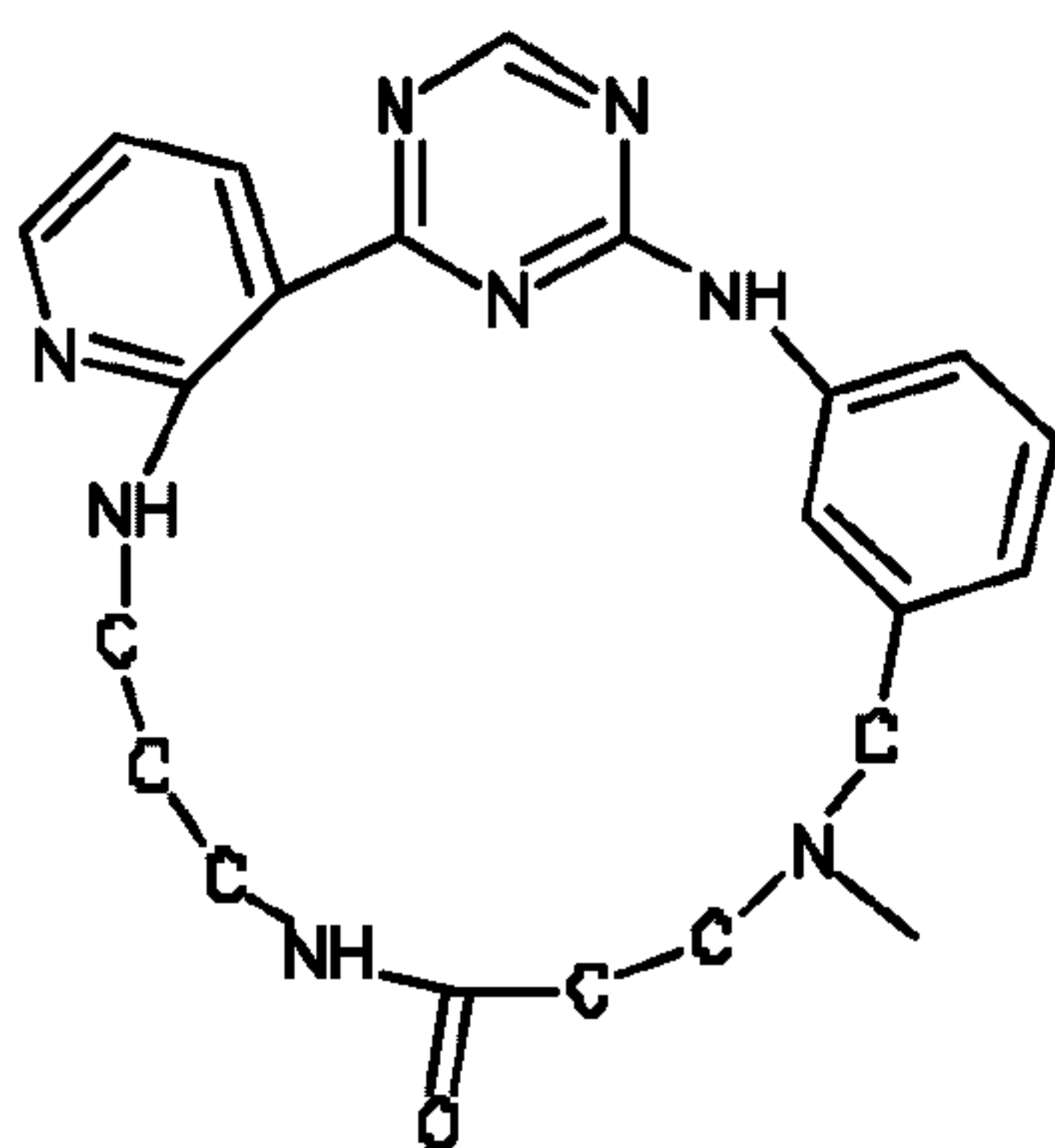
[0184] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (40).



Formula (40): 5-Chloro-

1,8,10,12,16,22,30-heptaazapentacyclo[22.2.2.1~3,7~.1~9,13~.1~14,18~]hentriaconta-3(31),4,6,9(30),10,12,14(29),15,17-nonaen-23-one. Referred to herein as “Compound 38”.

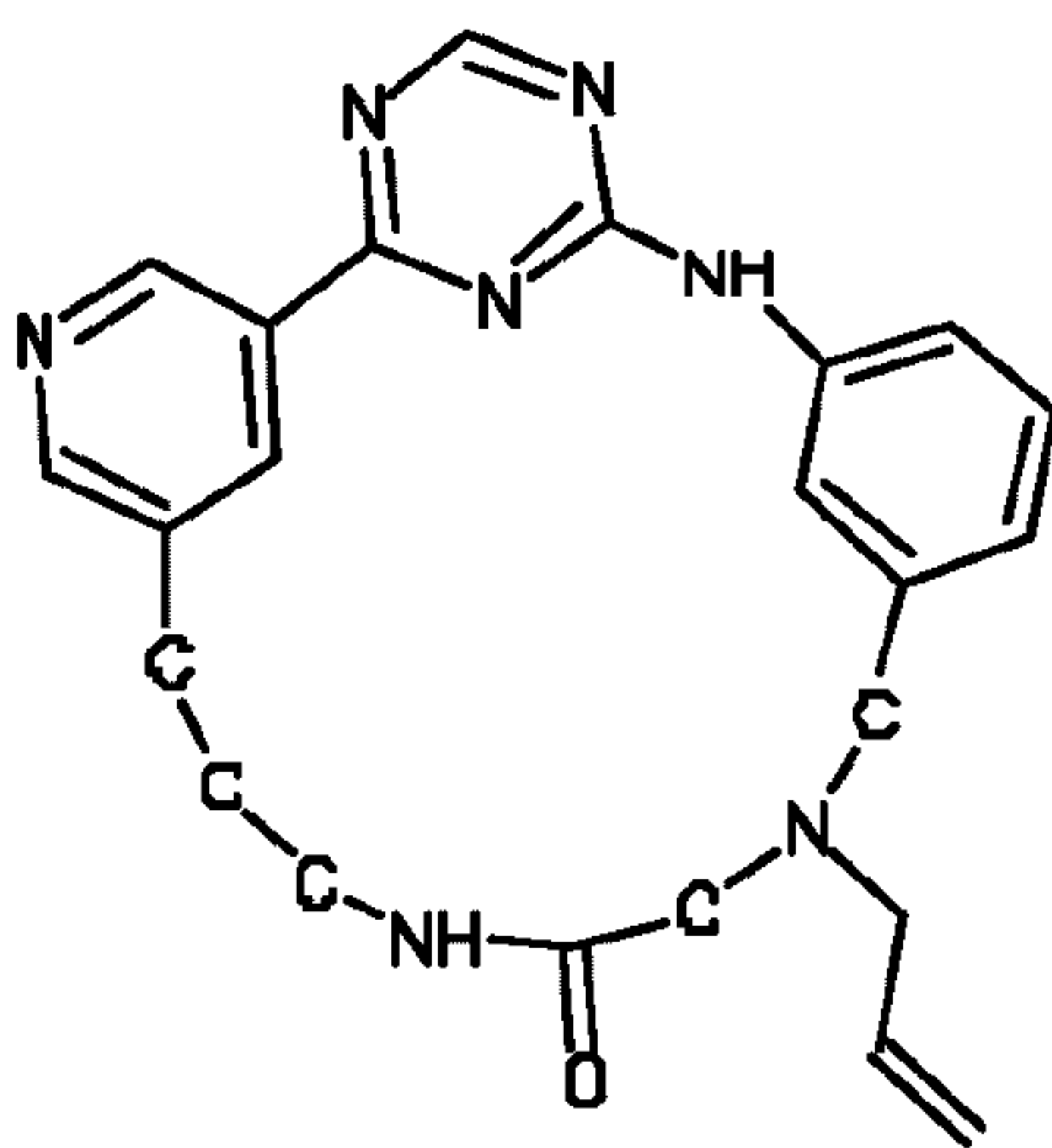
[0185] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (41).



Formula (41): 9-Methyl-

2,3,4,5,7,8,9,10-octahydro-16H-17,21-(azeno)-11,15-(metheno)pyrido[3,2-g][1,3,5,9,13,17]hexaazacyclotricosin-6(1H)-one. Referred to herein as “Compound 39”.

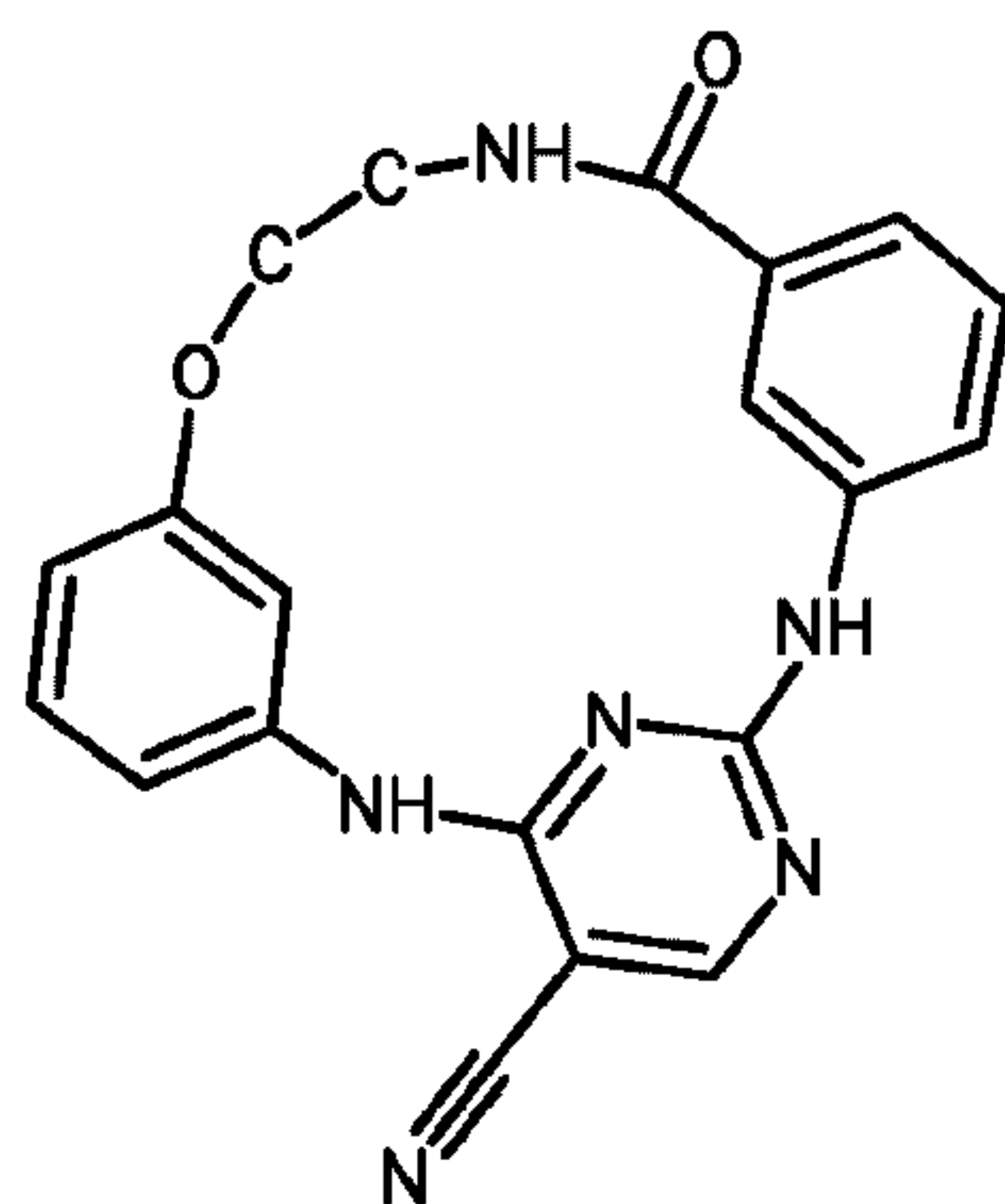
[0186] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (42).



Formula (42): 14-Prop-2-en-1-yl-

3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosal-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one. Referred to herein as “Compound 40”.

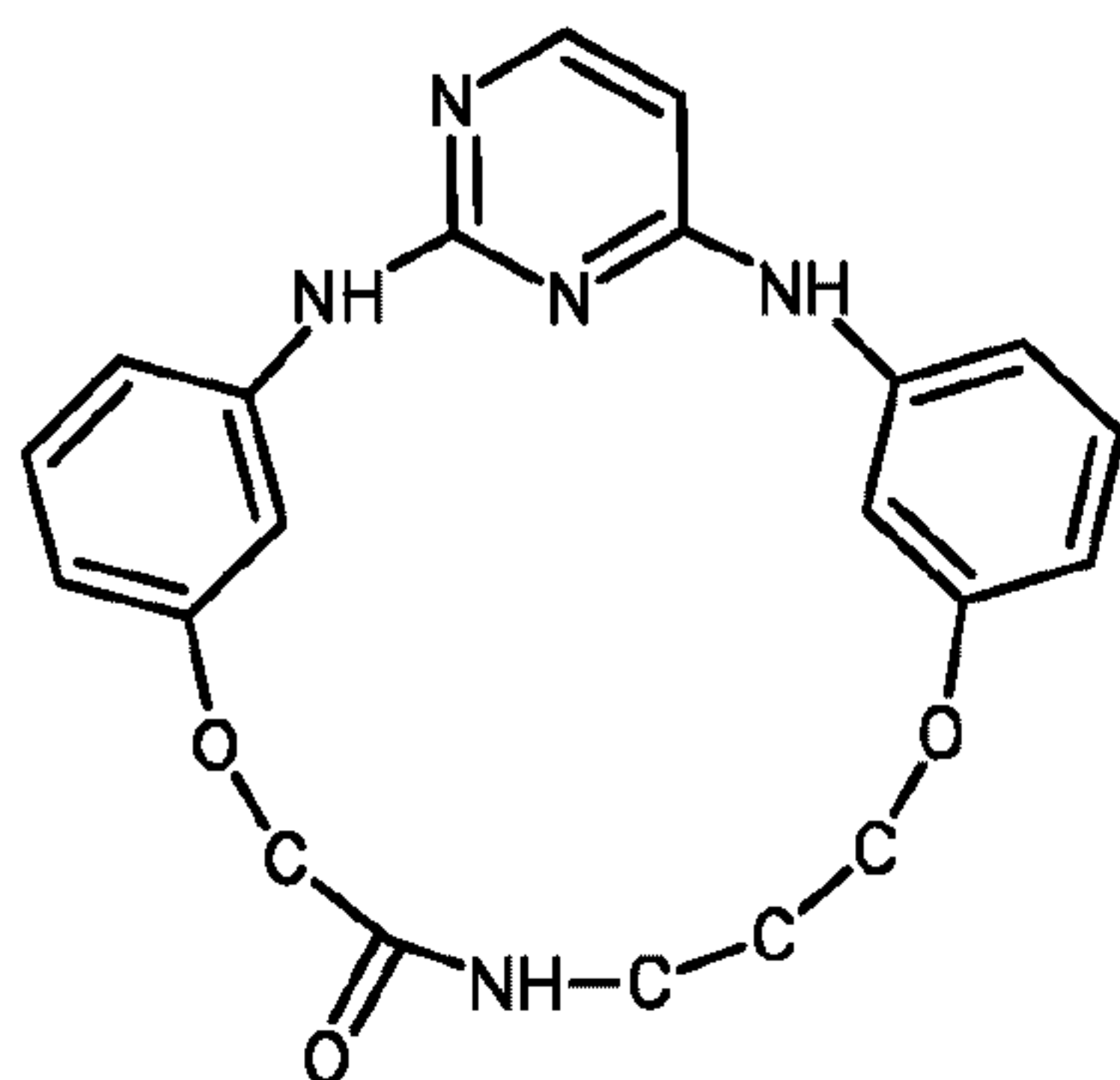
[0187] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (43).



Formula (43): 18-Oxo-14-oxa-

2,4,8,17,25-pentaazatetracyclo[17.3.1.1~3,7~.1~9,13~]pentacosa-
1(23),3(25),4,6,9(24),10,12,19,21-nonaene-6-carbonitrile. Referred to herein as
“Compound 41”.

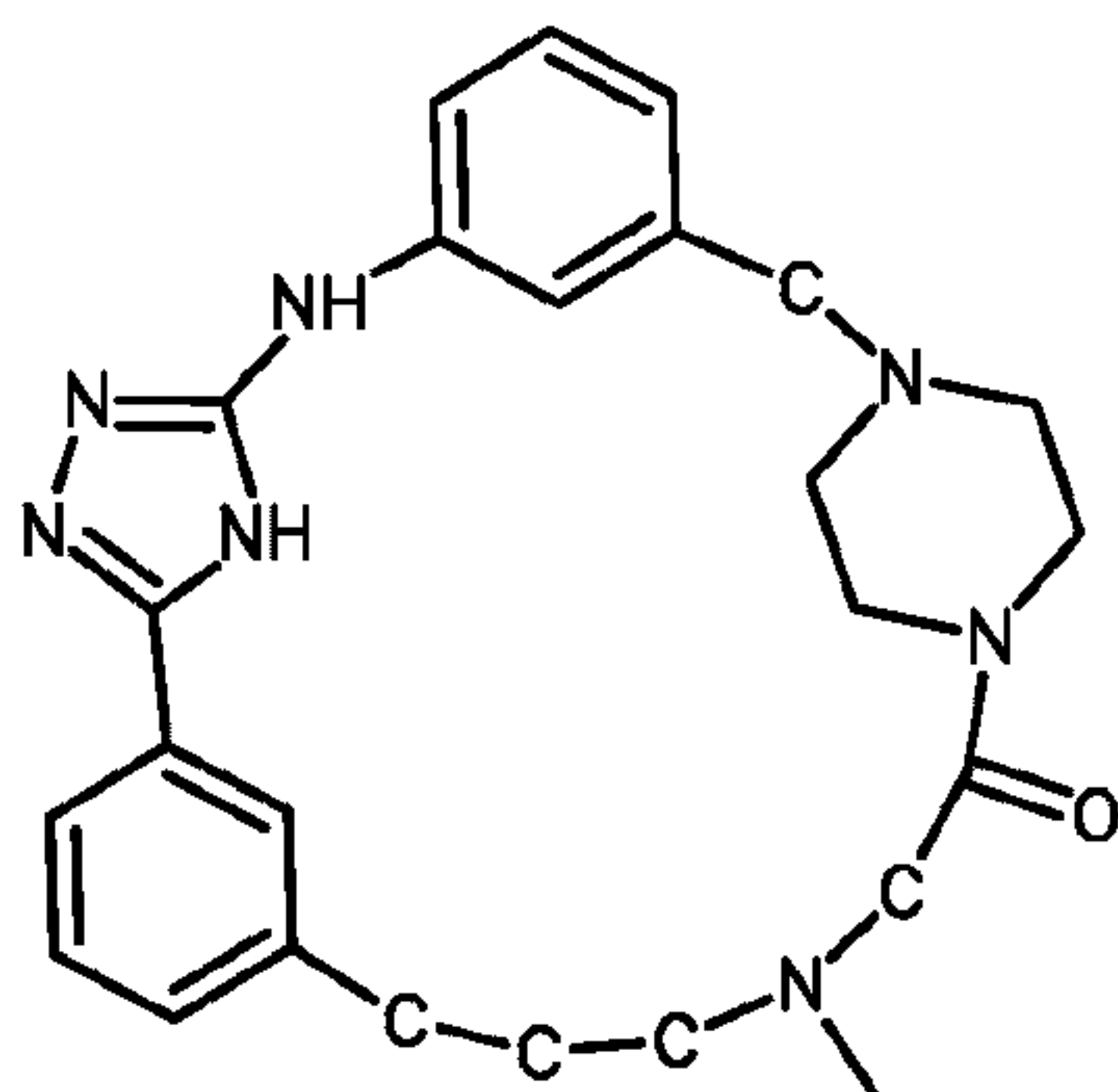
[0188] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (44).



Formula (44): 14,21-Dioxa-

2,4,8,18,28-pentaazatetracyclo[20.3.1.1~3,7~.1~9,13~]octacosa-
1(26),3(28),4,6,9(27),10,12,22,24-nonaen-19-one. Referred to herein as “Compound
42”.

[0189] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (45).



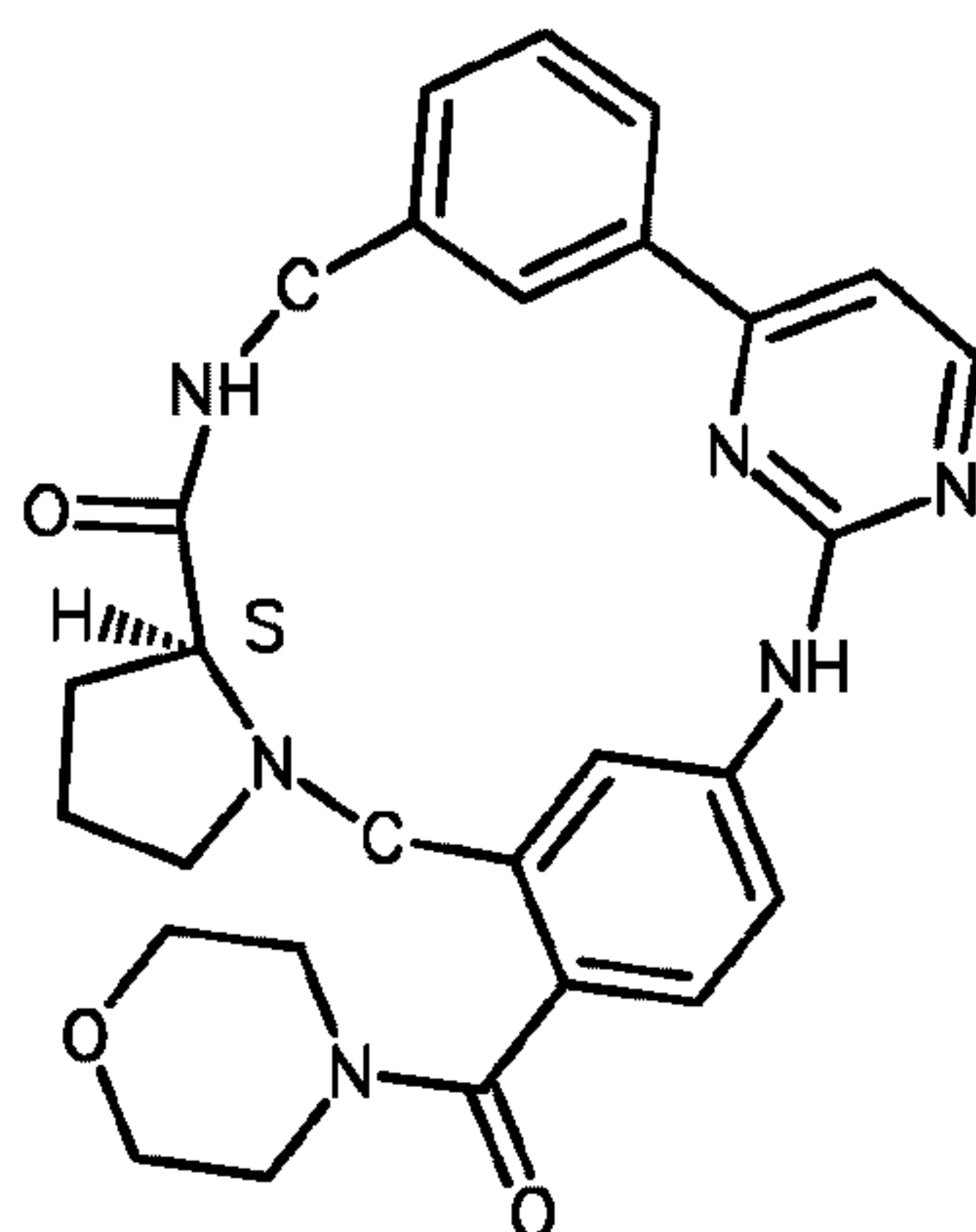
Formula (45): 21-Methyl-

1,8,10,11,21,24,30-

heptaazapentacyclo[22.2.2.1~3,7~.1~9,12~.1~13,17~]hentriaconta-

3(31),4,6,9,11,13(29),14,16-octaen-23-one. Referred to herein as "Compound 43".

[0190] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (46).



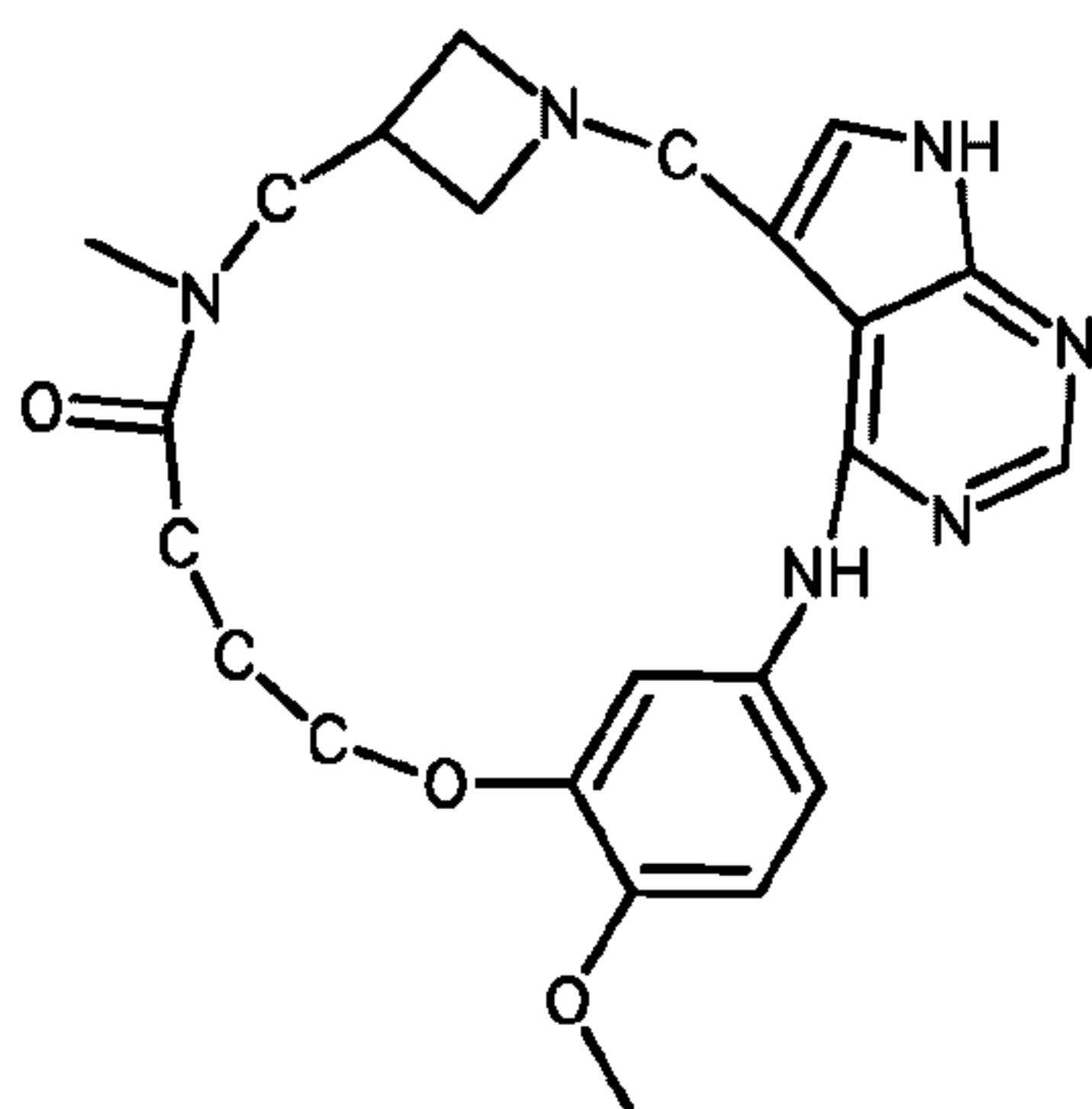
Formula (46): (18S)-11-

(Morpholin-4-ylcarbonyl)-5,7,14,20,28-

pentaazapentacyclo[20.3.1.1~2,6~.1~8,12~.0~14,18~]octacosa-

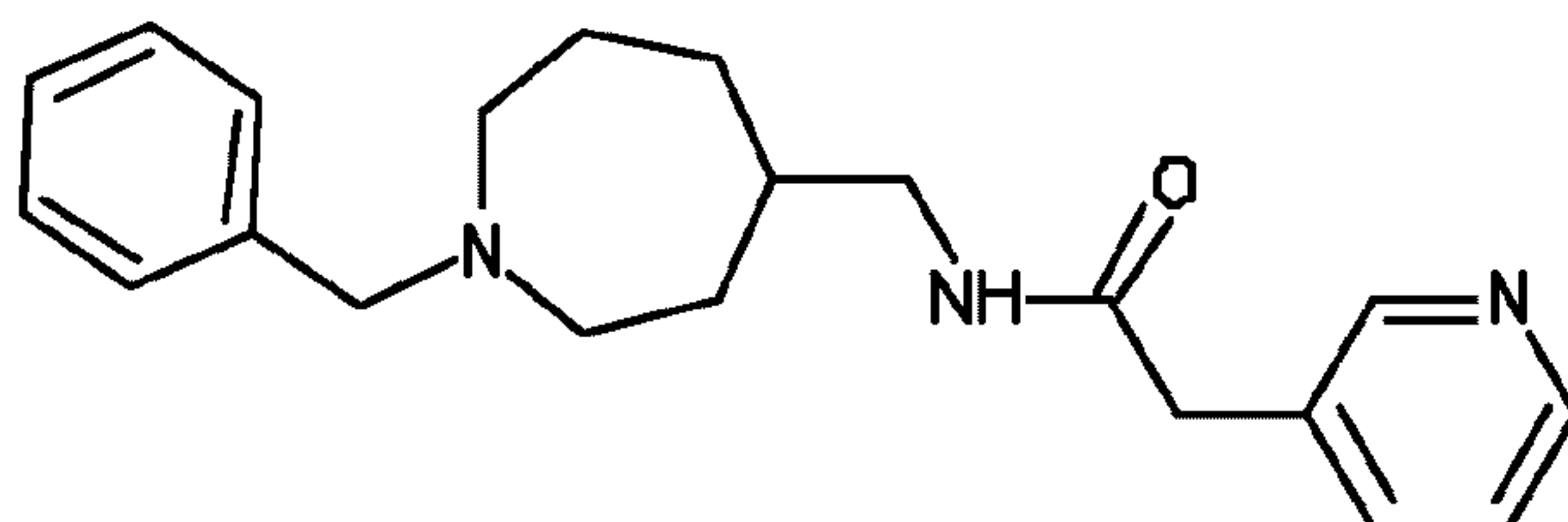
1(26),2(28),3,5,8(27),9,11,22,24-nonaen-19-one. Referred to herein as "Compound 44".

[0191] In one embodiment, the cyclic aniline-pyridinotriazine is a compound of the Formula (47).



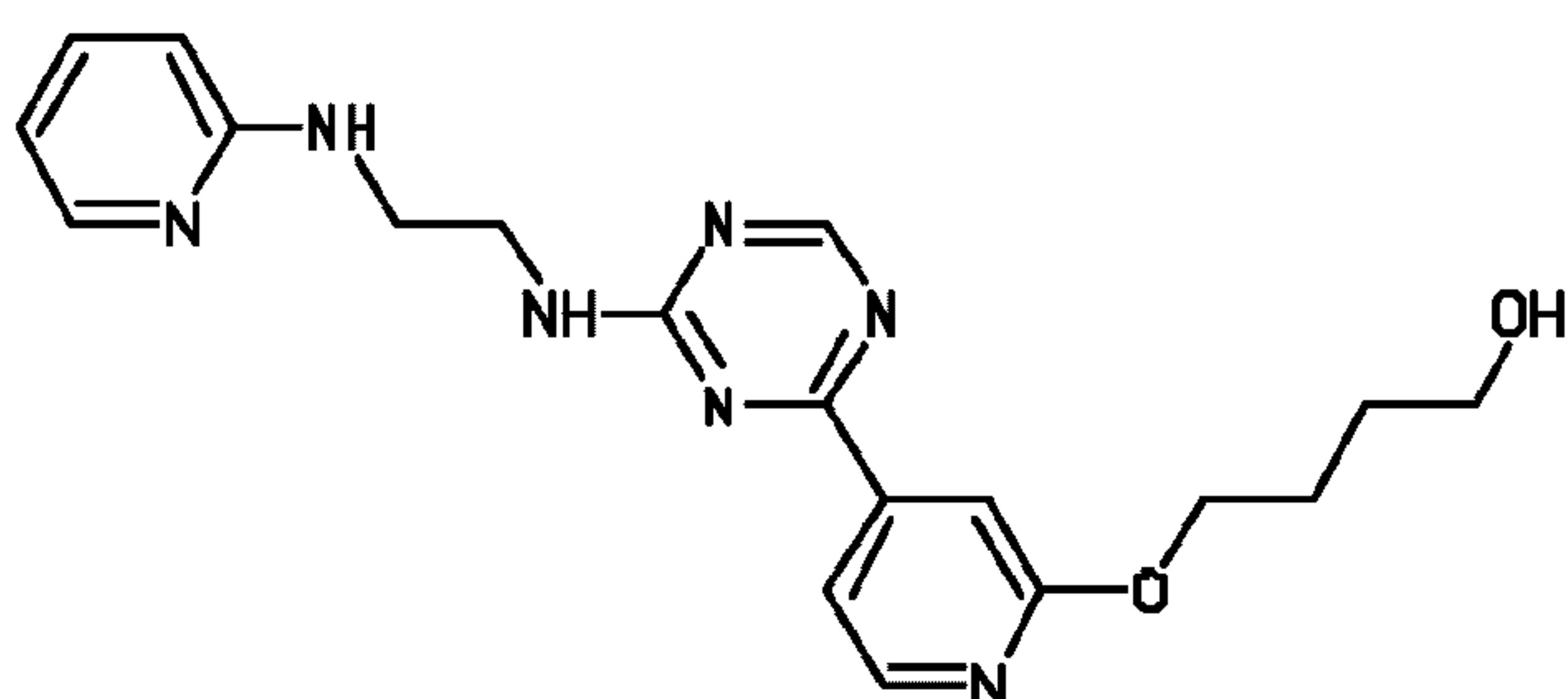
Formula (47): 10-Methoxy-17-methyl-2,14,15,17,18,19,20,22-octahydro-6H-19,21-methano-7,11-(metheno)-12-oxa-2,3,5,6,17,21-hexaazacycloicosa[1,2,3-cd]inden-16(13H)-one. Referred to herein as “Compound 45”.

[0192] In one embodiment, the at least one other factor is a compound of the Formula (48):



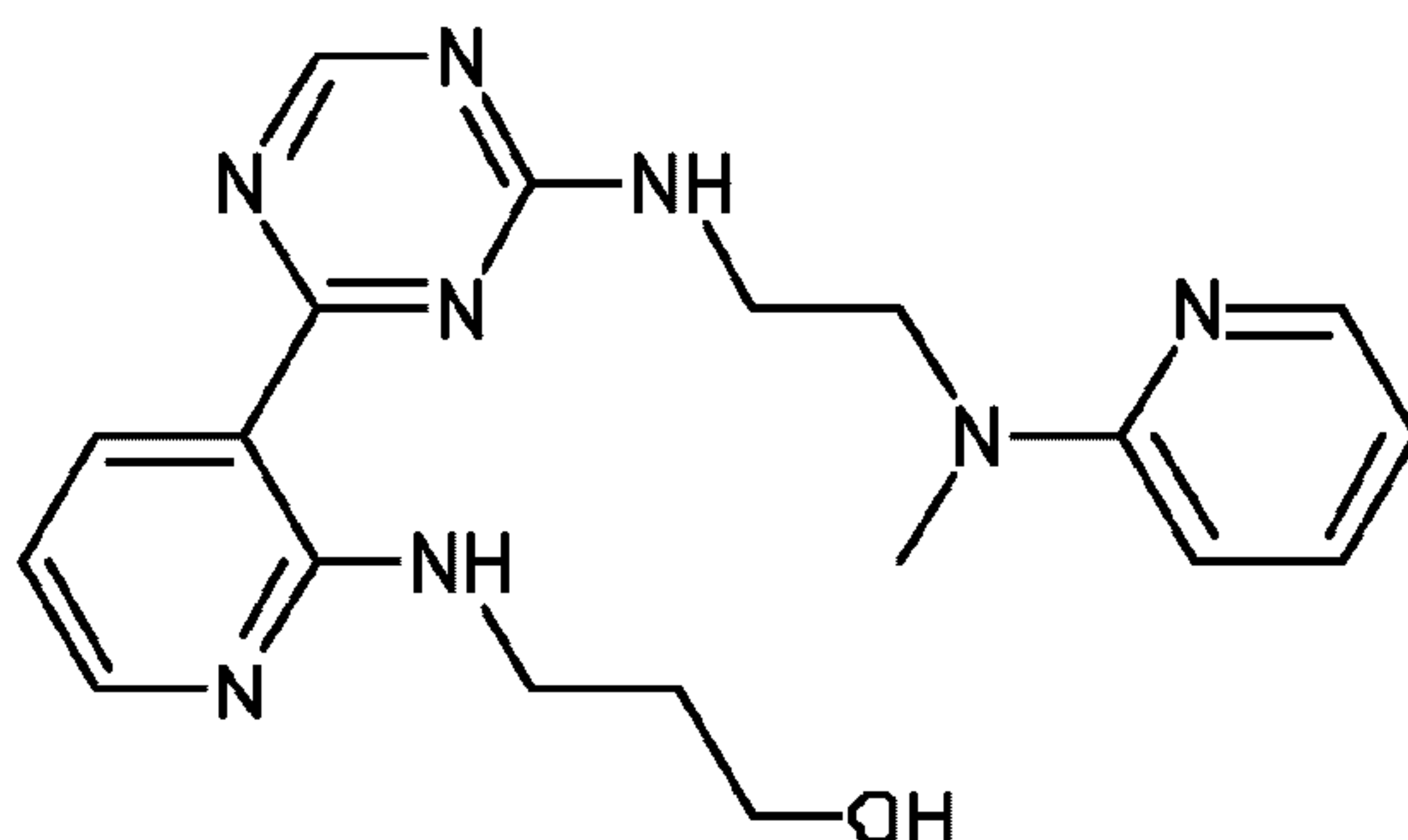
Formula (48). N-{[1-(Phenylmethyl)azepan-4-yl]methyl}-2-pyridin-3-ylacetamide. Referred herein as “Compound 46”.

[0193] In one embodiment, the at least one other factor is a compound of the Formula (49):



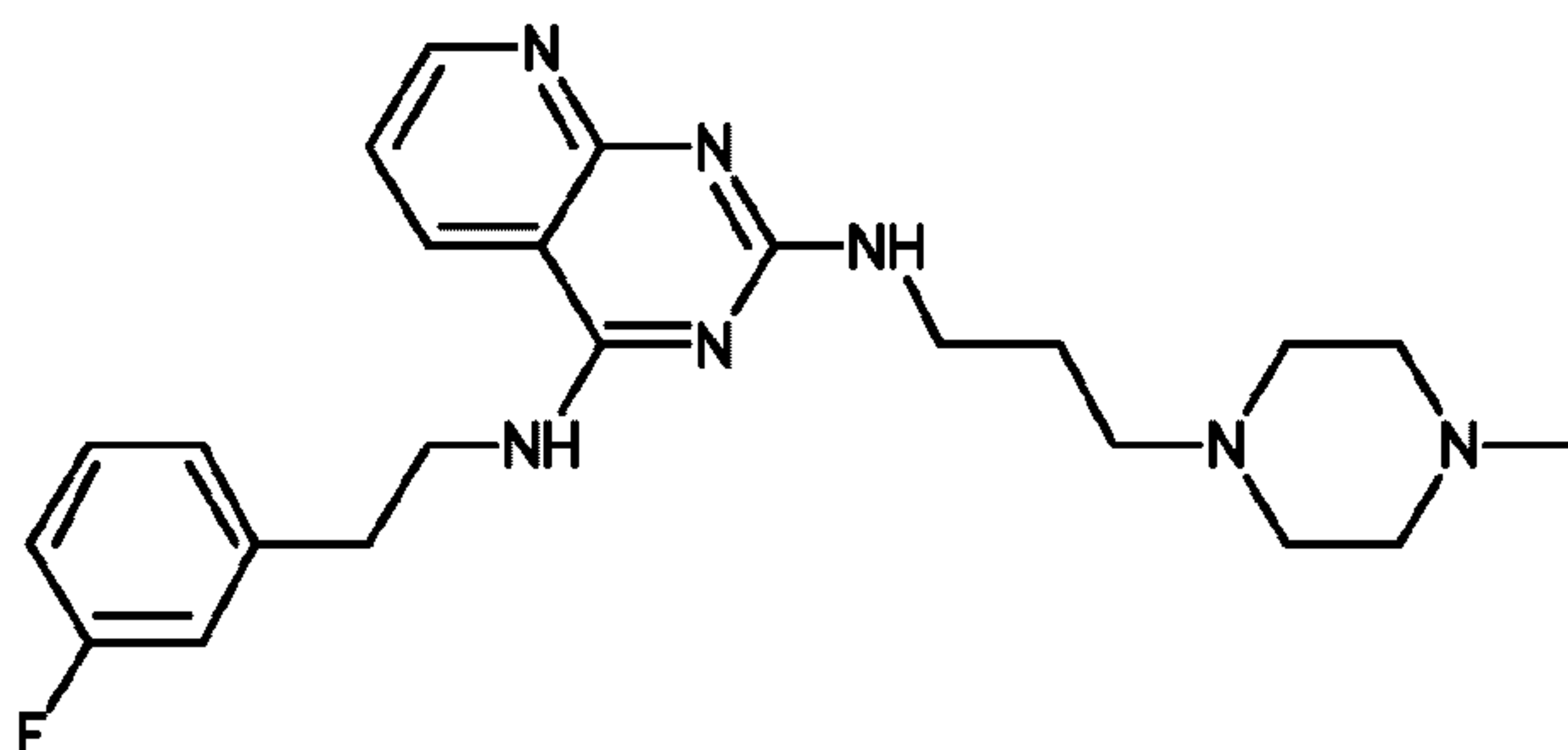
Formula (49). 4-{{4-([2-(pyridin-2-ylamino)ethyl]amino)-1,3,5-triazin-2-yl}pyridin-2-yl}oxy}butan-1-ol. Referred herein as “Compound 47”.

[0194] In one embodiment, the at least one other factor is a compound of the Formula (50):



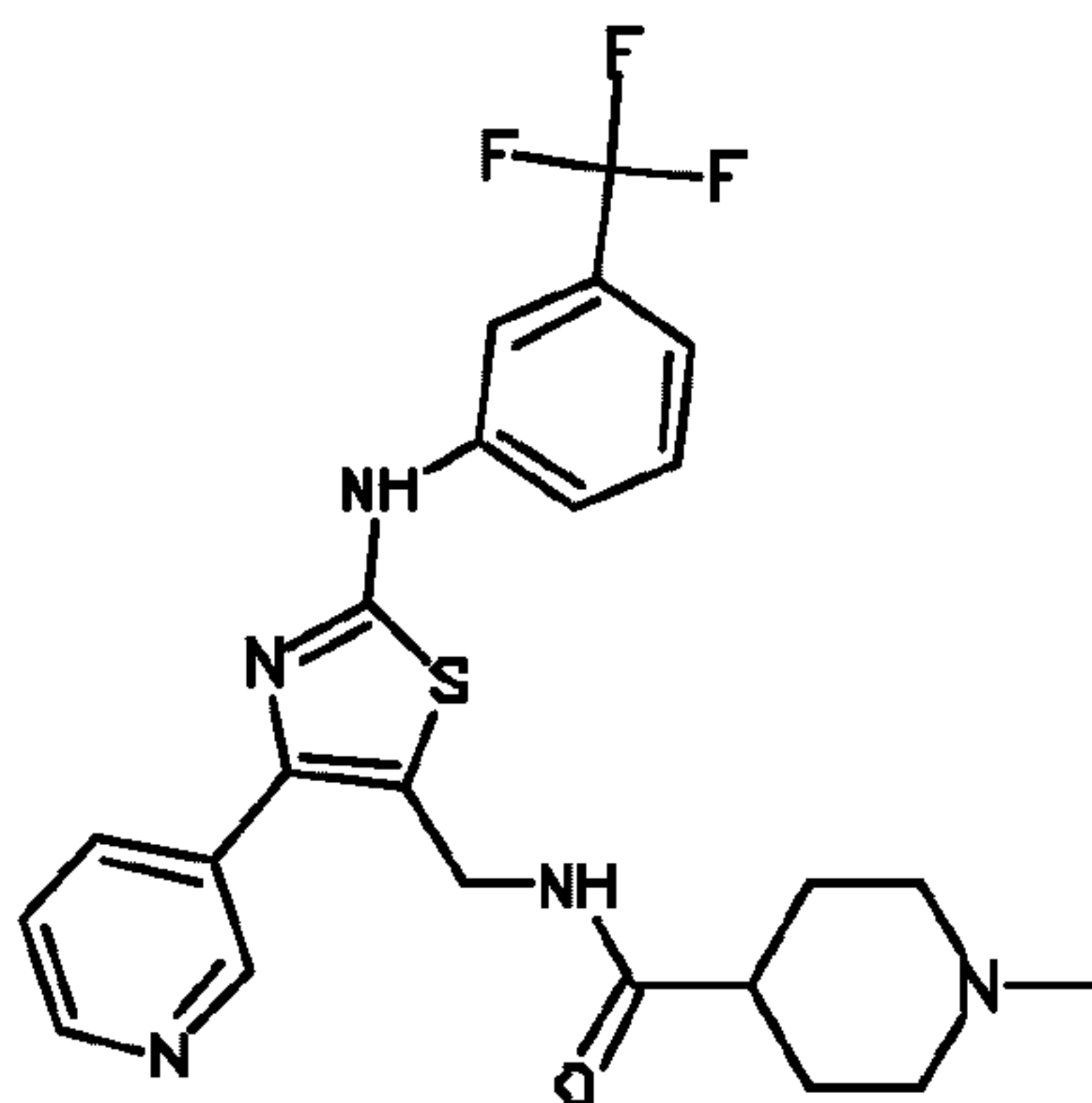
Formula (50). 3-({3-[4-({2-[Methyl(pyridin-2-yl)amino]ethyl}amino)-1,3,5-triazin-2-yl]pyridin-2-yl}amino)propan-1-ol. Referred herein as “Compound 48”.

[0195] In one embodiment, the at least one other factor is a compound of the Formula (51):



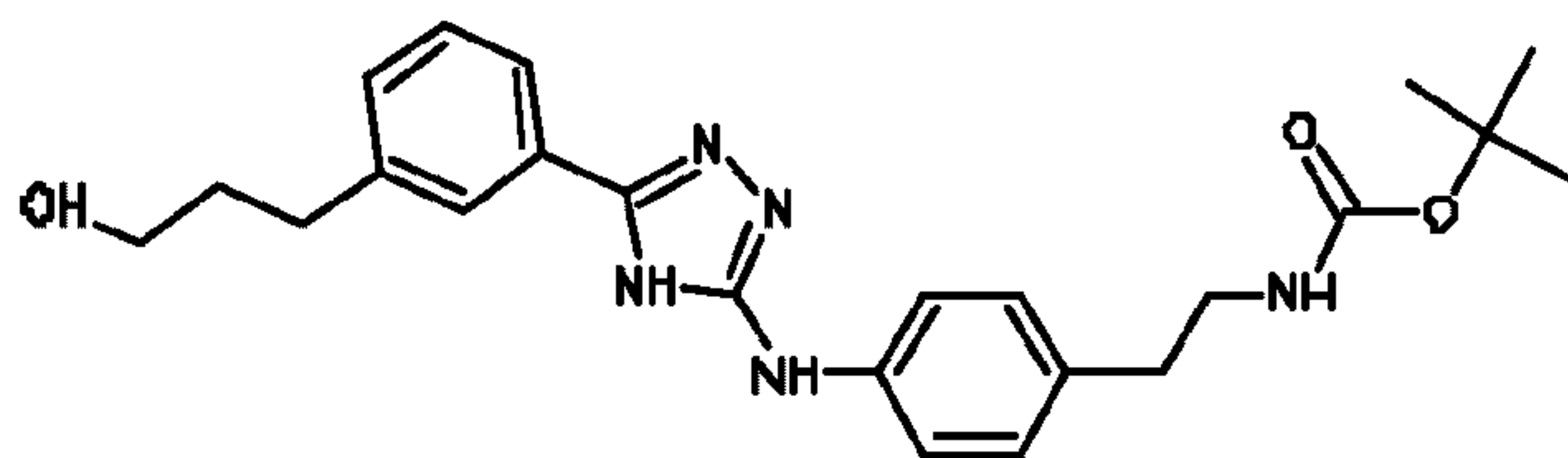
Formula (51). N~4~-[2-(3-Fluorophenyl)ethyl]-N~2~-[3-(4-methylpiperazin-1-yl)propyl]pyrido[2,3-d]pyrimidine-2,4-diamine. Referred herein as “Compound 49”.

[0196] In one embodiment, the at least one other factor is a compound of the Formula (52):



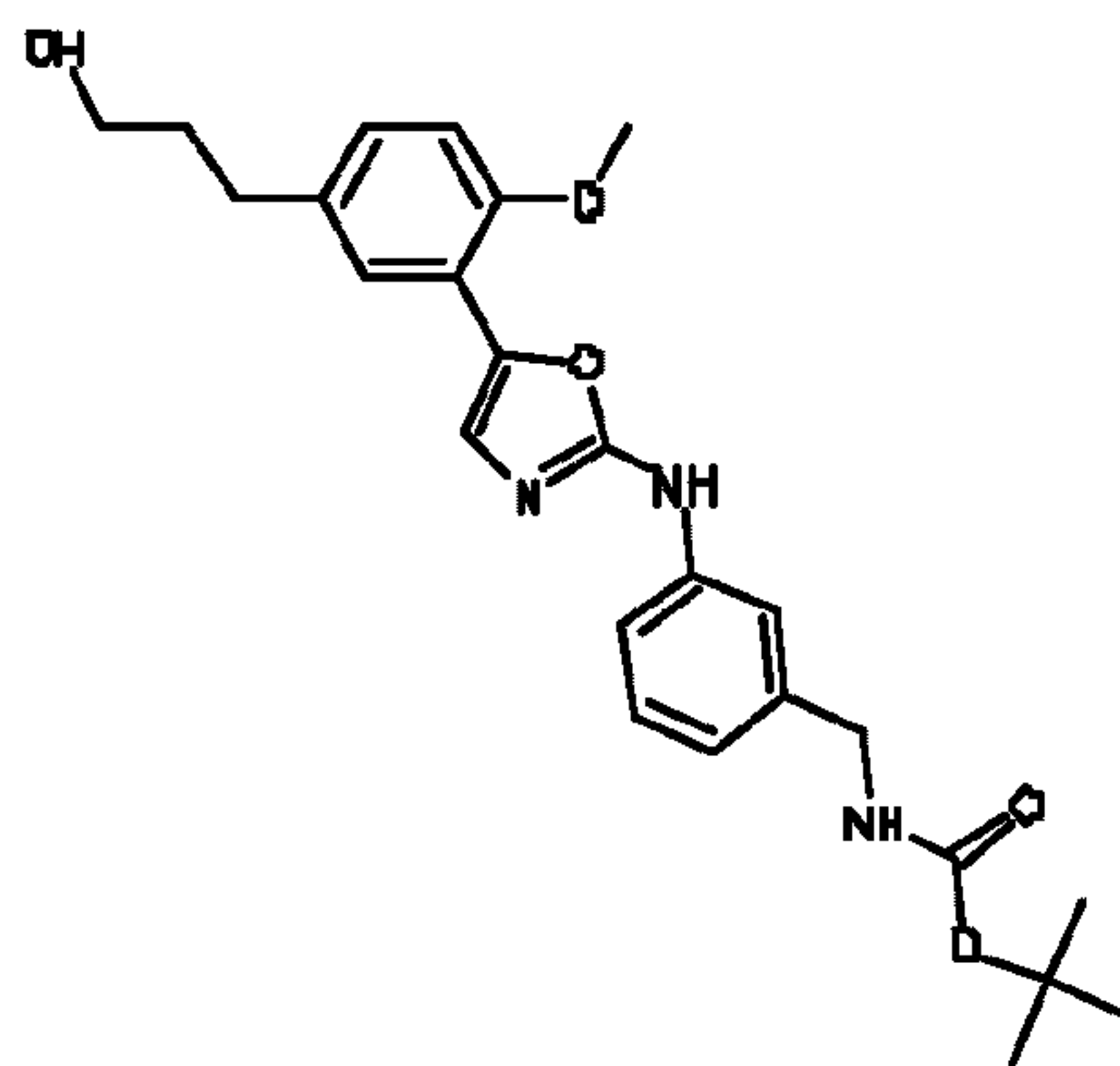
Formula (52). 1-Methyl-N-[(4-pyridin-3-yl-2- {[3-(trifluoromethyl)phenyl]amino}-1,3-thiazol-5-yl)methyl]piperidine-4-carboxamide. Referred herein as “Compound 50”.

[0197] In one embodiment, the at least one other factor is a compound of the Formula (53):



Formula (53). 1,1-Dimethylethyl {2-[4-({5-[3-(3-hydroxypropyl)phenyl]-4H-1,2,4-triazol-3-yl}amino)phenyl]ethyl}carbamate. Referred herein as “Compound 51”.

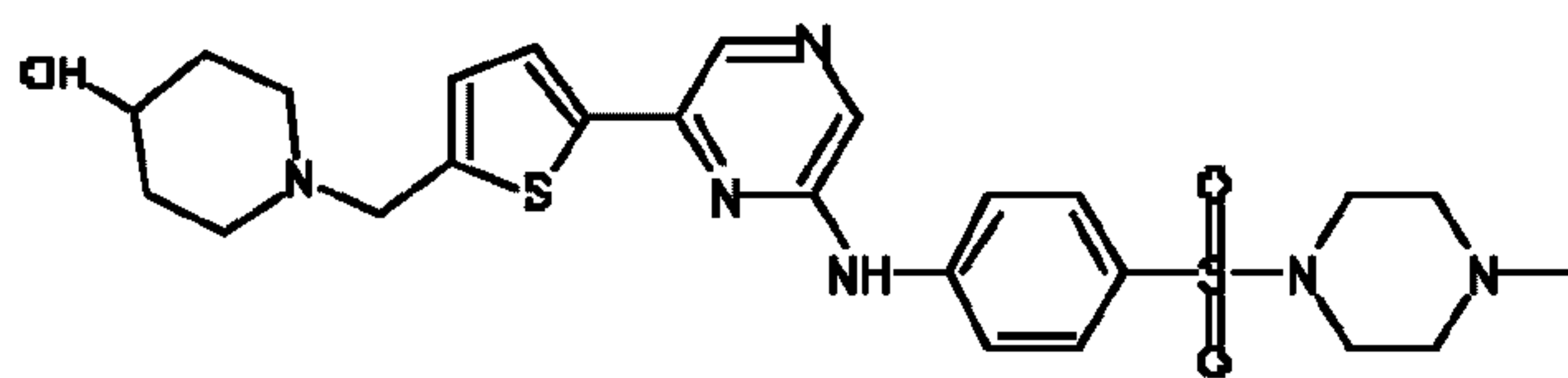
[0198] In one embodiment, the at least one other factor is a compound of the Formula (54):



Formula (54). 1,1-Dimethylethyl

{[3-({5-[5-(3-hydroxypropyl)-2-(methoxy)phenyl]-1,3-oxazol-2-yl}amino)phenyl]methyl} carbamate. Referred herein as “Compound 52”.

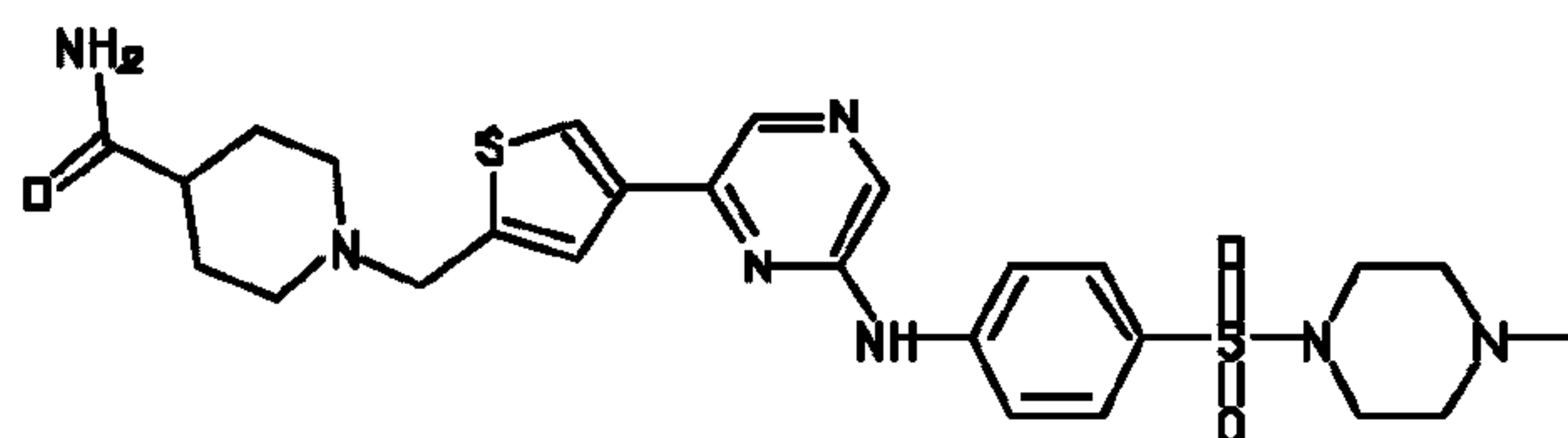
[0199] In one embodiment, the at least one other factor is a compound of the Formula (55):



Formula (55). 1-({5-[6-({4-[(4-

Methylpiperazin-1-yl)sulfonyl]phenyl} amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidin-4-ol. Referred herein as “Compound 53”.

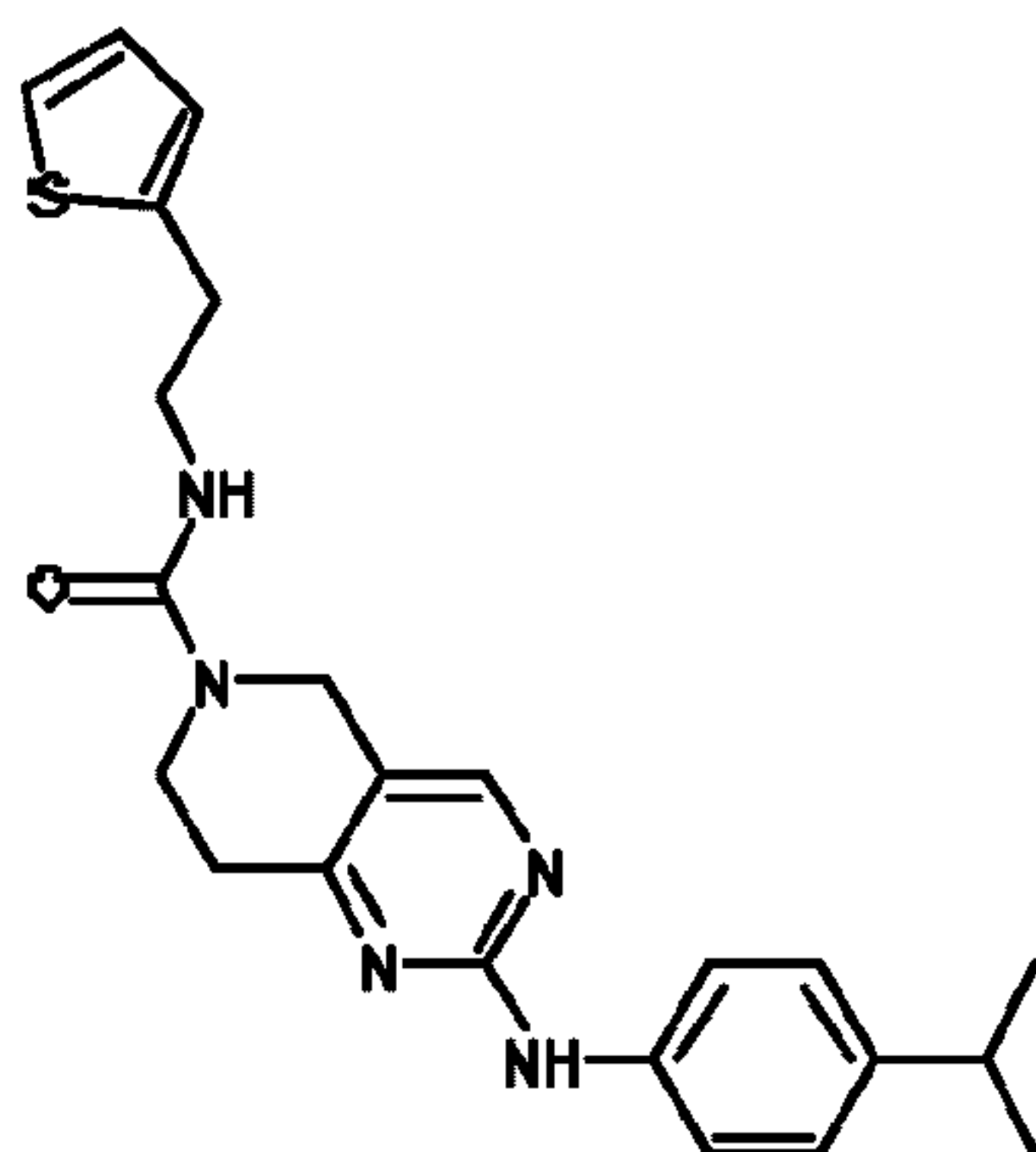
[0200] In one embodiment, the at least one other factor is a compound of the Formula (56):



Formula (56). 1-({4-[6-({4-[(4-

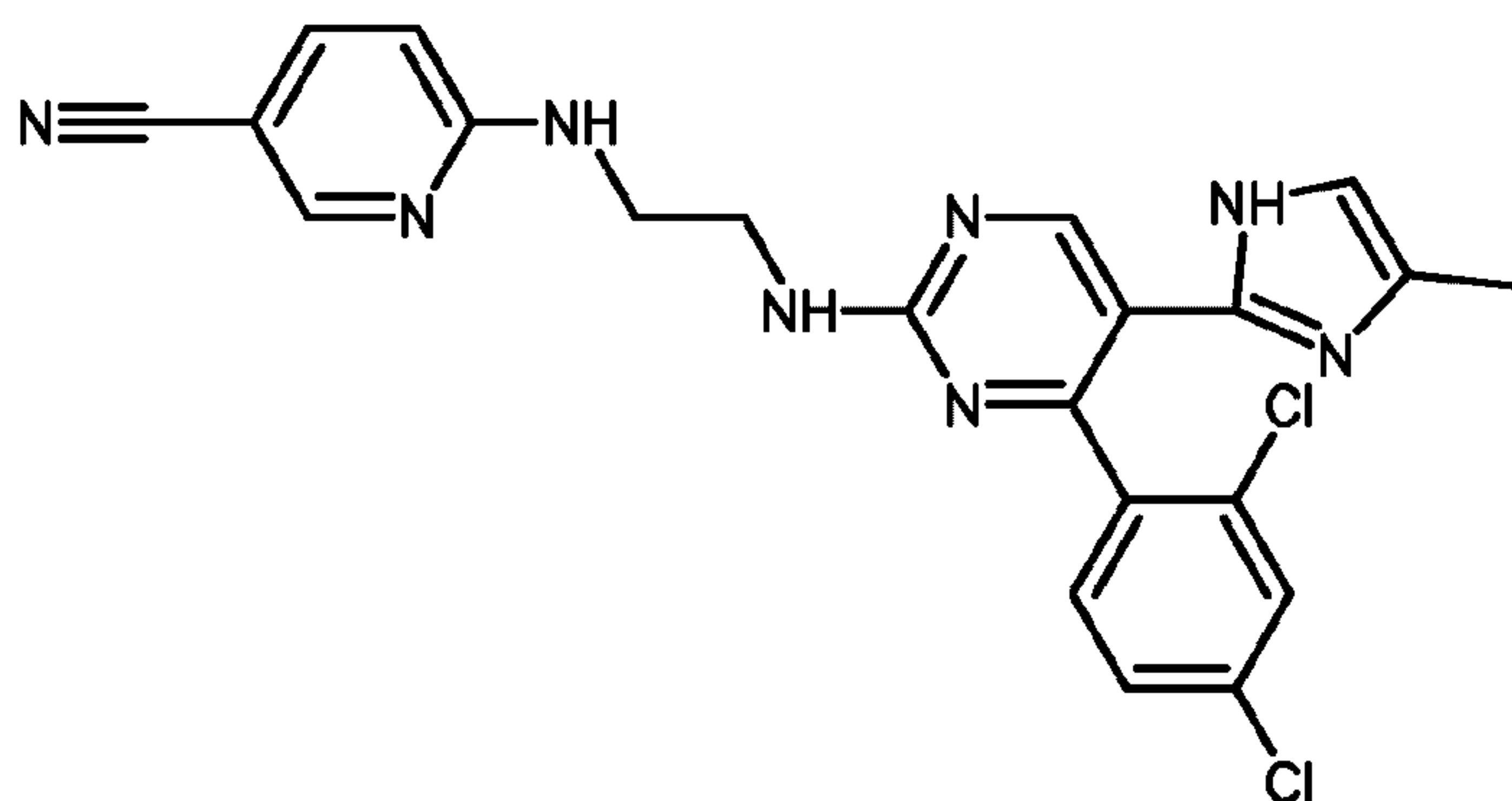
Methylpiperazin-1-yl)sulfonyl]phenyl} amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidine-4-carboxamide. Referred herein as “Compound 54”.

[0201] In one embodiment, the at least one other factor is a compound of the Formula (57):



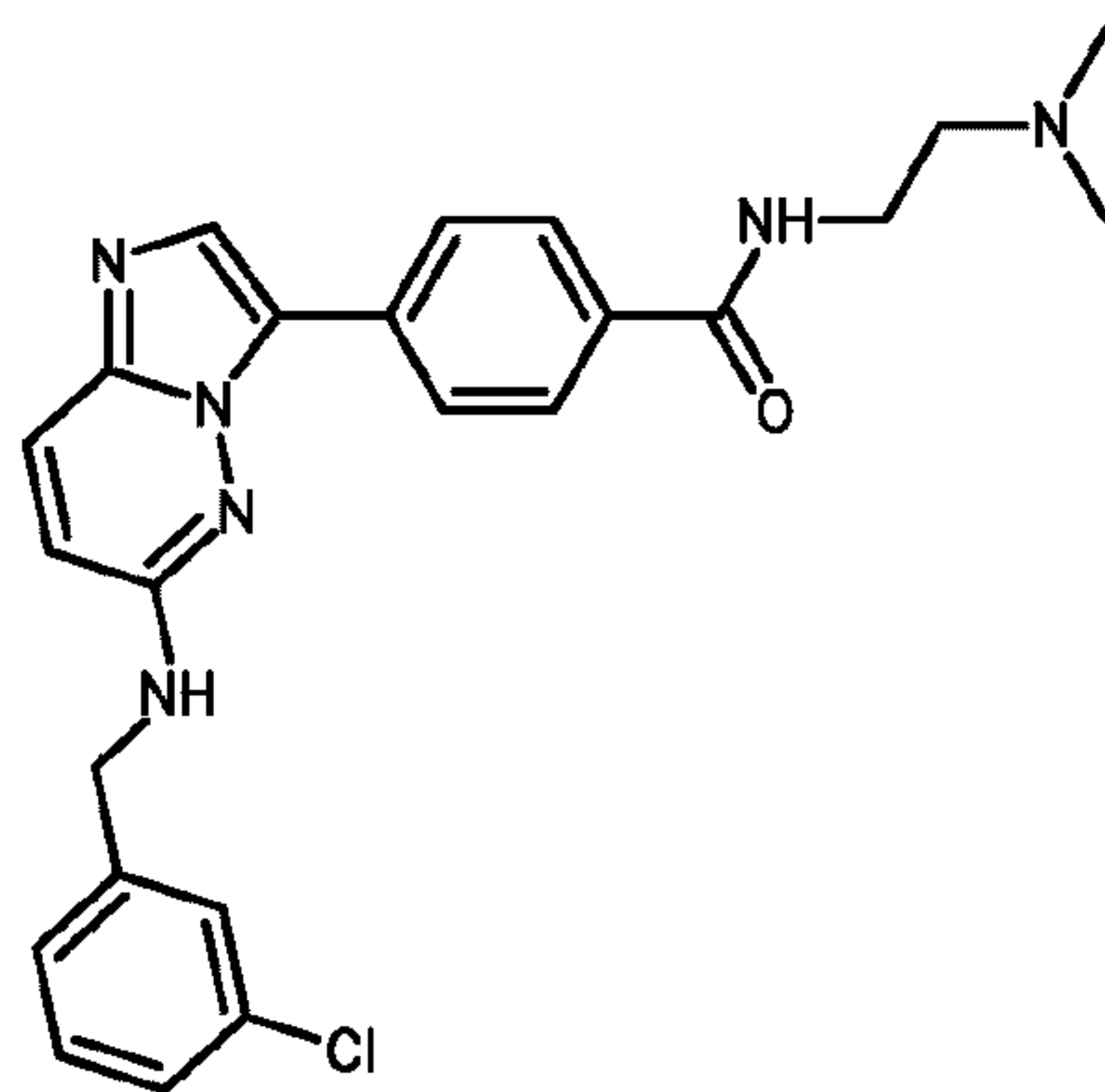
Formula (57). 2-{[4-(1-Methylethyl)phenyl]amino}-N-(2-thiophen-2-ylethyl)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxamide. Referred herein as “Compound 55”.

[0202] In one embodiment, the at least one other factor is a compound of the Formula (58):



Formula (58). 6-[(2-{[4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl]amino}ethyl)amino]pyridine-3-carbonitrile. Referred herein as “Compound 56”.

[0203] In one embodiment, the at least one other factor is a compound of the Formula (59):



Formula (59). 4-(6-{(3-Chlorophenyl)methyl}amino}imidazo[1,2-b]pyridazin-3-yl)-N-[2-(dimethylamino)ethyl]benzamide. Referred herein as “Compound 57”.

Detection of cells expressing markers characteristic of the definitive endoderm lineage

- [0204] Formation of cells expressing markers characteristic of the definitive endoderm lineage may be determined by testing for the presence of the markers before and after following a particular protocol. Pluripotent stem cells typically do not express such markers. Thus, differentiation of pluripotent cells is detected when cells begin to express them.
- [0205] The efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the definitive endoderm lineage.
- [0206] Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These include quantitative reverse transcriptase polymerase chain reaction (RT-PCR), Northern blots, *in situ* hybridization (see, e.g., Current Protocols in Molecular Biology (Ausubel *et al.*, eds. 2001 supplement)), and immunoassays such as immunohistochemical analysis of sectioned material, Western blotting, and for markers that are accessible in intact cells, flow cytometry analysis (FACS) (see, e.g., Harlow and Lane, Using Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press (1998)).
- [0207] For example, characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be

identified. Pluripotent stem cell markers include, for example, the expression of one or more of the following: ABCG2, *cripto*, FOXD3, Connexin43, Connexin45, OCT4, SOX2, Nanog, hTERT, UTF-1, ZFP42, SSEA-3, SSEA-4, Tra1-60, or Tra1-81.

- [0208] After treating pluripotent stem cells with the methods of the present invention, the differentiated cells may be purified by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker, such as CXCR4, expressed by cells expressing markers characteristic of the definitive endoderm lineage.

Formation of cells expressing markers characteristic of the pancreatic endoderm lineage

- [0209] Cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage by any method in the art or by any method proposed in this invention.
- [0210] For example, cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).
- [0211] For example, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with a fibroblast growth factor and the hedgehog signaling pathway inhibitor KAAD-cyclopamine, then removing the medium containing the fibroblast growth factor and KAAD-cyclopamine and subsequently culturing the cells in medium containing retinoic acid, a fibroblast growth factor and KAAD-cyclopamine. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).
- [0212] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid and at

least one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

- [0213] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid and at least one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.
- [0214] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/990,529.
- [0215] Cells expressing markers characteristic of the definitive endoderm lineage may be treated with at least one other additional factor that may enhance the formation of cells expressing markers characteristic of the pancreatic endoderm lineage. Alternatively, the at least one other additional factor may enhance the proliferation of the cells expressing markers characteristic of the pancreatic endoderm lineage formed by the methods of the present invention. Further, the at least one other additional factor may enhance the ability of the cells expressing markers characteristic of the pancreatic endoderm lineage formed by the methods of the present invention to form other cell types, or improve the efficiency of any other additional differentiation steps.
- [0216] The at least one additional factor may be, for example, nicotinamide, members of TGF- β family, including TGF- β 1, 2, and 3, serum albumin, members of the fibroblast growth factor family, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor (such as, for example, GDF-5, -6, -8, -10, -11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, retinoic acid, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, beta mercaptoethanol, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, activin, betacellulin, ITS,

noggin, neurite growth factor, nodal, valproic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine-1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA), keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, or combinations thereof.

- [0217] The at least one other additional factor may be supplied by conditioned media obtained from pancreatic cells lines such as, for example, PANC-1 (ATCC No: CRL-1469), CAPAN-1 (ATCC No: HTB-79), BxPC-3 (ATCC No: CRL-1687), HPAF-II (ATCC No: CRL-1997), hepatic cell lines such as, for example, HepG2 (ATCC No: HTB-8065), and intestinal cell lines such as, for example, FHs 74 (ATCC No: CCL-241).

Detection of cells expressing markers characteristic of the pancreatic endoderm lineage

- [0218] Markers characteristic of the pancreatic endoderm lineage are well known to those skilled in the art, and additional markers characteristic of the pancreatic endoderm lineage continue to be identified. These markers can be used to confirm that the cells treated in accordance with the present invention have differentiated to acquire the properties characteristic of the pancreatic endoderm lineage. Pancreatic endoderm lineage specific markers include the expression of one or more transcription factors such as, for example, Hlxb9, PTF-1a, PDX-1, HNF-6, HNF-1beta.
- [0219] The efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the pancreatic endoderm lineage.
- [0220] Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These include quantitative reverse transcriptase polymerase chain reaction (RT-PCR), Northern blots, *in situ* hybridization (see, e.g., Current Protocols in Molecular Biology (Ausubel *et al.*, eds. 2001 supplement)), and immunoassays such as immunohistochemical analysis of sectioned material, Western

blotting, and for markers that are accessible in intact cells, flow cytometry analysis (FACS) (see, e.g., Harlow and Lane, Using Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press (1998)).

Formation of cells expressing markers of the pancreatic endocrine lineage

- [0221] Cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art or by any method disclosed in this invention.
- [0222] For example, cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).
- [0223] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing DAPT and exendin 4, then removing the medium containing DAPT and exendin 4 and subsequently culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).
- [0224] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4, then removing the medium containing exendin 4 and subsequently culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in D'Amour *et al*, Nature Biotechnology, 2006.
- [0225] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing DAPT and exendin 4. An example of this method is disclosed in D'Amour *et al*, Nature Biotechnology, 2006.

- [0226] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.
- [0227] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.
- [0228] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.
- [0229] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 60/953,178, assigned to LifeScan, Inc.
- [0230] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/990,529.
- [0231] Cells expressing markers characteristic of the pancreatic endoderm lineage may be treated with at least one other additional factor that may enhance the formation of cells expressing markers characteristic of the pancreatic endocrine lineage.

Alternatively, the at least one other additional factor may enhance the proliferation of the cells expressing markers characteristic of the pancreatic endocrine lineage formed by the methods of the present invention. Further, the at least one other additional factor may enhance the ability of the cells expressing markers characteristic of the pancreatic endocrine lineage formed by the methods of the present invention to form other cell types or improve the efficiency of any other additional differentiation steps.

[0232] The at least one additional factor may be, for example, nicotinamide, members of TGF- β family, including TGF- β 1, 2, and 3, serum albumin, members of the fibroblast growth factor family, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor (such as, for example, GDF-5, -6, -8, -10, -11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, retinoic acid, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, beta mercaptoethanol, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, activin, betacellulin, ITS, noggin, neurite growth factor, nodal, valproic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine-1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA), keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, or combinations thereof.

[0233] The at least one other additional factor may be supplied by conditioned media obtained from pancreatic cells lines such as, for example, PANC-1 (ATCC No: CRL-1469), CAPAN-1 (ATCC No: HTB-79), BxPC-3 (ATCC No: CRL-1687), HPAF-II (ATCC No: CRL-1997), hepatic cell lines such as, for example, HepG2 (ATCC No: HTB-8065), and intestinal cell lines such as, for example, FHs 74 (ATCC No: CCL-241).

Detection of cells expressing markers characteristic of the pancreatic endocrine lineage

- [0234] Markers characteristic of cells of the pancreatic endocrine lineage are well known to those skilled in the art, and additional markers characteristic of the pancreatic endocrine lineage continue to be identified. These markers can be used to confirm that the cells treated in accordance with the present invention have differentiated to acquire the properties characteristic of the pancreatic endocrine lineage. Pancreatic endocrine lineage specific markers include the expression of one or more transcription factors such as, for example, NGN3, NEURO, or ISL1.
- [0235] Markers characteristic of cells of the β cell lineage are well known to those skilled in the art, and additional markers characteristic of the β cell lineage continue to be identified. These markers can be used to confirm that the cells treated in accordance with the present invention have differentiated to acquire the properties characteristic of the β -cell lineage. β cell lineage specific characteristics include the expression of one or more transcription factors such as, for example, PDX1, NKX2.2, NKX6.1, ISL1, PAX6, PAX4, NEUROD, HNF1 beta, HNF6, HNF3 beta, or MAFA, among others. These transcription factors are well established in the art for identification of endocrine cells. See, e.g., Edlund (Nature Reviews Genetics 3: 524-632 (2002)).
- [0236] The efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the pancreatic endocrine lineage. Alternatively, the efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the β cell lineage.
- [0237] Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These include quantitative reverse transcriptase polymerase chain reaction (RT-PCR), Northern blots, *in situ* hybridization (see, e.g., Current Protocols in Molecular Biology (Ausubel *et al.*, eds. 2001 supplement)), and immunoassays such as immunohistochemical analysis of sectioned material, Western blotting, and for markers that are accessible in intact cells, flow cytometry analysis

(FACS) (see, e.g., Harlow and Lane, Using Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press (1998)).

[0238] In one aspect of the present invention, the efficiency of differentiation is determined by measuring the percentage of insulin positive cells in a given cell culture following treatment. In one embodiment, the methods of the present invention produce about 100% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 90% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 80% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 70% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 60% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 50% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 40% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 30% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 20% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 10% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 5% insulin positive cells in a given culture.

[0239] In one aspect of the present invention, the efficiency of differentiation is determined by measuring glucose-stimulated insulin secretion, as detected by measuring the amount of C-peptide released by the cells. In one embodiment, cells produced by the methods of the present invention produce about 1000ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 900ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 800ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 700ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 600ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce

about 500ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 400ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 500ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 400ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 300ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 200ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 100ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 90ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 80ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 70ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 60ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 50ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 40ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 30ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 20ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 10ng C-peptide/pg DNA.

Therapies

- [0240] In one aspect, the present invention provides a method for treating a patient suffering from, or at risk of developing, Type 1 diabetes. This method involves culturing pluripotent stem cells, differentiating the pluripotent stem cells *in vitro* into a β -cell lineage, and implanting the cells of a β -cell lineage into a patient.
- [0241] In yet another aspect, this invention provides a method for treating a patient suffering from, or at risk of developing, Type 2 diabetes. This method involves culturing pluripotent stem cells, differentiating the cultured cells *in vitro* into a β -cell lineage, and implanting the cells of a β -cell lineage into the patient.

- [0242] If appropriate, the patient can be further treated with pharmaceutical agents or bioactives that facilitate the survival and function of the transplanted cells. These agents may include, for example, insulin, members of the TGF- β family, including TGF- β 1, 2, and 3, bone morphogenic proteins (BMP-2, -3, -4, -5, -6, -7, -11, -12, and -13), fibroblast growth factors-1 and -2, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II) growth differentiation factor (such as, for example, GDF-5, -6, -7, -8, -10, -15), vascular endothelial cell-derived growth factor (VEGF), pleiotrophin, endothelin, among others. Other pharmaceutical compounds can include, for example, nicotinamide, glucagon like peptide-I (GLP-1) and II, GLP-1 and -2 mimetibody, Exendin-4, retinoic acid, parathyroid hormone, MAPK inhibitors, such as, for example, compounds disclosed in U.S. Published Application 2004/0209901 and U.S. Published Application 2004/0132729.
- [0243] The pluripotent stem cells may be differentiated into an insulin-producing cell prior to transplantation into a recipient. In a specific embodiment, the pluripotent stem cells are fully differentiated into β -cells prior to transplantation into a recipient. Alternatively, the pluripotent stem cells may be transplanted into a recipient in an undifferentiated or partially differentiated state. Further differentiation may take place in the recipient.
- [0244] Definitive endoderm cells or, alternatively, pancreatic endoderm cells, or, alternatively, β cells, may be implanted as dispersed cells or formed into clusters that may be infused into the hepatic portal vein. Alternatively, cells may be provided in biocompatible degradable polymeric supports, porous non-degradable devices or encapsulated to protect from host immune response. Cells may be implanted into an appropriate site in a recipient. The implantation sites include, for example, the liver, natural pancreas, renal subcapsular space, omentum, peritoneum, subserosal space, intestine, stomach, or a subcutaneous pocket.
- [0245] To enhance further differentiation, survival or activity of the implanted cells, additional factors, such as growth factors, antioxidants or anti-inflammatory agents, can be administered before, simultaneously with, or after the administration of the cells. In certain embodiments, growth factors are utilized to differentiate the administered cells *in vivo*. These factors can be secreted by endogenous cells and exposed to the administered cells *in situ*. Implanted cells can be induced to

differentiate by any combination of endogenous and exogenously administered growth factors known in the art.

- [0246] The amount of cells used in implantation depends on a number of various factors including the patient's condition and response to the therapy, and can be determined by one skilled in the art.
- [0247] In one aspect, this invention provides a method for treating a patient suffering from, or at risk of developing diabetes. This method involves culturing pluripotent stem cells, differentiating the cultured cells *in vitro* into a β -cell lineage, and incorporating the cells into a three-dimensional support. The cells can be maintained *in vitro* on this support prior to implantation into the patient. Alternatively, the support containing the cells can be directly implanted in the patient without additional *in vitro* culturing. The support can optionally be incorporated with at least one pharmaceutical agent that facilitates the survival and function of the transplanted cells.
- [0248] Support materials suitable for use for purposes of the present invention include tissue templates, conduits, barriers, and reservoirs useful for tissue repair. In particular, synthetic and natural materials in the form of foams, sponges, gels, hydrogels, textiles, and nonwoven structures, which have been used *in vitro* and *in vivo* to reconstruct or regenerate biological tissue, as well as to deliver chemotactic agents for inducing tissue growth, are suitable for use in practicing the methods of the present invention. See, for example, the materials disclosed in U.S. Patent 5,770,417, U.S. Patent 6,022,743, U.S. Patent 5,567,612, U.S. Patent 5,759,830, U.S. Patent 6,626,950, U.S. Patent 6,534,084, U.S. Patent 6,306,424, U.S. Patent 6,365,149, U.S. Patent 6,599,323, U.S. Patent 6,656,488, U.S. Published Application 2004/0062753 A1, U.S. Patent 4,557,264 and U.S. Patent 6,333,029.
- [0249] To form a support incorporated with a pharmaceutical agent, the pharmaceutical agent can be mixed with the polymer solution prior to forming the support. Alternatively, a pharmaceutical agent could be coated onto a fabricated support, preferably in the presence of a pharmaceutical carrier. The pharmaceutical agent may be present as a liquid, a finely divided solid, or any other appropriate physical form. Alternatively, excipients may be added to the support to alter the release rate of the pharmaceutical agent. In an alternate embodiment, the support is incorporated with at least one

pharmaceutical compound that is an anti-inflammatory compound, such as, for example, compounds disclosed in U.S. Patent 6,509,369.

- [0250] The support may be incorporated with at least one pharmaceutical compound that is an anti-apoptotic compound, such as, for example, compounds disclosed in U.S. Patent 6,793,945.
- [0251] The support may also be incorporated with at least one pharmaceutical compound that is an inhibitor of fibrosis, such as, for example, compounds disclosed in U.S. Patent 6,331,298.
- [0252] The support may also be incorporated with at least one pharmaceutical compound that is capable of enhancing angiogenesis, such as, for example, compounds disclosed in U.S. Published Application 2004/0220393 and U.S. Published Application 2004/0209901.
- [0253] The support may also be incorporated with at least one pharmaceutical compound that is an immunosuppressive compound, such as, for example, compounds disclosed in U.S. Published Application 2004/0171623.
- [0254] The support may also be incorporated with at least one pharmaceutical compound that is a growth factor, such as, for example, members of the TGF- β family, including TGF- β 1, 2, and 3, bone morphogenic proteins (BMP-2, -3, -4, -5, -6, -7, -11, -12, and -13), fibroblast growth factors-1 and -2, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II) growth differentiation factor (such as, for example, GDF-5, -6, -8, -10, -15), vascular endothelial cell-derived growth factor (VEGF), pleiotrophin, endothelin, among others. Other pharmaceutical compounds can include, for example, nicotinamide, hypoxia inducible factor 1-alpha, glucagon like peptide-I (GLP-1), GLP-1 and GLP-2 mimetibody, and II, Exendin-4, nodal, noggin, NGF, retinoic acid, parathyroid hormone, tenascin-C, tropoelastin, thrombin-derived peptides, cathelicidins, defensins, laminin, biological peptides containing cell- and heparin-binding domains of adhesive extracellular matrix proteins such as fibronectin and vitronectin, MAPK inhibitors, such as, for example, compounds disclosed in U.S. Published Application 2004/0209901 and U.S. Published Application 2004/0132729.

[0255] The incorporation of the cells of the present invention into a scaffold can be achieved by the simple depositing of cells onto the scaffold. Cells can enter into the scaffold by simple diffusion (J. Pediatr. Surg. 23 (1 Pt 2): 3-9 (1988)). Several other approaches have been developed to enhance the efficiency of cell seeding. For example, spinner flasks have been used in seeding of chondrocytes onto polyglycolic acid scaffolds (Biotechnol. Prog. 14(2): 193-202 (1998)). Another approach for seeding cells is the use of centrifugation, which yields minimum stress to the seeded cells and enhances seeding efficiency. For example, Yang *et al.* developed a cell seeding method (J. Biomed. Mater. Res. 55(3): 379-86 (2001)), referred to as Centrifugational Cell Immobilization (CCI).

[0256] The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

[0257] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments, or applications of the present invention.

Example 1

Human embryonic stem cell culture

[0258] The human embryonic stem cell lines H1, H7, and H9 were obtained from WiCell Research Institute, Inc., (Madison, WI) and cultured according to instructions provided by the source institute. The human embryonic stem cells were also seeded on plates coated with a 1:30 dilution of reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231) and cultured in MEF-conditioned medium supplemented with 8 ng/ml bFGF (R&D Systems; Cat # 233-FB). The cells cultured on MATRIGEL™ were routinely passaged as clusters using collagenase IV (Invitrogen/GIBCO; Cat # 17104-019), DispaseTM (Invitrogen; Cat # 17105-041), or Liberase CI enzyme (Roche; Cat # 11814435001). In some instances, the cells were passaged as single cells using ACCUTASE (Sigma; Cat # A6964).

[0259] Human embryonic stem cells used in these examples were maintained in an undifferentiated, pluripotent state with passage on average every four-days. Passage was performed by exposing cell cultures to a solution of collagenase (1 or 10 mg/ml; Sigma-Aldrich) for 10 to 30 minutes at 37°C followed by gentle scraping with a pipette tip to recover cell clusters. Clusters were allowed to sediment by gravity, followed by washing to remove residual collagenase. Cell clusters were split at a 1:3 ratio for routine maintenance culture or a 1:1 ratio for later assay. All human ES cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotypic phenotype and absence of mycoplasma contamination.

Example 2

Bioassay for the Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

[0260] Activin A is an important mediator of differentiation in a broad range of cell types, including differentiation of embryonic stem cells to definitive endoderm. When human embryonic stem cells are treated with a combination of activin A and Wnt3a, various genes representative of definitive endoderm are up-regulated. A bioassay that measures this differentiation in human embryonic stem cells was adapted in miniaturized format to 96-well plates for screening purposes. Validation was completed using treatment with commercial sources of activin A and Wnt3a recombinant proteins and measuring protein expression of the transcription factor SOX17, considered to be a representative marker of definitive endoderm.

[0261] *Live Cell Assay:* Briefly, clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated in a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; Perkin Elmer; Cat #6005182). Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with 100µl per well mouse embryonic fibroblast (MEF) conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB).

- [0262] The assay was initiated by washing the wells of each plate twice in PBS (Invitrogen; Cat # 14190), followed by adding an aliquot (100 μ l) of test sample in DMEM:F12 basal medium (Invitrogen; Cat # 11330-032) to each well. Test conditions were performed in triplicate, feeding on alternate days by aspirating and replacing the medium from each well with test samples over a total four-day assay period. On the first and second day of assay, test samples added to the assay wells were diluted in DMEM:F12 with 0.5% FCS (HyClone; Cat # SH30070.03) and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN). On the third and fourth day of assay, test samples added to the assay wells were diluted in DMEM:F12 with 2% FCS, without any Wnt3a. Positive control samples consisted of recombinant human activin A (PeproTech; Cat #120-14) added at a concentration of 100ng/ml throughout assay plus Wnt3a (20ng/ml) on days 1 and 2. Negative control samples omitted treatment with both activin A and Wnt3a.
- [0263] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% TritonTM X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat #AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counter stain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.
- [0264] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and

subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0265] **Figure 1** shows validation of the screening assay, testing a two-fold dilution curve of a commercial source of activin A (PeproTech) and measuring both cell number (**Figure 1A**) and SOX17 intensity (**Figure 1B**). Optimal activin A effects for induction of SOX17 expression were generally observed in the 100-200ng/ml range with an EC₅₀ of 30-50ng/ml. Omitting Wnt3a from treatment on days 1 and 2 of assay failed to produce measurable SOX17 expression (**Figure 1B**, white bars). Absence of activin A also failed to yield SOX17 expression (**Figure 1B**).

Example 3

Primary Screening: Effects of the Compounds of the Present Invention on the Differentiation of Human Embryonic Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage in the Absence of Activin A

[0266] Differentiation of pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage is mediated through a series of receptor–ligand interactions that in turn activate receptor kinases leading to phosphorylation and nuclear translocation of downstream substrates, eventually regulating expression of specific target genes. Optimal activation of these signaling cascades in some cell types may require inhibition of opposing default pathways. In other cases, redundant pathways involving alternative members of a larger kinase family may substitute in part for one or more signaling molecules. In other cases, canonical and non-canonical

pathways may diverge with different initiating stimuli but may lead to a similar functional outcome.

- [0267] Cell-based functional screens are one approach to identify novel targets and methods that can impact specific cellular responses. One very powerful approach involves a series of iterative screens whereby leads or hits from one screen are integrated into a subsequent screen. Alternatively, a series of different variables are integrated in a combinatorial fashion (for example, growth factors with kinase inhibitors) to identify novel effects on cellular differentiation. In this case, a library of small molecules comprising aniline-pyridinotriazines, cyclic aniline-pyridinotriazines and intermediate structures in their synthesis was tested for properties important during definitive endoderm differentiation of human embryonic stem cells, specifically for effects to retain or enhance cell number at the conclusion of a 'first' differentiation step in low serum and in the absence of the growth factor activin A.

Screening Assay

- [0268] *Cell assay seeding:* Briefly, clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 µl/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0269] *Preparation of compounds and assay:* The compounds tested were made available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO (Sigma; Cat # D2650) and stored at -80°C. The library compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. Test conditions were performed in triplicate, feeding on alternate days over a four-day assay period. Primary screening assays

were initiated by aspirating culture medium from each well followed by three washes in PBS (Invitrogen; Cat # 14190) to remove residual growth factors and serum. On the first day of assay, test volumes of 200 μ l per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.5% FCS (HyClone; Cat # SH30070.03) and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN) plus 2.5 μ M test compound. On the third day of assay, test volumes of 200 μ l per well were added back containing DMEM:F12 base medium supplemented with 2% FCS plus 2.5 μ M test compound, without Wnt3a. Positive control samples contained the same base medium supplemented with FCS, substituting 100ng/ml recombinant human activin A (PeproTech; Cat #120-14) for the test compound throughout the four-day assay along with Wnt3a (20ng/ml) added only on days 1 and 2. Negative control samples contained DMEM:F12 base medium supplemented with FCS, adding Wnt3a on days 1 and 2 but omitting activin A.

[0270] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counter stain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0271] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and

subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

- [0272] **Table 1** shows results of primary screening for the compounds tested, showing their effects on the differentiation of human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage in the absence of activin A. The results include quantitative measures of both cell number and SOX17 intensity, where respective data points were averaged from triplicate wells and analyzed for each parameter using identical fields in each well. Expression of the transcription factor SOX17 is considered indicative of definitive endoderm differentiation. Primary screening results were captured from eight 96-well screening plates. Plate to plate variability was reduced with inclusion of individual positive and negative controls on each plate. Results are normalized and expressed as a percentage of the positive control. Emphasis was placed on retention or amplification of cell number at the conclusion of assay.
- [0273] **Table 2** lists a subset of 27 compounds and their analyzed results from the primary screening, where these hits appeared to retain cell number at a level equivalent to or better than the positive control despite the absence of activin A in the screening assay.
- [0274] In some cases, SOX17 expression was induced in the absence of activin A (for example, the cyclic aniline-pyridinotriazines Compound 35 and Compound 22.
- [0275] The compounds shown in **Table 2** were selected for further evaluation for effects on the differentiation of human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage in the absence of activin A.

Example 4

Secondary Screening: Effects of the Compounds of the Present Invention on the Differentiation of Human Embryonic Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage with EGF/FGF4 in the Absence of Activin A

- [0276] A titration curve for activin A with a constant amount of Wnt3a showed at least two effects during DE differentiation: 1) maintaining cell numbers or preventing cell loss; and 2) inducing a marker of DE, for example, SOX17 expression (**Example 2**). Primary screening from **Example 3** identified compounds that could maintain similar or improved cell numbers in assay relative to addition of activin A/Wnt3a alone. A secondary screening assay was conducted to evaluate the effect of combinations of the identified compounds with other growth factors, specifically EGF and FGF4, on the generation of definitive endoderm.
- [0277] *Cell assay seeding:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100µl/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0278] *Preparation of compounds and growth factors:* Stock concentrations for EGF (R&D Systems; Cat # 236-EG) and FGF4 (R&D Systems; Cat #235-F4) were 250 ng/ml, each solubilized in PBS with 0.1% BSA (Sigma; Cat # A7888). Compounds were available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO (Sigma; Cat # D2650) and stored at -80°C. The compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. All growth factors and inhibitors were prepared

in a deep well, 96-well polypropylene plate, diluted to 5x intermediate stocks in DMEM:F12 base medium at the beginning of assay and stored at 4°C.

- [0279] A secondary screening assay was conducted, testing in triplicate and feeding on alternate days over the four-day assay timeframe. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS to remove residual growth factors and serum. Test volumes of 80µl per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.625% FCS (HyClone; Cat # SH30070.03), 25ng/ml Wnt3a (R&D Systems), and 3.125µM compound plus 20µl 5x stock of growth factors to yield a final concentration of 0.5% FCS, 20ng/ml Wnt3a, and 2.5µM compound plus 50ng/ml EGF and 50ng/ml FGF4 in the assay. Positive control wells (100µl/well) contained the same base medium supplemented with 0.5% FCS, 20ng/ml Wnt3a and 100ng/ml activin A. Negative control wells (100µl/well) contained the same base medium with 0.5% FCS and 20ng/ml Wnt3a, omitting activin A.
- [0280] On day 3, wells were aspirated and fed with 80µl DMEM:F12 base medium supplemented with 2.5% FCS (HyClone) and 3.125 µM compound plus 20µl 5x stock of growth factors per well to yield a final concentration of 2% FCS and 2.5µM compound (omitting Wnt3a) plus 50ng/ml EGF and FGF4 in the assay. Positive control wells (100µl/well) contained the same base medium supplemented with 2% FCS and 100ng/ml activin A, omitting Wnt3a. Negative control wells (100µl/ well) contained the same base medium with 2% FCS, omitting both activin A and Wnt3a.
- [0281] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS, fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added

to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0282] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488.

Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0283] **Table 3A** shows the results for two growth factors, EGF and FGF 4 (50ng/ml each) tested in combination with the aniline-pyridinotriazine compounds shown in **Table 2** for their effects on the differentiation of human embryonic stem cells into cells expressing markers characteristic of the definitive endoderm lineage in the absence of activin A. Results are ranked in descending order for best effects on SOX17 expression. Although the effects of these compounds on SOX17 expression were considered weak relative to the activin A/Wnt3a positive control, the responses for some of these compounds were considered significant. For example a selection of the compounds appear to have unique properties with respect to retaining high cell numbers per well during assay, presumably either by preventing apoptosis or by modulating cell cycle. In addition, these compounds appear to synergize with EGF and FGF4 to promote modest definitive endoderm differentiation, as measured by

SOX17 expression. The most potent compounds are listed in **Table 3B**. Other compounds tested in combination with EGF and FGF4 in this assay were ineffective at inducing SOX17 expression but could retain cell numbers in assay (e.g. Compound 90: 85% cell number; 2% SOX17 expression).

Example 5

Effects of Compounds of the Present Invention in Combination with Other Factors on the Differentiation of Human Embryonic Stem Cells to Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage in the Absence of Activin A

- [0284] A secondary assay was conducted to evaluate the effect of the compounds of the present invention with combinations of other individual growth factors or compounds known from the literature to regulate definitive endoderm differentiation.
- [0285] *Cell assay seeding:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100µl/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0286] *Preparation of compounds and growth factors:* Stocks of growth factors purchased from R&D Systems were EGF (Cat # 236-EG), FGF4 (Cat #235-F4), PDGF-A (Cat #221-AA), PDGF-B (Cat #220-BB), PDGF-C (Cat #1687-CC), PDGF-D (Cat #1159-SB), PDGF-A/B (Cat #222-AB), VEGF (Cat #293-VE), BMP-1 (Cat #1927-ZN)BMP-2 (Cat #355-BM), BMP-4 (Cat #314-BP), BMP-6 (Cat #507-BP), BMP-7 (Cat #222-AB), BMP-2/7 (Cat #3229-BM). Other agents tested were purchased as follows: BMP-7 (Sigma; Cat # B1434), LY294002 (Cayman; Cat 70920), PD98059, U0126, U0124 (EMD Biosciences; Cat # 453710), muscimol (Tocris; Cat # 0289),

biuculline (Tocris; Cat # 0130), sodium butyrate (Sigma; Cat # B5887). All growth factors were solubilized in PBS with 0.1% BSA (Sigma; Cat # A7888) and stored frozen at -80°C . Small molecules were solubilized in 100% DMSO (Sigma; Cat # D2650) and stored frozen at -80°C . The compounds were available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at -80°C . The compounds of the present invention were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C . All growth factors and inhibitors were prepared in a deep well, 96-well polypropylene plate, diluted to 5x intermediate stocks in DMEM:F12 base medium at the beginning of assay and stored at 4°C .

[0287] A secondary screening assay was conducted, testing in triplicate and feeding on alternate days over the four-day assay timeframe. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS to remove residual growth factors and serum. Test volumes of 80 μl per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.625% FCS (HyClone; Cat # SH30070.03), 25ng/ml Wnt3a (R&D Systems), and 3.125 μM compound plus 20 μl 5x stock of growth factor or small molecule to yield a final concentration of 0.5% FCS, 20ng/ml Wnt3a, and 2.5 μM compound. All remaining growth factors were tested at a final assay concentration of 50ng/ml (EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF-A/B, VEGF, BMP-1, BMP-2, BMP-4, BMP-6, BMP-7, BMP-2/7). Final assay concentrations of small molecules tested were as follows: muscimol (20 μM), PD98059 (1 μM), LY294002 (2.5 μM), U0124 (1 μM), U0126 (1 μM), sodium butyrate (0.5mM). Positive control wells (100 μl /well) contained the same base medium supplemented with 0.5% FCS, 20ng/ml Wnt3a and 100ng/ml activin A. Negative control wells (100 μl /well) contained the same base medium with 0.5% FCS and 20ng/ml Wnt3a, omitting activin A.

[0288] On day 3, wells were aspirated and fed with 80 μl DMEM:F12 base medium supplemented with 2.5% FCS (HyClone) and 3.125 μM cyclic aniline-pyridinotriazine compound plus 20 μl 5x stock of growth factors or small molecules per well to yield a final concentration of 2% FCS and 2.5 μM compound (omitting Wnt3a) and as denoted on day one for all remaining growth factors or small molecules. Positive control wells (100 μl /well) contained the same base medium

supplemented with 2% FCS and 100ng/ml activin A, omitting Wnt3a. Negative control wells (100 μ l/well) contained the same base medium with 2% FCS, omitting both activin A and Wnt3a.

[0289] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS, fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0290] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the

average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0291] **Table 4** shows the results for the differentiation of human embryonic stem cells into cells expressing markers characteristic of the definitive endoderm lineage following treatment with the compounds of the present invention in combination with individual growth factors or other small molecules. In general, members of the BMP family (BMP-1, BMP-2, BMP-4, BMP-6, BMP-7, BMP-2/7) inhibited or had negligible effects on SOX17 expression. The same was true for most of the small molecule enzyme inhibitors tested in this assay (LY294002, PD98059, U0126, U0124, sodium butyrate). However, some members of the PDGF family (PDGF-A, -AB, -C, and -D) provided an increase in SOX17 expression (10-25% of the activin A/Wnt3a control). Other growth factors showing similar increases in SOX17 expression included EGF (34%), VEGF (18%), and FGF4 (17%), although FGF4 was not able to support retention of cell numbers. The small molecule muscimol (GABA_A receptor agonist) tested in combination with Compound 35 also provided a modest increase in SOX17 expression; the GABA_A antagonist bicuculline had no effect on SOX17 expression. EGF, FGF4, PDGF-A, PDGF-B, PDGF-AB, PDGF-C, and PDGF-D and muscimol were selected for additional evaluation during definitive endoderm differentiation.

Example 6

Effects of the Compounds of the Present Invention in Combination with Other Factors on the Differentiation of Human Embryonic Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage in the Absence of Activin A

[0292] A secondary assay was conducted to evaluate the effects of combinations of different compounds with other individual agents on definitive endoderm differentiation. The other agents selected for this screen had previously shown a modest increase in definitive endoderm formation, as tested with Compound 17 and as denoted in **Table 5**. In this screen, a broader panel of compounds was evaluated in with these agents, either in single pair-wise comparisons or pooled combinations.

[0293] *Cell assay seeding:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGELTM (Invitrogen; Cat # 356231) -coated tissue

culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGELTM-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100µl/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF-conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of the assay.

[0294] *Preparation of compounds and growth factors:* Stocks of growth factors purchased from R&D Systems were EGF (Cat # 236-EG), FGF4 (Cat #235-F4), PDGF-A (Cat #221-AA), PDGF-D (Cat #1159-SB), PDGF-A/B (Cat #222-AB), and VEGF (Cat #293-VE). Muscimol was purchased from Tocris (Cat # 0289). All growth factors were solubilized in PBS with 0.1% BSA (Sigma; Cat # A7888) and stored frozen at –80°C. Muscimol was solubilized in 100% DMSO (Sigma; Cat # D2650) and stored frozen at –80°C. Compounds were available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at –80°C. Compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. All growth factors and inhibitors were prepared in a deep well, 96-well polypropylene plate, diluted to 5x intermediate stocks in DMEM:F12 base medium at the beginning of assay and stored at 4°C.

[0295] A secondary screening assay was conducted, testing in triplicate and feeding on alternate days over the four-day assay timeframe. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS to remove residual growth factors and serum. Test volumes of 80µl per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.625% FCS (HyClone; Cat # SH30070.03), 25ng/ml Wnt3a (R&D Systems), and 3.125µM compound plus 20µl 5x stock of growth factor or small molecule to yield a final concentration of 0.5% FCS, 20ng/ml Wnt3a, and 2.5µM. All remaining growth factors were tested at a final assay concentration of 50ng/ml (EGF, FGF4, PDGF-A, PDGF-A/B, VEGF). Final assay concentration of muscimol was 20µM. Positive control wells (100µl/well) contained the same base medium supplemented with 0.5%

FCS, 20ng/ml Wnt3a and 100ng/ml activin A. Negative control wells (100 μ l/well) contained the same base medium with 0.5% FCS and 20ng/ml Wnt3a, omitting activin A.

[0296] On day 3, wells were aspirated and fed with 80 μ l DMEM:F12 base medium supplemented with 2.5% FCS (HyClone) and 3.125 μ M compound plus 20 μ l 5x stock of growth factors or small molecules per well to yield a final concentration of 2% FCS and 2.5 μ M compound (omitting Wnt3a) and as denoted on day one for all remaining growth factors or small molecules. Positive control wells (100 μ l/well) contained the same base medium supplemented with 2% FCS and 100ng/ml activin A, omitting Wnt3a. Negative control wells (100 μ l/well) contained the same base medium with 2% FCS, omitting both activin A and Wnt3a.

[0297] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS, fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0298] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE

Healthcare) software. Segmentation for the nuclei was determined based on grayscale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0299] **Table 5** shows compounds previously identified as hits (**Table 2**) tested in a definitive endoderm bioassay in various combinations with growth factors and muscimol, without activin A. Some compounds had minimal or weak effects on SOX17 expression with all growth factor combinations tested. However, some compounds were able to induce significant SOX17 expression with some but not all growth factor combinations. One compound in particular, Compound 34, had significant synergistic responses with all growth factors tested and mediated increases in both cell numbers as well as SOX17 expression in this assay: Compound 39 with 1) EGF+FGF4 = 77% of positive control response; or 2) EGF+FGF4+PDGF-AB = 68% of positive control response; or 3) EGF+FGF4+PDGF-A+VEGF = 31% of positive control response.

Example 7

Effects of Compound 34 in Combination with Other Factors on the Differentiation of Human Embryonic Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage in the Absence of Activin A

[0300] In this example, an effort was made to analyze the minimum number of growth factors required in combination with the best cyclic aniline-pyridinotriazine compound, Compound 34 to yield a robust SOX17 response in the absence of activin A. Also in this example, a new growth factor, GDF-8, was added for evaluation. GDF-8, also known as myostatin, is a member of the TGF- β family and has been shown to use the activin type II and TGF- β type I receptors (ALK4/5) to induce SMAD 2/3 phosphorylation.

[0301] *Cell assay seeding:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100µl/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.

[0302] *Preparation of compounds and growth factors:* Stocks of growth factors purchased from R&D Systems were EGF (Cat # 236-EG), FGF4 (Cat #235-F4), PDGF-A (Cat #221-AA), PDGF-D (Cat #1159-SB), PDGF-A/B (Cat #222-AB), VEGF (Cat #293-VE), and GDF-8 (Cat # 788-G8). Muscimol was purchased from Tocris (Cat # 0289). All growth factors were solubilized in PBS with 0.1% BSA (Sigma; Cat # A7888) and stored frozen at -80°C. Muscimol was solubilized in 100% DMSO (Sigma; Cat # D2650) and stored frozen at -80°C. Cyclic aniline-pyridinotriazine compounds were available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at -80°C. Compound 34 was further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. All growth factors and inhibitors were prepared in a deep well, 96-well polypropylene plate, diluted to 5x intermediate stocks in DMEM:F12 base medium at the beginning of assay and stored at 4°C.

[0303] A secondary screening assay was conducted, testing in triplicate and feeding on alternate days over the four-day assay timeframe. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS to remove residual growth factors and serum. Test volumes of 80µl per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.625% FCS (HyClone; Cat # SH30070.03), 25ng/ml Wnt3a (R&D Systems), and 3.125µM Compound 27 plus 20µl 5x stock of growth factor or small molecule to yield a final concentration of 0.5% FCS, 20ng/ml Wnt3a, and 2.5µM Compound 34. All

remaining growth factors were tested at a final assay concentration of 50ng/ml (EGF, FGF4, PDGF-A, PDGF-A/B, VEGF) with the exception of GDF-8 tested at 25ng/ml. Final assay concentration of muscimol was 20 μ M. Positive control wells (100 μ l/well) contained the same base medium supplemented with 0.5% FCS, 20ng/ml Wnt3a and 100ng/ml activin A. Negative control wells (100 μ l/well) contained the same base medium with 0.5% FCS and 20ng/ml Wnt3a, omitting activin A.

[0304] On day 3, wells were aspirated and fed with 80 μ l DMEM:F12 base medium supplemented with 2.5% FCS (HyClone) and 3.125 μ M Compound 34 plus 20 μ l 5x stock of growth factors or small molecules per well to yield a final concentration of 2% FCS and 2.5 μ M Compound 34 (omitting Wnt3a) and as denoted on day one for all remaining growth factors or small molecules. Positive control wells (100 μ l/well) contained the same base medium supplemented with 2% FCS and 100ng/ml activin A, omitting Wnt3a. Negative control wells (100 μ l/well) contained the same base medium with 2% FCS, omitting both activin A and Wnt3a.

[0305] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS, fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0306] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated

negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0307] **Table 6** shows results of this assay. Where GDF-8 was present in any combination with the Compound 34, a substantial increase in SOX17 expression was observed. Furthermore, GDF-8 and Wnt3a with Compound 34 were sufficient to yield SOX17 expression (88% of control) in a range similar to that seen with 100ng/ml activin A/Wnt3a treatment. It appears that the growth factor GDF-8 can serve as a replacement for activin A during definitive endoderm differentiation of human embryonic stem cells.

Example 8

Additional Screening for Compounds Capable of Differentiating Pluripotent Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

[0308] Based on the compound structures for hits identified thus far, an analog search was conducted to find additional related compounds to test in the definitive endoderm bioassay. The substructure search yielded compounds for screening. Screening parameters for this assay were designed with the combination of factors that had yielded optimal results in previous assays, specifically combining EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, and GDF-8 with the small molecule compound.

- [0309] *Cell assay seeding:* Briefly, clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 µl/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0310] *Preparation of compounds and assay:* Growth factors purchased from R&D Systems were EGF (Cat # 236-EG), FGF4 (Cat #235-F4), PDGF-A (Cat #221-AA), PDGF-D (Cat #1159-SB), PDGF-A/B (Cat #222-AB), VEGF (Cat #293-VE), and GDF-8 (Cat # 788-G8). Muscimol was purchased from Tocris (Cat # 0289). Screening was conducted using a library of compounds that were made available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO (Sigma; Cat # D2650) and stored at -80°C. The compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. Test conditions were performed in single wells, feeding on alternate days over a four-day assay period. Primary screening assays were initiated by aspirating culture medium from each well followed by three washes in PBS (Invitrogen; Cat # 14190) to remove residual growth factors and serum. On the first day of assay, test volumes of 200µl per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.5% FCS (HyClone; Cat # SH30070.03) and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN) plus 2.5µM compound. All remaining growth factors were tested at a final assay concentration of 50ng/ml (EGF, FGF4, PDGF-A, PDGF-A/B, VEGF) with the exception of GDF-8 tested at 25ng/ml. Final assay concentration of muscimol was 20 µM. Positive control samples contained the same base medium supplemented with 0.5% FCS plus 20 ng/ml Wnt3a and 100 ng/ml recombinant human activin A (PeproTech; Cat #120-14). Negative control samples contained DMEM:F12 base medium supplemented with 0.5% FCS and 20ng/ml Wnt3a. On the third day of assay, test volumes of 200µl per well were

added back containing DMEM:F12 base medium supplemented with 2% FCS plus 2.5 μ M compound, without Wnt3a. All remaining growth factors were tested at a final assay concentration of 50ng/ml (EGF, FGF4, PDGF-A, PDGF-A/B, VEGF) with the exception of GDF-8 tested at 25ng/ml. Final assay concentration of muscimol was 20 μ M. Positive control samples contained the same base medium supplemented with 2% FCS and 100ng/ml recombinant human activin A (PeproTech; Cat #120-14). Negative control samples contained DMEM:F12 base medium supplemented with 2% FCS. Positive control samples contained the same base medium supplemented with FCS, substituting 100ng/ml recombinant human activin A (PeproTech; Cat #120-14) for the aniline-pyridinotriazine compound throughout the four-day assay along with Wnt3a (20ng/ml) on days 1 and 2. Negative control samples contained DMEM:F12 base medium supplemented with FCS, adding Wnt3a on days 1 and 2 but omitting treatment with activin A.

[0311] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0312] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and

subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell times area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0313] In **Table 7**, GDF-8 and a combination of growth factors/agonists (EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol) were tested with a new set of aniline-pyridinotriazine compounds. Results from two assay plates in this single experiment are ranked with respect to SOX17 responses (as a percentage of the positive control treatment with activin A and Wnt3a). Additional compounds were identified that show significant synergistic activity with the growth factor/agonist pool. These compounds were effective in both retaining assay cell number and yielding SOX17 expression during human embryonic stem cell differentiation in the absence of activin A. A list of these hits with greater than 25% activity of the positive control is shown in **Table 8**.

[0314] Of note, four hits from the initial primary screening (**Table 2**) were duplicated in the analog library. Two of these compounds repeated as hits with the analog screening (Compound 34 and Compound 35; shown boxed in **Table 8**); one was a weak hit in the analog screening, and one compound did not repeat.

Example 9

Effects of the Compounds of the Present Invention on the Differentiation of Human Embryonic Stem Cells to Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage in the Presence of Low Concentrations of Activin A

[0315] It was important to determine if the compounds that had been identified as hits in the definitive endoderm bioassays above could also show synergistic activity with very

low doses of activin A. An initial evaluation was performed using the short hit list of cyclic aniline-pyridinotriazine compounds denoted in **Table 3B**.

- [0316] *Cell assay seeding:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGELTM (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGELTM-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 μ l/well. Cells were allowed to attach as clusters and then recover log phase growth over a 1 to 3 day period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0317] *Preparation of compounds and growth factors:* Stocks of growth factors purchased from R&D Systems were EGF (Cat # 236-EG), FGF4 (Cat #235-F4), PDGF-A (Cat #221-AA), PDGF-D (Cat #1159-SB), PDGF-A/B (Cat #222-AB), VEGF (Cat #293-VE), and GDF-8 (Cat #788-G8). Activin A was purchased from PeproTech (Cat #). Muscimol was purchased from Tocris (Cat # 0289). All growth factors were solubilized in PBS with 0.1% BSA (Sigma; Cat # A7888) and stored frozen at -80°C. Muscimol was solubilized in 100% DMSO (Sigma; Cat # D2650) and stored frozen at -80°C. The compounds were available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO and stored at -80°C. The compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. All growth factors and inhibitors were prepared in a deep well, 96-well polypropylene plate, diluted to 5x intermediate stocks in DMEM:F12 base medium at the beginning of assay and stored at 4°C.
- [0318] A secondary screening assay was conducted, testing in triplicate and feeding on alternate days over the four-day assay timeframe. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS to remove residual growth factors and serum. Test volumes of 80 μ l per well were added back containing DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.625% FCS (HyClone; Cat # SH30070.03), 25ng/ml Wnt3a (R&D Systems), 12.5ng/ml

activin A, and 3.125 μ M compound plus 20 μ l 5x stock of growth factor or small molecule to yield a final concentration of 0.5% FCS, 20ng/ml Wnt3a, 10ng/ml activin A, and 2.5 μ M compound. All remaining growth factors were tested at a final assay concentration of 50ng/ml (EGF, FGF4, PDGF-A, PDGF-A/B, VEGF), with the exception of GDF-8 used at 25ng/ml. Final assay concentration of muscimol was 20 μ M. Positive control wells (100 μ l/well) contained the same base medium supplemented with 0.5% FCS, 20ng/ml Wnt3a and 10ng/ml (low dose) or 100ng/ml (high dose) activin A. Negative control wells (100 μ l/well) contained the same base medium with 0.5% FCS and 20ng/ml Wnt3a, omitting activin A.

[0319] On day 3, wells were aspirated and fed with 80 μ l DMEM:F12 base medium supplemented with 2.5% FCS (HyClone), 12.5ng/ml activin A, and 3.125 μ M compound plus 20 μ l 5x stock of growth factors or small molecules per well to yield a final concentration of 2% FCS, 10ng/ml activin A, and 2.5 μ M compound (omitting Wnt3a) and as denoted on day one for all remaining growth factors or small molecules. Positive control wells (100 μ l/well) contained the same base medium supplemented with 2% FCS and 10ng/ml or 100ng/ml activin A, omitting Wnt3a. Negative control wells (100 μ l/ well) contained the same base medium with 2% FCS, omitting both activin A and Wnt3a.

[0320] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS, fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

- [0321] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.
- [0322] **Table 9** shows results from assay of various compounds and different combinations of growth factors with low doses of activin A. Some compounds showed robust synergistic responses with various growth factors. In other cases, the synergistic effects were more modest but significant relative to a low dose activin A control. Other compounds had no activity relative to the low dose activin A control.

Example 10

Effects of the Compounds of the Present Invention on the Differentiation of Single Human Embryonic Stem Cells to Cells Expressing Markers of the Definitive Endoderm Lineage in the Absence of Activin A

- [0323] Cyclic aniline-pyridinotriazine compounds were also tested in a screening format using cells dispersed through enzymatic treatment to single cells and plated in monolayer for assay. The assay also made changes to eliminate serum that can provide growth factors even at low doses. To that end the basal medium was changed and serum was replaced with fatty acid free BSA. The assay was shortened from four days to three days to provide a more narrow timeframe to measure results. Finally,

the assay included two growth factors, EGF and FGF4 that had previously shown significant but sub-optimal effects on definitive endoderm differentiation in the absence of activin A.

Screening Assay

- [0324] *Cell assay seeding:* Briefly, clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cultures were treated with Accutase (Sigma; Cat # A6964), using equivalent volumes of 10ml per 10cm² surface area for 5 minutes at 37°C, then gently resuspended, pelleted by centrifugation, and resuspended in MEF conditioned medium for counting. For assay seeding, cells were plated at 50,000 cells/cm² on reduced growth factor MATRIGEL™-coated 96-well black plates (Packard ViewPlates; Cat #6005182) using volumes of 100µl/well. Cells were allowed to attach and recover log phase growth over a 3 to 5 day period, feeding daily with MEF conditioned medium supplemented with 8 ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0325] *Preparation of compounds and assay:* Stocks of EGF and FGF4 were prepared in a 96-well polypropylene plate (Corning, Inc.; Cat # 3960). Compound 22 was available as a 5 mM stock solubilized in 100% DMSO (Sigma; Cat # D2650) and stored at – 80°C. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS to remove residual growth factors and serum. Test volumes of 80µl per well were added back containing RPMI 1640 base medium (Invitrogen; Cat # 22400-089) supplemented with 2.5% fatty acid free BSA (MP Biomedicals LLC; Cat # 152401), 10ng/ml bFGF (PeproTech Inc; Cat # 100-18B), 25ng/ml Wnt3a (R&D Systems; Cat # 1324-WN) and 3.125µM Compound 22 plus 20µl 5x stock of growth factors to yield a final concentration of 2% fatty acid free BSA, 8ng/ml bFGF(PeproTech Inc; Cat # 100-18B), 20ng/ml Wnt3a, and 2.5µM Compound 22 in assay. Positive control wells contained the same base medium supplemented with 2% fatty acid free BSA, 8ng/ml bFGF, 20ng/ml Wnt3a, and 100ng/ml recombinant human activin A (PeproTech; Cat #120-14). Negative control wells contained the same base medium supplemented with 2% fatty acid free BSA, 8ng/ml bFGF, 20ng/ml Wnt3a but omitted treatment with activin A.

- [0326] On the second day of assay, wells were again aspirated and fed with 80µl per well were added back containing RPMI 1640 base medium supplemented with 2.5% fatty acid free BSA, 10ng/ml bFGF, and 3.125µM Compound 22 plus 20µl 5x stock of growth factors to yield a final concentration of 2% fatty acid free BSA, 8ng/ml bFGF and 2.5µM Compound 22 in assay. Positive control wells contained the same base medium supplemented with 2% fatty acid free BSA, 8ng/ml bFGF and 100ng/ml recombinant human activin A. Negative control samples contained the same base medium supplemented with 2% fatty acid free BSA and 8ng/ml bFGF but omitted treatment with activin A.
- [0327] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS, fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4µg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100µl/well PBS for imaging.
- [0328] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard

deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

[0329] **Table 10** shows results of this assay with Compound 34. Control samples with EGF and/or FGF4 alone without the Compound 34 had low SOX17 expression. Addition of Compound 34 added significant enhancement of SOX17 expression.

Example 11

A Comparison of the Ability of Activin A and GDF-8 to Differentiate Human Embryonic Stem Cells to Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

[0330] A previous example showed that GDF-8 is able to replace activin A to differentiate human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage. It was important to know the relative potencies of GDF-8 and activin A with respect to their ability to differentiate human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage. A dose response assay was conducted using equivalent concentrations of each growth factor to compare results during embryonic stem cell differentiation.

[0331] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen, Cat #: 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human embryonic stem cell lines were maintained at

passage numbers less than 50 and routinely evaluated for normal karyotypic phenotype and for absence of mycoplasma contamination.

[0332] Cell clusters used in the assay were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and seeded onto reduced growth factor MATRIGELTM-coated 96-well Packard VIEWPLATES (PerkinElmer; Cat # 6005182) in volumes of 100µl/well. MEF conditioned medium supplemented with 8ng/ml bFGF was used for initial plating and expansion. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.

[0333] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back an aliquot (100µl) of test medium. Test conditions were performed in quadruplicate over a total three-day assay period, feeding on day 1 and day 2 by aspirating and replacing the medium from each well with fresh test medium. Two 12-channel polypropylene basins (Argos technologies, Inc, Cat #: B3135) were used to make the test media containing different concentrations of Activin A (PeproTech; Cat #120-14) or GDF-8 (R&D Systems, Cat # 788-G8). Channels numbered 2 through 12 of each basin contained 1ml assay medium composed of RPMI-1640 medium (Invitrogen; Cat #: 22400) supplemented with 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (MP Biomedicals, Inc; Cat # 152401) and 8ng/ml bFGF (PeproTech Inc.; Cat #: 100-18B), and with 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF) added on day 1, omitted on day 2 and 3. Channel number 1 of each basin contained 1600 ng/ml Activin A or 1600 ng/ml GDF-8, diluted into the same assay medium. One ml of medium was transferred from channel number 1 to channel number 2 and mixed well. A fresh pipette tip was used to transfer one ml of medium from channel number 2 to channel number 3, followed by thorough mixing. The same procedure was repeated in sequence through channel number 11 for each respective basin. Channel number 12 of each basin contained medium without Activin A or GDF-8. By doing this, a series of two-fold test dilutions was created, containing Activin A or GDF-8 at concentrations ranging from 1.6ng/ml to 1600 ng/ml, for addition to the respective assay wells.

[0334] *High Content Analysis:* At the conclusion of three days of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat #A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0335] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total SOX17 intensity in each well were obtained using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each quadruplicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 to 4500. Total SOX17 intensity data were calculated using GraphPad Prism 4.02 (GraphPad Software, Inc., Lo Jolla, CA). Data were normalized to define the smallest and largest values in each data set as 0% and 100%, respectively. **Table 11** shows the normalized values for each of the activin A and GDF-8 data sets. Two sigmoidal dose-response curves are shown in **Figure 2** as generated using the normalized values shown in **Table 11**. The R^2 values, indicating curve fit, were calculated using GraphPad Prism and determined to be 0.9944 for activin A and 0.9964 for GDF-8. Using GraphPad Prism, EC_{50} values for each growth factor were calculated and determined to be 13.9 ng/ml for activin A and 184.8 ng/ml for GDF-8. These data indicate that GDF-8 is less potent than activin A

with respect to inducing human embryonic stem cells to differentiate to cells expressing markers characteristic of the definitive endoderm lineage. Nonetheless, GDF-8 can substitute for activin A and at specific concentrations, can induce an equivalent population of definitive endoderm cells, as denoted by SOX17 expression.

Example 12

Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage that were Formed According to the Methods of the Present Invention are able to Further Differentiate into Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0336] Parallel populations of human embryonic stem cells were differentiated to cells expressing markers characteristic of the definitive endoderm lineage using GDF-8 in combination with either Compound 34 or Compound 56. Thereafter, a step-wise differentiation protocol was applied to treated cells to promote differentiation toward pancreatic endoderm and endocrine lineages. A parallel control consisting of cells treated with Activin A and Wnt3a was maintained for comparison purposes throughout the step-wise differentiation process. Samples were taken at every stage of the differentiation to determine the appearance of proteins and mRNA biomarkers representative of the various stages of differentiation.

[0337] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGELTM-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human ES cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma.

- [0338] Cell clusters were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and seeded onto reduced growth factor MATRIGELTM-coated 24-well, black wall culture plates (Arctic White; Cat # AWLS-303012) in volumes of 0.5ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout the duration of assay.
- [0339] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back an aliquot (0.5ml) of test medium. Test conditions for the first step of differentiation were conducted over a three-day period, feeding daily by aspirating and replacing the medium from each well with fresh test medium. On the first day of assay, 100ng/ml activin A (PeproTech; Cat #120-14) or 200ng/ml GDF-8 (R&D Systems, Cat # 788-G8) was added to respective assay wells where each growth factor was diluted into RPMI-1640 medium (Invitrogen; Cat # 22400) with 1% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (MP Biomedicals, Inc; Cat # 152401), 1% Probumin (Millipore; Cat # 81-068-3) and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF). On the second day of assay, 100ng/ml activin A or 200ng/ml GDF-8 was diluted into RPMI-1640 medium supplemented with 2% FAF BSA without Wnt3a. In some test samples using GDF-8, Wnt3a was replaced with a either Compound 34 or Compound 56 at a concentration of 2.5μM, and either Compound 34 or Compound 56 was added daily during all three days of definitive endoderm differentiation. At the conclusion of the first step of differentiation, cells from some wells were harvested for flow cytometry analysis to evaluate levels of CXCR4, a marker of definitive endoderm formation. Additional wells were harvested for RT-PCR analysis to measure other markers of differentiation.
- [0340] At the conclusion of the first step of differentiation, replicate sets of parallel wells from each treatment group were subjected to further step-wise differentiation. It is important to note that after the first differentiation step, all wells undergoing continuing culture and differentiation received the same treatment. The protocol for this continuing differentiation is described below.
- [0341] Step 2 of the differentiation protocol was carried out over two days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM:F12 medium (Invitrogen; Cat # 11330-032) containing 2%

Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (MP Biomedicals, Inc; Cat # 152401), 50ng/ml FGF7 (PeproTech; Cat # 100-19), and 250nM cyclopamine (Calbiochem; Cat # 239804).

[0342] Step 3 of the differentiation protocol was carried out over four days. Cells were fed daily by aspirating medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 1% B27 (Invitrogen; Cat # 17504-044), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine (Calbiochem; Cat # 239804), and 2 μ M all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625). At the conclusion of the third step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of Pdx1, a transcription factor associated with pancreatic endoderm, and Cdx2, a transcription factor associated with intestinal endoderm.

[0343] Step 4 of the differentiation protocol was carried out over three days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM-high glucose supplemented with 1% B27, 100 ng/ml Noggin, 100ng/ml Netrin-4, 1 μ M DAPT (EMD Biosciences; Cat #565770), and 1 μ M Alk 5 inhibitor (Axxora; Cat # ALX-270-445). At the conclusion of the fourth step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of PDX1.

[0344] Step 5 of the differentiation protocol was carried out over seven days in DMEM-high glucose with 1% B27, and 1 μ M Alk 5 inhibitor. Medium in each well was aspirated and replaced with a fresh aliquot (0.5ml) on all days. At the conclusion of the fifth step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of insulin and glucagon.

[0345] Step 6 of the differentiation protocol was carried out over seven days in DMEM-high glucose with 1% B27. Medium in each well was aspirated and replaced with a fresh aliquot (0.5ml) on alternating days. At the conclusion of the sixth step of

differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation.

[0346] *FACS Analysis:* Cells for FACS analysis were blocked in a 1:5 solution of 0.5% human gamma-globulin (Sigma; Cat# G-4386) in PBS (Invitrogen; Cat # 14040-133): BD FACS staining buffer – BSA (BD; Cat #554657) for 15 minutes at 4°C. Cells were then stained with antibodies for CD9 PE (BD; Cat # 555372), CD99 PE (Caltag; Cat # MHCD9904) and CXCR4 APC (R&D Systems; Cat# FAB173A) for 30 minutes at 4°C. After a series of washes in BD FACS staining buffer, the cells were stained for viability with 7-AAD (BD; Cat # 559925) and run on a BD FACSArray. A mouse IgG1K Isotype control antibody for both PE and APC was used to gate percent positive cells.

[0347] *RT-PCR Analysis:* RNA samples were purified by binding to a silica-gel membrane (Rneasy Mini Kit, Qiagen, CA) in the presence of an ethanol-containing, high-salt buffer followed by washing to remove contaminants. The RNA was further purified using a TURBO DNA-free kit (Ambion, INC), and high-quality RNA was then eluted in water. Yield and purity were assessed by A260 and A280 readings on a spectrophotometer. CDNA copies were made from purified RNA using an ABI (ABI, CA) high capacity cDNA archive kit.

[0348] Unless otherwise stated, all reagents were purchased from Applied Biosystems. Real-time PCR reactions were performed using the ABI PRISM® 7900 Sequence Detection System. TAQMAN® UNIVERSAL PCR MASTER MIX® (ABI, CA) was used with 20 ng of reverse transcribed RNA in a total reaction volume of 20 µl. Each cDNA sample was run in duplicate to correct for pipetting errors. Primers and FAM-labeled TAQMAN®probes were used at concentrations of 200 nM. The level of expression for each target gene was normalized using a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control previously developed by Applied Biosystems. Primer and probe sets are listed in **Table 12**. After an initial incubation at 50°C for 2 min followed by 95°C for 10 min, samples were cycled 40 times in two stages - a denaturation step at 95°C for 15 sec followed by an annealing/extension step at 60°C for 1 min. Data analysis was carried out using GENEAMP®7000 Sequence Detection System software. For each primer/probe set, a Ct value was determined as the cycle number at which the fluorescence intensity

reached a specific value in the middle of the exponential region of amplification. Relative gene expression levels were calculated using the comparative Ct method. Briefly, for each cDNA sample, the endogenous control Ct value was subtracted from the gene of interest Ct to give the delta Ct value (ΔCt). The normalized amount of target was calculated as $2^{-\Delta Ct}$, assuming amplification to be 100% efficiency. Final data were expressed relative to a calibrator sample.

[0349] *High Content Analysis:* At the conclusion of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat # A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging. Other primary antibodies used for analysis included 1:100 dilution mouse anti-human CDX2 (Invitrogen; Cat # 397800), 1:100 dilution goat anti-human Pdx1 (Santa Cruz Biotechnology; Cat # SC-14664), 1:200 dilution rabbit anti-human insulin (Cell Signaling; Cat # C27C9), and 1:1500 dilution mouse anti-human glucagon (Sigma-Aldrich; Cat # G2654). Secondary antibodies used for analysis included 1:400 dilution Alexa Fluor 647 chicken anti-mouse IgG (Invitrogen; Cat # A-21463), 1:200 dilution Alexa Fluor 488 donkey anti-goat IgG (Invitrogen; Cat # A11055), 1:1000 dilution Alexa Fluor 647 chicken anti-rabbit IgG (Invitrogen; Cat # A21443), and 1:1000 dilution Alexa Fluor 488 chicken anti-mouse IgG (Invitrogen; Cat # A21200).

[0350] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total intensity were

obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 and 4500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

- [0351] PCR results for representative differentiation markers are shown in **Table 13** for cells harvested from each step of differentiation. Samples treated with GDF-8 and Wnt3a or with GDF-8 and either Compound 34 or Compound 56 showed similar, or in some instances, improved expression levels of expression markers associated with endodermal and endocrine differentiation.
- [0352] **Figure 3** shows the results of the FACS analysis, showing the expression of the definitive endoderm marker, CXCR4, after the first step of differentiation. Treatment of human embryonic stem cells with GDF-8 and Wnt3a yielded an equivalent percentage of CXCR4 positive cells compared to treatment with activin A and Wnt3a. Similarly, treatment of human embryonic stem cells with GDF-8 and a small molecule (Compound 34 or Compound 56) also yielded an equivalent or higher percentage of CXCR4 positive cells. **Figure 4** shows high content image analysis for normalized SOX17 protein expression in human embryonic stem cells after three days differentiation to definitive endoderm. Levels of expression for treatment groups using GDF-8 with Wnt3a or GDF-8 with a small molecule are similar to treatment with Activin A and Wnt3a.
- [0353] **Figure 5** shows high content image analysis for normalized Pdx1 and Cdx2 protein expression in human embryonic stem cells after the third step of differentiation to pancreatic endoderm. Levels of expression for treatment groups using GDF-8 with Wnt3a or GDF-8 with Wnt3a or GDF-8 with a compound of the present invention show equivalent levels of PDX1 and CDX2. In some treatment groups the cell number retained after differentiation decreased thereby increasing the ratio of PDX1 expressing cells. Similar results were obtained showing equivalent normalized PDX1 expression in all treatment groups after the fourth step of differentiation as shown in

Figure 6. In **Figure 7**, normalized protein levels of insulin and glucagon are shown, demonstrating equivalent expression between the Activin A and GDF-8 treatment groups.

- [0354] These collective results demonstrate that GDF-8, in combination with Wnt3a or Compound 34 or Compound 56, can substitute for activin A during definitive endoderm differentiation and subsequent pancreatic endoderm and endocrine differentiation.

Example 13

Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage with Other Members of the GDF Family of Proteins

- [0355] It was important to determine if treating human embryonic stem cells with other GDF family members could the formation of cells expressing markers characteristic of the definitive endoderm lineage. Wnt3a in combination with either Compound 34 or Compound 56 were tested on human embryonic stem cells in combination with six different GDF growth factors [GDF-3, GDF-5, GDF-8, GDF-10, GDF-11, and GDF-15] to determine the ability of members of the GDF family of proteins to differentiate human embryonic stem cells toward cells expressing markers characteristic of the definitive endoderm lineage. A parallel control of cells treated with activin A and Wnt3a was maintained for comparison purposes.
- [0356] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human ES cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma.

[0357] Cell clusters were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and seeded onto reduced growth factor MATRIGEL™-coated 96-well Packard VIEWPLATES (PerkinElmer; Cat # 6005182) in volumes of 0.1ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout the duration of assay.

[0358] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back aliquots (100µl) of test medium. Test conditions were performed in triplicate over a total four-day assay period, feeding on day 1 and day 3 by aspirating and replacing the medium from each well with fresh test medium. Various members of the GDF family of proteins were obtained for testing as follows: GDF-3 (PeproTech; Cat # 120-22); GDF-5 (DePuy Orthopaedics, Inc., a Johnson & Johnson company); GDF-8 (R&D Systems; Cat # 788-G8); GDF-10 (R&D Systems; Cat # 1543-BP); GDF11 (PeproTech; Cat # 120-11); GDF-15 (R&D Systems; Cat # 957-GD). On the first day of assay, all wells received an aliquot (80µl) of basal medium DMEM:F12 medium (Invitrogen; Cat # 11330-032) supplemented with 0.5% fetal bovine serum (Hyclone; Cat # SH30070.03). A series of five different control or experimental test samples was created to evaluate activin A or various GDFs in combination with Wnt3a or Compound 34 or Compound 56. These test samples were added in 20µl aliquots (5x concentrated) to appropriately matched assay wells to yield a final assay volume of 100µl in each well at the final assay conditions indicated. In the first set of control samples, the following conditions were tested: 1) no additive (i.e. no supplementary growth factor or small molecule); 2) 100ng/ml activin A (PeproTech; Cat #120-14) in combination with 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF); 3) 20ng/ml Wnt3a alone; 4) Compound 34 alone (2.5µM) without any growth factor or small molecule; 5) Compound 56 alone (2.5µM) without any growth factor or small molecule. In the second set of test samples, the following conditions were tested in combination with 100ng/ml GDF3: 1) no additive (i.e. GDF-3 alone); 2) 20ng/ml Wnt3a; 3) 20ng/ml Wnt3a with Compound 34 (2.5µM); 4) Compound 34 (2.5µM); 5) Compound 56 (2.5µM); and 6) 20ng/ml Wnt3a with Compound 56 (2.5µM). In the third set of test samples, each of the six conditions was combined with 100ng/ml GDF-5. In the fourth set of test samples, each of the six conditions

was combined with 100ng/ml GDF-8. In the fifth set of test samples, each of the six conditions was combined with 100ng/ml GDF-10. In the sixth set of test samples, each of the six conditions was combined with 100ng/ml GDF-11. In the seventh set of test samples, each of the six conditions was combined with 100ng/ml GDF-15. On the third day of assay, all wells for all test samples, received 100ng/ml Activin A or 100ng/ml respective GDF growth factor, without Wnt3a or Compound 34 or Compound 56, diluted into DMEM:F12 medium supplemented with 2% FBS.

[0359] *High Content Analysis:* At the conclusion of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat #A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5µg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at room temperature. Plates were washed once with PBS and left in 100µl/well PBS for imaging.

[0360] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 and 4500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

- [0361] **Figure 8** shows high content image analysis for SOX17 protein expression in human embryonic stem cells after four days differentiation to definitive endoderm. In each case, results are normalized to the positive control treatment with activin A and Wnt3a. In **Figure 8A**, only the positive control treatment yielded significant expression of SOX17; treatment with Wnt3a alone or either Compound 34 or Compound 56 alone failed to induce SOX17 expression. In **Figure 8**, panels **B** through **G**, normalized SOX17 expression levels are shown for each GDF growth factor substituting for activin A in the respective treatments. GDF-3 (**Figure 8B**) and GDF-5 (**Figure 8C**) induced weak expression of SOX17 and only in test samples where one of the compounds of the present invention was present. GDF10 (**Figure 8D**), GDF11 (**Figure 8E**) and GDF15 (**Figure 8G**) induced significant levels of SOX17 expression, more than observed with GDF3 or 5 treatments but less than observed that observed with activin A and Wnt3a treatment. In general, SOX17 expression was negligible when GDF-10, GDF-11, or GDF-15 was combined with Wnt3a, but improved in combination with one of the compounds of the present invention; in particular when combined with Compound 34. **Figure 8D** shows results for treatment groups using GDF-8 where GDF-8 in combination with either Compound 34 or Compound 56 caused a robust induction of SOX17, exceeding results seen with the activin A/Wnt3a positive control. In some of these examples, the presence of Compound 34 or Compound 56 combined with a GDF growth factor also caused an increase in cell number during differentiation.
- [0362] These collective results demonstrate that GDF-8 was superior to all other GDF family members tested when used in combination with Compound 34 or Compound 56, and could substitute for activin A during definitive endoderm differentiation.

Example 14

Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage with Other Members of the TGF Superfamily of Proteins

- [0363] It was important to determine if treating human embryonic stem cells with other TGF superfamily members could facilitate the formation of cells expressing markers characteristic of the definitive endoderm lineage. Compound 34 and Wnt3a were tested on human embryonic stem cells in combination with either TGF β -1, BMP2,

BMP3, or BMP4 to determine the ability of members of the TGF superfamily members to differentiate human embryonic stem cells toward cells expressing markers characteristic of the definitive endoderm lineage. In parallel, two different commercial sources of GDF-8 were tested with Wnt3a for their ability to differentiate human embryonic stem cells toward cells expressing markers characteristic of the definitive endoderm lineage. A positive control using activin A with Wnt3a was maintained for comparison purposes.

- [0364] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGELTM-(BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma.
- [0365] Cell clusters were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and seeded onto reduced growth factor MATRIGELTM-coated 96-well Packard VIEWPLATES (PerkinElmer; Cat # 6005182) in volumes of 0.1ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout assay.
- [0366] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back aliquots (100μl) of test medium. Test conditions were performed in triplicate over a total three day assay period, feeding on day 1 and day 2 by aspirating and replacing the medium from each well with fresh test medium. Various growth factor proteins were obtained for testing as follows: BMP-2 (R&D Systems; Cat # 355-BM); BMP-3 (R&D Systems; Cat # 113-BP); BMP-4 (R&D Systems; Cat # 314-BP); TGFβ-1 (R&D Systems; Cat # 240-B); GDF-8 (PeproTech; Cat # 120-00);

GDF-8 (Shenandoah; Cat #100-22); and activin A (PeproTech; Cat #120-14). On the first day of assay, each well was treated with 80µl of growth medium [RPMI-1640 (Invitrogen; Cat #: 22400) containing 2.5% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (MP Biomedicals, Inc; Cat # 152401), and 10ng/ml bFGF]. In some wells, 25ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF) was added to the growth medium to yield a final assay concentration of 20ng/ml. In some wells, activin A was added to the growth medium to yield a final assay concentration of 100ng/ml. In some wells, 3.125µM Compound 34 was added to the growth medium to yield a final assay concentration of 2.5µM. A dose titration of additional growth factors (5x concentrated, diluted in RPMI-1640) was also added to respective test wells to yield a final assay volume of 100µl in each well for all treatment conditions. On the second day of assay, Wnt3a and Compound 34 were omitted from assay. All wells received 80µl of growth medium [RPMI-1640 containing 2.5% FAF BSA, and 10ng/ml bFGF] and 20µl of respective growth factor dilution (5x concentrated, diluted in RPMI-1640). Comparative controls for this assay included: 1) no added growth factors; 2) Wnt3a alone; and 3) activin A with Wnt3a. Each commercial source of GDF-8 was tested in combination with Wnt3a. Each of the BMP growth factors, as well as TGFβ-1, was tested in combination with Wnt3a, with Compound 34, and with both Wnt3a in combination with Compound 34.

[0367] *High Content Analysis:* At the conclusion of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat # A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5µg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at

room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

[0368] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 and 4500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

[0369] **Figure 9** shows high content image analysis for SOX17 protein expression in human embryonic stem cells after three days differentiation to definitive endoderm. In each case, results are normalized to the positive control treatment for activin A with Wnt3a. The results in **Figure 9A**, show that treatment with growth medium alone, or Wnt3a alone failed to induce SOX17 expression; only the addition of activin A caused a robust expression of SOX17. In **Figure 9, panels B and C**, results for each of the commercial sources of GDF-8 are depicted, showing differences in potency between the two vendors. Although less potent than activin A, there was significant induction of SOX17 expression in cells treated with GDF-8 in combination with Wnt3a. In **Figure 9, panels D, E, F and G**, results are shown for definitive endoderm differentiation using BMP2, BMP3, BMP4, and TGF β -1, incorporating a dose titration for each growth factor in combination with Wnt3a, or Compound 34, or both Wnt3a with Compound 34. Although some treatments had a significant effect on cell numbers at the conclusion of assay (e.g. BMP2 and BMP4), induction of SOX17 expression resulting from any of these growth factors and treatment combinations was weak or negligible compared to the Wnt3a treatment alone.

Example 15

Dose Ranging Studies for Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage with a Selection of the Compounds of the Present Invention

- [0370] It was important to know the optimal working concentrations for Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, and Compound 34 that would mediate the formation of cells expressing markers characteristic of the definitive endoderm lineage. In conjunction, side-by-side comparisons were performed for titrations of each compound in combination with activin A or GDF-8 in the definitive endoderm assay. Finally, the duration of exposure for each compound was tested in assay, also in combination with activin A or GDF-8, adding compound only on the first day of assay or throughout all three days of definitive endoderm formation.
- [0371] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium supplemented with 8ng/ml bFGF (PeproTech Inc.; Cat # 100-18B) with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma.
- [0372] Cell clusters were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and seeded onto reduced growth factor MATRIGEL™-coated 96-well Packard VIEWPLATES (PerkinElmer; Cat # 6005182) in volumes of 0.1ml/well. Daily feeding was conducted by aspirating spent culture medium from

each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout the duration of assay.

[0373] *Assay:* Assay was initiated by aspirating the culture medium from each well and adding back aliquots (100µl) of test medium. Test conditions were performed in quadruplicate over a total four-day assay period, feeding daily by aspirating and replacing the medium from each well with fresh test medium. Each well was treated with 80µl of growth medium [RPMI-1640 (Invitrogen; Cat #: 22400) containing 2.5% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (MP Biomedicals, Inc; Cat # 152401), 10ng/ml bFGF, and additional growth factors (1.25x concentrated)] and 20µl of test compound (5x concentrated diluted in RPMI-1640) to yield a final assay volume of 100ul in each well. Test compounds in this assay included six of the compounds of the present invention: Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, and Compound 34, and a commercial GSK3i inhibitor BIO (EMD Chemicals, Inc.; Cat # 361550). On the first day of assay, wells were treated with various control or experimental conditions. Control conditions, with final assay concentrations as indicated, were as follows: 1) growth medium alone; 2) 20ng/ml Wnt3a only R&D Systems; Cat # 1324-WN/CF); 3) 100ng/ml activin A (PeproTech; Cat #120-14); 4) 100ng/ml activin A and 20ng/ml Wnt3a; 5) 100ng/ml GDF-8 (R&D Systems, Cat # 788-G8); 6) 100ng/ml GDF-8 and 20ng/ml Wnt3a. Test compounds were diluted two-fold in series to yield a concentration range from 78nM to 10µM in the final assay. Experimental test samples combined each individual compound dilution series with 100ng/ml activin A or 100ng/ml GDF-8, both treatment sets in the absence of Wnt3a. On the second and third day of assay, some wells continued to be treated with 20ng/ml Wnt3a or diluted test compound in combination with either activin A or GDF-8. In other wells, activin A or GDF-8 treatment continued on the second and third day of assay, but Wnt3a or diluted test compound was removed.

[0374] *High Content Analysis:* At the conclusion of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times

with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat #A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5µg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at room temperature. Plates were washed once with PBS and left in 100µl/well PBS for imaging.

[0375] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 and 4500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.

[0376] *Results:* High content analysis results are shown for SOX17 expression in **Figures 10 - 14** and resulting cell number at the conclusion of assay in **Figures 15 - 19**. In **Figure 10**, results are shown for SOX 17 expression resulting from control treatments using activin A or GDF-8, either alone or in combination with Wnt3a. Activin A treatments resulted in significantly higher SOX17 expression than was observed with GDF-8 treatment. Similarly, as seen in **Figure 15**, activin A treatment resulted in a higher number of cells at the conclusion of assay than was seen with GDF-8 treatment, regardless of whether Wnt3a was present for one or three days during assay. Adding any of Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, or Compound 34 with activin A treatment did not enhance SOX 17 expression (**Figures 11 -12**) or increase cell numbers (**Figures 17-18**), regardless of whether the compound was present for one day at the initiation of assay or three

days throughout the duration of assay. However, treatment with either Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, or Compound 34 in combination with GDF-8 significantly improved SOX17 expression (**Figures 13-14**) and also enhanced cell numbers at the end of assay (**Figures 18-19**). When either Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, or Compound 34 and GDF-8 were used in combination, the improvements to SOX17 expression and cell number in many cases were equivalent to results observed with activin A treatment. Improved differentiation in combination with GDF-8 was apparent in a dose titration effect for many of the compounds, although toxicity was sometimes observed at the highest concentrations. In most cases, optimal beneficial effects from treatment with the compound and GDF-8 were apparent with only one day of compound exposure at the initiation of assay. In some cases, presence of the compound throughout the duration of assay had no detrimental effect or had a slight beneficial effect. From these collective results an optimal working concentration range for each compound in combination with GDF8 treatment was determined. Results were compound specific, generally in the 1-10 μ M range as tested in this assay.

Example 16

Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage that were Formed Without According to the Methods of the Present Invention are able to Further Differentiate into Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0377] Additional small molecules were tested in combination with GDF-8 for definitive endoderm differentiation. These included a commercial inhibitor of GSK3 as well as compounds of the present invention. A step-wise differentiation protocol was applied to cells treated with GDF-8 in combination with various small molecules. The efficacy of differentiation was determined by gene expression for biomarkers representative the pancreatic endoderm, or pancreatic endocrine lineages. A parallel control sample of cells treated with activin A and Wnt3a was maintained for comparison purposes throughout the step-wise differentiation process.

- [0378] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen, Cat #: 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma.
- [0379] Cell clusters were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and plated onto reduced growth factor MATRIGEL-coated 24-well, black wall culture plates (Arctic White; Cat # AWLS-303012) in volumes of 0.5ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout the duration of assay.
- [0380] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back an aliquot (0.5ml) of test medium. Test conditions for the first step of differentiation were conducted over a three-day period, feeding daily by aspirating and replacing the medium from each well with fresh test medium. On the first day of assay, 100ng/ml activin A (PeproTech; Cat #120-14) or 100ng/ml GDF-8 (R&D Systems, Cat # 788-G8) was added to respective assay wells where each growth factor was diluted into RPMI-1640 medium (Invitrogen; Cat #: 22400) with 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (Proliant Inc. Cat #: SKU 68700), and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF). On the second day of assay, 100ng/ml activin A or 100ng/ml GDF-8 was diluted into RPMI-1640 medium supplemented with 2% FAF BSA without Wnt3a. In some test samples using GDF-8, Wnt3a was replaced with a small molecule compound, added only on the first day of definitive endoderm differentiation. These small molecules included Compound 19 (2.5µM in assay), Compound 202 (2.5µM in assay), Compound 40 (2.5µM in assay),

or a commercially available GSK3 inhibitor BIO (0.5 μ M in assay) (EMD Chemicals, Inc.; Cat # 361550). At the conclusion of the first step of differentiation, cells from some wells were harvested for flow cytometry analysis to evaluate levels of CXCR4, a marker of definitive endoderm formation. Additional wells were harvested for RT-PCR analysis to measure other markers of differentiation.

[0381] At the conclusion of the first step of definitive endoderm differentiation, replicate sets of parallel wells from each treatment group were subjected to further step-wise differentiation. It is important to note that after the first differentiation step, all wells undergoing subsequent culture and differentiation received the same treatment. The protocol for this continuing differentiation is described below.

[0382] Step 2 of the differentiation protocol was carried out over two days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM:F12 medium (Invitrogen; Cat # 11330-032) containing 2% FAF BSA, 50ng/ml FGF7 (PeproTech; Cat # 100-19), and 250nM cyclopamine-KAAD (Calbiochem; Cat # 239804).

[0383] Step 3 of the differentiation protocol was carried out over seven days. Cells were fed daily by aspirating medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 0.1% Albumax (Invitrogen; Cat #: 11020-021), 0.5x Insulin-Transferrin-Selenium (ITS-X; Invitrogen; Cat # 51500056), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine, and 2 μ M all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625). At the conclusion of the third step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of Pdx1, and Cdx2.

[0384] Step 4 of the differentiation protocol was carried out over three days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM-high glucose supplemented with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, 100 ng/ml Noggin, and 1 μ M Alk 5 inhibitor (Axxora; Cat # ALX-270-445). At the conclusion of the fourth step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of

differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of Pdx1.

[0385] Step 5 of the differentiation protocol was carried out over seven days in DMEM-high glucose with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, and 1 μ M Alk 5 inhibitor. Medium in each well was aspirated and replaced with a fresh aliquot (0.5ml) on all days. At the conclusion of the fifth step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of insulin and glucagon.

[0386] *FACS Analysis:* Cells for FACS analysis were blocked in a 1:5 solution of 0.5% human gamma-globulin (Sigma; Cat# G-4386) in PBS (Invitrogen; Cat # 14040-133): BD FACS staining buffer – BSA (BD; Cat #554657) for 15 minutes at 4°C. Cells were then stained with antibodies for CD9 PE (BD; Cat # 555372), CD99 PE (Caltag; Cat # MHCD9904) and CXCR4 APC (R&D Systems; Cat# FAB173A) for 30 minutes at 4°C. After a series of washes in BD FACS staining buffer, the cells were stained for viability with 7-AAD (BD; Cat # 559925) and run on a BD FACSArray. A mouse IgG1K Isotype control antibody for both PE and APC was used to gate percent positive cells.

[0387] *RT-PCR Analysis:* RNA samples were purified by binding to a silica-gel membrane (Rneasy Mini Kit, Qiagen, CA) in the presence of an ethanol-containing, high-salt buffer followed by washing to remove contaminants. The RNA was further purified using a TURBO DNA-free kit (Ambion, INC), and high-quality RNA was then eluted in water. Yield and purity were assessed by A260 and A280 readings on a spectrophotometer. CDNA copies were made from purified RNA using an ABI (ABI, CA) high capacity cDNA archive kit.

[0388] Unless otherwise stated, all reagents were purchased from Applied Biosystems. Real-time PCR reactions were performed using the ABI PRISM® 7900 Sequence Detection System. TAQMAN® UNIVERSAL PCR MASTER MIX® (ABI, CA) was used with 20 ng of reverse transcribed RNA in a total reaction volume of 20 μ l. Each cDNA sample was run in duplicate to correct for pipetting errors. Primers and FAM-labeled TAQMAN®probes were used at concentrations of 200 nM. The level

of expression for each target gene was normalized using a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control previously developed by Applied Biosystems. Primer and probe sets are listed in **Table 12**. After an initial incubation at 50°C for 2 min followed by 95°C for 10 min, samples were cycled 40 times in two stages - a denaturation step at 95°C for 15 sec followed by an annealing/extension step at 60°C for 1 min. Data analysis was carried out using GENEAMP®7000 Sequence Detection System software. For each primer/probe set, a Ct value was determined as the cycle number at which the fluorescence intensity reached a specific value in the middle of the exponential region of amplification. Relative gene expression levels were calculated using the comparative Ct method. Briefly, for each cDNA sample, the endogenous control Ct value was subtracted from the gene of interest Ct to give the delta Ct value (ΔCt). The normalized amount of target was calculated as $2^{-\Delta Ct}$, assuming amplification to be 100% efficiency. Final data were expressed relative to a calibrator sample.

[0389] *High Content Analysis:* At the conclusion of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat # A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging. Other primary antibodies used for analysis included 1:200 dilution rabbit anti-human insulin (Cell Signaling; Cat # C27C9), and 1:1500 dilution mouse anti-human glucagon (Sigma-Aldrich; Cat # G2654). Secondary antibodies used for analysis included 1:1000 dilution Alexa Fluor 647 chicken anti-rabbit IgG (Invitrogen; Cat # A21443), and 1:1000 dilution Alexa Fluor 488 chicken anti-mouse IgG (Invitrogen; Cat # A21200).

- [0390] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 and 4500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.
- [0391] PCR results for representative differentiation markers are shown in **Table 14** for cells harvested from each step of differentiation. Samples treated with GDF-8 and Wnt3a or with GDF-8 and a small molecule showed similar expression levels of markers associated with endodermal and endocrine differentiation.
- [0392] **Figure 20, panel A** shows FACS analysis for the definitive endoderm marker, CXCR4, after the first step of differentiation. Treatment of human embryonic stem cells with GDF-8 and Wnt3a yielded a similar percentage of CXCR4 positive cells compared to treatment with activin A and Wnt3a. Treatment of human embryonic stem cells with GDF-8 and a compound of the present invention (Compound 19, Compound 202, Compound 40, or GSK3 inhibitor IX BIO) also yielded an equivalent or slightly higher percentage of CXCR4 positive cells. **Figure 20, panel B** shows high content image analysis for normalized SOX17 protein expression in human embryonic stem cells after three days differentiation to definitive endoderm. In some cases, treatment with GDF-8 resulted in a lower cell number at the conclusion of the first step of differentiation. However, GDF-8 treatment in combination with Wnt3a or with the small molecule inhibitors clearly induced expression of SOX17, a marker of definitive endoderm. In one instance, treatment with GDF-8 and Compound 40 yielded cell numbers in culture and SOX17 expression equivalent to treatment with activin A and Wnt3a.
- [0393] **Figure 20, panel C** shows high content image analysis for relative cell numbers recovered from cultures treated through differentiation step 5. As observed earlier at

the end of step 1, some treatments caused a drop in cell recovery relative to treatment with activin A and Wnt3a. This decrease in cell number was seen specifically with treatment groups using GDF-8 with GSK3 inhibitor BIO and also using GDF-8 with Compound 19. Additional GDF-8 treatment groups had cell recoveries similar to treatment with activin A and Wnt3a. In **Figure 20, panels D-F**, normalized protein levels of insulin and glucagon are shown, along with their respective ratio for each treatment group. Similar levels of insulin and glucagon could be obtained with each of the GDF-8 treatments relative to treatment with activin A and Wnt3a, demonstrating that GDF-8, in combination with Wnt3a or a small molecule, can substitute for activin A during definitive endoderm differentiation and subsequent pancreatic endoderm and endocrine differentiation.

Example 17

Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage that were Formed Using GDF-8 and a Compound of the Present Invention are able to Further Differentiate into Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0394] Additional small molecules were tested in combination with GDF-8 and activin A for definitive endoderm differentiation. These included a commercial inhibitor of GSK3 as well as the compounds of the present invention. A step-wise differentiation protocol was applied to cells treated with GDF-8 in combination with various small molecules. The efficacy of differentiation was determined by gene expression for biomarkers representative of the pancreatic endoderm and pancreatic endocrine lineages. A parallel control sample of cells treated with activin A and Wnt3a was maintained for comparison purposes throughout the step-wise differentiation process.

[0395] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium supplemented with 8ng/ml bFGF (PeproTech Inc.; Cat # 100-18B) with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with

MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human ES cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma.

- [0396] Cell clusters were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and plated onto reduced growth factor MATRIGELTM-coated 24-well, black wall culture plates (Arctic White; Cat # AWLS-303012) in volumes of 0.5ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout assay.
- [0397] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back an aliquot (0.5ml) of test medium. Test conditions for the first step of differentiation were conducted over a three-day period, feeding daily by aspirating and replacing the medium from each well with fresh test medium. On the first day of assay, 100ng/ml activin A (PeproTech; Cat #120-14) or 100ng/ml GDF-8 (R&D Systems, Cat # 788-G8) was added to respective assay wells where each growth factor was diluted into RPMI-1640 medium (Invitrogen; Cat #: 22400) with 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (MP Biomedicals, Inc; Cat # 152401). In some samples, 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF) was also included. On the second day of assay, 100ng/ml activin A or 100ng/ml GDF-8 was diluted into RPMI-1640 medium supplemented with 2% FAF BSA, omitting Wnt3a from all samples. In some test samples using GDF-8, Wnt3a was replaced with a given concentration of small molecule compound, added only on the first day of definitive endoderm differentiation. These small molecules included: Compound 181 (1.25μM in assay), Compound 180 (2.5μM in assay), Compound 19 (10μM in assay), Compound 202 (2.5μM in assay), Compound 40 (5μM in assay), Compound 34 (2.5μM in assay), Compound 206 (2.5μM in assay), and a commercially available GSK3 inhibitor IX BIO (10μM in assay) (EMD Chemicals, Inc.; Cat # 361550). At the conclusion of the first step of differentiation, cells from some wells were harvested for flow cytometry analysis to evaluate levels of CXCR4, a marker of

definitive endoderm formation. Additional wells were harvested for RT-PCR analysis to measure other markers of differentiation.

- [0398] At the conclusion of the first step of definitive endoderm differentiation, replicate sets of parallel wells from each treatment group were subjected to further step-wise differentiation. It is important to note that after the first differentiation step, all wells undergoing subsequent culture and differentiation received the same treatment. The protocol for this continuing differentiation is described below.
- [0399] Step 2 of the differentiation protocol was carried out over two days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM:F12 medium (Invitrogen; Cat # 11330-032) containing 2% FAF BSA, 50ng/ml FGF7 (PeproTech; Cat # 100-19), and 250nM cyclopamine-KAAD (Calbiochem; Cat # 239804).
- [0400] Step 3 of the differentiation protocol was carried out over four days. Cells were fed daily by aspirating medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 0.1% Albumax (Invitrogen; Cat #: 11020-021), 0.5x Insulin-Transferrin-Selenium (ITS-X; Invitrogen; Cat # 51500056), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine, and 2 μ M all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625). At the conclusion of the third step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation.
- [0401] Step 4 of the differentiation protocol was carried out over three days. Cells were fed daily by aspirating the medium from each well and replacing with a fresh aliquot (0.5ml) of DMEM-high glucose supplemented with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, 100 ng/ml Noggin, and 1 μ M Alk 5 inhibitor (Axxora; Cat # ALX-270-445). At the conclusion of the fourth step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation.
- [0402] Step 5 of the differentiation protocol was carried out over seven days in DMEM-high glucose with 0.1% Albumax, 0.5x Insulin-Transferrin-Selenium, and 1 μ M Alk 5

inhibitor. Medium in each well was aspirated and replaced with a fresh aliquot (0.5ml) on all days. At the conclusion of the fifth step of differentiation, cells from some wells were harvested for analysis by RT-PCR to measure markers of differentiation. Other culture wells were subjected to high content image analysis for protein expression levels of insulin and glucagon.

[0403] *FACS Analysis:* Cells for FACS analysis were blocked in a 1:5 solution of 0.5% human gamma-globulin (Sigma; Cat# G-4386) in PBS (Invitrogen; Cat # 14040-133): BD FACS staining buffer – BSA (BD; Cat #554657) for 15 minutes at 4°C. Cells were then stained with antibodies for CD9 PE (BD; Cat # 555372), CD99 PE (Caltag; Cat # MHCD9904) and CXCR4 APC (R&D Systems; Cat# FAB173A) for 30 minutes at 4°C. After a series of washes in BD FACS staining buffer, the cells were stained for viability with 7-AAD (BD; Cat # 559925) and run on a BD FACSArray. A mouse IgG1K Isotype control antibody for both PE and APC was used to gate percent positive cells.

[0404] *RT-PCR Analysis:* RNA samples were purified by binding to a silica-gel membrane (Rneasy Mini Kit, Qiagen, CA) in the presence of an ethanol-containing, high-salt buffer followed by washing to remove contaminants. The RNA was further purified using a TURBO DNA-free kit (Ambion, INC), and high-quality RNA was then eluted in water. Yield and purity were assessed by A260 and A280 readings on a spectrophotometer. CDNA copies were made from purified RNA using an ABI (ABI, CA) high capacity cDNA archive kit.

[0405] Unless otherwise stated, all reagents were purchased from Applied Biosystems. Real-time PCR reactions were performed using the ABI PRISM® 7900 Sequence Detection System. TAQMAN® UNIVERSAL PCR MASTER MIX® (ABI, CA) was used with 20 ng of reverse transcribed RNA in a total reaction volume of 20 µl. Each cDNA sample was run in duplicate to correct for pipetting errors. Primers and FAM-labeled TAQMAN®probes were used at concentrations of 200 nM. The level of expression for each target gene was normalized using a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control previously developed by Applied Biosystems. Primer and probe sets are listed in **Table 12**. After an initial incubation at 50°C for 2 min followed by 95°C for 10 min, samples were cycled 40 times in two stages - a denaturation step at 95°C for 15 sec followed by an

annealing/extension step at 60°C for 1 min. Data analysis was carried out using GENEAMP®7000 Sequence Detection System software. For each primer/probe set, a Ct value was determined as the cycle number at which the fluorescence intensity reached a specific value in the middle of the exponential region of amplification. Relative gene expression levels were calculated using the comparative Ct method. Briefly, for each cDNA sample, the endogenous control Ct value was subtracted from the gene of interest Ct to give the delta Ct value (ΔCt). The normalized amount of target was calculated as $2^{-\Delta Ct}$, assuming amplification to be 100% efficiency. Final data were expressed relative to a calibrator sample.

[0406] *High Content Analysis:* At the conclusion of culture, assay plates were washed once with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for two hours at room temperature. After washing three times with PBS, Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Invitrogen; Cat # A21467) diluted 1:200 in PBS was added to each well. To counterstain nuclei, 5 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for fifteen minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging. Other primary antibodies used for analysis included 1:100 dilution mouse anti-human CDX2 (Invitrogen; Cat # 397800), 1:100 dilution goat anti-human Pdx1 (Santa Cruz Biotechnology; Cat # SC-14664), 1:200 dilution rabbit anti-human insulin (Cell Signaling; Cat # C27C9), and 1:1500 dilution mouse anti-human glucagon (Sigma-Aldrich; Cat # G2654). Secondary antibodies used for analysis included 1:400 dilution Alexa Fluor 647 chicken anti-mouse IgG (Invitrogen; Cat # A-21463), 1:200 dilution Alexa Fluor 488 donkey anti-goat IgG (Invitrogen; Cat # A11055), 1:1000 dilution Alexa Fluor 647 chicken anti-rabbit IgG (Invitrogen; Cat # A21443), and 1:1000 dilution Alexa Fluor 488 chicken anti-mouse IgG (Invitrogen; Cat # A21200).

- [0407] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Images were acquired from 25 fields per well. Measurements for total intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by the area of the cell. Background was eliminated based on acceptance criteria for gray-scale ranges between 200 and 4500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control.
- [0408] *Results:* Results for representative differentiation markers are shown in **Figure 21** and **Table 15** for cells harvested from each step of differentiation. In **Figure 21A** and **B**, flow cytometric results for CXCR4 are shown for various treatments during the first step of definitive endoderm differentiation. **Figure 21A** shows the effects on CXCR4 expression from treatment with various compounds in combination with activin A. **Figure 21B** shows the effects on CXCR4 from treatment with various compounds in combination with GDF-8. Compounds of the present invention in combination with activin A did not enhance CXCR4 expression. However, all of the compounds of the present invention tested in this Example enhanced CXCR4 expression in combination with GDF-8.
- [0409] In **Figures 21 C** and **21D**, normalized RT-PCR values for various differentiation markers at the end of step one of differentiation are shown for treatments applied during step one of the protocol, using selected compounds of the present invention in combination with activin A (**Figure 21C**) or in combination with GDF-8 (**Figure 21D**). Similar normalized RT-PCR values were evaluated at the conclusion of step three of the differentiation protocol (**Figures 21E** and **21F**) and at the end of step four of the differentiation protocol (**Figures 21G** and **21H**) and at the end of step 5 of the differentiation protocol (**Figures 21I** and **21J**). Treatments during differentiation step 1, which combined a compound of the present invention with GDF-8, had improved expression of various endoderm and pancreatic markers relative to GDF-8 treatment alone (**Figures 21 F** and **21H** and **21J**). Treatments combining compounds of the

present invention with activin A had minimal or no improvement in expression markers relative to treatment with activin A alone or activin A with Wnt3a (**Figures 21E, and 21G and 21I**). **Table 15** summarizes comparative CT values for additional gene markers at the end of each differentiation step, comparing treatments during step one that combined activin A or GDF-8 with or without a compound of the present invention. At the conclusion of step five of differentiation, high content analysis was performed to measure cell numbers (**Figure 21K and 21M**) and protein expression of insulin and glucagon (**Figures 21L and 21N**). Treatment with GDF-8 during the first step of differentiation, alone or in combination with a compound of the present invention, resulted in insulin and glucagon expression at the conclusion of step five of differentiation, demonstrating that GDF-8 was able to substitute for activin A during the initiation of definitive endoderm formation and subsequently led to pancreatic hormonal cells. Collectively, these data show that addition of any of the respective small molecules had minimal effects on differentiation markers for treatments in combination with activin A. However, addition of a small molecule in combination with GDF8 treatment had significant improved effects on immediate definitive endoderm differentiation at the conclusion of step 1 differentiation and also on downstream differentiation markers at the conclusion of steps 3, 4, and 5. Variability was observed within the panel of small molecules, perhaps attributable to the concentration of compound used in assay and/or mechanism of action.

Example 18

Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage that were Formed Using GDF-8 and a Compound of the Present Invention are able to Release C-Peptide Following Transplantation into a Rodent

- [0410] It was important to determine whether cells expressing markers characteristic of the pancreatic endoderm lineage generated *in vitro* by treatment with GDF-8 and a small molecule could produce functional endocrine cells *in vivo*. An *in vivo* transplant study was done to compare cells differentiated by treatment with activin A and Wnt3a versus treatment with GDF-8 and small molecule compounds.
- [0411] *Preparation of cells:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGELTM (Invitrogen; Cat # 356231) -coated tissue

culture plastic with passage on average every four days. MEF conditioned medium supplemented with 8ng/ml bFGF was used for initial seeding and expansion. All human ES cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma contamination.

[0412] Cell passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the cell monolayer with MEF conditioned medium and gentle scraping to recover cell clusters. Cell clusters were centrifuged at low speed in MEF conditioned medium to remove residual dispase and then evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF (PeproTech Inc.; Cat # 100-18B) for seeding on reduced growth factor MATRIGEL (BD Biosciences; Cat # 356231)-coated 6-well plates (Nunc; Cat# 140685) at a 1:3 ratio using volumes of 2.5ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout the time in culture.

[0413] *Cell differentiation:* The differentiation process was started three days after the cells were seeded onto 6-well plates coated with reduced growth factor MATRIGELTM. A four-step protocol was used for the *in vitro* differentiation of H1 human embryonic stem cells to cells expressing markers characteristic of the pancreatic endoderm lineage. Step 1 was conducted over three days to generate definitive endoderm cells. On the first day of step 1, differentiation was initiated by aspirating spent culture medium and adding an equal volume of RPMI-1640 basal medium (Invitrogen; Cat # 22400) with 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (Proliant Biologicals; Cat # SKU 68700) and 8ng/ml bFGF. In one treatment group, cells were exposed to 100ng/ml activin A (PeproTech; Cat #120-14) with 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF). In a second treatment group, cells were exposed to 100 ng/ml GDF-8 (R&D Systems; Cat # 788-G8) with 2.5 µM Compound 40. In a third treatment group, cells were exposed to 100 ng/ml GDF-8 (R&D Systems; Cat # 788-G8) with 2.5 µM Compound 202. On the second and third day of step 1 of differentiation, cells in all treatment groups were fed with RPMI-1640 containing 2% FAF BSA, 8ng/ml bFGF and either 100ng/ml activin A (treatment group 1) or 100 ng/ml GDF-8 (treatment groups 2 and 3), without the addition of Wnt3a or a

compound of the present invention. At the end of the third day of culture, one well from each treatment group was collected for FACS analysis.

- [0414] Step 2 of the differentiation protocol was conducted over three days. Cells for all treatment groups were fed daily with DMEM:F12 (Invitrogen; Cat # 11330-032) supplemented with 2% FAF BSA and 50 ng/ml FGF7 (PeproTech; Cat # 100-19).
- [0415] Step 3 of the differentiation protocol was conducted over four days. Cells for all treatment groups were fed daily with DMEM-high glucose (Invitrogen; Cat #10569) supplemented with 1% B27 (Invitrogen; Cat #: 17504-044), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine (Calbiochem; Cat # 239804), and 2 μ M all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625).
- [0416] Step 4 of the differentiation protocol was conducted over three days. Cells for all treatment groups were fed daily for the first two days with DMEM-high glucose supplemented with 1% B27, 100 ng/ml Noggin, and 1 μ M ALK5 inhibitor (Axxora; Cat # ALX-270-445). On the third day, cells were lifted from the substratum by using a 20 μ l tip (Rainin; Cat # RT-L10F) and a cell scraper (Corning; Cat # 3008), then transferred to a 50 ml tube. The cells were allowed to sediment by gravity, and the supernatant was aspirated without disturbing the cell pellet. Cells were resuspended in DMEM-high glucose supplemented with 1% B27, 100 ng/ml Noggin and 1 μ M ALK5 inhibitor, then cultured overnight in six-well Costar Ultra Low Attachment Microplates (Corning Inc., Cat # 3471). On the following day, cells in suspension culture were collected and counted. Aliquots of 10×10^6 cells/ mouse were used for transplantation. Aliquots of 0.5×10^6 cells were collected for RT-PCR analysis.
- [0417] **Figure 22, panel A** shows flow cytometric results for definitive endoderm cells generated at the end of step 1 for each of the respective treatment groups. Treatment with activin A and Wnt3a or treatment with GDF-8 and a compound of the present invention resulted in cells expressing similar levels of CXCR4 (greater than 85%) at the end of step 1, suggesting that an equivalent definitive endoderm population of cells was derived from each treatment group.

- [0418] Results for RT-PCR analysis for cells from each treatment group at the conclusion of step 4 of the differentiation protocol are shown in **Figure 22, panel B**. Cells differentiated to pancreatic endoderm (PE) using Activin A and Wnt3a or using GDF-8 and Compound 40 or using GDF-8 and Compound 202 expressed equivalent levels of PE markers: CDX2, MAFA, NGN3, NKX6.1, PDX1 and Ptf1 alpha. These results suggest that the differentiation protocol utilizing GDF-8 and a small molecule was equally effective in creating a pancreatic endoderm precursor population of cells.
- [0419] *Transplantation of Human Embryonic Stem Cells Treated According to the Methods of the Present Invention into Mice:* Five to six-week-old male scid-beige mice (C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lysf*^{bg} N7) were purchased from Taconic Farms. Mice were housed in microisolator cages with free access to sterilized food and water. In preparation for surgery, mice were identified by ear tagging, their body weight was measured, and their blood glucose was determined using a hand held glucometer (LifeScan; One Touch). On the day of surgery, mice were anesthetized with a mixture of isoflurane and oxygen, and the surgical site was shaved with small animal clippers. Mice were dosed with 0.1 mg/kg Buprenex subcutaneously pre-operatively. The surgical site was prepared with successive washes of 70% isopropyl alcohol, 10% povidone-iodine, and 70% isopropyl alcohol, and a left lateral incision was made through the skin and muscle layers. The left kidney was externalized and kept moist with 0.9% sodium chloride. A 24G x 3/4" I.V. catheter was used to penetrate the kidney capsule, and the needle was removed. The catheter was then advanced under the kidney capsule to the distal pole of the kidney. During preoperative preparation of the mice, cells for transplant were centrifuged in a 1.5 mL microfuge tube, and most of the supernatant was removed, leaving sufficient medium to collect the pellet of cells. The cells were collected into a Rainin Pos-D positive displacement pipette tip, and the pipette was inverted to allow the cells to settle by gravity. Excess medium was dispensed leaving a packed cell preparation for transplant. For transplantation, the Pos-D pipette tip was placed firmly in the hub of the catheter, and the cells were dispensed from the pipette through the catheter under the kidney capsule for delivery to the distal pole of the kidney. The lumen of the catheter was flushed with a small volume of culture medium to deliver any remaining cells, and the catheter was withdrawn. The kidney capsule was sealed with a low temperature cautery, and the kidney was returned to its original anatomical position. The muscle was closed with

continuous sutures using 5-0 VICRYL sutures, and the skin was closed with wound clips. The mouse was removed from anesthesia and allowed to fully recover. Mice were dosed with 1.0 mg/kg Metacam subcutaneously post-operatively.

[0420] Following transplantation, mice were weighed once per week and blood glucose was measured twice per week. At various intervals following transplantation, mice were dosed with 3 g/kg glucose IP, and blood was drawn 60 minutes following glucose injection via the retro-orbital sinus into microfuge tubes containing a small amount of heparin. The blood was centrifuged, and the plasma was placed into a second microfuge tube, frozen on dry ice, for storage at -80°C until the human C-peptide assay was performed. Human C-peptide levels were determined using the Mercodia/ALPCO Diagnostics Ultrasensitive C-peptide ELISA according to the manufacturer's instructions.

[0421] ELISA results for human C-peptide are shown in **Figure 23** for mice transplanted with cells from each of the respective treatment groups. No circulating human C-peptide was detected at four weeks post-transplant for any mice receiving cells from any of the treatment groups. At 8-weeks post-transplant, detectable C-peptide was found in one of two mice receiving cells treated with activin A and Wnt3a; one of three mice receiving cells treated with GDF-8 and Compound 40; and two of three mice receiving cells treated with GDF-8 and Compound 202. These results suggest that an equivalent endocrine precursor cell population could be derived from the differentiation protocol with GDF-8 and a small molecule and that the cells further matured *in vivo* to a glucose responsive, insulin secreting cell.

Example 19

Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage that were Formed Using GDF-8 are able to Release C-Peptide Following Transplantation into a Rodent

[0422] It was important to demonstrate that cells differentiated with GDF-8 in the absence of activin A could also be further differentiated to an endocrine cell population capable of secreting human C-peptide in an *in vivo* rodent transplant model.

- [0423] *Preparation of cells:* Clusters of H1 human embryonic stem cells were grown on reduced growth factor MATRIGEL™ (Invitrogen; Cat # 356231) -coated tissue culture plastic with passage on average every four days. MEF conditioned medium supplemented with 8ng/ml bFGF was used for initial seeding and expansion. All human ES cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotype and absence of mycoplasma contamination.
- [0424] Cell passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen; Cat # 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the cell monolayer with MEF conditioned medium and gentle scraping to recover cell clusters. Cell clusters were centrifuged at low speed in MEF conditioned medium to remove residual dispase and then evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF (PeproTech Inc.; Cat # 100-18B) for seeding on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated 6-well plates (Nunc; Cat# 140685) at a 1:3 ratio using volumes of 2.5ml/well. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. Plates were maintained at 37°C, 5% CO₂ throughout culture.
- [0425] *Cell differentiation:* The differentiation process was started three days after the cells were seeded into 6-well plates. A four-step protocol was used for the *in vitro* differentiation of H1 human embryonic stem cells to cells expressing markers characteristic of the pancreatic endoderm lineage. Step 1 was conducted over three days to generate cells expressing markers characteristic of the definitive endoderm lineage. On the first day of step 1, differentiation was initiated by aspirating spent culture medium and adding an equal volume of RPMI-1640 basal medium (Invitrogen; Cat # 22400) with 2% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA) (Proliant Biologicals; Cat # SKU 68700) and 8ng/ml bFGF. In one treatment group, duplicate sets of cells were treated with 100 ng/ml GDF-8 (R&D Systems; Cat # 788-G8) and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF). In a second treatment group, duplicate sets of cells were treated with 100 ng/ml GDF-8 and 2.5 μM Compound 40. On the second and third day of step 1 differentiation, cells in all treatment groups were fed with RPMI-1640 containing 2% FAF BSA, 8ng/ml bFGF and 100 ng/ml GDF-8 but without the addition of Wnt3a or Compound 40. At the

end of the third day of culture, one well from each treatment group was collected for FACS analysis.

- [0426] Step 2 of the differentiation protocol was carried out over three days. Cells for all treatment groups were fed daily with DMEM:F12 (Invitrogen; Cat # 11330-032) supplemented with 2% FAF BSA and 50 ng/ml FGF7 (PeproTech; Cat # 100-19).
- [0427] Step 3 of the differentiation protocol was carried out over four days. Cells for all treatment groups were fed daily with DMEM-high glucose (Invitrogen; Cat # 10569) supplemented with 1% B27 (Invitrogen; Cat #: 17504-044), 50 ng/ml FGF7, 100 ng/ml Noggin (R&D Systems; Cat # 3344-NG), 250 nM KAAD-cyclopamine (Calbiochem; Cat # 239804), and 2 μ M all-trans retinoic acid (RA) (Sigma-Aldrich; Cat # R2625).
- [0428] Step 4 of the differentiation protocol was carried out over three days. Cells for all treatment groups were fed daily with DMEM-high glucose supplemented with 1% B27, 100 ng/ml Noggin and 1 μ M ALK5 inhibitor (Axxora; Cat # ALX-270-445), and 100 ng/ml GDF-8 (R&D Systems; Cat # 788-G8) during the first two days. On the third day of step 4, cells were harvested from the 6-well plates using a 20 μ l tip (Rainin; Cat # RT-L10F) and a cell scraper (Corning; Cat # 3008) and transferred to a 50 ml tube. Cells were allowed to sediment by gravity, and the supernatant was aspirated without disturbing the cell pellet. Cells were resuspended in DMEM-high glucose supplemented with 1% B27, 100 ng/ml Noggin, and 1 μ M ALK5 inhibitor, then cultured overnight in six-well Costar Ultra Low Attachment Microplates (Corning Inc., Cat # 3471). On the following day, cells in suspension culture were collected and counted. Aliquots of 10×10^6 cells/mouse were used for transplantation. Aliquots of 0.5×10^6 cells were collected for RT-PCR analysis.
- [0429] **Figure 24A** shows flow cytometric results for definitive endoderm cells generated at the end of step 1 for each of the respective treatment groups. Results for treatment with GDF-8 and Wnt3a or treatment with GDF-8 and Compound 40 expressed similar levels of CXCR4 at the end of step 1, suggesting that an equivalent and robust definitive endoderm population of cells resulted from each treatment group. Duplicate treatment sets were in strong agreement. Results prior to transplant for RT-PCR analysis at the conclusion of step 4 of the differentiation protocol are shown in

Figure 24B. Cells differentiated to pancreatic endoderm (PE) using GDF-8 and Wnt3a or GDF-8 and Compound 40 expressed equivalent levels of markers characteristic of the pancreatic endoderm lineage, such as: CDX2, MafA, Ngn3, NKX6.1, Pdx-1 and Ptf1A. These results demonstrate that the differentiation protocol utilizing GDF-8 and Wnt3a or GDF-8 and a compound of the present invention was effective in creating a pancreatic endoderm precursor population of cells. The differentiation protocol was conducted in two independent but identical treatment sets. Results from duplicate treatment sets were in strong agreement as shown by RT-PCR analysis.

[0430] *Human embryonic stem cell Transplantation into Mice:* Five to six-week-old male scid-beige mice (C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7) were purchased from Taconic Farms. Mice were housed in microisolator cages with free access to sterilized food and water. In preparation for surgery, mice were identified by ear tagging, their body weight was measured, and their blood glucose was determined using a hand held glucometer (LifeScan; One Touch). On the day of surgery, mice were anesthetized with a mixture of isoflurane and oxygen, and the surgical site was shaved with small animal clippers. Mice were dosed with 0.1 mg.kg Buprenex subcutaneously pre-operatively. The surgical site was prepared with successive washes of 70% isopropyl alcohol, 10% povidone-iodide, and 70% isopropyl alcohol, and a left lateral incision was made through the skin and muscle layers. The left kidney was externalized and kept moist with 0.9% sodium chloride. A 24G x 3/4" I.V. catheter was used to penetrate the kidney capsule, and the needle was removed. The catheter was then advanced under the kidney capsule to the distal pole of the kidney. During preoperative preparation of the mice, cells for transplant were centrifuged in a 1.5 mL microfuge tube, and most of the supernatant was removed, leaving sufficient medium to collect the pellet of cells. The cells were collected into a Rainin Pos-D positive displacement pipette tip, and the pipette was inverted to allow the cells to settle by gravity. Excess medium was dispensed leaving a packed cell preparation for transplant. For transplantation, the Pos-D pipette tip was placed firmly in the hub of the catheter, and the cells were dispensed from the pipette through the catheter under the kidney capsule for delivery to the distal pole of the kidney. The lumen of the catheter was flushed with a small volume of culture medium to deliver any remaining cells, and the catheter was withdrawn. The kidney capsule was sealed with a low

temperature cautery, and the kidney was returned to its original anatomical position. The muscle was closed with continuous sutures using 5-0 vicryl, and the skin was closed with wound clips. The mouse was removed from anesthesia and allowed to fully recover. Mice were dosed with 1.0 mg/kg Metacam subcutaneously post-operatively.

- [0431] Following transplantation, mice were weighed once per week and blood glucose was measured twice per week. At various intervals following transplantation, mice were dosed with 3 g/kg glucose IP, and blood was drawn 60 minutes following glucose injection via the retro-orbital sinus into microfuge tubes containing a small amount of heparin. The blood was centrifuged, and the plasma was placed into a second microfuge tube, frozen on dry ice, for storage at -80°C until the human C-peptide assay was performed. Human C-peptide levels were determined using the Mercodia/ALPCO Diagnostics Ultrasensitive C-peptide ELISA according to the manufacturer's instructions. ELISA results for human C-peptide are shown in **Figure 24C and D** for mice transplanted with cells from each of the respective treatment groups. Similar levels of human C-peptide were detectable at 8 weeks post-transplant for each treatment category, indicating that an equivalent endocrine precursor cell population could be derived from the differentiation protocol using GDF-8 and Wnt3a or GDF-8 and a compound of the present invention.

Example 20

Evaluation of the Potential of Inhibitors of CDK, GSK3, and TRK to Differentiate Human Embryonic Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

- [0432] A subset of 14 proprietary small molecules, known to have specificity for the CDK, GSK3, and/or TRK signaling pathways were evaluated for their potential to differentiate human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage.
- [0433] *Cell assay seeding:* Briefly, clusters of H1 human embryonic stem cells were grown on reduced growth factor MatrigelTM (Invitrogen; Cat # 356231) coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with

even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat # 6005182) using volumes of 100 µl/well. Cells were allowed to attach and then recover log phase growth over a 1 to 3 day time period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.

[0434] *Preparation of compounds and assay:* Screening was conducted using the compounds described in **Table 16**. In addition Compound 34 was included as a positive control, as demonstrated in previous examples. Compounds were made available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO (Sigma; Cat # D2650) and stored at -80°C. The library compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. Test conditions were performed in triplicate, feeding on alternating days over a four-day assay period. Assay was initiated by aspirating culture medium from each well followed by three washes in PBS (Invitrogen; Cat # 14190) to remove residual growth factors. On the first day of assay, test volumes of 200µl per well were added to each well using DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.5% FCS (HyClone; Cat # SH30070.03) and 100 ng/ml GDF-8 (R&D Systems, Cat # 788-G8) plus 2.5µM compound. A parallel set of test samples were treated in an identical manner but omitting GDF-8 from the medium. On the third day of assay, test volumes of 100µl per well were added to each well using DMEM:F12 base medium supplemented with 2% FCS plus 100 ng/ml GDF-8 (R&D Systems, Cat # 788-G8). GDF-8 was omitted from test samples that did not get treated with GDF-8 on the first day of assay. Positive control samples contained the same base medium supplemented with FCS and 100ng/ml recombinant human activin A (PeproTech; Cat #120-14) throughout the four day assay along with Wnt3a (20ng/ml) addition on days 1 and 2. Negative control samples contained DMEM:F12 base medium supplemented with FCS.

[0435] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then

washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4 μ g/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100 μ l/well PBS for imaging.

- [0436] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell multiplied by area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 and 3500. Average data from triplicate wells were collected. The percentage of treated wells relative to the positive control was calculated.
- [0437] Results for this screen are shown in **Table 17**. None of the small molecules induced significant SOX17 expression in the absence of GDF-8 during the four day differentiation process. Compound 34 served as an experimental control and induced significant SOX17 expression in the presence of GDF-8, equivalent to levels observed with the positive control using activin A and Wnt3a. The remaining compounds of the present invention tested in this example showed a range of activities with weak to

moderate induction of SOX17 expression. Of note, differentiation activity in this subset of compounds was observed in association with selectivity for all three enzymatic signal pathways, making it difficult to conclusively determine a clear mechanism of action.

Example 21

Screening for Analogues of the Compounds of the Present Invention that are Capable of Mediating the Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

- [0438] Based on the structures for the compounds of the present invention, an analog search was conducted and 118 analogues were found. Initial screening determined that some analogues were able to induce definitive endoderm differentiation in the absence of activin A in combination with other growth factors. It was important to determine if these analogues could also induce definitive endoderm differentiation in combination with only GDF-8.
- [0439] *Cell assay seeding:* Briefly, clusters of H1 human embryonic stem cells were grown on reduced growth factor MatrigelTM (Invitrogen; Cat # 356231) -coated tissue culture plastic. Cells were passaged using collagenase (Invitrogen; Cat # 17104-019) treatment and gentle scraping, washed to remove residual enzyme, and plated with even dispersal at a ratio of 1:1 (surface area) on reduced growth factor MATRIGELTM (BD Biosciences; Cat # 356231)-coated 96-well black plates (Packard ViewPlates; PerkinElmer; Cat #6005182) using volumes of 100 μ l/well. Cells were allowed to attach and then recover log phase growth over a 1 to 3 day time period, feeding daily with MEF conditioned medium supplemented with 8ng/ml bFGF (R&D Systems; Cat # 233-FB). Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.
- [0440] *Preparation of compounds and assay:* Screening was conducted using a library of the analogue compounds. Compounds from this library were made available as 5 mM stocks in 96-well plate format, solubilized in 100% DMSO (Sigma; Cat # D2650) and stored at -80°C. The library compounds were further diluted to an intermediate concentration of 0.2 mM in 50mM HEPES (Invitrogen; Cat # 15630-080), 20% DMSO and stored at 4°C. Test conditions were performed in triplicate, feeding on

alternating days over a four-day assay period. Assays were initiated by aspirating culture medium from each well followed by three washes in PBS (Invitrogen; Cat # 14190) to remove residual growth factors. On the first day of assay, test volumes of 200µl per well were added to each well using DMEM:F12 base medium (Invitrogen; Cat # 11330-032) supplemented with 0.5% FCS (HyClone; Cat # SH30070.03) and 200 ng/ml GDF-8 (R&D Systems, Cat # 788-G8) plus 2.5µM compound. On the third day of assay, test volumes of 100µl per well were added to each well using DMEM:F12 base medium supplemented with 2% FCS plus 200 ng/ml GDF-8 (R&D Systems, Cat # 788-G8). Positive control samples contained the same base medium supplemented with FCS and 100ng/ml recombinant human activin A (PeproTech; Cat #120-14) throughout the four-day assay along with Wnt3a (20ng/ml) on days 1 and 2. Negative control samples contained DMEM:F12 base medium supplemented with FCS, adding Wnt3a on days 1 and 2 but omitting treatment with activin A.

[0441] *High Content Analysis:* At the conclusion of four-days of culture, assay plates were washed twice with PBS (Invitrogen; Cat # 14190), fixed with 4% paraformaldehyde (Alexis Biochemical; Cat # ALX-350-011) at room temperature for 20 minutes, then washed three times with PBS and permeabilized with 0.5% Triton X-100 (Sigma; Cat # T8760-2) for 20 minutes at room temperature. Cells were washed again three times with PBS and blocked with 4% chicken serum (Invitrogen; Cat # 16110082) in PBS for 30 minutes at room temperature. Primary antibody (goat anti-human SOX17; R&D Systems; Cat # AF1924) was diluted 1:100 in 4% chicken serum and added to each well for one hour at room temperature. Alexa Fluor 488 conjugated secondary antibody (chicken anti-goat IgG; Molecular Probes; Cat # AZ1467) was diluted 1:200 in PBS and added to each sample well after washing three times with PBS. To counterstain nuclei, 4µg/ml Hoechst 33342 (Invitrogen; Cat # H3570) was added for ten minutes at room temperature. Plates were washed once with PBS and left in 100µl/well PBS for imaging.

[0442] Imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare) utilizing the 51008bs dichroic for cells stained with Hoechst 33342 and Alexa Fluor 488. Exposure times were optimized from positive control wells and from untreated negative control wells stained with secondary antibody alone. Images from 15 fields per well were acquired to compensate for any cell loss during the bioassay and

subsequent staining procedures. Measurements for total cell number and total SOX17 intensity were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Segmentation for the nuclei was determined based on gray-scale levels (baseline range 100-300) and nuclear size. Averages and standard deviations were calculated for each replicate data set. Total SOX17 protein expression was reported as total intensity or integrated intensity, defined as total fluorescence of the cell times area of the cell. Background was eliminated based on acceptance criteria of gray-scale ranges between 200 to 3500. Total intensity data were normalized by dividing total intensities for each well by the average total intensity for the positive control. Normalized data were calculated for averages and standard deviations for each replicate set.

- [0443] Screening results are shown in **Table 18** from four assay plates in this single experiment. Compounds are ranked with respect to SOX17 expression as a percentage of the positive control treatment with activin A and Wnt3a. This assay identified a list of 12 new analogue hits as shown in **Table 19**.

Example 22

Human Embryonic Stem Cells Grown on Microcarriers can be Differentiated into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage According to the Methods of the Present Invention

- [0444] For purposes of differentiation and production of large numbers of endocrine cells under scalable conditions, it was important to show that human embryonic stem cells could be grown and differentiated to definitive endoderm on microcarrier beads using the methods of the present invention.
- [0445] *Preparation of cells for assay and differentiation:* H1 p49C3 cells were routinely grown on Cytodex3 beads (GE Healthcare Life Sciences, NJ) in a 125ml spinner flask, according to the methods described in US Patent Application No. 61/116,447. After seven days, cells and beads were transferred to a 6 well plate at a ratio of 30cm² bead surface area per well, and the plate was placed on a rocking platform. Cells on beads in the positive control treatment well (designated AA/Wnt3a) were differentiated with addition of 100ng/ml activin A (PeproTech; Cat #120-14) and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF) for two days followed by 100ng/ml activin A and

8ng/ml bFGF (PeproTech Inc.; Cat #: 100-18B) for one day in RPMI-1640 (Invitrogen; Cat #: 22400) with 2% Fatty Acid Free BSA (MP Biomedicals, Inc; Cat # 152401) using volumes of 2ml/well. Compound 34, at a final concentration of 2.5 μ M was added to a negative control treatment well (designated CMP alone (JNJ alone)) in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) for three days in the absence of any other growth factor treatment. A third treatment well (designated CMP+8 (JNJ+8)) received Compound 34 at 2.5 μ M plus 50ng/ml GDF-8 (R&D Systems, Cat # 788-G8) in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) for three days. A fourth treatment well (designated CMP+ 8+D (JNJ+8+D)) received Compound 34 at 2.5 μ M with 50ng/ml GDF-8 and 50ng/ml PDGF-D in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) for three days. A fifth treatment well (designated CMP+8+D+V (JNJ+8+D+V)) received Compound 34 at 2.5 μ M with 50ng/ml GDF-8, 50ng/ml PDGF-D, and 50ng/ml VEGF in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) for three days. A sixth treatment well (designated CMP+8+D+V+M (JNJ+8+D+V+M)) received Compound 34 at 2.5 μ M with 50ng/ml GDF-8, 50ng/ml PDGF-D, 50ng/ml VEGF, and 20ng/ml Muscimol in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) for three days. All media and treatments were exchanged daily.

- [0446] At the conclusion of treatment and culture, cells were harvested from the beads, according to the methods described in US Patent Application No. 61/116,447. The harvested cells were counted and analyzed by flow cytometry, according to the methods described above.
- [0447] Results are shown in **Figure 25**. As shown in panel A, similar numbers of cells were recovered for all treatment groups undergoing differentiation. As shown in panel B, cells treated with the Compound 34 (JNJ) alone did not differentiate into CXCR4 positive cells. The positive control treatment, adding activin A and Wnt3a during differentiation, induced expression of CXCR4 in 68% of the resulting cell population. Compound 34 added with the various growth factor combinations induced CXCR4 expression in 50% of the cells, on average. Of note, equivalent levels of CXCR4 expression were observed during treatment with Compound 34 in combination with a single growth factor, GDF-8, or in combination with multiple growth factors that included GDF-8. This proves that Compound 34 in combination with at least GDF-8 can substitute for activin A and Wnt3a to promote definitive endoderm differentiation.

This example also shows that the treatment procedure is effective for cells grown and differentiated on microcarrier beads.

Example 23

The Compounds of the Present Invention, Together with GDF-8 Enhance Cell Proliferation

- [0448] A previous example showed that GDF-8 is able to replace activin A to differentiate human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage. It was important to know the relative potencies of GDF-8 and activin A with respect to definitive endoderm formation. A dose response assay was conducted using equivalent concentrations of each growth factor to compare results during human embryonic stem cell differentiation.
- [0449] The compounds of the present invention used in combination with GDF-8 during definitive endoderm differentiation were evaluated for their ability to induce cell proliferation. Results were compared to treatment with activin A or GDF-8 alone.
- [0450] *Preparation of cells for assay:* Stock cultures of human embryonic stem cells (H1 human embryonic stem cell line) were maintained in an undifferentiated, pluripotent state on reduced growth factor MATRIGEL™ (BD Biosciences; Cat # 356231)-coated dishes in MEF conditioned medium with passage on average every four days. Passage was performed by exposing cell cultures to a solution of 1 mg/ml dispase (Invitrogen, Cat #: 17105-041) for 5 to 7 minutes at 37°C followed by rinsing the monolayer with MEF conditioned culture medium and gentle scraping to recover cell clusters. Clusters were centrifuged at low speed to collect a cell pellet and remove residual dispase. Cell clusters were split at a 1:3 or 1:4 ratio for routine maintenance culture or a 1:1 ratio for immediate assay. All human embryonic stem cell lines were maintained at passage numbers less than 50 and routinely evaluated for normal karyotypic phenotype and for absence of mycoplasma contamination.
- [0451] Cell clusters used in the assay were evenly resuspended in MEF conditioned medium supplemented with 8ng/ml bFGF and seeded onto reduced growth factor MATRIGEL™-coated 96-well Packard VIEWPLATES (PerkinElmer; Cat # 6005182) in volumes of 100µl/well. MEF conditioned medium supplemented with

8ng/ml bFGF was used for initial seeding and expansion. Daily feeding was conducted by aspirating spent culture medium from each well and replacing with an equal volume of fresh medium. A background set of wells in each assay plate was not seeded with cells but was treated throughout assay with basal media conditions. Plates were maintained at 37°C, 5% CO₂ in a humidified box throughout the duration of assay.

- [0452] *Assay:* The assay was initiated by aspirating the culture medium from each well and adding back a final aliquot (100µl) of test medium. Test conditions were performed in triplicate over a total three-day assay period, feeding daily by aspirating and replacing the medium from each well with fresh test medium. Identical assays were set up simultaneously in parallel for evaluation at the end of 24, 48, and 72 hours.
- [0453] On the first day of assay, all wells containing cells received an aliquot (80µl) of RPMI-1640 medium (Invitrogen; Cat #: 22400) supplemented with 2.5% Albumin Bovine Fraction V, Fatty Acid Free (FAF BSA; 2% in final assay) (Proliant Inc. Cat #: SKU 68700). Various control and test samples were created at 5x concentration to be added to appropriate wells (20µl per well). Control conditions included the following, with final growth factor concentrations as indicated: 1) basal medium with 2% FAF BSA; 2) 100ng/ml activin A (PeproTech; Cat #120-14) with 8ng/ml bFGF (PeproTech; Cat # 100-18B); 3) 100ng/ml activin A with 8ng/ml bFGF and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF); 4) 100ng/ml GDF-8 (R&D Systems, Cat # 788-G8) with 8ng/ml bFGF; 5) GDF-8 with 8ng/ml bFGF and 20ng/ml Wnt3a. Cells in an additional set of control wells were treated with MEF conditioned medium throughout the assay. In some control samples using GDF-8, Wnt3a was replaced with a compound of the present invention. For experimental test samples, eight different compounds were diluted two-fold in series to create three different dose concentrations then combined with 100ng/ml GDF-8 and 8ng/ml bFGF. These small molecules included proprietary compounds Compound 181, Compound 180, Compound 19, Compound 202, Compound 40, Compound 34, Compound 56, and a commercially available GSK3 inhibitor BIO (EMD Chemicals, Inc.; Cat # 361550). On the second and third day of assay, all wells for control and experimental samples were aspirated and fed again using identical treatment conditions except that Wnt3a was removed from some control wells.

- [0454] *MTS Assay:* At the conclusion of 24, 48, or 72 hours of culture, one set of assay plates was subjected to a MTS assay (Promega; Cat# G3581), following the manufacturer's instructions. In brief, 20 μ l of MTS was added to each well, and assay plates were incubated at 37°C, 5% CO₂ for four hours prior to taking OD490 readings. Statistical measures were calculated minus background (i.e. treatment wells without cells) to determine mean values for each triplicate set in addition to a standard error of the mean.
- [0455] The MTS assay is a measure of cellular metabolic activity in the enzymatic reduction of a tetrazolium compound to a formazan product. At a single time point, the MTS assay can be used as a comparative indicator of cell viability. MTS assays evaluated in parallel at sequential time points can add additional information regarding increases in cellular metabolic activity which in turn can be correlated with cell proliferation for each treatment condition. **Figure 26, panel A** shows OD490 readings for all control treatments over the three day assay period. Cells treated with conditioned medium showed little change in OD490 over three days, indicating that cell numbers in this treatment group remained static. In contrast, cells cultured in basal medium without growth factors (no treatment), showed a steady decline in OD490 correlated with a loss in cell number over time. Activin A treatments during the differentiation process, with and without Wnt3a, showed incremental increases in OD490, indicating significant expansion of the cell population over time. GDF-8 treatment in the absence of Wnt3a resulted in a decrease in OD490 relative to activin A treatment; this was noticeable on the first day and sustained throughout all three days of culture. Addition of Wnt3a to the GDF-8 treatment group resulted in a recovery and increase in OD490 by the third day of culture.
- [0456] **Figures 26, panel B** through **Figure 26, panel I** show MTS assay results for treatment with a small molecule inhibitor in combination with GDF-8. OD490 readings from treatments with a compound of the present invention and GDF-8 were equivalent to or exceeded results from treatment with activin A. In all cases, an optimal concentration of each small molecule combined with GDF-8 resulted in improved OD490 readings over the three day assay relative to treatment with GDF-8 alone. This suggests that the compounds of the present invention are important for

inducing proliferation and expansion of a cell population during definitive endoderm differentiation.

Example 24

Human Embryonic Stem Cells Grown on Microcarriers can be Differentiated into Endocrine Progenitor Cells According to the Methods of the Present Invention

[0457] For purposes of differentiation and production of large numbers of endocrine cells under industrial conditions, it was important to show that human embryonic stem cells could be grown and differentiated to endocrine progenitor cells on microcarrier beads using a protocol without activin A.

[0458] *Preparation of cells for assay and differentiation:* H1 p45 cells were grown on Cytodex3 beads (GE Healthcare; Cat # 17-0485-01) in a 6 well ultra low attachment plate (Costar; Cat #3471) placed on a rocking platform at about 1 rotation every 10 seconds (Vari Mix, Thermo Scientific, Cat#M79735). MEF conditioned media was changed daily for six days. Then the media was changed to the following treatments to initiate endoderm differentiation. Cells on beads in the positive control treatment well (designated AA+Wnt) were differentiated with addition of 100ng/ml activin A (PeproTech; Cat #120-14), 8ng/ml bFGF (PeproTech Inc.; Cat #: 100-18B), and 20ng/ml Wnt3a (R&D Systems; Cat # 1324-WN/CF) for one day followed by 100ng/ml activin A and 8ng/ml bFGF (PeproTech Inc.; Cat #: 100-18B) for two days in RPMI-1640 (Invitrogen; Cat #: 22400) with 2% Fatty Acid Free BSA (Proliant Biomedicals, Inc; SKU # 68700) using volumes of 2ml/well. A second treatment well (designated GDF-8+MCX) received Compound 202 at 2.5 μ M plus 200ng/ml GDF-8 (R&D Systems, Cat # 788-G8) and 8ng/ml bFGF for one day followed by two days with 200ng/ml GDF-8 and 8ng/ml bFGF in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) media. A third treatment well (designated GDF-8+Wnt) received 200ng/ml GDF-8 with 20ng/ml Wnt3a and 8ng/ml bFGF for one day followed by two days with 200ng/ml GDF-8 and 8ng/ml bFGF in RPMI-1640 with 2% Fatty Acid Free BSA (2ml/well) media. All media and treatments were exchanged daily.

[0459] At the conclusion of treatment and culture, cells were harvested and counted to determine cell recovery and undergo flow cytometric analysis. High levels of

CXCR4 and CD99 was seen following all three treatment regiments (**Figure 27A**). Cell number varied between samples (**Figure 27B**). A lower cell number was observed in samples treated with GDF-8 and at the definitive endoderm and fourth stage than the other treatment groups. This suggests that the compounds of the present invention may increase proliferation of the cells during differentiation.

[0460] At the end of stage 3 the endodermal genes PDX1, HNF4 alpha, and CDX2 are expressed in the cells (**Figure 27C, D**). Treatment of the cells with GDF-8 and a compound of the present invention during stage one of differentiation resulted in better expression of Pdx1 than the control differentiation treatment. At the end of stage 4, endodermal genes were up regulated further (**Figure 27E, F**). These results conclude that GDF-8 plus Compound 202 can replace activin A and Wnt3a for definitive endoderm differentiation resulting in pancreatic endoderm formation.

[0461]

Although the various aspects of the invention have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the invention is defined not by the foregoing description but by the following claims properly construed under the principles of patent law.

Table 1					
Plate #	Compound #	Cell Number		Sox17 Expression	
		Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
plate 5	no Activin A (with Wnt3a)	7159	67.42	8.12E+06	2.51
plate 5	Activin A / Wnt3a	10619	100.00	3.23E+08	100.00
plate 5	Compound 58	4848	45.66	-1.60E+06	-0.49
plate 5	Compound 59	20	0.19	-4.62E+06	-1.43
plate 5	Compound 60	3348	31.52	-2.33E+05	-0.07
plate 5	Compound 61	2931	27.60	-3.05E+06	-0.94
plate 5	Compound 62	7171	67.53	-2.04E+06	-0.63
plate 5	Compound 3	14211	133.82	-2.34E+06	-0.73
plate 6	no Activin A (with Wnt3a)	3264	32.97	2.52E+06	0.80
plate 6	Activin A / Wnt3a	9902	100.00	3.14E+08	100.00
plate 6	Compound 63	1917	19.36	4.75E+05	0.15
plate 6	Compound 26	5434	54.88	-6.33E+05	-0.20
plate 6	Compound 27	6288	63.50	-1.13E+06	-0.36
plate 6	Compound 28	4121	41.62	-1.89E+06	-0.60

plate 6	Compound 29	5164	52.15	-1.66E+06	-0.53
plate 6	Compound 30	4726	47.73	-1.23E+06	-0.39
plate 7	no Activin A (with Wnt3a)	9545	47.57	-4.87E+06	-0.99
plate 7	Activin A / Wnt3a	20064	100.00	4.92E+08	100.00
plate 7	Compound 31	7230	36.03	-3.45E+06	-0.70
plate 7	Compound 32	14655	73.04	-3.03E+06	-0.62
plate 7	Compound 33	13891	69.23	-8.11E+06	-1.65
plate 7	Compound 34	11674	58.18	-2.24E+06	-0.46
plate 7	Compound 35	15379	76.65	-7.30E+06	-1.48
plate 7	Compound 36	8356	41.65	-4.57E+06	-0.93
plate 8	no Activin A (with Wnt3a)	6868	36.97	-2.31E+06	-0.52
plate 8	Activin A / Wnt3a	18575	100.00	4.47E+08	100.00
plate 8	Compound 37	9048	48.71	-3.51E+06	-0.79
plate 8	Compound 38	11361	61.16	-4.31E+06	-0.96
plate 8	Compound 39	7054	37.98	-3.83E+06	-0.86
plate 8	Compound 40	8104	43.63	-4.59E+06	-1.03
plate 1	no Activin A (with Wnt3a)	2972	27.98	1.64E+07	19.74
plate 1	Activin A / Wnt3a	3126	29.44	8.33E+07	100.00
plate 1	Compound 64	2201	20.72	1.71E+07	20.52
plate 1	Compound 65	3030	28.53	2.83E+07	33.95
plate 1	Compound 66	1990	18.74	2.36E+07	28.30
plate 1	Compound 67	2074	19.53	2.63E+07	31.55
plate 1	Compound 68	1432	13.48	1.03E+07	12.39
plate 1	Compound 69	2593	24.42	2.62E+07	31.43
plate 1	Compound 70	2236	21.05	2.59E+07	31.11
plate 1	Compound 71	2996	28.22	3.07E+07	36.92
plate 1	Compound 72	2179	20.52	1.21E+07	14.50
plate 1	Compound 73	2817	26.53	2.93E+07	35.25
plate 1	Compound 74	2853	26.86	2.25E+07	27.01
plate 1	Compound 75	1689	15.91	1.42E+07	17.05
plate 1	Compound 76	2324	21.89	1.48E+07	17.81
plate 1	Compound 77	2306	21.71	2.04E+07	24.55
plate 1	Compound 78	3298	31.06	2.58E+07	31.00
plate 1	Compound 79	2855	26.88	2.79E+07	33.47
plate 1	Compound 80	3603	33.93	3.22E+07	38.62
plate 1	Compound 81	2263	21.31	1.07E+07	12.91
plate 1	Compound 82	1210	11.39	1.36E+07	16.33
plate 1	Compound 83	1805	17.00	1.82E+07	21.87
plate 1	Compound 84	2024	19.06	2.48E+07	29.80
plate 1	Compound 85	2840	26.74	3.45E+07	41.44
plate 1	Compound 86	1447	13.63	8.43E+06	10.13
plate 1	Compound 87	5336	50.25	4.20E+07	50.38
plate 2	no Activin A (with Wnt3a)	4033	35.50	2.14E+07	21.70
plate 2	Activin A / Wnt3a	4292	37.78	9.86E+07	100.00
plate 2	Compound 88	3416	30.06	4.17E+07	42.28
plate 2	Compound 89	4751	41.82	2.11E+07	21.40

plate 2	Compound 90	4542	39.98	3.03E+07	30.70
plate 2	Compound 91	1401	12.33	1.29E+06	1.31
plate 2	Compound 92	4210	37.06	2.95E+07	29.90
plate 2	Compound 93	4157	36.59	2.29E+07	23.26
plate 2	Compound 94	4046	35.61	2.85E+07	28.91
plate 2	Compound 95	8368	73.66	4.02E+07	40.72
plate 2	Compound 96	3695	32.53	2.92E+07	29.57
plate 2	Compound 97	3437	30.26	2.41E+07	24.44
plate 2	Compound 98	4178	36.77	3.75E+07	38.07
plate 2	Compound 99	3739	32.91	2.10E+07	21.29
plate 2	Compound 100	2275	20.02	1.27E+07	12.86
plate 2	Compound 101	3496	30.77	2.98E+07	30.17
plate 2	Compound 102	4874	42.90	2.10E+07	21.32
plate 2	Compound 103	4228	37.22	2.69E+07	27.32
plate 2	Compound 104	6115	53.82	4.93E+07	49.99
plate 2	Compound 105	6484	57.07	5.03E+07	50.95
plate 2	Compound 106	4211	37.06	3.94E+07	40.00
plate 2	Compound 107	2853	25.11	1.78E+07	18.04
plate 2	Compound 108	3779	33.27	2.39E+07	24.26
plate 2	Compound 108	2869	25.26	2.04E+07	20.71
plate 2	Compound 110	4398	38.71	2.53E+07	25.65
plate 3	no Activin A (with Wnt3a)	2589	91.17	1.17E+07	5.89
plate 3	Activin A / Wnt3a	6933	244.13	1.98E+08	100.00
plate 3	Compound 111	6816	240.04	5.33E+07	26.90
plate 3	Compound 112	5357	188.66	3.52E+07	17.74
plate 3	Compound 113	6002	211.37	8.55E+07	43.11
plate 3	Compound 114	3308	116.49	3.85E+07	19.44
plate 3	Compound 115	5007	176.31	3.96E+07	19.95
plate 3	Compound 116	3802	133.89	3.12E+07	15.75
plate 3	Compound 117	6521	229.64	4.16E+07	20.97
plate 3	Compound 118	6128	215.81	5.53E+07	27.91
plate 3	Compound 119	4184	147.35	3.41E+07	17.21
plate 3	Compound 120	2489	87.66	2.87E+07	14.49
plate 3	Compound 121	4985	175.54	3.94E+07	19.87
plate 3	Compound 25	4151	146.17	4.03E+07	20.32
plate 3	Compound 122	6407	225.61	4.15E+07	20.95
plate 3	Compound 123	4465	157.24	5.35E+07	26.99
plate 3	Compound 124	4417	155.53	4.67E+07	23.55
plate 3	Compound 125	6367	224.23	5.73E+07	28.93
plate 3	Compound 126	6157	216.82	7.47E+07	37.70
plate 3	Compound 127	5593	196.97	5.61E+07	28.28
plate 3	Compound 128	4160	146.50	4.91E+07	24.77
plate 3	Compound 129	3778	133.03	3.54E+07	17.88
plate 3	Compound 130	4357	153.43	4.15E+07	20.92
plate 3	Compound 131	6135	216.05	4.28E+07	21.61
plate 3	Compound 132	4421	155.69	4.58E+07	23.12

plate 3	Compound 133	7069	248.94	6.52E+07	32.88
plate 4	no Activin A (with Wnt3a)	3274	86.62	1.25E+07	12.79
plate 4	Activin A / Wnt3a	4158	110.03	9.79E+07	100.00
plate 4	Compound 134	5277	139.62	3.43E+07	35.04
plate 4	Compound 64	5657	149.67	3.38E+07	34.48
plate 4	Compound 135	2790	73.83	1.63E+07	16.63
plate 4	Compound 34	4774	126.33	4.35E+07	44.47
plate 4	Compound 136	4881	129.16	3.20E+07	32.73
plate 4	Compound 137	1740	46.05	9.16E+06	9.35
plate 4	Compound 30	6367	168.46	4.22E+07	43.13
plate 4	Compound 37	5377	142.27	2.85E+07	29.14
plate 4	Compound 138	7722	204.32	3.07E+07	31.37
plate 4	Compound 139	3574	94.56	1.30E+07	13.32
plate 4	Compound 140	3893	103.00	1.12E+07	11.46
plate 4	Compound 39	6114	161.77	3.45E+07	35.22
plate 4	Compound 141	4310	114.04	1.61E+07	16.48
plate 4	Compound 142	5091	134.71	3.74E+07	38.22
plate 4	Compound 35	6601	174.65	8.50E+07	86.77
plate 4	Compound 143	3582	94.79	2.17E+07	22.14
plate 4	Compound 144	6787	179.57	5.45E+07	55.69
plate 4	Compound 145	3752	99.29	2.23E+07	22.81
plate 4	Compound 146	2554	67.59	1.83E+07	18.71
plate 4	Compound 112	3289	87.03	1.48E+07	15.11
plate 4	Compound 113	3819	101.06	2.34E+07	23.93
plate 4	Compound 114	1259	33.32	1.34E+07	13.67
plate 4	Compound 22	5517	145.98	7.09E+07	72.39
plate 4	Compound 150	5104	135.04	3.34E+07	34.11
plate 5	no Activin A (with Wnt3a)	7159	116.70	8.12E+06	2.51
plate 5	Activin A / Wnt3a	10619	173.09	3.23E+08	100.00
plate 5	Compound 151	2785	45.39	-1.03E+06	-0.32
plate 5	Compound 152	4693	76.50	-3.08E+06	-0.95
plate 5	Compound 153	9718	158.40	-1.20E+06	-0.37
plate 5	Compound 154	3479	56.70	-1.97E+06	-0.61
plate 5	Compound 155	9343	152.28	-3.45E+06	-1.07
plate 5	Compound 156	3813	62.16	-2.58E+05	-0.08
plate 6	no Activin A (with Wnt3a)	3264	68.37	2.52E+06	0.80
plate 6	Activin A / Wnt3a	9902	207.40	3.14E+08	100.00
plate 6	Compound 157	2480	51.94	-1.22E+06	-0.39
plate 6	Compound 158	5271	110.41	-1.30E+06	-0.41
plate 6	Compound 159	6478	135.68	-1.84E+06	-0.59
plate 6	Compound 160	4212	88.21	1.30E+05	0.04
plate 6	Compound 161	2439	51.09	-9.20E+05	-0.29
plate 6	Compound 162	1260	26.39	-1.35E+06	-0.43
plate 7	no Activin A (with Wnt3a)	9545	156.12	-4.87E+06	-0.99
plate 7	Activin A / Wnt3a	20064	328.17	4.92E+08	100.00
plate 7	Compound 163	16557	270.81	-7.31E+06	-1.49

plate 7	Compound 164	16472	269.42	-7.37E+06	-1.50
plate 7	Compound 165	3015	49.32	-7.34E+06	-1.49
plate 7	Compound 166	13845	226.45	-7.98E+06	-1.62
plate 7	Compound 167	10325	168.87	-7.35E+06	-1.49
plate 7	Compound 168	14139	231.26	-6.49E+06	-1.32
plate 7	Compound 169	4468	73.08	-6.38E+06	-1.30
plate 8	no Activin A (with Wnt3a)	6868	179.83	-2.31E+06	-0.52
plate 8	Activin A / Wnt3a	18575	486.35	4.47E+08	100.00
plate 8	Compound 170	13140	344.04	-4.13E+06	-0.93
plate 8	Compound 171	10894	285.22	-2.61E+06	-0.58
plate 8	Compound 172	3416	89.44	-4.72E+06	-1.06
plate 8	Compound 173	8815	230.81	-4.25E+06	-0.95
plate 8	Compound 174	11760	307.91	-3.33E+06	-0.75
plate 8	Compound 175	5	0.13	-4.91E+06	-1.10
plate 8	Compound 176	10139	265.47	-4.73E+06	-1.06
plate 8	Compound 177	9994	261.68	-2.95E+06	-0.66
plate 8	Compound 178	8998	235.58	-3.74E+06	-0.84
plate 5	no Activin A (with Wnt3a)	7159	67.42	8.12E+06	2.51
plate 5	Activin A / Wnt3a	10619	100.00	3.23E+08	100.00
plate 5	Compound 21	4719	44.44	-1.96E+06	-0.61
plate 5	Compound 22	2036	19.18	-1.79E+06	-0.55
plate 5	Compound 23	2563	24.13	-1.56E+06	-0.48
plate 5	Compound 24	4470	42.09	-7.05E+05	-0.22
plate 5	Compound 24	6085	57.30	-3.08E+06	-0.95
plate 5	Compound 26	7276	68.52	-2.38E+06	-0.74
plate 5	Compound 27	4588	43.20	-5.63E+05	-0.17
plate 5	Compound 28	2682	25.26	-1.37E+06	-0.43
plate 5	Compound 29	5778	54.41	-1.94E+06	-0.60
plate 5	Compound 30	620	5.84	-5.05E+06	-1.56
plate 5	Compound 31	3419	32.19	-1.42E+06	-0.44
plate 6	no Activin A (with Wnt3a)	3264	69.07	2.52E+06	0.80
plate 6	Activin A / Wnt3a	9902	209.51	3.14E+08	100.00
plate 6	Compound 32	2142	45.32	-1.33E+06	-0.42
plate 6	Compound 33	5564	117.73	-8.63E+05	-0.27
plate 6	Compound 34	5927	125.41	-2.01E+06	-0.64
plate 6	Compound 35	10068	213.01	-2.15E+06	-0.68
plate 6	Compound 36	5170	109.39	-1.22E+06	-0.39
plate 6	Compound 37	3098	65.55	1.91E+06	0.61
plate 6	Compound 38	1537	32.52	4.48E+04	0.01
plate 6	Compound 39	3650	77.23	-2.01E+06	-0.64
plate 6	Compound 40	5817	123.07	4.91E+05	0.16
plate 6	Compound 64	4359	92.23	-1.07E+05	-0.03
plate 6	Compound 30	4035	85.38	2.09E+06	0.66
plate 6	Compound 65	3279	69.37	-5.63E+05	-0.18
plate 6	Compound 67	2698	57.08	-1.95E+06	-0.62
plate 7	no Activin A (with Wnt3a)	9545	321.22	-4.87E+06	-0.99

plate 7	Activin A / Wnt3a	20064	675.20	4.92E+08	100.00
plate 7	Compound 68	10894	366.62	-5.15E+06	-1.05
plate 7	Compound 69	9734	327.58	-3.97E+06	-0.81
plate 7	Compound 70	16736	563.21	-6.51E+06	-1.32
plate 7	Compound 71	17999	605.71	-7.38E+06	-1.50
plate 7	Compound 72	7309	245.96	-6.47E+06	-1.32
plate 7	Compound 73	8888	299.10	-3.03E+06	-0.62
plate 7	Compound 74	11496	386.85	-2.67E+06	-0.54
plate 7	Compound 75	9739	327.74	-7.75E+06	-1.57
plate 7	Compound 76	14439	485.89	-4.19E+06	-0.85
plate 7	Compound 77	12331	414.95	-6.03E+06	-1.22
plate 7	Compound 78	9702	326.49	-6.57E+06	-1.33
plate 7	Compound 79	8535	287.22	-6.92E+06	-1.41
plate 8	no Activin A (with Wnt3a)	6868	295.49	-2.31E+06	-0.52
plate 8	Activin A / Wnt3a	18575	799.17	4.47E+08	100.00
plate 8	Compound 80	13939	599.68	-4.23E+06	-0.95
plate 8	Compound 81	10466	450.29	-4.91E+06	-1.10
plate 8	Compound 82	10323	444.14	-4.90E+06	-1.10
plate 8	Compound 83	14619	628.95	1.48E+06	0.33
plate 8	Compound 84	14105	606.84	-4.44E+06	-0.99
plate 8	Compound 85	12172	523.66	-3.48E+06	-0.78
plate 8	Compound 86	7218	310.54	-4.22E+06	-0.94
plate 8	Compound 87	5383	231.58	-4.07E+06	-0.91
plate 8	Compound 88	10419	448.27	-4.27E+06	-0.96
plate 8	Compound 89	11780	506.83	-3.94E+06	-0.88
plate 8	Compound 90	7002	301.25	-1.54E+06	-0.35
plate 8	Compound 91	6224	267.78	-4.53E+06	-1.01

Table 2		
Compound #	Cell Number	Sox17 Intensity
	% of positive control	% of positive control
Compound 17	133.8	-0.7
Compound 95	195.0	40.7
Compound 138	185.7	31.4
Compound 87	170.7	50.4
Compound 144	163.2	55.7
Compound 35	158.7	86.8
Compound 30	153.1	43.1
Compound 105	151.0	51.0
Compound 39	147.0	35.2
Compound 104	142.5	50.0
Compound 29	136.0	34.5
Compound 22	132.7	72.4
Compound 37	129.3	29.1

Compound 134	126.9	35.0
Compound 150	122.7	34.1
Compound 142	122.4	38.2
Compound 136	117.4	32.7
Compound 80	115.2	38.6
Compound 34	114.8	44.5
Compound 102	113.5	21.3
Compound 89	110.7	21.4
Compound 105	105.8	30.7
Compound 78	105.5	31.0
Compound 141	103.6	16.5
Compound 110	102.5	25.7
Compound 133	102.0	32.9

Table 3						
Compound #	Treatments		Cell Number		Sox17 Expression	
			Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
none	no Activin A, with Wnt3a		23253	124.16	1.97E+07	10.59
none	Activin A / Wnt3a		18728	100.00	1.86E+08	100.00
Compound 17	no AA (with Wnt3a)	EGF+FGF4	21445	114.51	3.43E+07	18.48
none	no Activin A, with Wnt3a		23253	124.16	1.97E+07	10.59
none	Activin A / Wnt3a		18728	100.00	1.86E+08	100.00
Compound 22	no AA (with Wnt3a)	EGF+FGF4	18336	97.91	3.72E+07	20.05
Compound 34	no AA (with Wnt3a)	EGF+FGF4	18891	100.87	3.26E+07	17.55
Compound 29	no AA (with Wnt3a)	EGF+FGF4	20221	107.97	2.83E+07	15.27
Compound 39	no AA (with Wnt3a)	EGF+FGF4	17095	91.28	2.82E+07	15.19
Compound 37	no AA (with Wnt3a)	EGF+FGF4	15605	83.32	2.67E+07	14.37
Compound 35	no AA (with Wnt3a)	EGF+FGF4	23823	127.20	2.54E+07	13.69
Compound 80	no AA (with Wnt3a)	EGF+FGF4	19864	106.07	2.33E+07	12.54
Compound 141	no AA (with Wnt3a)	EGF+FGF4	17719	94.61	2.24E+07	12.04
Compound 30	no AA (with Wnt3a)	EGF+FGF4	18063	96.45	2.18E+07	11.73
Compound 150	no AA (with Wnt3a)	EGF+FGF4	16833	89.88	2.16E+07	11.63
Compound 144	no AA (with Wnt3a)	EGF+FGF4	17100	91.31	2.04E+07	11.01

Compound 104	no AA (with Wnt3a)	EGF+FGF4	17863	95.38	1.89E+07	10.19
Compound 142	no AA (with Wnt3a)	EGF+FGF4	18955	101.21	1.84E+07	9.90
Compound 110	no AA (with Wnt3a)	EGF+FGF4	17534	93.62	1.76E+07	9.45
Compound 78	no AA (with Wnt3a)	EGF+FGF4	17703	94.52	1.71E+07	9.23
Compound 133	no AA (with Wnt3a)	EGF+FGF4	16521	88.22	1.67E+07	8.97
Compound 87	no AA (with Wnt3a)	EGF+FGF4	16495	88.07	1.55E+07	8.33
Compound 95	no AA (with Wnt3a)	EGF+FGF4	16900	90.24	1.43E+07	7.72
Compound 136	no AA (with Wnt3a)	EGF+FGF4	19167	102.34	7.91E+06	4.26
Compound 105	no AA (with Wnt3a)	EGF+FGF4	15217	81.25	7.45E+06	4.01
Compound 134	no AA (with Wnt3a)	EGF+FGF4	17208	91.88	7.40E+06	3.99
Compound 138	no AA (with Wnt3a)	EGF+FGF4	16695	89.14	6.65E+06	3.58
Compound 89	no AA (with Wnt3a)	EGF+FGF4	14652	78.24	3.89E+06	2.10
Compound 90	no AA (with Wnt3a)	EGF+FGF4	15903	84.92	3.53E+06	1.90
Compound 102	no AA (with Wnt3a)	EGF+FGF4	12943	69.11	2.85E+05	0.15
none	no Activin A, with Wnt3a		23253	124.16	1.97E+07	10.59
none	Activin A / Wnt3a		18728	100.00	1.86E+08	100.00
Compound 35	no AA (with Wnt3a)	EGF+FGF4	18294	97.68	1.99E+07	10.70

Table 3B		
	Cell Number	Sox17 Intensity
Compound #	% of positive control	% of positive control
Compound 22	97.91	20.05
Compound 34	100.87	17.55
Compound 29	107.97	15.27
Compound 39	91.28	15.19
Compound 37	83.32	14.37
Compound 35	127.20	13.69

Table 4						
	Treatments		Cell Number		Sox17 Expression	
Compound #			AverageTotal Cell Number	% of positive control	Average Total Intensity	% of positive control
none	no Activin A (with Wnt3a)		7107	67.96	-1.27E+07	-7.94
none	Activin A / Wnt3a		10459	100.00	1.60E+08	100.00
Compound 17	no AA (with Wnt3a)	EGF	6942	73.43	1.27E+06	0.74
Compound 17	no AA (with Wnt3a)	EGF+FGF4	5738	60.69	3.14E+06	1.83
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	4453	47.10	9.30E+05	0.54
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	10391	109.91	8.92E+06	5.20
Compound 17	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	5728	60.59	2.14E+06	1.24
Compound 17	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	13198	139.59	1.29E+07	7.54
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	10480	110.85	8.97E+06	5.23
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	13649	144.37	1.45E+07	8.43
none	no Activin A (with Wnt3a)		3117	34.86	-1.41E+06	-0.72
none	Activin A / Wnt3a		8942	100.00	1.95E+08	100.00

Compound 35	no AA (with Wnt3a)	EGF	19334	216.23	6.62E+07	33.86
Compound 35	no AA (with Wnt3a)	PDGF-AB	16662	186.34	4.95E+07	25.33
Compound 35	no AA (with Wnt3a)	PDGF-A	16885	188.84	4.48E+07	22.94
Compound 35	no AA (with Wnt3a)	VEGF	18263	204.25	3.51E+07	17.98
Compound 35	no AA (with Wnt3a)	FGF4	4410	49.32	3.33E+07	17.04
Compound 35	no AA (with Wnt3a)	Muscimol	18867	211.00	2.61E+07	13.35
Compound 35	no AA (with Wnt3a)	PDGF-C	16642	186.12	1.85E+07	9.46
Compound 35	no AA (with Wnt3a)	PDGF-D	17618	197.03	1.84E+07	9.41
Compound 35	no AA (with Wnt3a)	PDGF-B	14168	158.46	1.52E+07	7.76
Compound 35	no AA (with Wnt3a)	PD98059	18877	211.11	1.30E+07	6.64
Compound 35	no AA (with Wnt3a)	BMP1	18849	210.81	1.29E+07	6.59
Compound 35	no AA (with Wnt3a)	LY294002	18374	205.49	1.03E+07	5.28
Compound 35	no AA (with Wnt3a)	BMP4	16748	187.31	8.97E+06	4.59
Compound 35	no AA (with Wnt3a)	BMP2	16218	181.38	8.89E+06	4.55
Compound 35	no AA (with Wnt3a)	BMP7	20111	224.91	8.05E+06	4.12
Compound 35	no AA (with Wnt3a)	U0124	16539	184.97	7.54E+06	3.86
Compound 35	no AA (with Wnt3a)	BMP6	17838	199.50	7.32E+06	3.75
Compound 35	no AA (with Wnt3a)	BMP2/7	12042	134.67	7.08E+06	3.62
Compound 35	no AA (with Wnt3a)	bicuculline	19312	215.98	1.95E+06	1.00
Compound 35	no AA (with Wnt3a)	U0126	19961	223.24	-5.75E+05	-0.29
Compound 35	no AA (with Wnt3a)	Butyrate	14238	159.24	-1.85E+06	-0.94
none		no Activin A (with Wnt3a)	6049	45.2	-1.31E+07	-5.2
none		Activin A/Wnt3a	13392	100.0	2.50E+08	100.0
Compound 20		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	9434	70.4	1.48E+08	59.1
Compound 17		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	7988	59.6	1.13E+08	45.0
Compound 16		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	8303	62.0	9.20E+07	36.7
Compound 13		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	7045	52.6	7.22E+07	28.8

Compound 19		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	7799	58.2	6.82E+07	27.2
Compound 92		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	5886	44.0	5.63E+07	22.5
Compound 93		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	5463	40.8	4.38E+07	17.5
Compound 94		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	5100	38.1	4.18E+07	16.7
Compound 95		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	4510	33.7	3.32E+07	13.3
Compound 96		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	4570	34.1	3.09E+07	12.3
Compound 97		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	4561	34.1	2.15E+07	8.6
Compound 98		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3176	23.7	9.86E+06	3.9
Compound 99		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	1209	9.0	-1.56E+07	-6.2
none		no Activin A (with Wnt3a)	15494	98.0	-1.25E+07	-4.4
none		Activin A/Wnt3a	15807	100.0	2.86E+08	100.0
Compound 18		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	8742	55.3	1.01E+08	35.4
Compound 14		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	8464	53.5	8.33E+07	29.1
Compound 15		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	7234	45.8	7.95E+07	27.8
Compound 100		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	6805	43.0	5.88E+07	20.6
Compound 101		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	5668	35.9	5.34E+07	18.7
Compound 102		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	6195	39.2	5.29E+07	18.5
Compound 103		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	7545	47.7	5.13E+07	18.0

Compound 104		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	4757	30.1	4.58E+07	16.0
Compound 105		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	6285	39.8	4.29E+07	15.0
Compound 106		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	5622	35.6	2.86E+07	10.0
Compound 107		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3951	25.0	1.72E+07	6.0
Compound 108		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3226	20.4	1.58E+07	5.5
Compound 109		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3473	22.0	1.46E+07	5.1
Compound 110		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3703	23.4	1.32E+07	4.6
Compound 111		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2918	18.5	1.22E+07	4.3
Compound 112		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2975	18.8	1.04E+07	3.6
Compound 113		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2910	18.4	9.18E+06	3.2
Compound 114		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2734	17.3	6.13E+06	2.1
Compound 115		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2169	13.7	3.77E+06	1.3
Compound 116		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3107	19.7	3.52E+06	1.2
Compound 117		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3343	21.1	5.35E+05	0.2
Compound 118		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	3034	19.2	2.37E+05	0.1
Compound 119		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2263	14.3	-1.66E+06	-0.6
Compound 120		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	1771	11.2	-5.57E+06	-2.0

Compound 121		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	1136	7.2	-1.79E+07	-6.3
Compound 122		EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	2021	12.8	-2.09E+07	-7.3

Table 5						
Compound #	Treatments		Cell Number		Sox17 Expression	
			Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
none	no Activin A (with Wnt3a)		7107	67.96	-1.27E+07	-7.94
none	Activin A / Wnt3a		10459	100.00	1.60E+08	100.00
Compound 17	no AA (with Wnt3a)	EGF	6942	73.43	1.27E+06	0.74
Compound 17	no AA (with Wnt3a)	EGF+FGF4	5738	60.69	3.14E+06	1.83
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	4453	47.10	9.30E+05	0.54
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	10391	109.91	8.92E+06	5.20
Compound 17	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	5728	60.59	2.14E+06	1.24
Compound 17	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	13198	139.59	1.29E+07	7.54
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	10480	110.85	8.97E+06	5.23
Compound 17	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	13649	144.37	1.45E+07	8.43
none	no Activin A (with Wnt3a)		7107	67.96	-1.27E+07	-7.94
none	Activin A / Wnt3a		10459	100.00	1.60E+08	100.00
Compound 35	no AA (with Wnt3a)	EGF	23887	228.40	-1.01E+07	-6.32
Compound 35	no AA (with Wnt3a)	EGF+FGF4	21268	203.36	1.36E+06	0.85
Compound 35	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	17611	168.39	1.28E+07	8.03
Compound 35	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	17949	171.62	1.54E+06	0.96
Compound 35	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	23242	222.23	1.23E+07	7.72
Compound 35	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	16068	153.63	3.92E+07	24.57
Compound 35	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	16132	154.25	9.11E+07	57.04

Compound 35	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	15457	147.80	6.89E+07	43.15
Compound 29	no AA (with Wnt3a)	EGF	1971	18.84	-1.44E+07	-9.00
Compound 29	no AA (with Wnt3a)	EGF+FGF4	7436	71.10	-4.35E+06	-2.72
Compound 29	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	6535	62.48	-7.52E+06	-4.71
Compound 29	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	1376	13.15	-1.42E+07	-8.91
Compound 29	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	8880	84.91	-8.53E+06	-5.34
Compound 29	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	8146	77.89	-4.82E+06	-3.02
Compound 29	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	8858	84.70	-7.15E+06	-4.48
Compound 29	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	10071	96.30	2.95E+06	1.85
Compound 37	no AA (with Wnt3a)	EGF	7966	76.17	-1.19E+07	-7.42
Compound 37	no AA (with Wnt3a)	EGF+FGF4	6932	66.28	-4.62E+06	-2.89
Compound 37	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	7473	71.46	-2.61E+06	-1.63
Compound 37	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	7914	75.67	-1.91E+06	-1.20
Compound 37	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	12956	123.88	-1.25E+07	-7.82
Compound 37	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	6731	64.36	-1.10E+07	-6.89
Compound 37	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	8778	83.93	1.39E+05	0.09
Compound 37	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	5821	55.66	-1.22E+07	-7.64
Compound 34	no AA (with Wnt3a)	EGF	13062	124.89	2.78E+07	17.39
Compound 34	no AA (with Wnt3a)	EGF+FGF4	13133	125.58	1.23E+08	76.85
Compound 34	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	12532	119.83	1.09E+08	68.41
Compound 34	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	15811	151.18	6.90E+06	4.32
Compound 34	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	11801	112.84	4.04E+06	2.53
Compound 34	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	15262	145.93	1.15E+07	7.18
Compound 34	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	12901	123.36	5.01E+07	31.35
Compound 34	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	12208	116.72	5.56E+07	34.83
none	no Activin A (with Wnt3a)		10224	108.14	7.36E+05	0.43
none	Activin A / Wnt3a		9455	100.00	1.72E+08	100.00
Compound 39	no AA (with Wnt3a)	EGF	11615	122.85	1.49E+05	0.09
Compound 39	no AA (with Wnt3a)	EGF+FGF4	10456	110.59	5.11E+06	2.98
Compound 39	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	9972	105.47	1.62E+06	0.94

Compound 39	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	10540	111.48	2.22E+06	1.29
Compound 39	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	17050	180.34	4.84E+06	2.82
Compound 39	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	8856	93.67	7.01E+05	0.41
Compound 39	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	7973	84.33	5.30E+06	3.09
Compound 39	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	9103	96.28	7.32E+05	0.43
Compound 22	no AA (with Wnt3a)	EGF	14105	149.19	1.75E+06	1.02
Compound 22	no AA (with Wnt3a)	EGF+FGF4	12971	137.19	1.04E+07	6.05
Compound 22	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB	16580	175.36	8.60E+06	5.01
Compound 22	no AA (with Wnt3a)	EGF+FGF4+PDGF-AB+Muscimol	14676	155.23	5.61E+06	3.27
Compound 22	no AA (with Wnt3a)	EGF+PDGF-A+VEGF	20372	215.48	4.99E+06	2.91
Compound 22	no AA (with Wnt3a)	FGF4+PDGF-A+VEGF	12277	129.85	4.90E+06	2.86
Compound 22	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF	12522	132.44	7.88E+06	4.59
Compound 22	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+Muscimol	11610	122.80	1.33E+07	7.77

Table 6						
Compound #	Treatments		Cell Number		Sox17 Expression	
			AverageTotal Cell Number	% of positive control	Average Total Intensity	% of positive control
none	no Activin A (with Wnt3a)		477	6.64	7.4E+04	0.09
none	Activin A / Wnt3a		7185	100.00	8.0E+07	100.00
Compound 34	no AA (with Wnt3a)	none	4611	64.18	1.4E+07	17.21
Compound 34	no AA (with Wnt3a)	EGF	6145	85.53	1.5E+07	19.18
Compound 34	no AA (with Wnt3a)	FGF4	5323	74.09	2.7E+07	33.75
Compound 34	no AA (with Wnt3a)	PDGF-D	5017	69.84	1.5E+07	18.76
Compound 34	no AA (with Wnt3a)	PDGF-A	4175	58.11	1.1E+07	13.43

34						
Compound 34	no AA (with Wnt3a)	VEGF	4713	65.60	1.0E+07	12.49
Compound 34	no AA (with Wnt3a)	GDF8	6354	88.44	7.1E+07	88.59
Compound 34	no AA (with Wnt3a)	Muscimol	7286	101.41	3.1E+07	38.38
Compound 34	no AA (with Wnt3a)	PDGF-D+VEGF	5030	70.01	1.2E+07	14.58
Compound 34	no AA (with Wnt3a)	VEGF+Muscimol	776	10.81	1.3E+06	1.56
Compound 34	no AA (with Wnt3a)	PDGF-D+Muscimol	3490	48.57	6.5E+06	8.02
Compound 34	no AA (with Wnt3a)	GDF8+PDGF-D	6889	95.88	5.8E+07	72.59
Compound 34	no AA (with Wnt3a)	PDGF-D+Muscimol+VEGF	2133	29.68	2.7E+06	3.32
Compound 34	no AA (with Wnt3a)	GDF8+PDGF-D+VEGF	5585	77.74	6.6E+07	81.75
Compound 34	no AA (with Wnt3a)	GDF8+VEGF+Muscimol	6083	84.67	5.6E+07	69.62
Compound 34	no AA (with Wnt3a)	GDF8+PDGF-D+VEGF+Muscimol	9455	131.60	9.6E+07	119.24
Compound 34	no AA , no Wnt3a	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8	4757	66.21	3.9E+07	48.77
Compound 34	no AA (with Wnt3a)	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8	6028	83.90	7.0E+07	87.44

Table 7						
			Cell Number		Sox17 Expression	
Plate	Treatment	Compound #	Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
1	no Activin A (with Wnt3a)	none	6049	45.2	-1.31E+07	-5.2
1	Activin A/Wnt3a	none	13392	100.0	2.50E+08	100.0
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 18	13037	97.3	1.63E+08	65.2
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 14	9344	69.8	1.23E+08	49.0
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 15	8448	63.1	8.64E+07	34.5
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 16	5498	41.1	6.56E+07	26.2
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 64	5063	37.8	5.88E+07	23.5
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 65	4788	35.8	4.57E+07	18.2
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 66	8129	60.7	3.53E+07	14.1
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 67	6791	50.7	3.18E+07	12.7
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 68	3456	25.8	2.30E+07	9.2
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 69	3995	29.8	1.69E+07	6.8
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 70	474	3.5	-1.80E+07	-7.2
2	no Activin A (with Wnt3a)	none	15494	98.0	-1.25E+07	-4.4
2	Activin A/Wnt3a	none	15807	100.0	2.86E+08	100.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 19	8425	53.3	1.19E+08	41.6

2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 13	9123	57.7	1.13E+08	39.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 71	6048	38.3	5.51E+07	19.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 72	6060	38.3	5.46E+07	19.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 73	5545	35.1	3.99E+07	14.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 74	10898	68.9	3.91E+07	13.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 75	4117	26.0	3.01E+07	10.5
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 76	3825	24.2	2.74E+07	9.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 77	5928	37.5	2.44E+07	8.5
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 78	3303	20.9	2.03E+07	7.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 79	4767	30.2	1.85E+07	6.5
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 80	2194	13.9	1.22E+07	4.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 81	2920	18.5	9.16E+05	0.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 82	1819	11.5	-1.05E+07	-3.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 83	2153	13.6	-1.19E+07	-4.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 84	58	0.4	-2.94E+07	-10.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 85	57	0.4	-3.03E+07	-10.6
1	no Activin A (with Wnt3a)	none	6049	45.2	-1.31E+07	-5.2
1	Activin A/Wnt3a	none	13392	100.0	2.50E+08	100.0

1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 20	9434	70.4	1.48E+08	59.1
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 17	7988	59.6	1.13E+08	45.0
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 16	8303	62.0	9.20E+07	36.7
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 13	7045	52.6	7.22E+07	28.8
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 19	7799	58.2	6.82E+07	27.2
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 92	5886	44.0	5.63E+07	22.5
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 93	5463	40.8	4.38E+07	17.5
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 94	5100	38.1	4.18E+07	16.7
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 95	4510	33.7	3.32E+07	13.3
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 96	4570	34.1	3.09E+07	12.3
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 97	4561	34.1	2.15E+07	8.6
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 98	3176	23.7	9.86E+06	3.9
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 99	1209	9.0	-1.56E+07	-6.2
2	no Activin A (with Wnt3a)	none	15494	98.0	-1.25E+07	-4.4
2	Activin A/Wnt3a	none	15807	100.0	2.86E+08	100.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 18	8742	55.3	1.01E+08	35.4
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 14	8464	53.5	8.33E+07	29.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 15	7234	45.8	7.95E+07	27.8

2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 100	6805	43.0	5.88E+07	20.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 101	5668	35.9	5.34E+07	18.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 102	6195	39.2	5.29E+07	18.5
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 103	7545	47.7	5.13E+07	18.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 104	4757	30.1	4.58E+07	16.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 105	6285	39.8	4.29E+07	15.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 106	5622	35.6	2.86E+07	10.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 107	3951	25.0	1.72E+07	6.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 108	3226	20.4	1.58E+07	5.5
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 109	3473	22.0	1.46E+07	5.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 110	3703	23.4	1.32E+07	4.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 111	2918	18.5	1.22E+07	4.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 112	2975	18.8	1.04E+07	3.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 113	2910	18.4	9.18E+06	3.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 114	2734	17.3	6.13E+06	2.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 115	2169	13.7	3.77E+06	1.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 116	3107	19.7	3.52E+06	1.2

2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 117	3343	21.1	5.35E+05	0.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 118	3034	19.2	2.37E+05	0.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 119	2263	14.3	-1.66E+06	-0.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 120	1771	11.2	-5.57E+06	-2.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 121	1136	7.2	-1.79E+07	-6.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 122	2021	12.8	-2.09E+07	-7.3
1	no Activin A (with Wnt3a)	none	6049	45.2	-1.31E+07	-5.2
1	Activin A/Wnt3a	none	13392	100.0	2.50E+08	100.0
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 19	15878	118.6	2.67E+08	106.5
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 24	12714	94.9	2.46E+08	98.2
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 23	12165	90.8	2.15E+08	86.0
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 21	12640	94.4	1.65E+08	65.9
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 13	11491	85.8	1.61E+08	64.3
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 30	11396	85.1	1.34E+08	53.4
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 36	7964	59.5	9.47E+07	37.8
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 32	8066	60.2	9.29E+07	37.1
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 26	7415	55.4	8.30E+07	33.1
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 17	6994	52.2	7.76E+07	31.0

1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 31	6957	51.9	6.59E+07	26.3
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 179	3573	26.7	2.43E+07	9.7
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 180	922	6.9	-2.20E+07	-8.8
1	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 181	8	0.1	-2.68E+07	-10.7
2	no Activin A (with Wnt3a)	none	15494	98.0	-1.25E+07	-4.4
2	Activin A/Wnt3a	none	15807	100.0	2.86E+08	100.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 18	21102	133.5	4.18E+08	146.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 15	15373	97.3	3.74E+08	130.8
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 14	9008	57.0	2.62E+08	91.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 38	9650	61.0	2.46E+08	86.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 35	10461	66.2	1.59E+08	55.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 16	9064	57.3	1.48E+08	51.8
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 34	8907	56.3	9.99E+07	35.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 20	7346	46.5	8.90E+07	31.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 27	8044	50.9	8.81E+07	30.8
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 28	7591	48.0	8.77E+07	30.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 40	4049	25.6	8.23E+07	28.8
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 33	7485	47.4	8.10E+07	28.3

2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 25	6571	41.6	7.60E+07	26.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 182	7631	48.3	6.74E+07	23.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 183	6777	42.9	5.93E+07	20.8
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 184	5475	34.6	5.44E+07	19.0
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 185	4093	25.9	4.92E+07	17.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 186	5274	33.4	4.63E+07	16.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 187	5342	33.8	4.02E+07	14.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 188	5533	35.0	3.98E+07	13.9
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 189	5928	37.5	3.96E+07	13.9
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 190	4822	30.5	3.90E+07	13.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 191	4249	26.9	3.81E+07	13.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 192	5616	35.5	3.54E+07	12.4
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 193	4158	26.3	3.23E+07	11.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 194	3470	22.0	2.96E+07	10.4
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 195	3800	24.0	2.95E+07	10.3
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 196	4619	29.2	2.78E+07	9.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 197	4011	25.4	2.45E+07	8.6

2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 198	4367	27.6	1.92E+07	6.7
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 199	3162	20.0	1.20E+07	4.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 200	2087	13.2	4.43E+06	1.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 201	1568	9.9	-6.17E+06	-2.2
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 202	5213	33.0	-1.41E+07	-4.9
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 203	7	0.0	-3.04E+07	-10.6
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 204	11	0.1	-3.18E+07	-11.1
2	EGF, FGF, PDGF-A, VEGF, PDGF-D, muscimol, GDF8	Compound 205	10	0.1	-3.20E+07	-11.2

Table 8				
	Cell Number		Sox17 Expression	
Compound #	AverageTotal Cell Number	% of positive control	Average Total Intensity	% of positive control
Compound 18	21102	133.5	4.18E+08	146.3
Compound 15	15373	97.3	3.74E+08	130.8
Compound 19	15878	118.6	2.67E+08	106.5
Compound 24	12714	94.9	2.46E+08	98.2
Compound 14	9008	57.0	2.62E+08	91.6
Compound 38	9650	61.0	2.46E+08	86.2
Compound 23	12165	90.8	2.15E+08	86.0
Compound 21	12640	94.4	1.65E+08	65.9
Compound 13	11491	85.8	1.61E+08	64.3
Compound 35	10461	66.2	1.59E+08	55.7
Compound 30	11396	85.1	1.34E+08	53.4
Compound 16	9064	57.3	1.48E+08	51.8
Compound 36	7964	59.5	9.47E+07	37.8
Compound 32	8066	60.2	9.29E+07	37.1
Compound 34	8907	56.3	9.99E+07	35.0
Compound 26	7415	55.4	8.30E+07	33.1
Compound 20	7346	46.5	8.90E+07	31.2
Compound 17	6994	52.2	7.76E+07	31.0
Compound 27	8044	50.9	8.81E+07	30.8
Compound 28	7591	48.0	8.77E+07	30.7
Compound 40	4049	25.6	8.23E+07	28.8
Compound 33	7485	47.4	8.10E+07	28.3
Compound 25	6571	41.6	7.60E+07	26.6
Compound 31	6957	51.9	6.59E+07	26.3
Compound 20	9434	70.4	1.48E+08	59.1

Compound 17	7988	59.6	1.13E+08	45.0
Compound 16	8303	62.0	9.20E+07	36.7
Compound 18	8742	55.3	1.01E+08	35.4
Compound 14	8464	53.5	8.33E+07	29.1
Compound 13	7045	52.6	7.22E+07	28.8
Compound 15	7234	45.8	7.95E+07	27.8
Compound 19	7799	58.2	6.82E+07	27.2

Table 9							
	Treatments			Cell Number		Sox17 Expression	
Plate #	Activin A	Compound #	Growth Factors	Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
1	none	none	none	9164	149.46	-5.91E+06	-5.17
1	10 ng/ml AA	none	none	6132	100.00	1.52E+06	1.33
1	100 ng/ml AA	none	none	9658	157.51	1.14E+08	100.00
1	10 ng/ml AA	Compound 22	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8+Wnt3a	8556	139.53	8.78E+07	76.82
1	10 ng/ml AA	Compound 22	EGF+FGF4+PDGF-AB+VEGF+Wnt3a	7657	124.87	4.70E+07	41.09
1	10 ng/ml AA	Compound 22	EGF+FGF4+PDGF-A+Muscimol+Wnt3a	8100	132.10	4.42E+07	38.65
1	10 ng/ml AA	Compound 22	EGF+FGF4+PDGF-AB+Wnt3a	7975	130.06	3.43E+07	30.03
1	10 ng/ml AA	Compound 22	EGF+FGF4+Wnt3a	9800	159.83	4.59E+07	40.13
1	10 ng/ml AA	Compound 22	FGF4+Wnt3a	6490	105.84	4.28E+07	37.43
1	10 ng/ml AA	Compound 22	EGF+Wnt3a	5001	81.55	2.80E+07	24.45
1	10 ng/ml AA	Compound 22	Wnt3a	4543	74.09	3.05E+07	26.65
1	10 ng/ml AA	Compound 35	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8+Wnt3a	2522	41.14	-4.86E+06	-4.25
1	10 ng/ml AA	Compound 35	EGF+FGF4+PDGF-AB+VEGF+Wnt3a	3479	56.74	-3.96E+06	-3.46

1	10 ng/ml AA	Compound 35	EGF+FGF4+PDGF-A+Muscimol+Wnt3a	3820	62.29	-1.67E+06	-1.46
1	10 ng/ml AA	Compound 35	EGF+FGF4+PDGF-AB+Wnt3a	3263	53.21	-4.56E+06	-3.99
1	10 ng/ml AA	Compound 35	EGF+FGF4+Wnt3a	2704	44.10	-4.17E+06	-3.65
1	10 ng/ml AA	Compound 35	FGF4+Wnt3a	284	4.64	-7.54E+06	-6.59
1	10 ng/ml AA	Compound 35	EGF+Wnt3a	155	2.53	-7.82E+06	-6.84
1	10 ng/ml AA	Compound 35	Wnt3a	173	2.83	-7.61E+06	-6.66
1	10 ng/ml AA	Compound 29	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8+Wnt3a	2737	44.63	2.41E+07	21.10
1	10 ng/ml AA	Compound 29	EGF+FGF4+PDGF-AB+VEGF+Wnt3a	2283	37.23	5.59E+06	4.88
1	10 ng/ml AA	Compound 29	EGF+FGF4+PDGF-A+Muscimol+Wnt3a	4676	76.26	2.41E+07	21.11
1	10 ng/ml AA	Compound 29	EGF+FGF4+PDGF-AB+Wnt3a	3964	64.65	2.27E+07	19.89
1	10 ng/ml AA	Compound 29	EGF+FGF4+Wnt3a	1736	28.31	1.98E+06	1.73
1	10 ng/ml AA	Compound 29	FGF4+Wnt3a	2139	34.89	6.98E+06	6.10
1	10 ng/ml AA	Compound 29	EGF+Wnt3a	365	5.96	-4.86E+06	-4.25
1	10 ng/ml AA	Compound 29	Wnt3a	2090	34.09	4.89E+06	4.28
2	none	none	none	9325	121.89	-3.35E+06	-3.01
2	10 ng/ml AA	none	none	5177	67.67	3.89E+06	3.49
2	100 ng/ml AA	none	none	7650	100.00	1.11E+08	100.00
2	10 ng/ml AA	Compound 34	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8+Wnt3a	18362	240.02	3.45E+08	309.74
2	10 ng/ml AA	Compound 34	EGF+FGF4+PDGF-AB+VEGF+Wnt3a	15574	203.58	2.59E+08	232.70
2	10 ng/ml AA	Compound 34	EGF+FGF4+PDGF-A+Muscimol+Wnt3a	17890	233.85	2.88E+08	258.30
2	10 ng/ml AA	Compound 34	EGF+FGF4+PDGF-AB+Wnt3a	17875	233.65	2.68E+08	241.07
2	10 ng/ml AA	Compound 34	EGF+FGF4+Wnt3a	14158	185.07	2.40E+08	215.35
2	10 ng/ml AA	Compound 34	FGF4+Wnt3a	13323	174.15	2.19E+08	196.86
2	10 ng/ml AA	Compound 34	EGF+Wnt3a	14527	189.89	2.28E+08	204.84
2	10 ng/ml AA	Compound 34	Wnt3a	3589	46.91	7.02E+07	63.08
2	10 ng/ml AA	Compound 39	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8+Wnt3a	5738	75.00	2.14E+07	19.24
2	10 ng/ml AA	Compound 39	EGF+FGF4+PDGF-AB+VEGF+Wnt3a	2531	33.08	2.82E+06	2.53

2	10 ng/ml AA	Compound 39	EGF+FGF4+PDGF-A+Muscimol+Wnt3a	2879	37.64	3.61E+06	3.24
2	10 ng/ml AA	Compound 39	EGF+FGF4+PDGF-AB+Wnt3a	2989	39.07	-1.78E+04	-0.02
2	10 ng/ml AA	Compound 39	EGF+FGF4+Wnt3a	734	9.59	-3.93E+06	-3.53
2	10 ng/ml AA	Compound 39	FGF4+Wnt3a	521	6.81	-4.46E+06	-4.01
2	10 ng/ml AA	Compound 39	EGF+Wnt3a	211	2.75	-4.54E+06	-4.08
2	10 ng/ml AA	Compound 39	Wnt3a	518	6.78	-2.37E+06	-2.13
2	10 ng/ml AA	Compound 37	EGF+FGF4+PDGF-A+VEGF+PDGF-D+Muscimol+GDF8+Wnt3a	5711	74.65	1.21E+07	10.82
2	10 ng/ml AA	Compound 37	EGF+FGF4+PDGF-AB+VEGF+Wnt3a	4767	62.31	-5.16E+05	-0.46
2	10 ng/ml AA	Compound 37	EGF+FGF4+PDGF-A+Muscimol+Wnt3a	4540	59.34	9.23E+05	0.83
2	10 ng/ml AA	Compound 37	EGF+FGF4+PDGF-AB+Wnt3a	4223	55.20	-6.15E+05	-0.55
2	10 ng/ml AA	Compound 37	EGF+FGF4+Wnt3a	3501	45.77	5.60E+05	0.50
2	10 ng/ml AA	Compound 37	FGF4+Wnt3a	3930	51.37	-1.88E+06	-1.69
2	10 ng/ml AA	Compound 37	EGF+Wnt3a	1431	18.70	-2.75E+06	-2.47
2	10 ng/ml AA	Compound 37	Wnt3a	791	10.34	-2.99E+06	-2.68

Table 10							
Compound #	Treatments			Cell Number		Sox17 Expression	
				Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
none	No Activin A (with Wnt3a)			4273	33.70	4.75E+07	17.49
none	Activin A (with Wnt3a)			12676	100.00	2.72E+08	100.00
Compound 34	No AA (without Wnt3a)	FGF(50ng/ml)	EGF(50ng/ml)	13317	105.06	2.01E+08	74.08
Compound 34	No AA (without Wnt3a)	FGF(50ng/ml)	EGF(100ng/ml)	14189	111.93	2.01E+08	73.90
Compound 34	No AA (without Wnt3a)	FGF(100ng/ml)	EGF(50ng/ml)	12616	99.52	1.80E+08	66.21
Compound 34	No AA (without Wnt3a)	FGF(100ng/ml)	EGF(100ng/ml)	8269	65.23	1.13E+08	41.73
Compound 34	No AA (with Wnt3a)	none	none	11711	92.38	1.65E+08	60.68
Compound 34	No AA (with Wnt3a)	none	EGF(25ng/ml)	16052	126.63	2.14E+08	78.82

Compound 34	No AA (with Wnt3a)	none	EGF(50ng/ml)	13593	107.23	1.94E+08	71.52
Compound 34	No AA (with Wnt3a)	none	EGF(100ng/ml)	13170	103.90	1.93E+08	71.04
Compound 34	No AA (with Wnt3a)	FGF(25ng/ml)	none	18433	145.41	2.49E+08	91.72
Compound 34	No AA (with Wnt3a)	FGF(25ng/ml)	EGF(25ng/ml)	18841	148.63	2.60E+08	95.72
Compound 34	No AA (with Wnt3a)	FGF(25ng/ml)	EGF(50ng/ml)	16232	128.05	2.30E+08	84.79
Compound 34	No AA (with Wnt3a)	FGF(25ng/ml)	EGF(100ng/ml)	9309	73.44	1.39E+08	51.00
Compound 34	No AA (with Wnt3a)	FGF(50ng/ml)	none	12757	100.64	1.66E+08	61.10
Compound 34	No AA (with Wnt3a)	FGF(50ng/ml)	EGF(25ng/ml)	17720	139.79	2.31E+08	85.01
Compound 34	No AA (with Wnt3a)	FGF(50ng/ml)	EGF(50ng/ml)	16331	128.83	2.26E+08	83.11
Compound 34	No AA (with Wnt3a)	FGF(50ng/ml)	EGF(100ng/ml)	16336	128.87	2.32E+08	85.24
Compound 34	No AA (with Wnt3a)	FGF(100ng/ml)	none	19853	156.61	2.59E+08	95.45
Compound 34	No AA (with Wnt3a)	FGF(100ng/ml)	EGF(25ng/ml)	19880	156.83	2.59E+08	95.47
Compound 34	No AA (with Wnt3a)	FGF(100ng/ml)	EGF(50ng/ml)	18166	143.30	2.35E+08	86.30
Compound 34	No AA (with Wnt3a)	FGF(100ng/ml)	EGF(100ng/ml)	11241	88.68	1.55E+08	57.10
none	No AA (with Wnt3a)	none	EGF(50ng/ml)	5558	43.85	5.01E+07	18.44
none	No AA (with Wnt3a)	none	EGF(100ng/ml)	6818	53.79	6.42E+07	23.62
none	No AA (with Wnt3a)	FGF(50ng/ml)	none	8494	67.01	6.62E+07	24.35
none	No AA (with Wnt3a)	FGF(50ng/ml)	EGF(50ng/ml)	10138	79.98	7.30E+07	26.87
none	No AA (with Wnt3a)	FGF(50ng/ml)	EGF(100ng/ml)	10219	80.62	7.75E+07	28.51
none	No AA (with Wnt3a)	FGF(100ng/ml)	none	9944	78.45	6.68E+07	24.59
none	No AA (with Wnt3a)	FGF(100ng/ml)	EGF(50ng/ml)	11046	87.14	8.17E+07	30.07
none	No AA (with Wnt3a)	FGF(100ng/ml)	EGF(100ng/ml)	7695	60.71	6.87E+07	25.28

TABLE 11				
	Normalized SOX17 Intensity			
	Activin A		GDF8	
ng/ml	Average	SD	Average	SD
1600	100.00	9.20	100.00	9.00
800	100.00	6.60	84.90	6.30
400	100.00	3.30	72.20	7.50
200	100.00	1.90	51.30	5.30
100	90.70	8.70	32.70	5.10
50	85.20	4.70	17.60	4.80
25	73.10	2.80	5.10	3.60
12.50	50.90	6.20	0.90	0.80
6.25	18.40	4.80	0.70	1.40
3.13	3.00	1.90	0.10	0.20
1.56	0.10	0.00	0.00	0.20
0.00	0.00	0.20	0.30	0.30

TABLE 12	
Marker name	Catalog # *
AFP	Hs00173490 ml
CD99	Hs00365982 ml
CD9	Hs00233521 ml
CDH1	Hs00170423 ml
CDH2	Hs00169953 ml
CDX2	Hs00230919 ml
CER1	Hs00193796 ml
CXCR4	Hs00237052 ml
FGF17	Hs00182599 ml
FGF4	Hs00173564 ml
FOXA2	Hs00232764 ml
GAPDH	Hs99999905 ml
GATA4	Hs00171403 ml
GATA6	Hs00232018 ml
GSC	Hs00418279 ml
HLXB9	Hs00232128 ml
KIT	Hs00174029 ml
MIXL1	Hs00430824 gl
NANOG	Hs02387400 gl
OTX2	Hs00222238 ml
POU5F1	Hs00742896 sl
SOX17	Hs00751752 sl
SOX7	Hs00846731 sl

T	Hs00610080_ml
ALB	Hs00609411_ml
AMY2A	Hs00420710_gl
ARX	Hs00292465_ml
CDX2	Hs00230919_ml
GAPDH	Hs99999905_ml
GCG	Hs00174967_ml
HNF4A	Hs00230853_ml
INS	Hs00355773_ml
ISL1	Hs00158126_ml
MAFA	Hs01651425_sl
MAFB	Hs00534343_sl
NEUROD1	Hs00159598_ml
NEUROG3	Hs00360700_gl
NKX2-2	Hs00159616_ml
NKX2-5	Hs00231763_ml
NKX6-1	Hs00232355_ml
PAX4	Hs00173014_ml
PAX6	Hs00240871_ml
PDX1	Hs00236830_ml
PECAM1	Hs00169777_ml
POU3F4	Hs00264887_sl
PTF1A	Hs00603586_gl
SST	Hs00356144_ml
ZIC1	Hs00602749_ml

TABLE 13																								
Differentiation Step 1	CT Values																							
Treatment	GAPDH	AFP	CD9	CD99	CDH1	CDH2	CDX2	CER1	CXCR4	FGF17	FGF4	FOXA2	GATA4	GATA6	GSC	KIT	MXL1	MXL1	NANOG	OTX2	POU5F1	SOX17	SOX7	T
AA/Wnt3a	19.5	34.7	33.3	34.1	24.5	21.5	35.8	18.4	22.7	20	33.5	24.7	23.7	22.1	21.3	25	23.4	28	23.8	22.6	31.4	23.5	32.2	32.3
GDF8/Wnt3a	18.7	36.1	23	23.5	23.3	21	35.2	17.8	21.9	19.9	33.1	23.8	23.7	21.9	22.1	23.9	23.1	28.6	23	21.9	29.6	23.4	31.9	32.2
GDF8/Compound 34	18.5	32	23	23.1	23.6	20.9	35.2	17.9	21.3	19.7	32.6	24	23.2	21.7	21.9	24	23	27.5	23.2	21.5	30.1	23.2	31.7	32.1
GDF8/Compound 56	17	31.2	20.3	20.9	21.2	18.4	35.3	15.5	19.4	17.2	29.7	21.1	20.8	19.6	20	21.5	21.5	25	21.1	19.2	27.9	21.8	31.1	36.3
Differentiation Step 3	CT Values																							
Treatment/CTs	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM1	POU3F4	PTF1A	SST	ZIC1
AA/Wnt3a	18.7	22.1	30.2	30.3	22.4	34.1	21.1	34.9	27.9	35.1	26.3	30.6	23	29.3	33.1	38.5	30.6	36	25.6	28.2	29.5	38.4	30.3	32.7
GDF8/Wnt3a	18.4	22.1	29.7	30.9	22.5	34.5	21.1	34.7	27.4	34.6	26.9	30.5	23	30	36.4	36	31.1	33.5	25.4	30.4	30.1	38.5	27.9	32.4
GDF8/Compound 34	18.4	22.3	29.7	34.7	22.5	36.6	21.1	38.1	27.5	34.2	26.3	33.3	31	32.2	31.8	37.8	32.2	36.5	26.3	28.1	31.1	36.4	27.6	32.2
GDF8/Compound 56	18.2	22.5	29.7	31.6	22.5	36.3	21.2	35.7	27.3	34.4	27	30.6	27.9	30	31.8	33.2	30.3	32.7	25	28.9	30.4	34.8	27.5	32.7
Differentiation Step 4	CT Values																							
Treatment/CTs	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM1	POU3F4	PTF1A	SST	ZIC1
AA/Wnt3a	18.3	18.3	27.1	23.3	21.7	19.5	20.6	20.5	22.3	31	23.3	23	27.9	24.6	31.5	31.5	27.5	26.4	24.1	26.9	27.6	40	25.2	31.4
GDF8/Wnt3a	18.9	19.3	27.7	24.1	22.2	19.9	21.2	20.7	22.7	31.2	24.2	23.5	27.8	25	31.4	31.5	27.7	26.3	24.5	29.5	29.3	38.2	24.9	31.4
GDF8/Compound 34	18.9	18.9	27.1	25	22.3	21.5	21	22.2	24.4	31.3	24.6	24.6	29.7	25.8	31.6	29.6	27.9	26.4	23.9	27.5	28.1	37.1	24.7	25.5
GDF8/Compound 56	18.3	18.8	27.1	22.3	22.6	17.6	21.3	18.6	22.4	29.9	22.9	22.7	25.5	23.2	32	27.9	25.5	23.3	23.2	27.8	27.5	30.9	22.8	32.1
Differentiation Step 5	CT Values																							
Treatment/CTs	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM1	POU3F4	PTF1A	SST	ZIC1
AA/Wnt3a	18.3	18.5	27.3	24	22.3	18.2	21.4	16.5	22.4	31.3	23.7	24.1	32.5	25.4	29.7	32	28	25.1	24.2	26.9	29	37.5	22.5	26.9
GDF8/Wnt3a	19.3	19.7	28.5	24.2	23.1	18.2	22	16.7	22.4	31.1	23.4	24.6	32.3	25.6	29.9	30.5	28.1	25.4	24.6	29.9	29.9	33.4	22.1	32.1
GDF8/Compound 34	19.9	20.6	29	24.1	23.7	17.8	22.4	16.3	22.7	31.3	24	24.4	32.4	25.8	31.3	32	28.4	25.6	24.6	29.7	30.7	34.5	21.6	35
GDF8/Compound 56	20	21.1	29	25.1	24.8	18.3	23.2	17.1	24.6	32.1	24.9	24.8	33.8	26.3	34	30.2	29.7	27.3	25.9	29.7	31.5	34.7	22.1	33.3
Differentiation Step 6	CT Values																							
Treatment/CTs	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM1	POU3F4	PTF1A	SST	ZIC1
AA/Wnt3a	20.4	24.3	30.7	27.6	25.7	19.4	24.7	20.5	26.4	34.6	27.2	27.1	40	29.6	30.7	33.4	30.9	28.6	29.2	31.3	32.8	38.4	22.2	34.3
GDF8/Wnt3a	20.7	22.5	30.4	26.3	25.2	18.4	24.2	19.3	26.2	35.2	26.3	26.5	35	29	32.3	30.5	30.7	27.3	28.5	32.2	31.5	33.9	22.4	27.4
GDF8/Compound 34	21.3	24.6	31.3	27.1	25	18.4	24.7	20.1	26.3	34.3	26.4	27	34.5	29.2	31.6	33.1	30.4	28.1	29	32.9	33.9	37.7	22.1	34.5
GDF8/Compound 56	21.2	25	30.9	26	25.9	17.4	24.4	19.6	25.7	34.7	25.9	26.1	34.2	28.1	31.8	30.7	29.3	27.2	27.9	33.6	33.2	35.2	21	34.9

TABLE 14

Differentiation Step 1												Ct Value												
Treatment	GAPDH	AFP	CD9	CD99	CDH1	CDH	CDX2	CER1	CXCR4	FGF17	FGF4	FOXA2	GATA4	GATA6	GSC	HLXB9	KIT	MIXL1	NANOG	OTX2	POU5F1	SOX17	SOX7	T
AAWnt3a	20	35.6	24.1	24.2	26	20.9	40	17.5	22.7	19.8	35.8	24.7	23.8	22.1	21.6	23.4	23.2	28.1	24.5	22	32.6	23.2	33	36.8
GDF8/Wnt3a	20.1	34	23.8	24.5	24.6	21.6	40	19.5	23.3	21	34.8	25.1	24.5	23.3	23.3	17.6	25.5	28.3	24.9	23	31	23.7	33.3	34.2
GDF8/GSK3 inh BIO	19	34.4	23.7	24.1	24.3	21.3	36	18.7	23	20.1	33.5	24.2	24.2	22.4	21.8	23.4	24.2	28.4	23.7	21.8	30.8	23	33.7	33.1
GDF8/Compound 19	19.8	34.8	23.8	24	24.6	20.7	37.7	18.8	22.3	20	34.4	24.2	23.6	22.5	21.9	23.1	24.3	28	24.3	21.8	31.3	22.3	33	32.9
GDF8/Compound 202	19.8	40	24.5	23.5	25.9	20.8	40	18.8	22.2	20.3	36.5	24.4	23.4	22.3	22.3	24	24.8	27.3	26	21.9	33.3	22.7	32.6	32.1
GDF8/Compound 40	19.8	36.1	24.3	22.9	26.2	21.6	33.3	18.8	22.5	20.3	38.1	25.3	23.4	23	23	25	25.7	27.8	26	22.3	32.8	23.2	27.2	29.3
Differentiation Step 3												Ct Value												
Treatment	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4A	INS	ISL1	MAFA	MAFB	NEURO D1	NEURO G3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM	POU3F4	PTF1A	SST	ZIC1
AAWnt3a	17.9	25.4	29.5	28.4	23.3	34.1	21.8	29.2	29.4	34	27	25.8	25.2	27.3	34.1	28.3	27.8	35.2	22.7	28.3	28.6	30.8	32.2	37.4
GDF8/Wnt3a	18.5	26.5	30.4	29.4	23.9	34.2	22.6	29	29	34.4	27.1	27.2	26.4	27.9	37.8	29	29.2	31.4	23.3	32.2	30.1	30.7	31	30.1
GDF8/GSK3 inh BIO	18.5	25.2	30.3	29.4	23.6	32.8	22.6	28.8	29.3	34.7	27.6	26.8	26.2	27.6	35	28.8	28.7	32.9	23.2	32.2	29.5	30.6	31.3	31.1
GDF8/Compound 19	18.4	26.1	30.2	29.1	24	33.1	22.5	28.5	30	34.4	27.3	26.6	25.9	27.5	37.6	27.8	28.3	33.8	22.9	31.7	29.7	30	32.4	33.4
GDF8/Compound 202	18.7	26.7	31.1	29.6	24	34.9	22.7	30.3	31.6	34.2	27.8	27.2	27	28	40	30	29	36.2	23.7	30.9	30.2	32.4	32.4	34.6
GDF8/Compound 40	18.6	25.8	30.5	29.6	23.8	37.6	22.5	30	31.1	34.5	27.9	27.2	26.2	27.8	37.2	29.5	29	37.1	23.2	31.5	30.2	31.5	32.4	35.5
Differentiation Step 4												Ct Value												
Treatment	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4A	INS	ISL1	MAFA	MAFB	NEURO D1	NEURO G3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM	POU3F4	PTF1A	SST	ZIC1
AAWnt3a	18.9	21.3	28.8	24.6	23.4	21.7	21.9	21.6	25.2	32.4	24.9	23.7	23.8	24.2	33.9	25.6	25.6	27	23	29.2	27	28.1	25.3	32.6
GDF8/Wnt3a	18.3	21.3	28.5	25.3	23.1	22.6	21.9	21.9	25.7	33.1	24.9	24.3	24.2	24.7	35.4	25.8	26.2	27.3	23.2	31.2	27	28.7	24.7	24.6
GDF8/GSK3 inh BIO	19	21.1	28.7	25.3	23.3	22.3	21.9	22	25.7	32.5	25.4	24	24	24.7	35.4	26.1	26	27.7	23.2	30.7	27.4	28.5	25.6	31.5
GDF8/Compound 19	18.9	21.7	28.9	25.2	23.5	22.4	22.2	22	25.6	34	25.4	24.1	23.9	24.6	35.9	25.7	25.7	27.8	23.1	31.4	27.1	28.6	25.5	31.4
GDF8/Compound 202	19	20.9	29.2	25.1	23.6	22.4	22.1	22	25.5	33.3	25.5	23.9	24.1	24.5	35.7	26	25.8	27.6	23.4	30.2	27.5	28.8	26.1	35.7
GDF8/Compound 40	19.2	21.1	29.4	25.5	23.7	22.8	22.3	22.3	26	33.5	25.8	24.2	24.2	24.6	37.3	25.9	25.9	28.4	23.1	30.4	27.6	28.4	26.3	34.4
Differentiation Step 5												Ct Value												
Treatment	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4A	INS	ISL1	MAFA	MAFB	NEURO D1	NEURO G3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM	POU3F4	PTF1A	SST	ZIC1
AAWnt3a	19.1	19.5	28.6	23.1	23.9	16.2	21.9	16.9	23.4	33.7	23.3	21.7	27.4	24	33.1	25	26.4	24.7	22.6	27.1	28.4	27.5	22	34.1
GDF8/Wnt3a	18.4	19.9	28.4	23.8	23.8	17.2	22.3	17.4	24	32.6	23.9	22.6	28.6	24.2	33.1	25.8	27.2	25.6	23.6	29.2	28.9	29.1	22.7	25.6
GDF8/GSK3 inh BIO	19.1	19.2	29.1	24	24.2	17.2	22.4	17.6	24	33.5	23.8	22.9	28.4	24.4	40	25.1	27.3	25.6	23.6	29.2	28.1	28.7	23	26.3
GDF8/Compound 19	19	20	28.8	23.4	24.2	17	22.6	17.1	23.8	33.2	23.8	22.8	28.6	24.1	34.6	25	26.8	25.6	23.4	29.8	28.2	28.2	22.9	28.8
GDF8/Compound 202	19.2	20	29	23	23.9	16.7	22.2	16.8	23.2	32.7	23.8	22.3	28.2	23.2	40	26	27.2	25.8	23.4	29.9	29.1	28.4	22.6	33.8
GDF8/Compound 40	19.6	19.5	29	23.7	24.2	16.9	22.2	17.1	23.9	33.2	23.9	22.5	28.1	23.5	34.8	25	27.3	26.4	23.4	29.9	29.2	27.5	22.3	34.8

TABLE 15																								
Step 1	RT-PCR CT Values																							
Treatment	GAPDH	AFP	CD9	CD99	CDH1	CDH	CDX2	CER1	CXCR4	FGF17	FGF4	FOXA2	GATA4	GATA6	GSC	HLXB9	KIT	MXL1	NANOG	OTX2	POU5F1	SOX17	SOX7	T
AA	19.4	32.8	25.2	24.0	26.1	21.3	36.0	18.3	20.4	20.7	34.0	25.1	25.0	22.9	22.4	24.8	22.7	28.9	24.1	22.5	32.2	21.6	32.3	36.1
AA+Wnt3a	18.2	40.0	23.5	22.0	24.1	20.9	40.0	17.1	22.0	18.7	33.4	22.9	23.1	21.8	22.8	23.3	22.9	27.7	22.7	19.9	31.0	21.2	31.2	34.1
AA+Compound 181	20.1	40.0	24.5	23.3	26.0	20.7	35.9	18.2	22.0	20.1	35.1	25.6	24.7	22.9	20.8	25.5	19.7	27.8	24.8	22.3	32.8	22.2	33.0	33.8
AA+Compound 180	18.4	34.2	23.6	21.6	25.9	21.4	35.2	17.2	22.0	18.9	34.0	24.0	23.1	22.6	22.5	24.8	22.6	27.9	23.7	20.3	32.1	21.4	32.0	32.7
AA+Compound 19	20.1	35.4	24.4	24.9	26.3	20.3	40.0	17.9	17.7	20.5	32.8	25.6	25.1	22.7	21.3	26.0	22.6	29.5	24.1	22.9	32.1	22.3	33.0	29.6
AA+Compound 202	20.3	40.0	25.1	23.7	25.9	21.4	40.0	18.3	22.0	20.4	36.0	25.7	24.5	23.0	21.8	25.2	23.9	27.7	24.5	22.5	32.7	22.6	32.1	33.5
AA+Compound 40	19.9	40.0	24.6	23.6	25.8	20.4	40.0	17.7	22.7	20.2	35.1	25.5	24.6	22.5	20.9	25.5	22.9	28.0	23.8	22.0	33.6	22.3	32.5	32.8
AA-GSK3 inhib BIO	20.2	35.0	25.4	23.7	27.2	21.9	35.5	18.5	22.2	20.9	36.0	25.8	25.0	23.2	22.2	25.2	23.7	28.5	24.9	22.8	34.3	23.4	32.7	33.5
AA+Compound 206	19.8	40.0	24.9	23.7	25.5	21.1	40.0	18.3	19.6	20.7	36.2	24.4	24.8	22.7	22.6	24.1	23.7	27.6	23.9	22.0	33.2	23.3	32.4	34.6
GDF8	21.6	40.0	25.5	25.9	25.2	22.3	40.0	20.1	24.6	22.1	34.9	25.9	27.5	24.8	24.2	25.3	25.4	30.6	25.0	24.3	29.5	25.2	29.8	32.9
GDF8+Wnt3a	21.2	40.0	25.0	25.8	25.1	22.6	40.0	19.7	23.6	22.0	34.8	25.7	27.3	24.4	23.8	25.0	25.1	30.5	24.8	24.4	31.1	25.0	34.5	32.6
GDF8+Compound 181	20.7	40.0	25.1	23.6	25.5	22.4	40.0	20.0	23.0	21.5	36.3	25.2	25.4	23.9	23.6	24.3	26.3	28.1	25.4	23.3	31.8	23.7	32.8	31.9
GDF8+Compound 180	20.9	40.0	25.6	24.0	26.9	22.1	34.7	19.9	22.7	21.1	36.6	25.0	25.2	23.9	23.6	24.2	25.8	28.3	26.3	23.0	33.2	24.2	32.8	32.3
GDF8+Compound 19	19.6	40.0	23.9	23.7	24.6	20.7	40.0	18.0	21.8	20.4	33.2	24.2	25.0	22.8	22.1	24.0	22.6	28.5	23.1	22.7	30.3	22.6	32.1	29.3
GDF8+Compound 202	18.5	30.6	22.1	20.2	22.8	19.7	35.8	18.5	22.4	19.9	34.3	23.6	22.7	22.5	23.5	23.1	25.9	27.7	25.1	21.5	30.0	22.3	32.0	32.5
GDF8+Compound 40	19.7	40.0	23.0	22.6	24.7	20.5	33.8	18.2	22.4	20.5	35.4	24.9	24.2	22.6	23.4	23.4	25.4	28.5	24.0	22.2	31.6	23.7	31.2	32.7
GDF8-GSK3 inhib BIO	19.6	30.1	23.1	21.8	24.3	20.0	33.4	17.7	23.3	20.1	34.8	24.7	24.5	22.4	22.7	24.1	25.0	29.3	24.8	21.8	31.7	23.3	33.7	34.6
GDF8+Compound 206	19.7	40.0	22.7	22.5	23.2	21.0	29.9	18.4	23.0	20.3	34.5	25.3	24.9	22.9	23.6	23.8	25.7	29.7	24.7	22.2	30.7	24.4	32.8	33.8
Step 3	RT-PCR CT Values																							
Treatment	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4A	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM	POU3F4	PTF1A	SST	ZIC1
AA	18.5	26.9	30.6	32.0	22.9	34.1	22.4	34.8	29.3	36.4	27.4	30.1	28.0	29.3	34.6	32.2	31.4	40.0	23.3	31.0	30.5	34.6	33.2	34.8
AA+Wnt3a	18.4	27.3	30.2	33.0	23.0	34.7	22.4	40.0	28.8	33.9	27.7	30.4	28.1	29.0	40.0	34.9	31.9	38.1	23.8	30.9	30.7	40.0	32.8	40.0
AA+Compound 181	18.6	26.0	30.1	34.2	22.3	40.0	22.3	40.0	30.4	34.6	28.5	31.4	29.0	30.3	33.9	33.8	33.3	40.0	25.0	30.0	30.8	36.0	34.6	34.8
AA+Compound 180	18.8	25.5	30.0	33.3	22.5	35.1	22.5	40.0	29.4	34.3	28.6	32.6	30.3	31.1	32.5	33.3	33.3	35.2	26.3	28.9	31.3	40.0	34.7	34.4
AA+Compound 19	34.1	40.0	40.0	40.0	40.0	40.0	37.4	40.0	40.0	40.0	34.9	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
AA+Compound 202	18.5	26.2	30.7	33.0	22.5	34.8	22.6	35.1	29.8	35.1	28.2	30.0	28.5	29.5	33.6	33.9	32.0	35.6	24.1	29.7	31.9	40.0	34.2	40.0
AA+Compound 40	18.5	25.8	30.1	34.9	22.2	40.0	22.3	40.0	29.7	34.1	28.1	30.8	29.1	30.3	33.6	34.4	32.9	40.0	24.8	30.3	31.3	35.6	35.3	35.4
AA-GSK3 inhib BIO	18.5	24.9	30.1	34.6	22.0	40.0	21.5	40.0	30.2	34.9	27.8	34.0	31.0	31.8	32.6	40.0	34.9	40.0	26.1	31.0	32.4	40.0	35.2	34.9
AA+Compound 206	18.3	27.0	30.3	33.7	22.7	35.7	22.5	40.0	28.4	34.4	27.8	30.7	28.6	29.8	32.8	35.0	31.5	30.4	23.9	29.7	31.2	35.0	31.9	40.0
GDF8	18.0	28.7	30.4	35.3	23.8	40.0	23.6	40.0	28.5	33.6	27.2	30.3	28.7	30.3	34.4	35.0	32.4	24.8	25.5	30.0	30.8	34.2	29.5	26.1
GDF8+Wnt3a	17.4	27.0	29.5	33.8	23.1	35.1	22.4	40.0	26.3	30.5	26.1	30.1	27.5	29.1	40.0	32.9	31.5	25.8	23.1	32.2	29.8	34.0	27.7	28.2
GDF8+Compound 181	18.8	27.8	30.2	31.3	23.4	40.0	22.7	34.5	28.8	35.5	27.3	28.9	27.0	28.0	35.4	32.5	30.5	35.4	23.5	29.4	30.5	33.9	32.4	34.5
GDF8+Compound 180	18.8	27.3	30.6	32.4	22.8	34.9	22.7	40.0	29.5	35.9	27.7	30.0	27.5	29.0	35.4	32.6	31.2	40.0	23.7	31.6	30.8	34.6	34.4	40.0
GDF8+Compound 19	18.3	24.9	29.7	33.4	22.0	40.0	22.2	40.0	29.7	34.5	28.4	33.3	31.5	32.2	34.4	35.2	34.4	35.0	25.0	29.4	31.6	40.0	34.1	33.5
GDF8+Compound 202	18.7	27.8	30.4	32.8	23.8	40.0	22.9	34.6	28.5	34.2	27.6	29.6	27.1	28.4	35.7	30.5	30.4	31.8	23.0	30.1	30.2	31.6	30.9	34.0
GDF8+Compound 40	18.4	27.7	30.1	32.5	23.0	35.1	22.4	40.0	29.1	34.3	27.5	30.0	27.3	28.8	35.3	32.9	31.5	40.0	23.2	29.2	30.5	36.3	33.7	40.0
GDF8-GSK3 inhib BIO	18.4	24.9	30.3	31.5	22.2	34.7	21.7	34.5	29.9	35.3	27.6	29.2	26.8	27.9	34.6	33.9	30.6	35.1	24.0	30.4	30.0	34.8	33.6	40.0
GDF8+Compound 206	18.2	27.6	30.2	33.5	23.8	40.0	22.9	40.0	27.9	35.1	27.1	29.9	27.7	29.3	40.0	32.3	31.3	27.4	23.3	32.7	30.8	33.0	29.2	30.3

Step 4	RT-PCR CT Values																							
Treatment	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4A	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM	POU3F4	PTF1A	SST	ZIC1
AA	19.0	22.4	28.6	23.9	23.7	22.8	22.1	23.6	24.1	30.6	23.5	23.7	23.3	24.0	31.9	27.4	25.7	27.1	23.7	29.8	26.5	29.6	24.3	32.5
AA+Win3a	19.5	23.5	29.3	24.6	24.1	25.1	22.7	25.2	25.1	31.1	24.4	24.4	24.1	24.5	31.8	27.5	26.1	27.9	24.1	30.4	27.2	31.1	25.9	32.4
AA+Compound 181	18.0	21.0	27.7	24.0	22.1	24.5	21.0	24.6	24.5	31.6	24.0	24.2	24.6	24.6	30.9	28.1	26.3	28.0	24.0	27.9	27.5	31.0	25.5	31.2
AA+Compound 180	19.4	21.0	27.3	26.3	21.6	25.4	20.6	26.7	26.1	34.7	24.6	25.5	25.8	25.0	29.4	28.9	27.7	28.9	24.0	26.7	28.5	32.6	27.1	31.2
AA+Compound 19	(insufficient RNA sample)																							
AA+Compound 202	19.2	20.7	29.1	24.0	23.3	21.8	21.9	22.7	24.4	31.1	23.9	24.0	24.1	24.4	32.1	27.4	26.3	26.9	24.3	29.6	27.7	29.5	24.0	35.1
AA+Compound 40	19.2	20.8	29.4	24.7	22.9	22.2	21.8	23.5	25.3	31.5	24.7	24.7	25.3	25.0	32.2	28.7	27.1	28.2	25.1	30.3	28.5	31.2	25.6	32.7
AA+GSK3 inhib BIO	19.0	19.1	29.2	26.7	22.7	25.2	21.1	26.3	26.5	33.0	25.4	26.3	27.7	26.2	31.5	32.9	29.4	30.0	26.5	29.6	30.5	33.7	27.2	32.4
AA+Compound 206	18.8	20.9	28.4	23.3	22.9	21.2	21.7	22.8	24.0	30.4	23.5	23.3	23.3	23.8	31.9	27.2	25.5	26.3	23.6	29.7	26.6	29.3	23.6	32.9
GDF8	18.0	25.5	29.1	29.8	24.6	31.3	23.8	30.9	27.6	32.8	24.1	29.2	28.7	29.0	32.3	31.2	31.1	27.9	26.5	29.4	29.4	40.0	26.0	22.8
GDF8+Win3a	19.0	24.3	29.0	25.4	24.4	27.7	23.0	25.5	25.4	32.7	25.1	25.4	24.0	25.0	33.3	28.0	26.7	28.8	23.8	33.4	27.7	30.5	24.1	29.0
GDF8+Compound 181	18.0	22.8	28.1	23.5	23.1	24.6	21.4	22.7	23.8	30.6	23.0	22.9	21.8	23.2	32.1	25.7	24.2	27.0	22.3	27.6	25.4	28.6	24.2	31.6
GDF8+Compound 180	19.5	24.0	29.3	24.4	23.9	25.7	22.5	24.5	24.7	31.4	24.4	24.3	23.7	24.4	33.5	27.7	26.0	28.3	23.8	30.6	26.9	30.7	26.3	33.4
GDF8-Compound 19	19.1	22.6	28.7	25.5	22.9	26.7	22.1	27.2	25.8	32.6	25.5	26.2	26.4	25.6	34.0	30.2	28.2	30.0	25.1	30.2	29.2	32.6	27.9	31.1
GDF8+Compound 202	19.0	22.0	28.9	23.7	24.5	21.8	22.3	21.7	24.3	30.3	23.5	22.9	22.2	23.4	33.4	25.9	24.8	26.5	23.0	29.5	26.1	27.9	22.4	34.1
GDF8-Compound 40	19.0	21.4	29.0	23.4	23.6	21.0	22.0	21.5	23.8	30.2	23.3	23.2	22.7	23.7	33.2	26.6	24.8	26.1	23.4	29.3	26.4	28.4	22.7	32.6
GDF8+GSK3 inhib BIO	19.1	19.4	29.1	24.3	23.0	21.5	21.4	21.8	24.6	31.0	24.0	23.8	23.8	24.2	33.3	27.9	26.1	27.0	24.2	30.0	27.8	29.5	23.4	32.7
GDF8+Compound 206	18.9	21.6	28.9	24.4	24.0	22.6	22.3	22.7	24.9	30.9	24.2	23.7	23.0	24.0	35.3	26.3	25.5	27.2	23.5	31.4	26.9	27.7	24.0	28.9
Step 5	RT-PCR CT Values																							
Treatment	GAPDH	ALB	AMY2A	ARX	CDX2	GCG	HNF4A	INS	ISL1	MAFA	MAFB	NEUROD1	NEUROG3	NKX2-2	NKX2-5	NKX6-1	PAX4	PAX6	PDX1	PECAM	POU3F4	PTF1A	SST	ZIC1
AA	18.3	20.2	27.6	22.0	23.4	14.1	21.3	14.9	22.4	31.8	22.2	22.8	28.1	23.7	34.1	25.5	27.3	24.0	23.2	30.1	28.8	27.3	19.6	34.4
AA+Win3a	18.0	20.0	27.7	21.9	23.1	14.0	20.9	14.6	22.3	31.6	22.0	21.6	28.0	23.4	34.8	26.1	27.3	23.7	23.3	29.6	28.8	27.5	19.4	32.5
AA+Compound 181	18.0	18.8	27.6	22.0	22.9	14.3	20.9	14.5	22.1	31.4	22.2	21.5	28.9	23.3	32.2	26.1	26.8	24.0	23.1	27.5	28.8	28.0	18.8	31.2
AA+Compound 180	18.0	18.8	27.6	22.4	22.9	14.9	21.0	14.7	22.4	31.9	22.6	21.7	29.5	23.8	30.2	26.5	27.2	24.3	23.2	26.7	29.0	28.7	18.7	30.3
AA+Compound 19	17.9	23.6	28.6	28.2	25.4	27.0	24.2	26.9	26.2	32.0	24.4	27.2	31.2	28.0	30.1	25.8	35.1	28.6	29.4	31.5	28.2	32.4	23.1	24.0
AA+Compound 202	18.6	19.2	28.0	22.6	23.4	14.9	21.3	15.0	22.7	31.8	22.6	21.9	28.6	23.7	29.9	25.8	26.9	24.7	23.6	27.7	29.2	27.9	19.4	32.8
AA+Compound 40	18.3	18.9	27.9	22.3	23.0	14.6	21.1	14.7	22.5	31.5	22.4	21.6	29.0	23.5	32.9	26.1	27.1	24.4	23.2	28.1	29.2	28.1	19.1	31.9
AA+GSK3 inhib BIO	18.3	17.1	28.0	23.0	22.6	15.1	20.5	15.1	22.8	31.8	22.8	22.1	29.5	24.2	33.8	27.5	27.4	24.9	23.8	28.3	29.9	29.7	19.5	32.0
AA+Compound 206	18.2	19.5	27.9	22.2	23.4	14.4	21.3	14.8	22.5	31.1	22.4	21.7	28.0	23.6	35.8	25.9	27.1	24.1	23.3	29.0	28.7	27.5	19.7	32.7
GDF8	17.4	20.5	28.2	25.2	24.4	18.1	22.9	17.7	24.3	31.8	23.3	24.2	30.1	25.9	31.4	26.6	29.4	26.6	25.6	29.7	27.8	29.5	21.1	22.5
GDF8+Win3a	17.8	20.6	28.2	24.8	24.3	17.7	22.9	17.5	24.2	31.9	23.5	24.0	30.2	25.6	31.9	27.0	29.3	26.5	25.6	29.8	28.1	30.7	21.5	22.8
GDF8+Compound 181	18.0	19.1	27.6	22.4	23.4	14.5	21.2	14.8	22.5	31.5	22.6	21.7	27.4	23.4	33.8	25.0	26.8	24.4	23.0	27.5	28.8	27.3	19.5	31.9
GDF8+Compound 180	18.0	18.0	27.3	22.2	22.9	14.2	20.9	14.4	22.2	31.4	22.1	21.2	27.9	23.2	40.0	25.1	26.1	23.8	22.9	29.6	28.7	27.3	18.8	31.5
GDF8-Compound 19	18.3	18.5	27.8	23.4	23.0	16.2	21.2	15.6	23.2	32.2	23.2	22.6	31.2	24.3	33.9	28.6	27.3	25.2	24.2	29.0	30.1	31.0	20.5	31.9
GDF8+Compound 202	18.7	19.6	28.3	23.1	24.0	15.7	21.9	15.8	23.5	32.8	23.3	22.3	27.8	23.8	31.3	24.8	27.1	25.2	23.2	29.0	29.5	27.1	20.8	30.9
GDF8-Compound 40	18.1	18.7	27.9	22.3	23.0	14.8	21.1	14.8	22.6	31.5	22.5	21.5	27.3	23.2	33.2	25.0	26.6	24.1	22.9	27.3	28.9	27.4	20.4	32.0
GDF8+GSK3 inhib BIO	18.4	17.1	27.6	23.2	22.8	15.2	20.6	15.5	23.1	32.6	22.6	22.1	28.2	24.2	35.0	26.7	27.3	24.8	24.0	28.1	30.1	28.5	19.3	32.4
GDF8+Compound 206	18.0	20.0	27.9	23.4	24.0	16.0	22.1	16.0	23.6	32.1	22.9	22.8	28.5	24.4	30.5	25.9	27.5	25.4	24.0	29.7	28.3	28.0	20.8	23.9

TABLE 16	
Compound #	Primary Selectivity
Compound 6	Selective for GSK
Compound 7	Selective for GSK
Compound 8	Selective for GSK
Compound 9	Selective for CDK
Compound 57	Selective for Trk
Compound 41	Selective for GSK
Compound 42	Selective for CDK
Compound 10	Selective for CDK
Compound 34	Positive Control
Compound 11	Selective for CDK
Compound 43	Selective for Trk
Compound 44	Selective for GSK
Compound 12	Selective for CDK
Compound 45	Selective for Trk

TABLE 17							
				Cell Number		Sox17 Expression	
Plate	Treatment	Compound #	Compound Selectivity	Average Total Cell Number	% of positive control	Average Total Intensity	% of positive control
1	no Activin A	none	n/a	9809	67.8	-4.0E+05	-0.2
1	Activin A/Wnt3a	none	n/a	14476	100.0	2.3E+08	100.0
1	No GDF8	Compound 11	Selective for CDK	565	3.9	-1.1E+06	-0.5
1	No GDF8	Compound 44	Selective for GSK	14	0.1	-1.1E+06	-0.5

1	No GDF8	Compound 43	Selective for Trk	8610	59.5	-2.1E+05	-0.1
1	No GDF8	Compound 42	Selective for CDK	8700	60.1	-2.4E+05	-0.1
1	No GDF8	Compound 57	Selective for Trk	1222	8.4	-7.1E+05	-0.3
1	No GDF8	Compound 10	Selective for CDK	7011	48.4	-6.6E+05	-0.3
1	No GDF8	Compound 41	Selective for GSK	9995	69.0	5.9E+04	0.0
1	No GDF8	Compound 7	Selective for CDK	3	0.0	-1.4E+06	-0.6
1	No GDF8	Compound 45	Selective for Trk	8857	61.2	-4.5E+05	-0.2
1	No GDF8	Compound 6	Selective for GSK	14827	102.4	-1.8E+05	-0.1
1	No GDF8	Compound 9	Selective for CDK	7156	49.4	-4.2E+04	0.0
1	No GDF8	Compound 12	Selective for GSK	13124	90.7	-2.3E+05	-0.1
1	No GDF8	Compound 8	Selective for GSK	13235	91.4	3.8E+05	0.2
1	GDF8	Compound 34	Positive Control	13926	96.2	2.6E+08	111.8
1	GDF8	Compound 45	Selective for Trk	9540	65.9	1.1E+08	47.9
1	GDF8	Compound 7	Selective for GSK	5296	36.6	7.0E+07	30.4
1	GDF8	Compound 10	Selective for CDK	4627	32.0	6.6E+07	28.6
1	GDF8	Compound 6	Selective for GSK	5118	35.4	5.8E+07	25.2
1	GDF8	Compound 43	Selective for Trk	6682	46.2	5.4E+07	23.4
1	GDF8	Compound 42	Selective for CDK	5686	39.3	4.9E+07	21.2
1	GDF8	Compound 8	Selective for GSK	5018	34.7	4.7E+07	20.4
1	GDF8	Compound 9	Selective for CDK	4816	33.3	4.5E+07	19.4
1	GDF8	Compound 41	Selective for GSK	4455	30.8	3.4E+07	14.8
1	GDF8	n/a	n/a	2856	19.7	2.2E+07	9.4
1	GDF8	Compound 57	Selective for Trk	2110	14.6	1.1E+07	4.8
1	GDF8	Compound 11	Selective for CDK	210	1.4	-4.9E+05	-0.2
1	GDF8	Compound 44	Selective for GSK	226	1.6	-9.5E+05	-0.4
1	GDF8	Compound 12	Selective for CDK	31	0.2	-1.3E+06	-0.6

Table 18										
			Cell Number				Sox17 Expression			
Plate	Treatment	Compound #	Average Total Cell Number	SD	CV%	% of positive control	Average Total Intensity	SD	CV%	% of positive control
1	no Activin A (with Wnt3a)	none	15489	0	0.00	103.2	3.75E+07	0.00E+00	0.00	10.9
1	Activin A/Wnt3a	none	15007	1991	13.27	100.0	3.45E+08	7.16E+07	20.75	100.0
1	GDF8	Compound 206	20568	1683	8.18	137.1	5.19E+08	4.41E+07	8.51	150.3
1	GDF8	Compound 207	19224	1091	5.68	128.1	2.54E+08	5.69E+07	22.41	73.6
1	GDF8	Compound 19	12569	1524	12.13	83.8	2.40E+08	6.34E+07	26.44	69.5
1	GDF8	Compound 23	8758	474	5.41	58.4	1.16E+08	9.07E+06	7.80	33.7
1	GDF8	Compound 170	6460	2305	35.68	43.0	9.44E+07	6.98E+07	73.93	27.4
1	GDF8	Compound 208	4848	1225	25.27	32.3	2.26E+07	2.15E+07	94.96	23.6
1	GDF8	Compound 209	4831	1243	25.74	32.2	3.97E+07	1.61E+07	40.56	11.5
1	GDF8	Compound 32	4338	1520	35.04	28.9	3.63E+07	3.27E+07	90.14	10.5
1	GDF8	Compound 30	4679	435	9.29	31.2	3.47E+07	1.04E+07	30.03	10.1
1	GDF8	Compound 223	3704	1077	29.08	24.7	3.45E+07	2.74E+07	79.43	10.0
1	GDF8	Compound 2	4538	632	13.93	30.2	2.95E+07	2.81E+06	9.50	8.6
1	GDF8	Compound 210	2645	817	30.88	17.6	2.90E+07	2.45E+07	84.73	8.4
1	GDF8	Compound 24	5012	1263	25.21	33.4	2.64E+07	1.66E+07	62.95	7.7
1	GDF8	Compound 211	5165	796	15.41	34.4	2.61E+07	5.02E+06	19.23	7.6
1	GDF8	Compound 212	5476	1445	26.39	36.5	2.54E+07	1.18E+07	46.53	7.4
1	GDF8	Compound 224	5188	761	14.67	34.6	2.46E+07	8.26E+06	33.56	7.1
1	GDF8	Compound 225	4431	1149	25.92	29.5	2.45E+07	2.65E+07	108.19	7.1
1	GDF8	Compound 13	3123	1508	48.27	20.8	2.44E+07	2.30E+07	94.13	7.1
1	GDF8	Compound 213	1261	1028	81.49	8.4	2.07E+07	1.97E+07	95.03	6.0
1	GDF8	Compound 52	4932	386	7.82	32.9	1.99E+07	6.90E+06	34.67	5.8

1	GDF8	Compound 214	3345	335	10.01	22.3	1.93E+07	1.39E+07	72.18	5.6
1	GDF8	Compound 51	4289	940	21.91	28.6	1.70E+07	1.10E+07	64.86	4.9
1	GDF8	Compound 26	4896	545	11.14	32.6	1.65E+07	5.93E+06	36.02	4.8
1	GDF8	Compound 226	3617	577	15.94	24.1	1.59E+07	4.96E+06	31.21	4.6
1	GDF8	Compound 215	4326	165	3.81	28.8	1.45E+07	2.69E+06	18.53	4.2
1	GDF8	Compound 31	3619	1011	27.92	24.1	1.36E+07	4.63E+06	34.15	3.9
1	GDF8	Compound 216	3364	629	18.70	22.4	8.75E+06	2.30E+06	26.32	2.5
1	GDF8	Compound 217	2859	544	19.03	19.1	8.75E+06	1.94E+06	22.16	2.5
1	GDF8	Compound 218	1327	118	8.92	8.8	6.44E+06	9.70E+05	15.05	1.9
1	GDF8	Compound 219	368	168	45.67	2.5	1.79E+06	1.29E+06	72.17	0.5
2	no Activin A (with Wnt3a)	none	15778	0	0.00	103.2	2.24E+07	0.00E+00	0.00	6.7
2	Activin A/Wnt3a	none	15290	1119	7.32	100.0	3.37E+08	2.84E+07	8.44	100.0
2	GDF8	Compound 202	20177	987	4.89	132.0	4.85E+08	1.94E+07	4.00	144.0
2	GDF8	Compound 227	2911	4619	158.69	19.0	3.89E+07	6.69E+07	172.00	11.5
2	GDF8	Compound 15	4383	1775	40.49	28.7	3.57E+07	3.57E+07	100.03	10.6
2	GDF8	Compound 228	4043	1253	30.98	26.4	3.10E+07	2.53E+07	81.62	9.2
2	GDF8	Compound 229	3451	892	25.85	22.6	1.80E+07	1.46E+07	81.07	5.4
2	GDF8	Compound 4	3163	805	25.44	20.7	1.58E+07	3.54E+06	22.32	4.7
2	GDF8	Compound 220	2791	1453	52.05	18.3	1.40E+07	9.00E+06	64.28	4.2
2	GDF8	Compound 5	3137	1172	37.34	20.5	1.30E+07	7.52E+06	57.85	3.9
2	GDF8	Compound 230	2624	248	9.46	17.2	1.24E+07	1.55E+07	124.73	3.7
2	GDF8	Compound 231	4773	2651	55.55	31.2	1.22E+07	6.51E+06	53.37	3.6
2	GDF8	Compound 232	3273	1290	39.41	21.4	1.18E+07	1.51E+07	127.98	3.5
2	GDF8	Compound 221	1950	361	18.52	12.8	1.18E+07	1.54E+07	131.11	3.5
2	GDF8	Compound 233	3041	180	5.93	19.9	1.12E+07	1.09E+07	97.44	3.3
2	GDF8	Compound 147	3434	1199	34.91	22.5	1.12E+07	9.80E+06	87.75	3.3
2	GDF8	Compound 234	2835	623	21.98	18.5	9.47E+06	5.67E+06	59.84	2.8
2	GDF8	Compound 235	3391	2269	66.91	22.2	9.10E+06	6.51E+06	71.52	2.7
2	GDF8	Compound 236	2868	561	19.57	18.8	6.73E+06	6.32E+06	93.82	2.0
2	GDF8	Compound 33	2362	511	21.66	15.4	6.60E+06	2.45E+06	37.20	2.0

2	GDF8	Compound 1	3213	166	5.16	21.0	6.48E+06	3.09E+06	47.67	1.9
2	GDF8	Compound 53	2783	441	15.86	18.2	6.36E+06	2.89E+06	45.36	1.9
2	GDF8	Compound 237	2973	292	9.83	19.4	6.02E+06	3.00E+06	49.79	1.8
2	GDF8	Compound 238	2739	485	17.70	17.9	5.97E+06	6.10E+06	102.07	1.8
2	GDF8	Compound 239	3156	667	21.15	20.6	5.60E+06	2.42E+06	43.24	1.7
2	GDF8	Compound 240	3002	287	9.55	19.6	4.68E+06	3.13E+06	66.80	1.4
2	GDF8	Compound 200	2308	209	9.04	15.1	4.39E+06	1.88E+06	42.83	1.3
2	GDF8	Compound 222	1776	719	40.47	11.6	3.33E+06	2.52E+06	75.78	1.0
2	GDF8	Compound 241	2949	446	15.14	19.3	3.29E+06	1.55E+06	47.03	1.0
2	GDF8	Compound 242	385	184	47.83	2.5	1.08E+06	8.85E+05	81.61	0.3
2	GDF8	Compound 243	249	55	22.21	1.6	2.53E+05	3.07E+05	121.25	0.1
2	GDF8	Compound 204	250	21	8.38	1.6	1.36E+05	2.27E+04	16.66	0.0
3	no Activin A (with Wnt3a)	none	15796	0	0.00	99.6	2.82E+07	0.00E+00	0.00	8.0
3	Activin A/Wnt3a	none	15867	785	4.95	100.0	3.54E+08	2.40E+07	6.77	100.0
3	GDF8	Compound 34	6974	3723	53.38	44.0	2.07E+08	9.51E+07	45.85	58.6
3	GDF8	Compound 185	10892	1552	14.24	68.6	1.53E+08	4.08E+07	26.72	43.1
3	GDF8	Compound 35	7746	1873	24.17	48.8	1.35E+08	4.86E+07	36.08	38.0
3	GDF8	Compound 22	6727	1927	28.64	42.4	1.06E+08	5.04E+07	47.73	29.8
3	GDF8	Compound 34	4889	1152	23.57	30.8	4.31E+07	2.11E+07	48.95	12.2
3	GDF8	Compound 184	4173	1758	42.14	26.3	3.94E+07	2.24E+07	56.78	11.1
3	GDF8	Compound 223	4234	1604	37.88	26.7	3.55E+07	2.51E+07	70.56	10.0
3	GDF8	Compound 37	4187	338	8.06	26.4	3.11E+07	1.56E+07	50.18	8.8
3	GDF8	Compound 244	4479	1229	27.43	28.2	2.73E+07	1.52E+07	55.71	7.7
3	GDF8	Compound 245	4725	99	2.09	29.8	2.59E+07	1.03E+07	39.90	7.3
3	GDF8	Compound 246	3820	1091	28.57	24.1	2.30E+07	2.69E+07	117.08	6.5
3	GDF8	Compound 247	3730	966	25.90	23.5	2.14E+07	1.04E+07	48.63	6.1
3	GDF8	Compound 248	3875	445	11.48	24.4	2.13E+07	9.45E+06	44.45	6.0
3	GDF8	Compound 25	3879	658	16.95	24.4	1.76E+07	1.21E+07	69.04	5.0
3	GDF8	Compound 195	3703	405	10.94	23.3	1.61E+07	3.27E+06	20.34	4.5
3	GDF8	Compound 227	2904	397	13.68	18.3	1.43E+07	1.35E+07	94.25	4.0

3	GDF8	Compound 183	3306	969	29.32	20.8	1.35E+07	1.14E+07	84.25	3.8
3	GDF8	Compound 187	2768	1426	51.51	17.4	1.35E+07	9.02E+06	66.67	3.8
3	GDF8	Compound 201	3213	1114	34.66	20.3	1.35E+07	1.69E+07	125.02	3.8
3	GDF8	Compound 197	3268	211	6.46	20.6	1.30E+07	5.25E+06	40.51	3.7
3	GDF8	Compound 249	3840	348	9.06	24.2	1.29E+07	6.79E+06	52.72	3.6
3	GDF8	Compound 141	2404	213	8.86	15.1	1.12E+07	4.95E+06	44.30	3.2
3	GDF8	Compound 194	3177	354	11.14	20.0	9.75E+06	2.11E+06	21.63	2.8
3	GDF8	Compound 250	3683	420	11.40	23.2	9.14E+06	4.78E+06	52.32	2.6
3	GDF8	Compound 251	3021	668	22.10	19.0	8.41E+06	4.59E+06	54.60	2.4
3	GDF8	Compound 20	2793	205	7.35	17.6	6.77E+06	1.86E+06	27.45	1.9
3	GDF8	Compound 252	2580	135	5.24	16.3	6.20E+06	2.31E+05	3.72	1.8
3	GDF8	Compound 253	2485	820	32.98	15.7	5.83E+06	1.47E+06	25.20	1.6
3	GDF8	Compound 202	2095	518	24.71	13.2	5.75E+06	2.62E+06	45.66	1.6
3	GDF8	Compound 21	371	294	79.19	2.3	2.36E+06	3.07E+06	129.78	0.7
4	no Activin A (with Wnt3a)	none	16629	0	0.00	119.3	2.42E+07	0.00E+00	0.00	7.8
4	Activin A/Wnt3a	none	13945	1535	11.01	100.0	3.09E+08	4.77E+07	15.46	100.0
4	GDF8	Compound 34	7416	6482	87.41	53.2	2.10E+08	1.82E+08	86.70	68.0
4	GDF8	Compound 240	11283	2023	17.93	80.9	1.61E+08	4.41E+07	27.34	52.2
4	GDF8	Compound 28	5236	1787	34.12	37.5	4.03E+07	3.08E+07	76.36	13.1
4	GDF8	Compound 198	3985	2674	67.10	28.6	3.89E+07	5.55E+07	142.91	12.6
4	GDF8	Compound 196	4861	1501	30.87	34.9	3.03E+07	1.98E+07	65.37	9.8
4	GDF8	Compound 18	1921	1759	91.56	13.8	2.94E+07	3.65E+07	123.90	9.5
4	GDF8	Compound 186	3486	425	12.19	25.0	2.34E+07	1.42E+07	60.78	7.6
4	GDF8	Compound 254	3960	1521	38.42	28.4	2.31E+07	2.27E+07	98.10	7.5
4	GDF8	Compound 168	3460	324	9.36	24.8	2.28E+07	7.13E+06	31.23	7.4
4	GDF8	Compound 190	3402	1318	38.74	24.4	1.87E+07	1.58E+07	84.61	6.1
4	GDF8	Compound 255	4006	1625	40.57	28.7	1.52E+07	1.05E+07	68.91	4.9
4	GDF8	Compound 50	2666	743	27.86	19.1	1.48E+07	8.30E+06	56.15	4.8
4	GDF8	Compound 27	3721	721	19.37	26.7	1.19E+07	9.69E+06	81.29	3.9
4	GDF8	Compound 256	2922	1275	43.64	21.0	9.41E+06	8.65E+06	92.01	3.0

4	GDF8	Compound 257	3182	705	22.14	22.8	8.06E+06	4.49E+06	55.75	2.6
4	GDF8	Compound 258	2731	472	17.29	19.6	7.89E+06	7.24E+06	91.70	2.6
4	GDF8	Compound 189	2350	1625	69.16	16.9	7.72E+06	5.36E+06	69.41	2.5
4	GDF8	Compound 259	2195	955	43.49	15.7	6.92E+06	2.58E+06	37.29	2.2
4	GDF8	Compound 260	2468	741	30.04	17.7	6.64E+06	3.33E+06	50.18	2.2
4	GDF8	Compound 261	2965	456	15.38	21.3	6.23E+06	2.10E+06	33.61	2.0
4	GDF8	Compound 192	2377	572	24.08	17.0	6.17E+06	2.76E+06	44.65	2.0
4	GDF8	Compound 262	2894	399	13.78	20.8	5.75E+06	3.00E+06	52.20	1.9
4	GDF8	Compound 188	3005	759	25.26	21.6	5.02E+06	3.97E+06	79.06	1.6
4	GDF8	Compound 263	2129	230	10.79	15.3	4.77E+06	1.14E+06	23.93	1.5
4	GDF8	Compound 264	2630	342	13.00	18.9	4.28E+06	2.17E+06	50.73	1.4
4	GDF8	Compound 265	2636	1372	52.04	18.9	4.27E+06	1.15E+06	26.86	1.4
4	GDF8	Compound 14	274	14	5.02	2.0	1.56E+05	9.51E+04	60.91	0.1
4	GDF8	Compound 205	241	3	1.20	1.7	1.36E+05	6.83E+04	50.42	0.0
4	GDF8	Compound 266	271	7	2.67	1.9	1.18E+05	3.34E+04	28.43	0.0
4	GDF8	Compound 203	253	4	1.49	1.8	1.09E+05	3.49E+04	32.09	0.0

TABLE 19	
	Sox17 Expression
Compound #	% of positive control
Compound 181	150.3
Compound 202	144.0
Compound 180	73.6
Compound 19	69.5
Compound 34	68.0
Compound 40	52.2
Compound 185	43.1
Compound 185	38.0
Compound 35	33.7
Compound 23	29.8
Compound 22	27.4
Compound 17	23.6

What is claimed is:

1. A method of differentiating pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage comprising culturing the pluripotent stem cells in a medium lacking activin A, and supplemented with (i) GDF-8 and (ii) one or more compound selected from the group consisting of: an aniline-pyridinotriazine, a cyclic aniline-pyridinotriazine, EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D, VEGF, muscimol, PD98059, LY294002, U0124, U0126, 6-[(2-{[4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl]amino}ethyl)amino]pyridine-3-carbonitrile and sodium butyrate, for a period of time sufficient for the pluripotent stem cells to differentiate into cells expressing markers characteristic of the definitive endoderm lineage.
2. The method of claim 1 wherein the cells expressing markers characteristic of the definitive endoderm lineage are definitive endoderm cells.
3. The method of claim 1 or 2, wherein the medium lacking activin A is further lacking Wnt3a.
4. The method of any one of claims 1 to 3, wherein the medium lacking activin A is supplemented with GDF-8 and an aniline-pyridinotriazine.
5. The method of any one of claims 1 to 3, wherein the medium lacking activin A is supplemented with GDF-8 and a cyclic aniline-pyridinotriazine.
6. The method of claim 5 wherein the cyclic aniline-pyridinotriazine is 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosal-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one.
7. The method of claim 5 wherein the cyclic aniline-pyridinotriazine is 5-Chloro-1,8,10,12,16,22,26,32-octaazapentacyclo[24.2.2.1~3,7~.1~9,13~.1~14,18~]tritriacontal-3(33),4,6,9(32),10,12,14(31),15,17-nonaen-23-one.
8. The method of any one of claims 1 to 3, wherein the medium is supplemented with GDF-8 and 6-[(2-{[4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-yl]amino}ethyl)amino]pyridine-3-carbonitrile.

9. The method of any one of claims 1 to 8, wherein the medium lacking activin A comprises at least one factor selected from the group consisting of: EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D, VEGF, muscimol, PD98059, LY294002, U0124, U0126, and sodium butyrate.
10. The method of any one of claims 1 to 3, wherein the medium lacking activin A is further supplemented with at least one other compound selected from the group consisting of: N-{[1-(Phenylmethyl)azepan-4-yl]methyl}-2-pyridin-3-ylacetamide, 4-{[4-(4-{[2-(Pyridin-2-ylamino)ethyl]amino}-1,3,5-triazin-2-yl)pyridin-2-yl]oxy}butan-1-ol, 3-{[3-[4-({2-[Methyl(pyridin-2-yl)amino]ethyl}amino)-1,3,5-triazin-2-yl]pyridin-2-yl]amino}propan-1-ol, N~4~-[2-(3-Fluorophenyl)ethyl]-N~2~-[3-(4-methylpiperazin-1-yl)propyl]pyrido[2,3-d]pyrimidine-2,4-diamine, 1-Methyl-N-[(4-pyridin-3-yl-2-{[3-(trifluoromethyl)phenyl]amino}-1,3-thiazol-5-yl)methyl]piperidine-4-carboxamide, 1,1-Dimethylethyl {2-[4-({5-[3-(3-hydroxypropyl)phenyl]-4H-1,2,4-triazol-3-yl}amino)phenyl]ethyl}carbamate, 1,1-Dimethylethyl {[3-({5-[5-(3-hydroxypropyl)-2-(methyloxy)phenyl]-1,3-oxazol-2-yl}amino)phenyl]methyl}carbamate, 1-({5-[6-({4-[(4-Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidin-4-ol, 1-({4-[6-({4-[(4-Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidine-4-carboxamide, and 2-{[4-(1-Methylethyl)phenyl]amino}-N-(2-thiophen-2-ylethyl)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxamide.
11. The method of any one of claims 1 to 3, wherein GDF-8 is used at a concentration from 5 ng/ml to 500 ng/ml.
12. The method of any one of claims 1 to 3, wherein GDF-8 is used at a concentration from 5 ng/ml to 25 ng/ml.
13. The method of any one of claims 1 to 3, wherein GDF-8 is used at a concentration at a concentration of 25 ng/ml.
14. The method of any one of claims 1 to 3, wherein EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D and/or VEGF is used at a concentration from 5 ng/ml to 500 ng/ml.

15. The method of any one of claims 1 to 3, wherein EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D and/or VEGF is used at a concentration of 5 ng/ml to 50 ng/ml.
16. The method of any one of claims 1 to 3, wherein EGF, FGF4, PDGF-A, PDGF-B, PDGF-C, PDGF-D and/or VEGF is used at a concentration of 50 ng/ml.
17. The method of any one of claims 1 to 3, wherein muscimol is used at a concentration from 1 μ M to 200 μ M.
18. The method of claim 17, wherein the concentration is 20 μ M.
19. The method of any one of claims 1 to 3, wherein PD98059, U0124 and/or U0126 is used at a concentration from 0.1 μ M to 10 μ M.
20. The method of claim 19, wherein the concentration is 1 μ M.
21. The method of any one of claims 1 to 3, wherein LY294002 is used at a concentration from 0.25 μ M to 25 μ M.
22. The method of claim 21, wherein the concentration is 2.5 μ M.
23. The method of any one of claims 1 to 3, wherein sodium butyrate is used at a concentration from 0.05 μ M to 5 μ M.
24. The method of claim 23, wherein the concentration is 0.5 μ M.
25. The method of any one of claims 1 to 24, wherein the pluripotent stem cells are cultured in the medium lacking activin A for between one day and seven days.
26. The method of any one of claims 1 to 25, wherein the pluripotent stem cells are human pluripotent stem cells.
27. The method of claim 26 wherein the human pluripotent stem cells are human embryonic stem cells.

Figure 1A

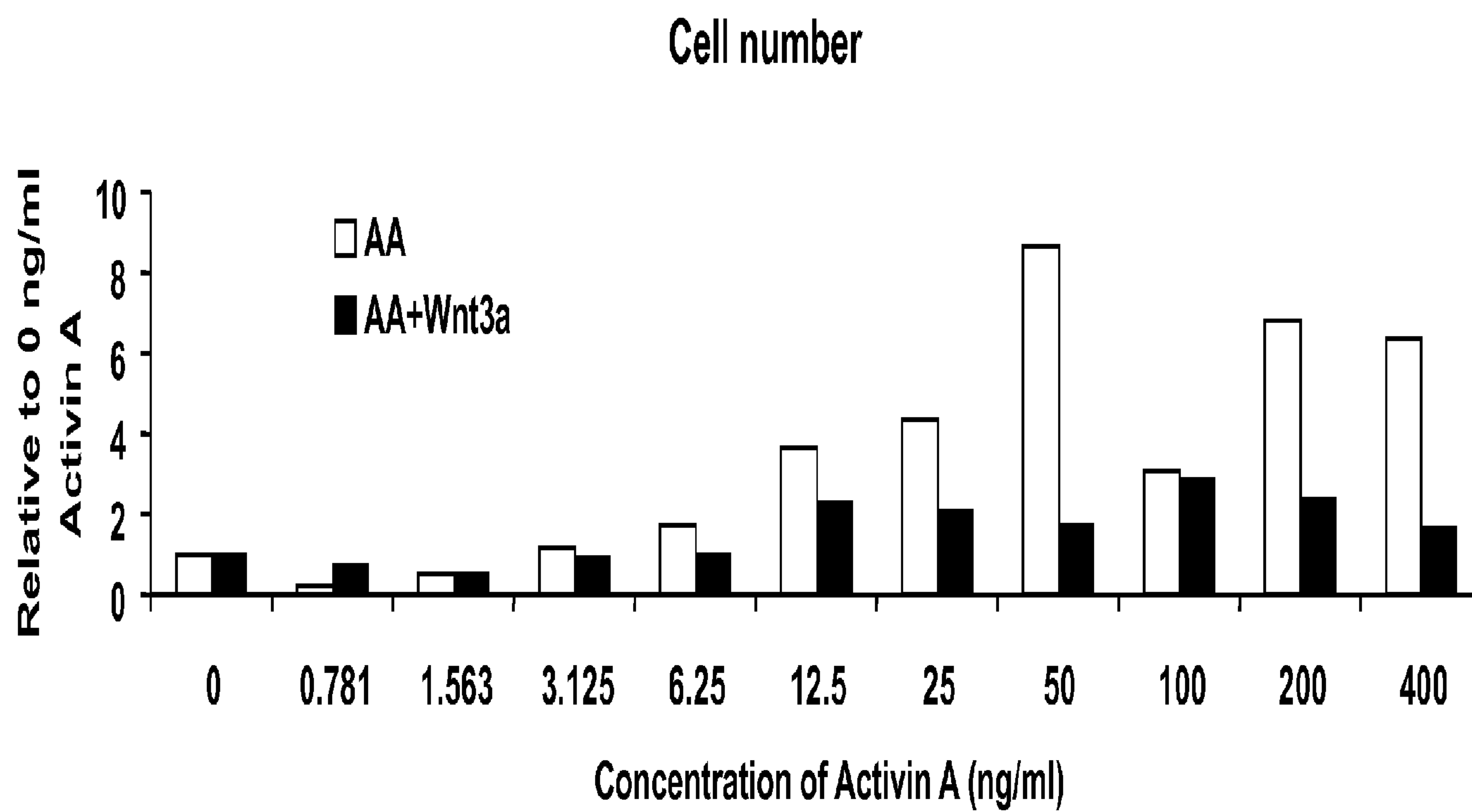


Figure 1B

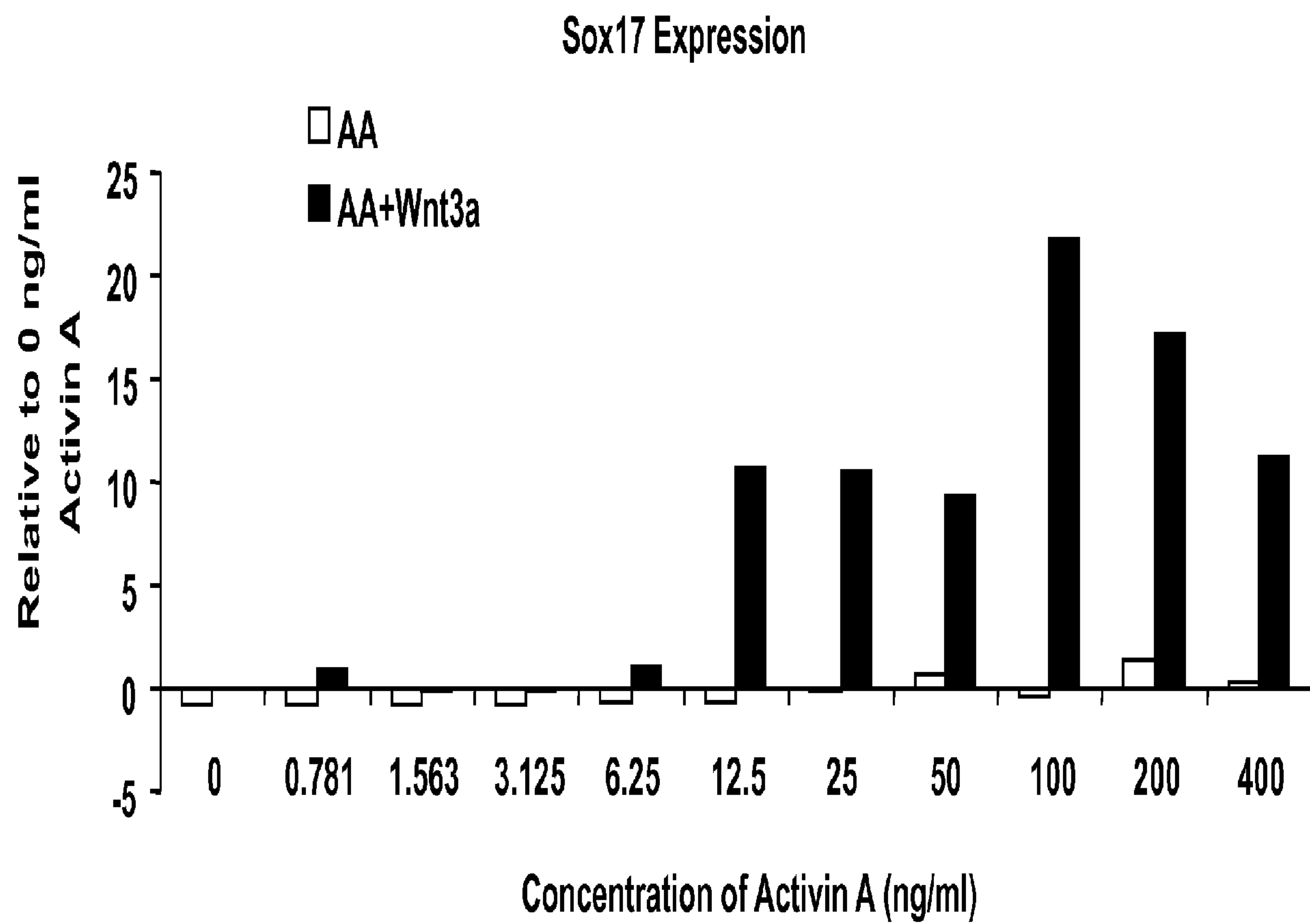


Figure 2

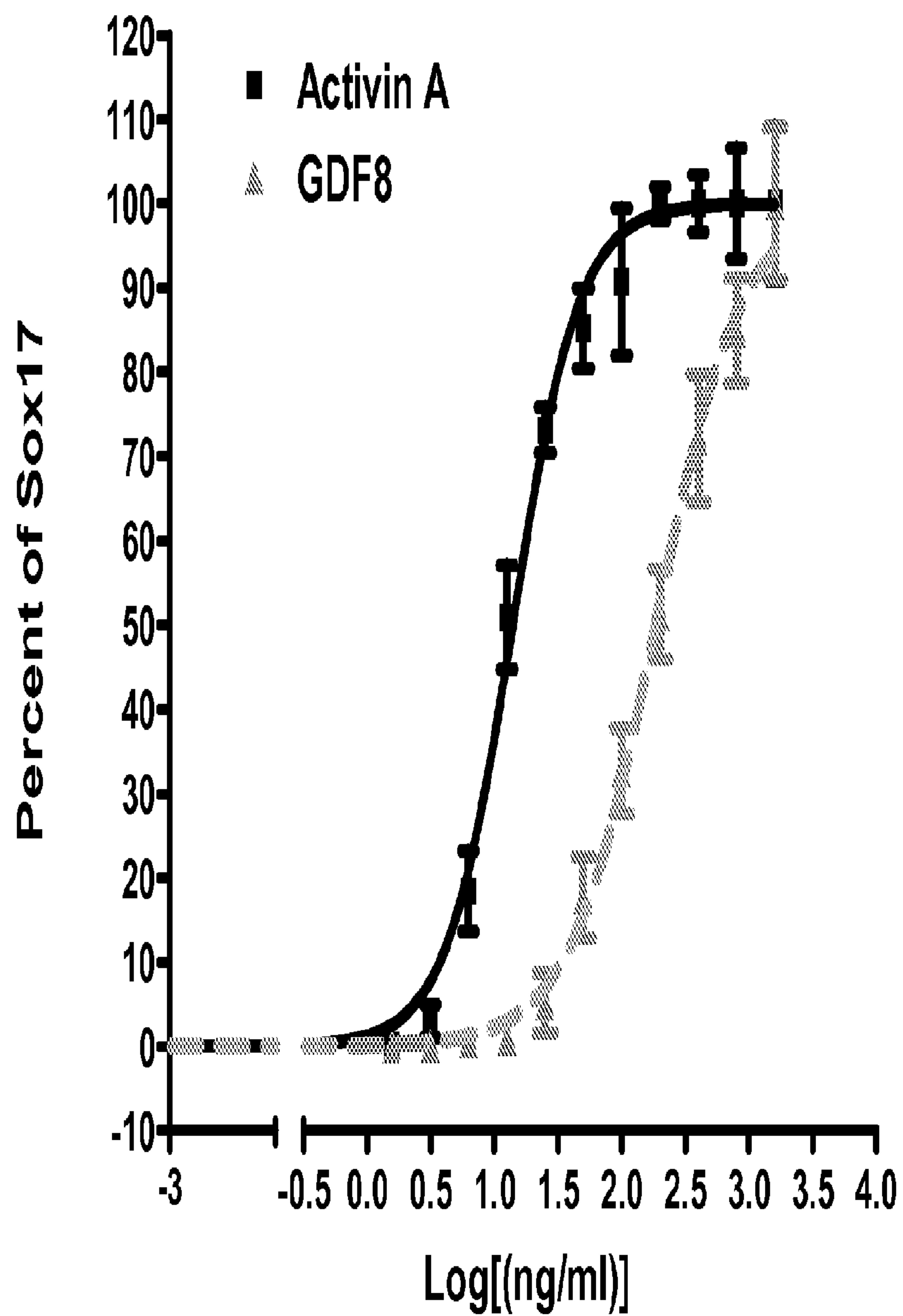


Figure 3

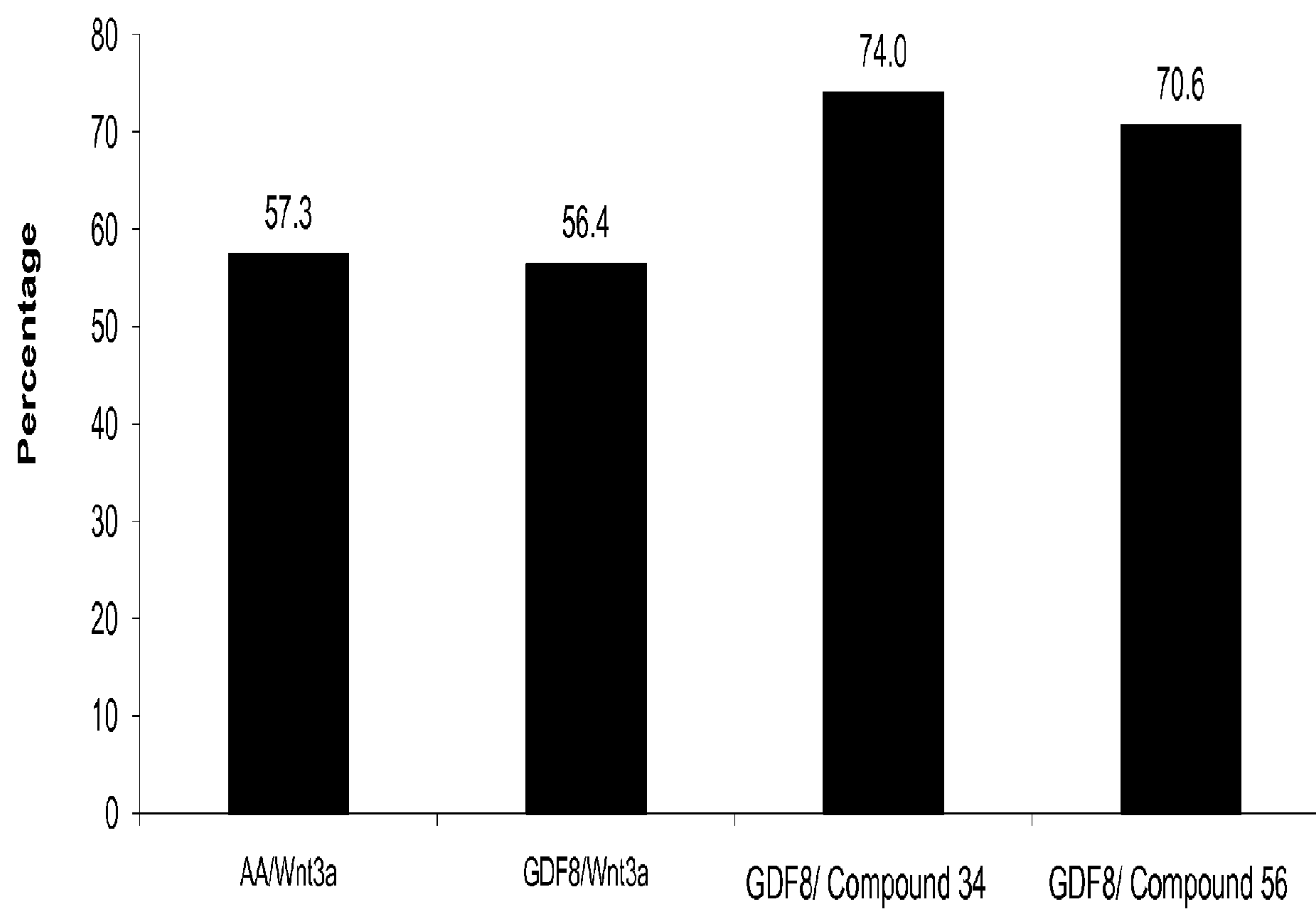
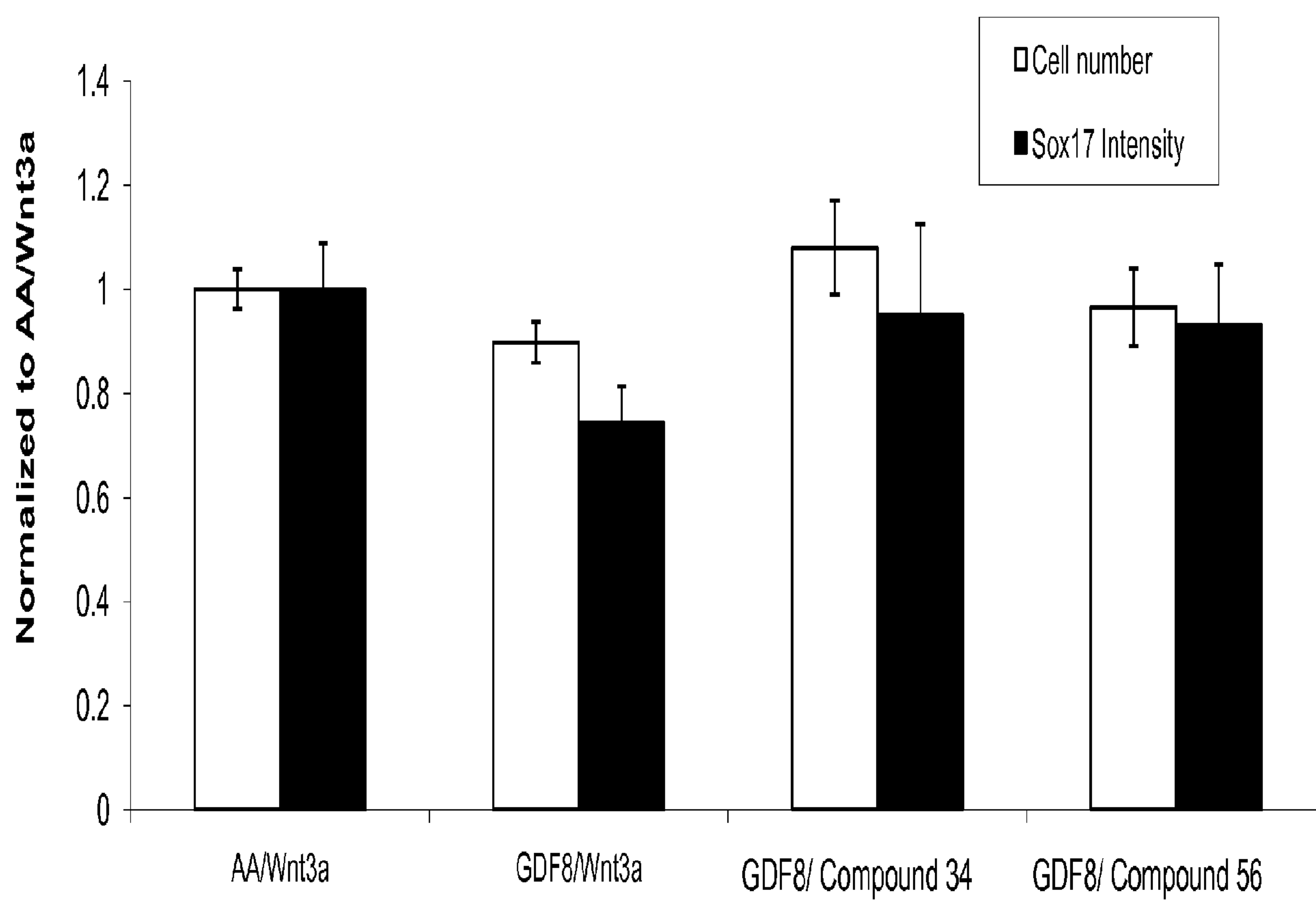


Figure 4



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Figure 5

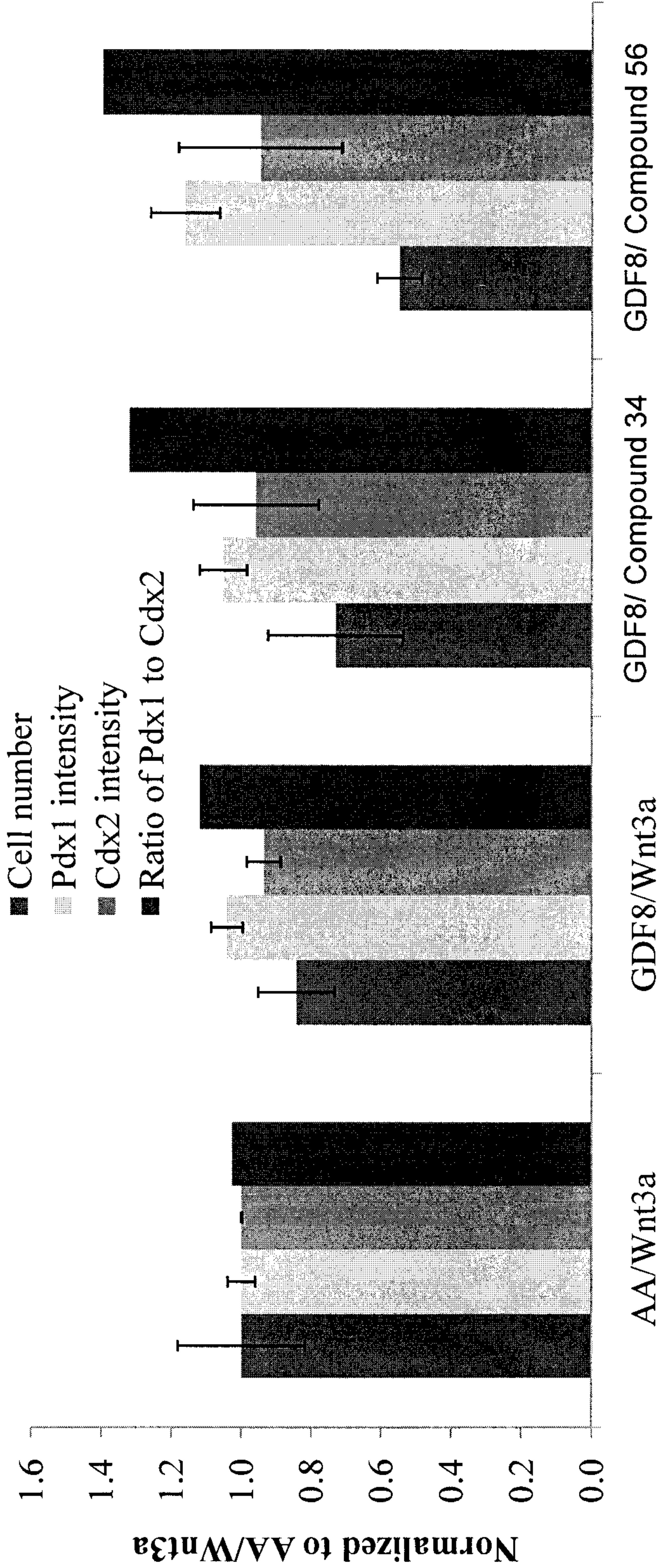


Figure 6

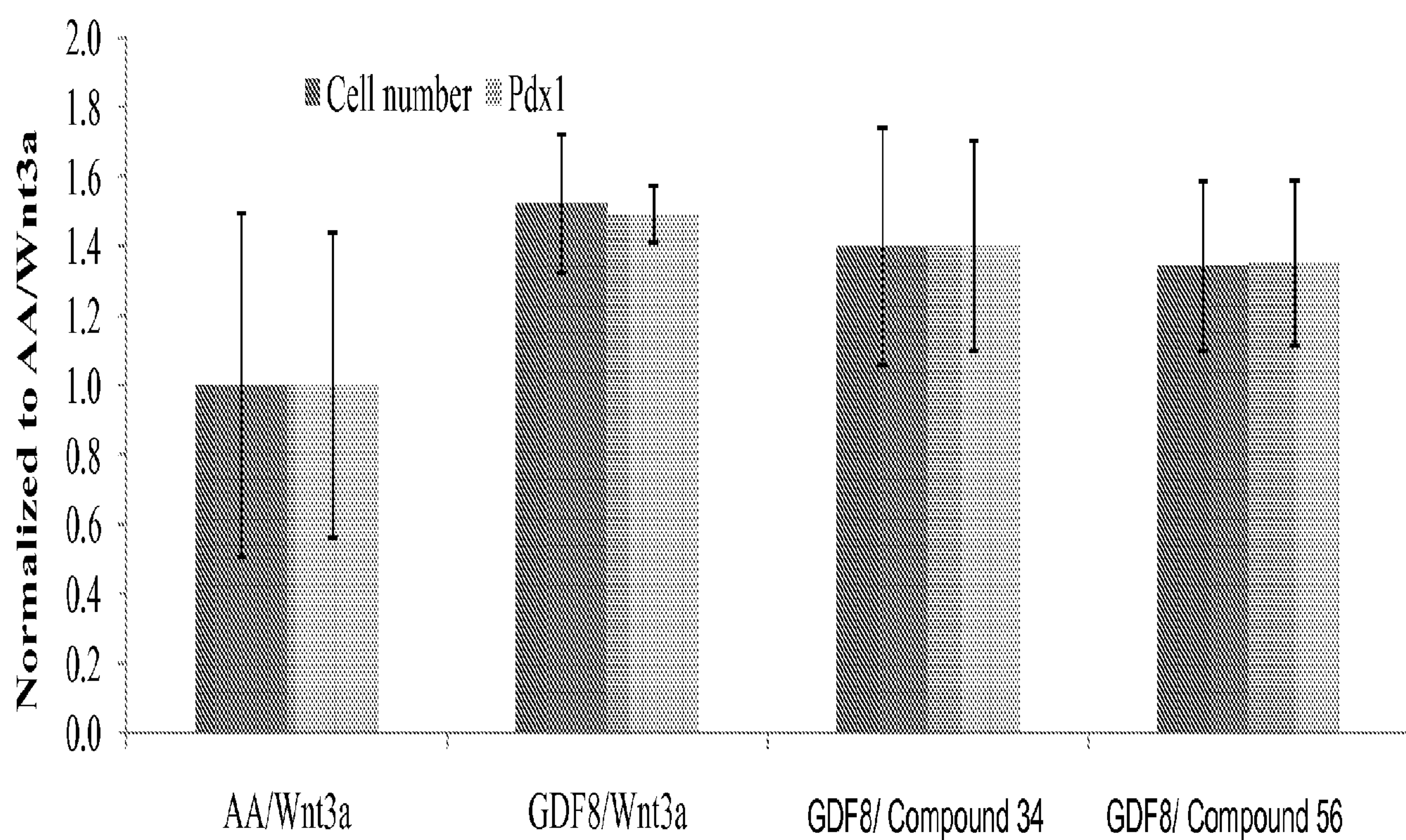


Figure 7

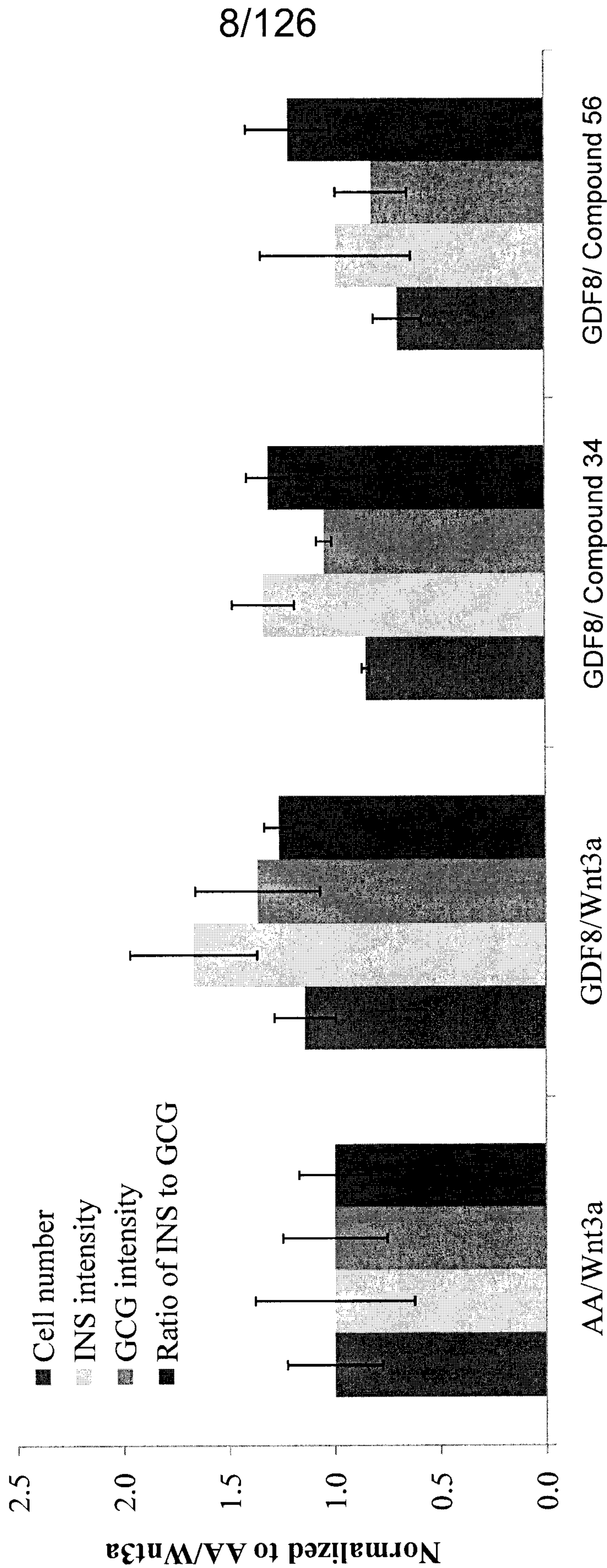


Figure 8A

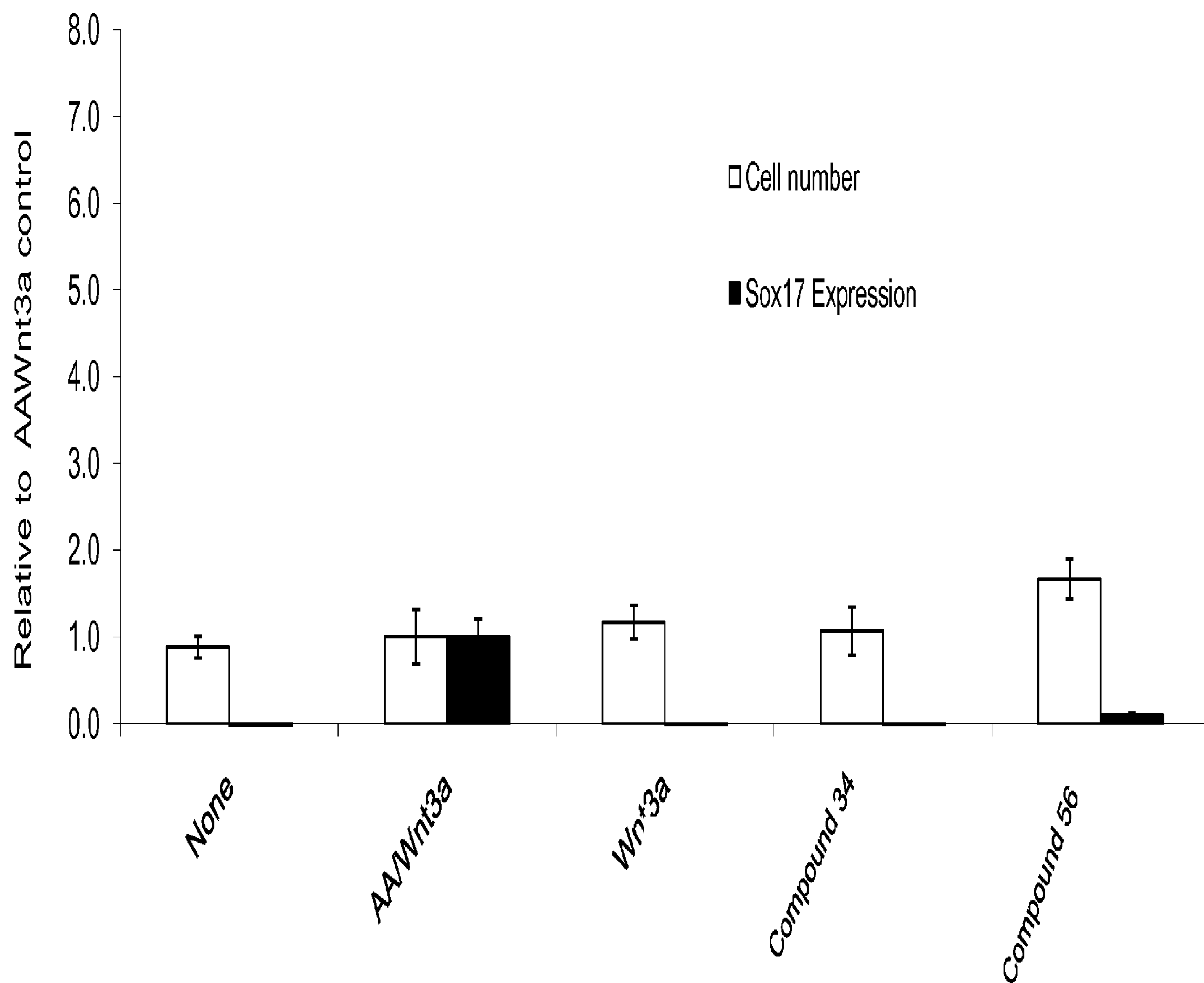


Figure 8B

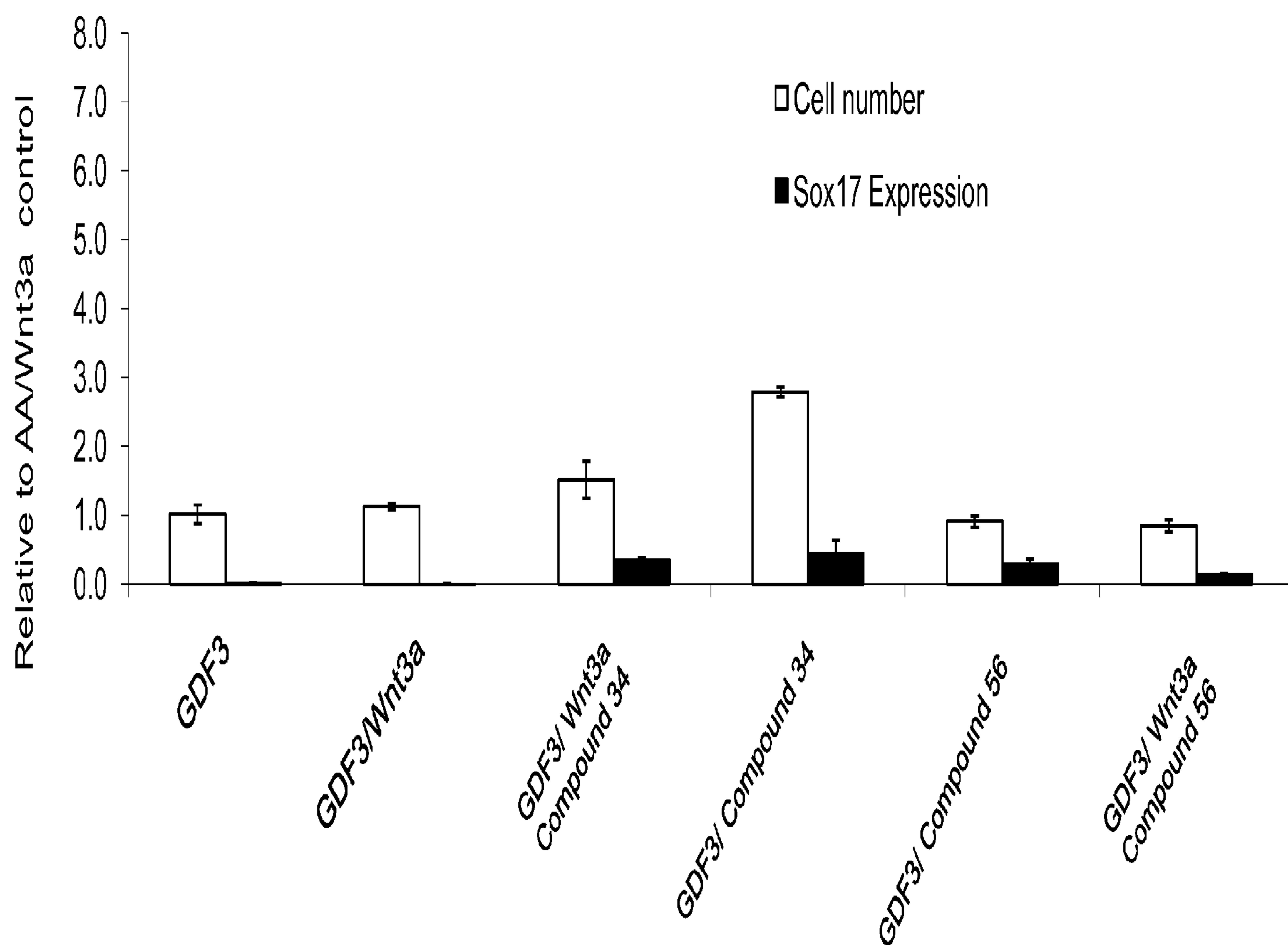


Figure 8C

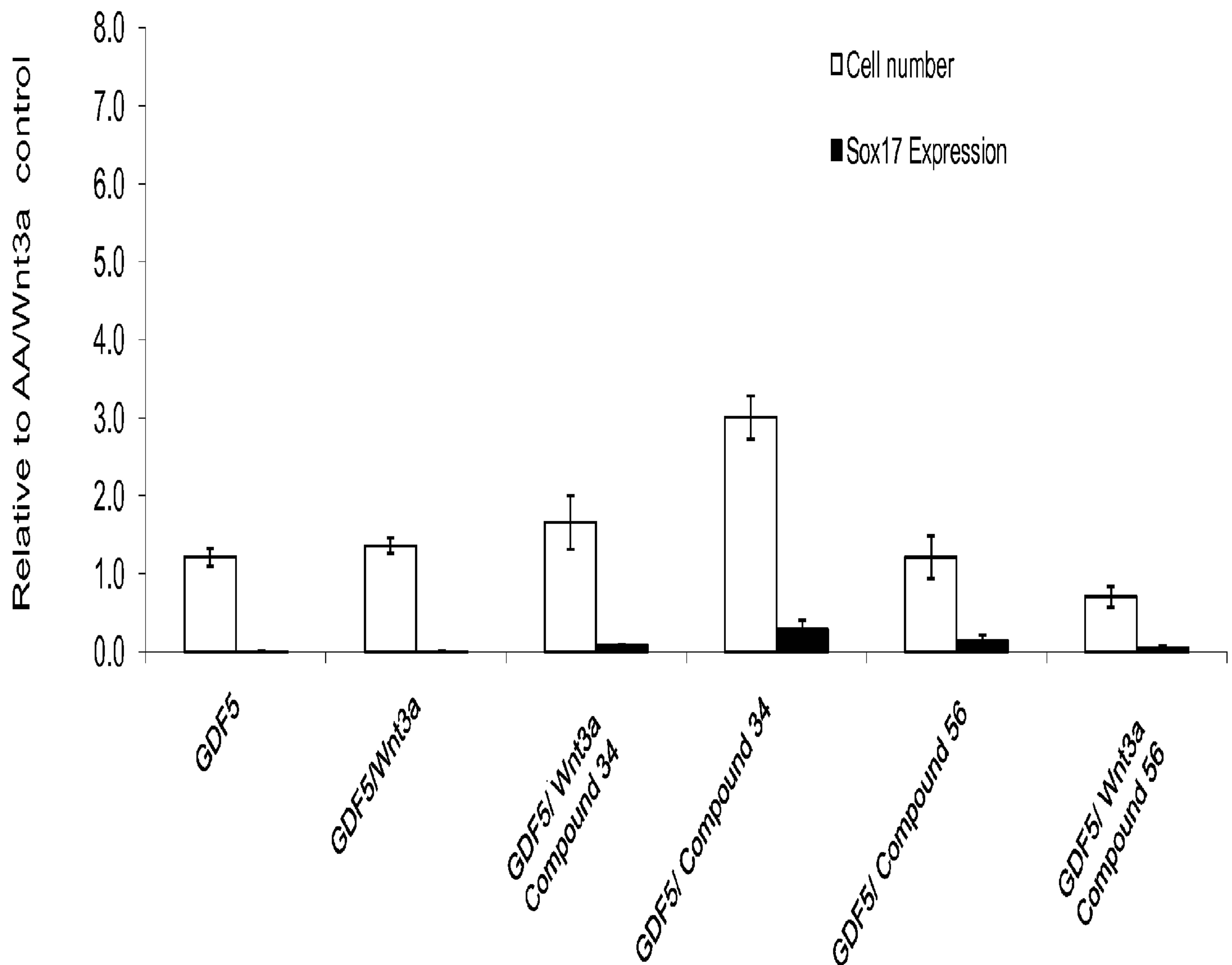


Figure 8D

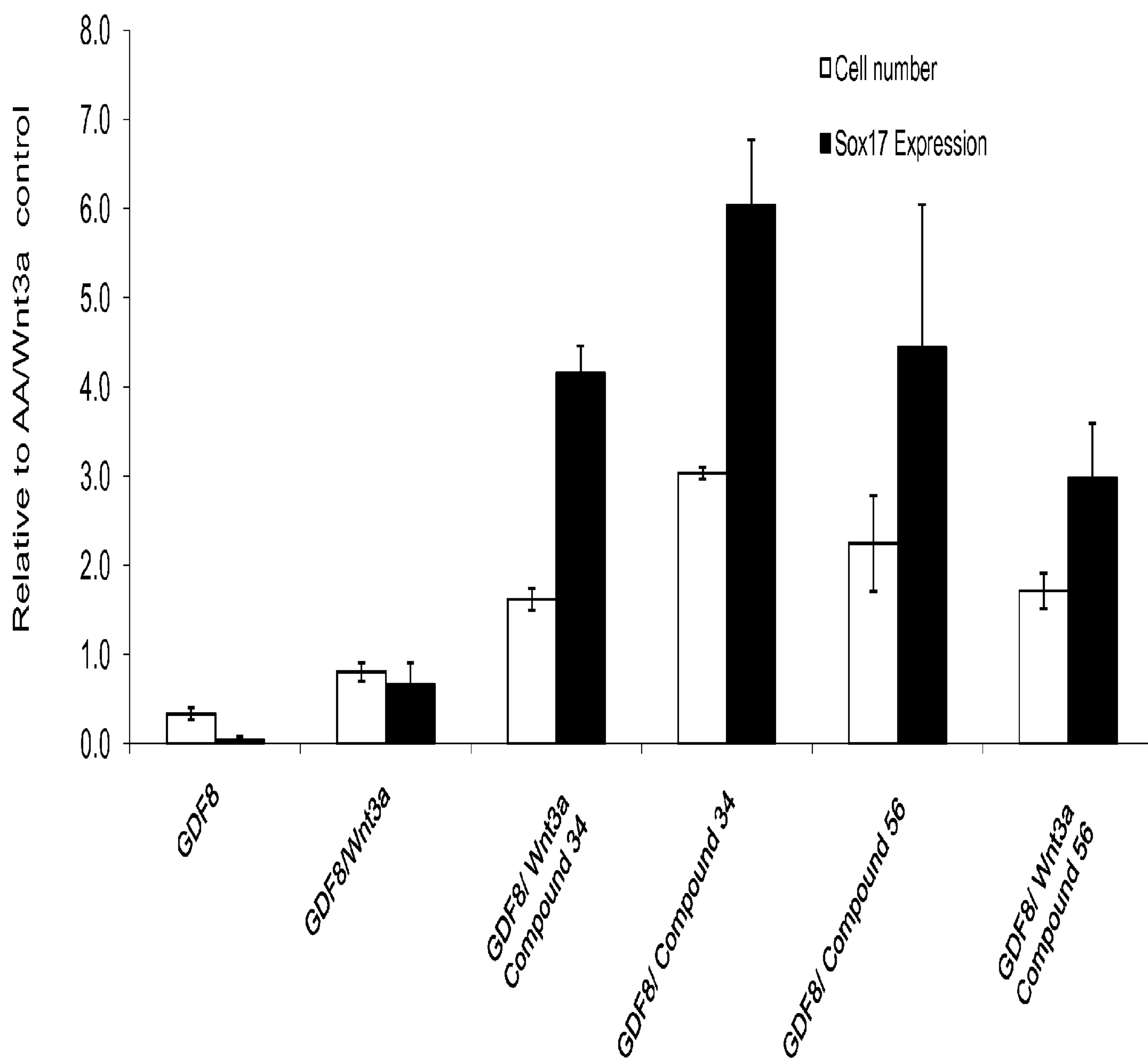
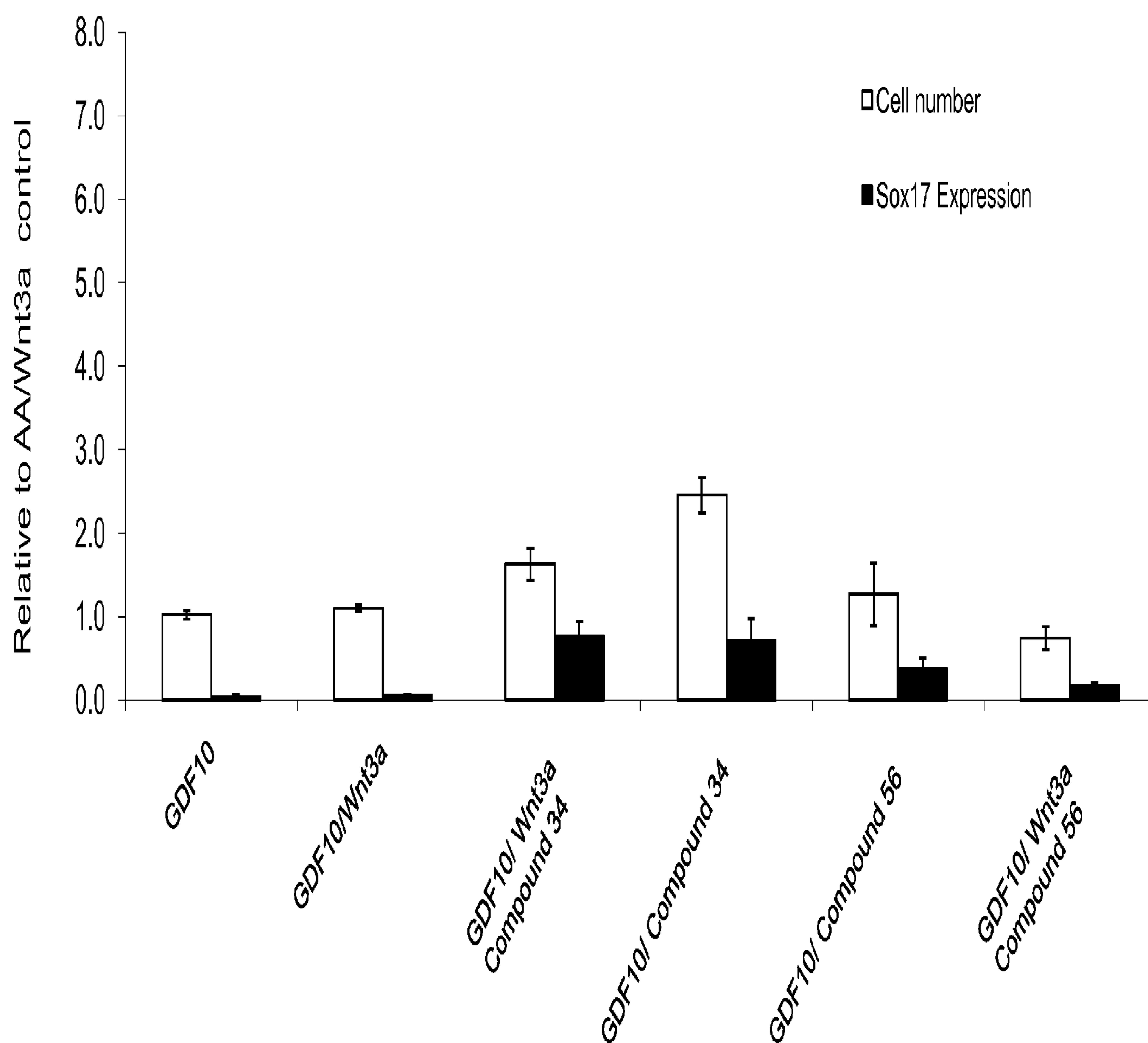
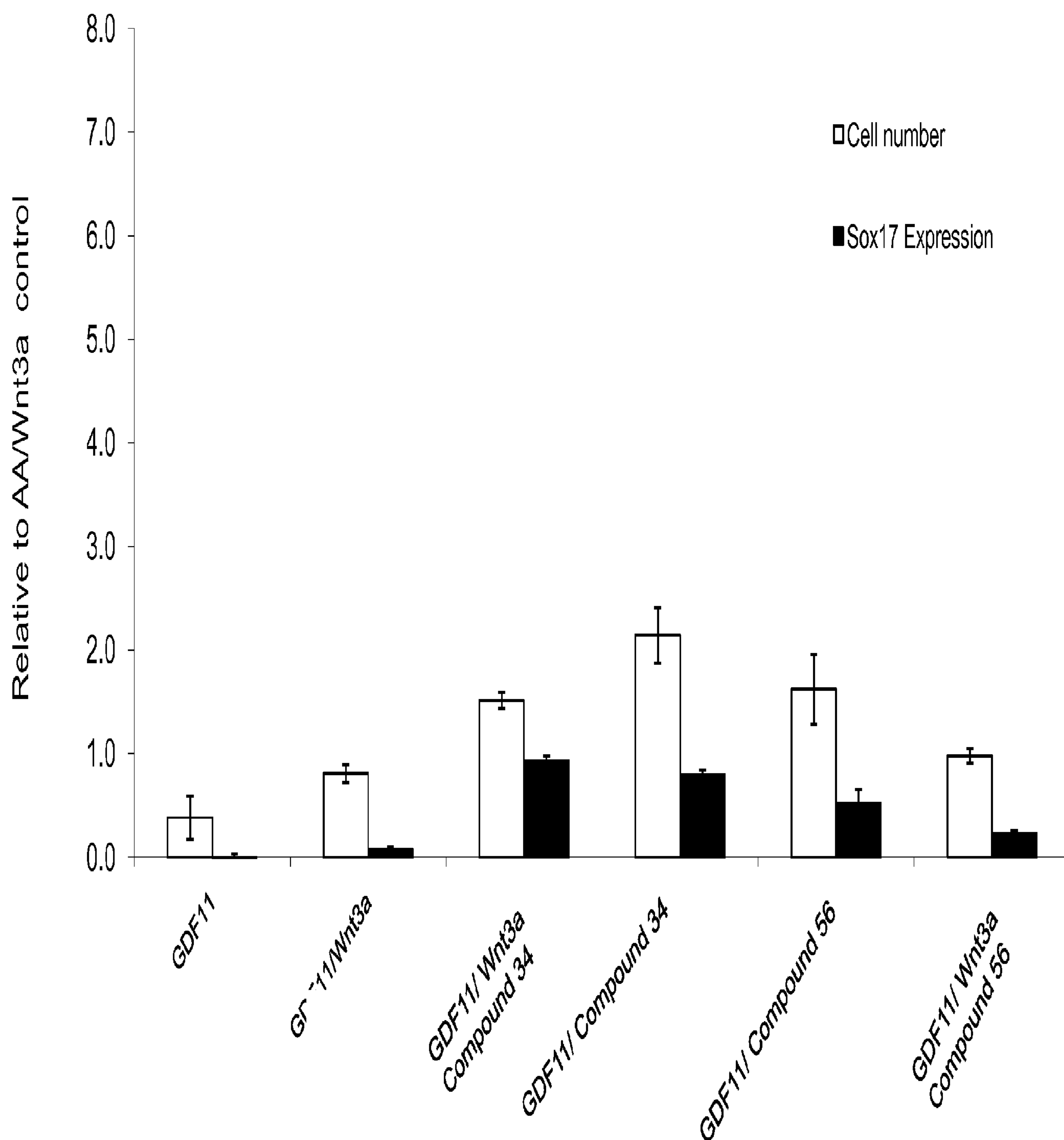


Figure 8E



CEN5227WOPCT: DIFFERENTIATION OF PLURIPOTENT STEM CELLS
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Figure 8F



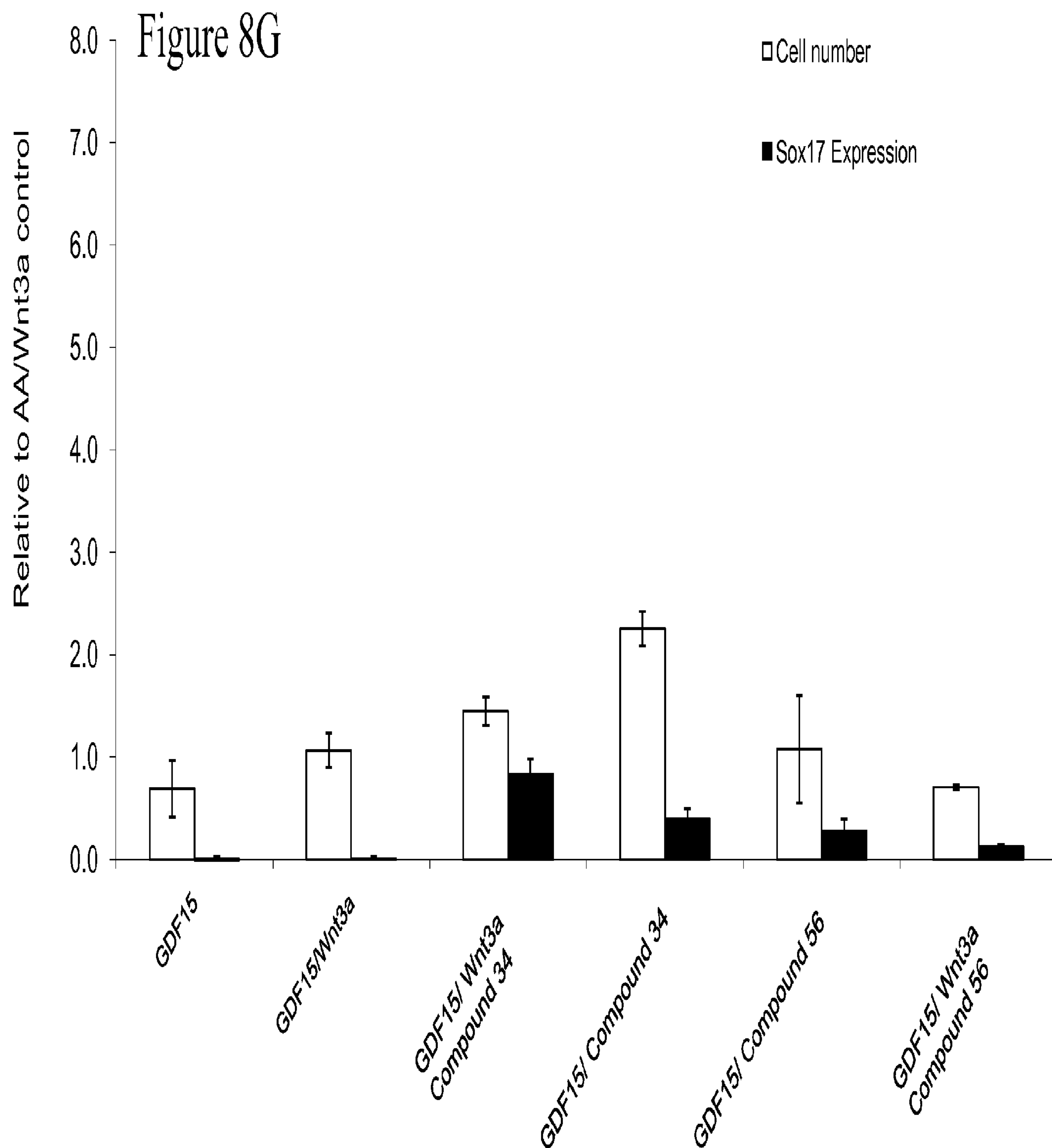


Figure 9A

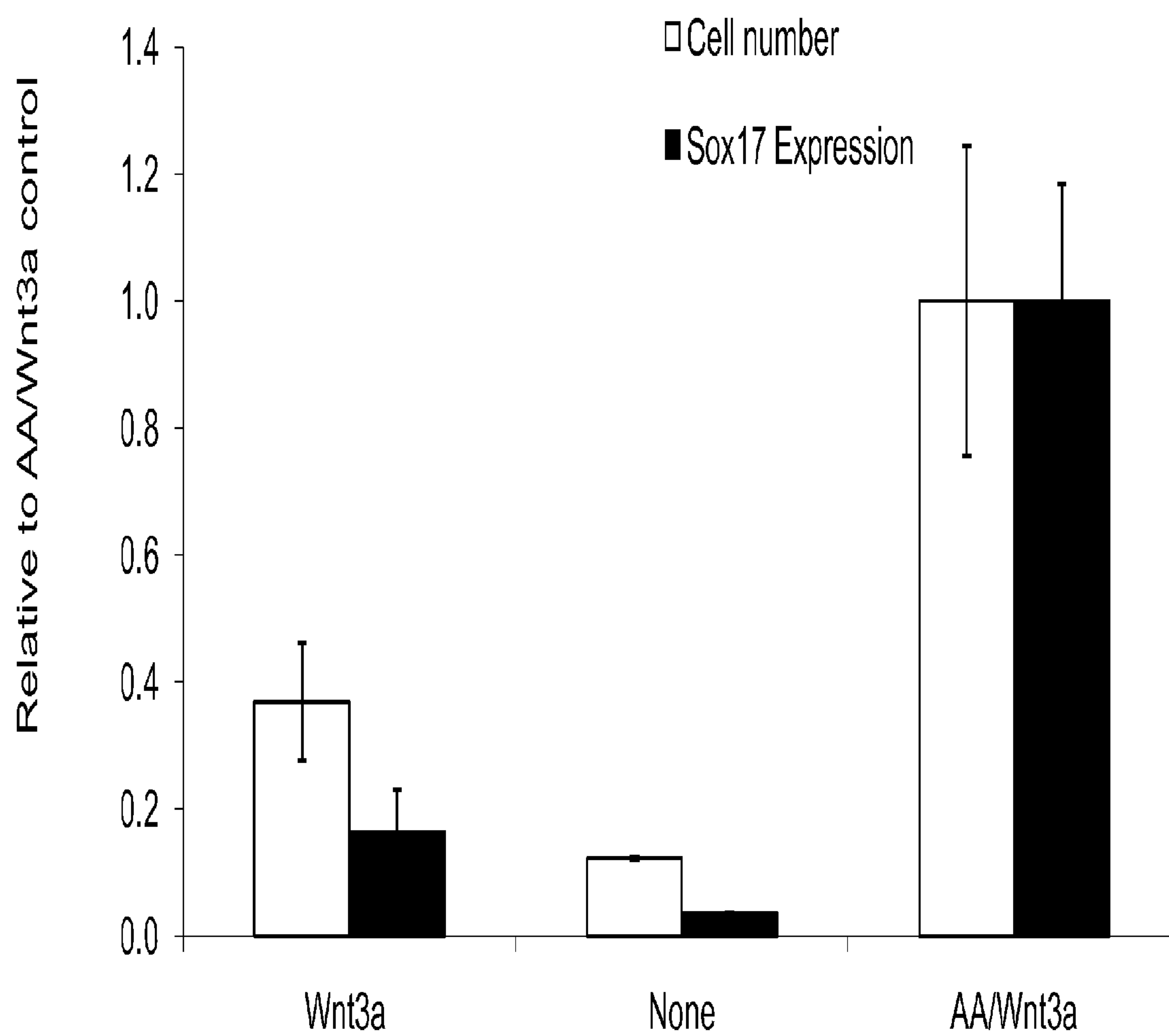


Figure 9B

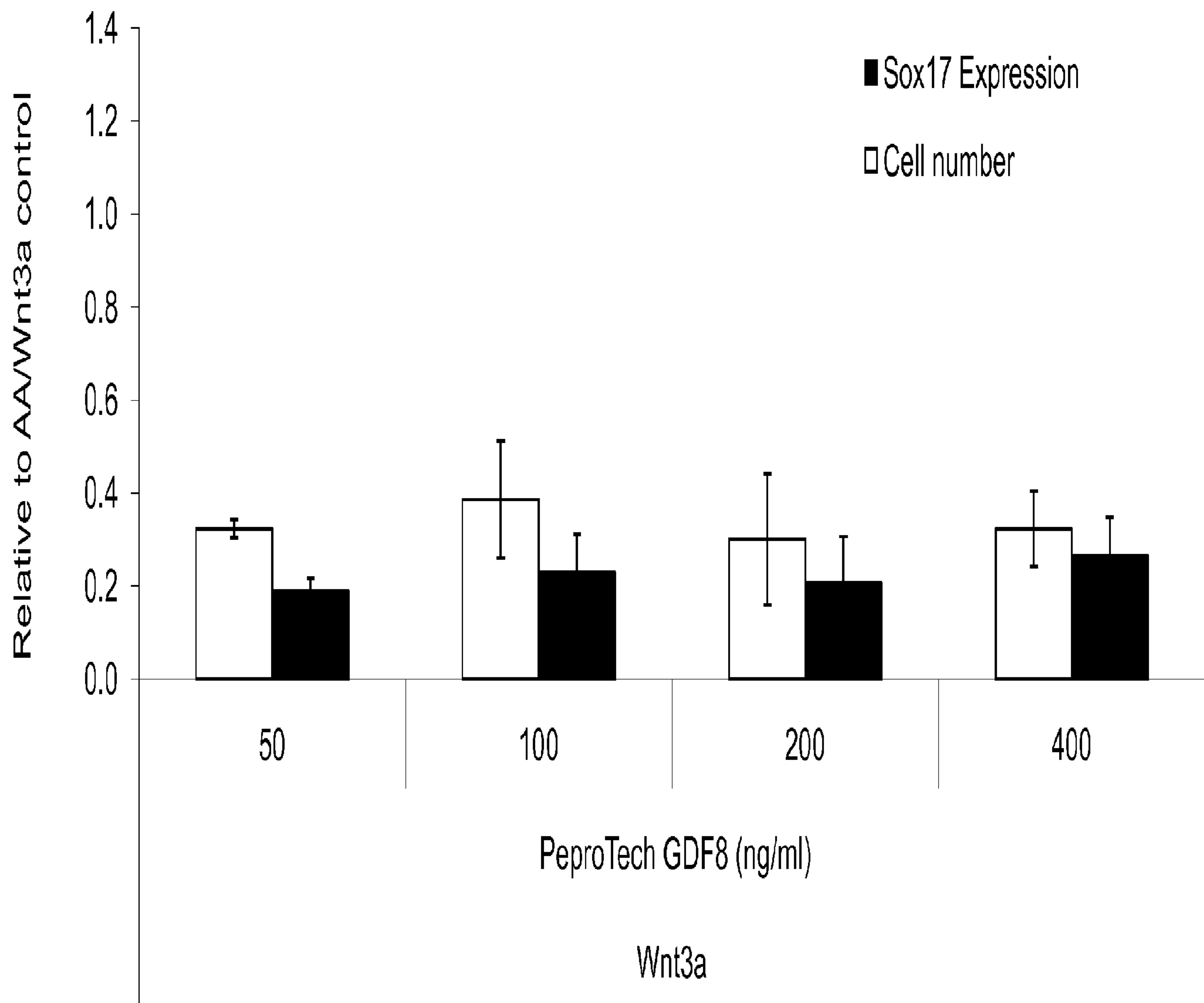


Figure 9C

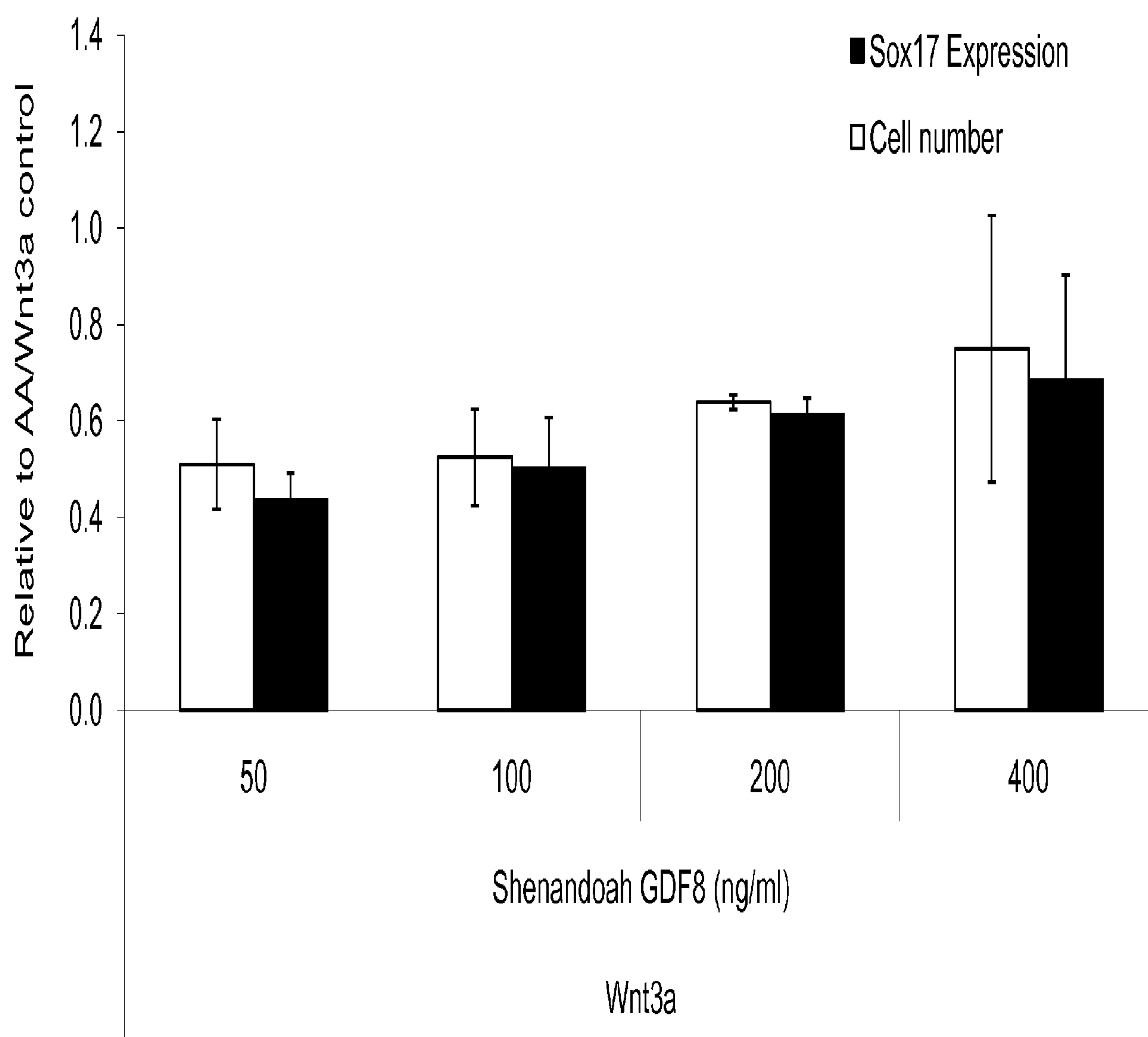


Figure 9D

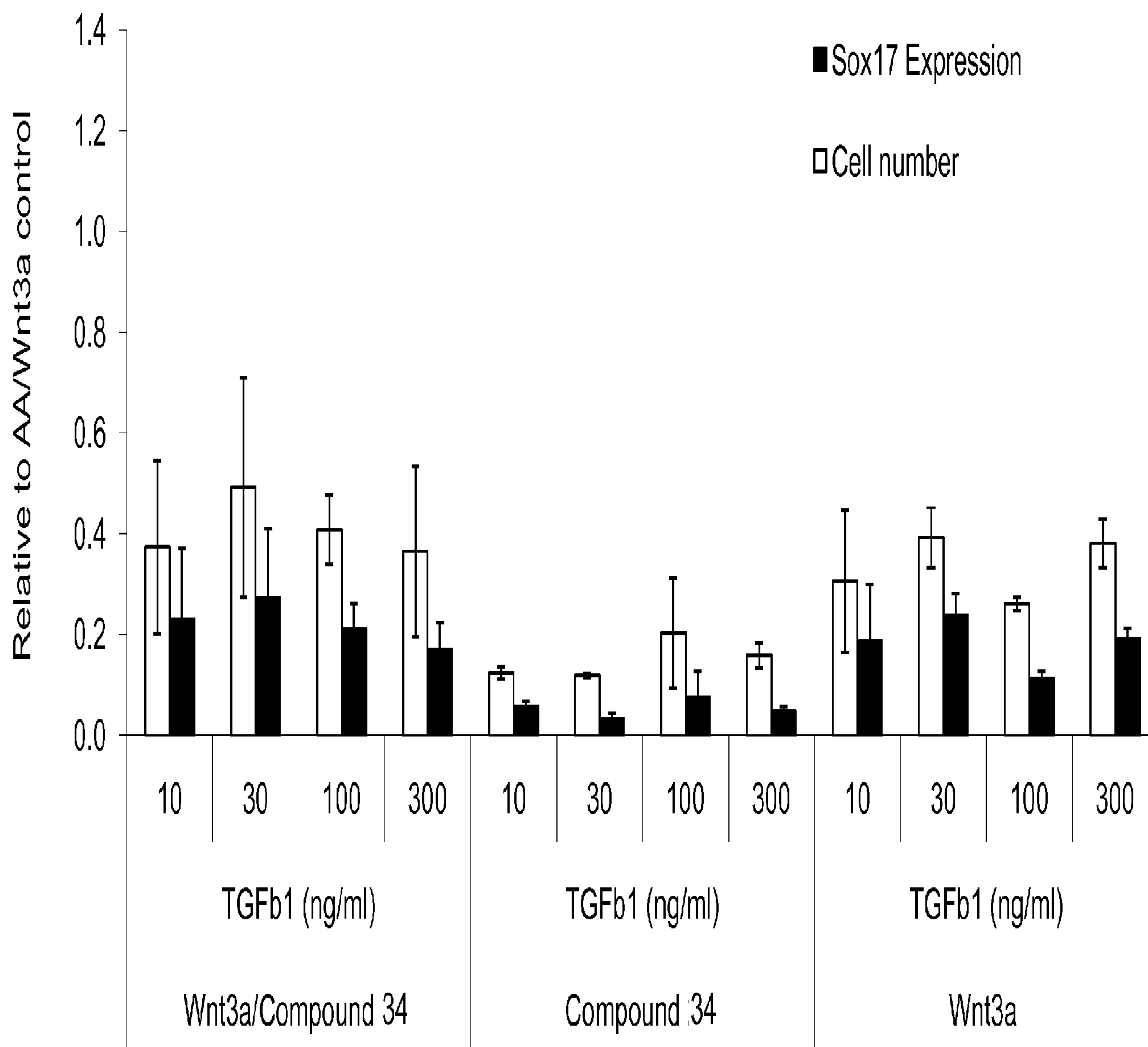


Figure 9E

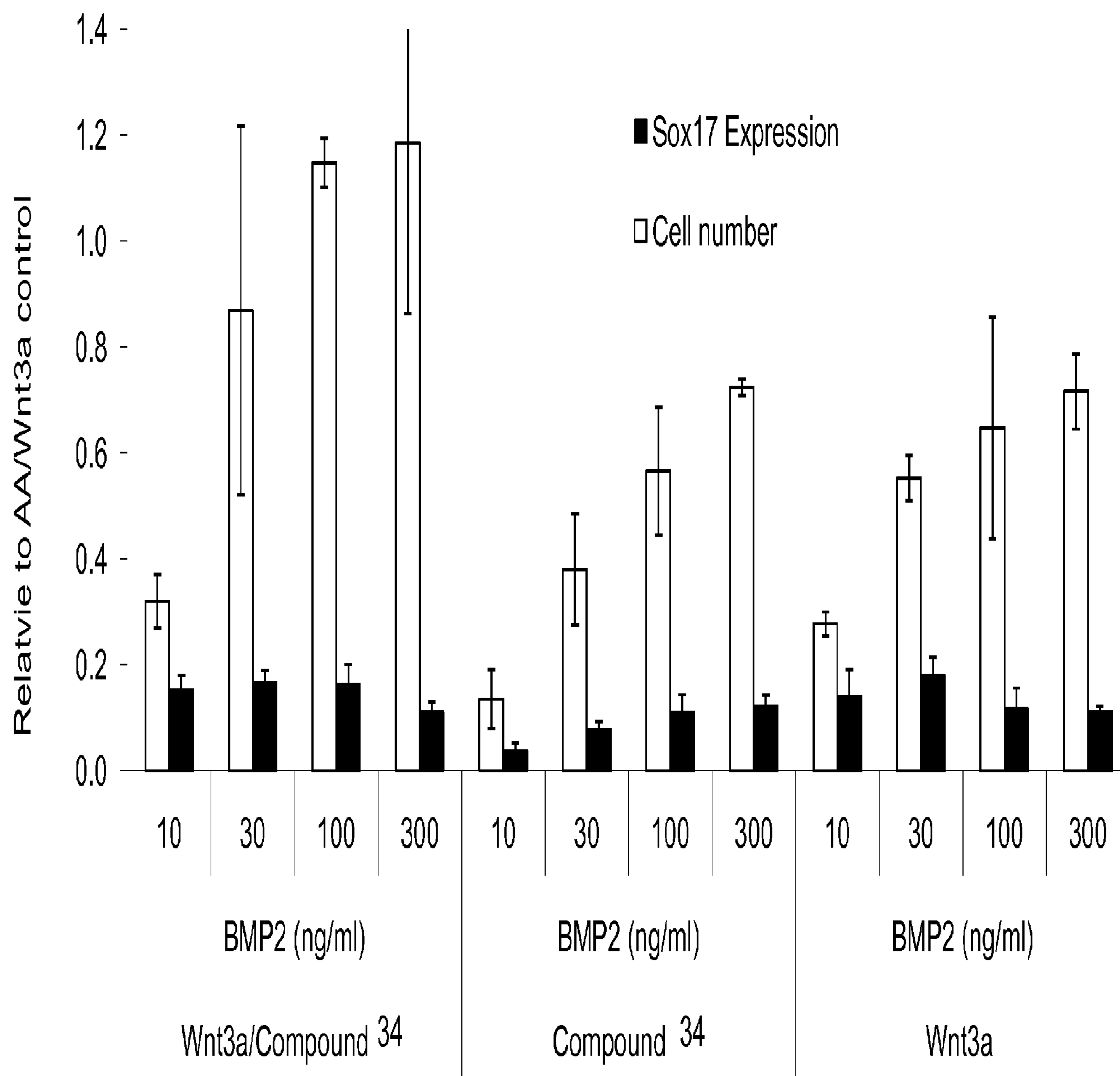


Figure 9F

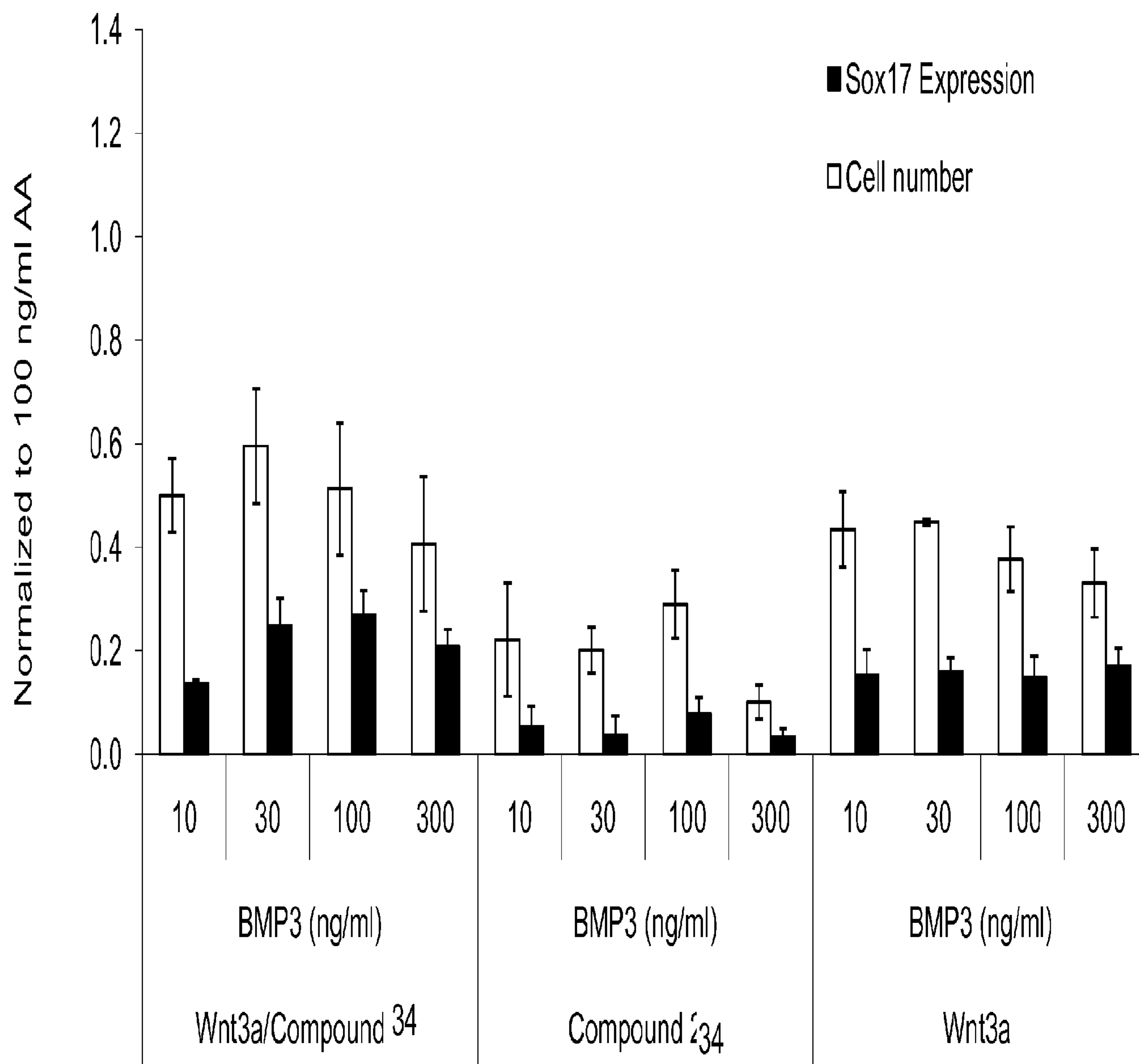


Figure 9G

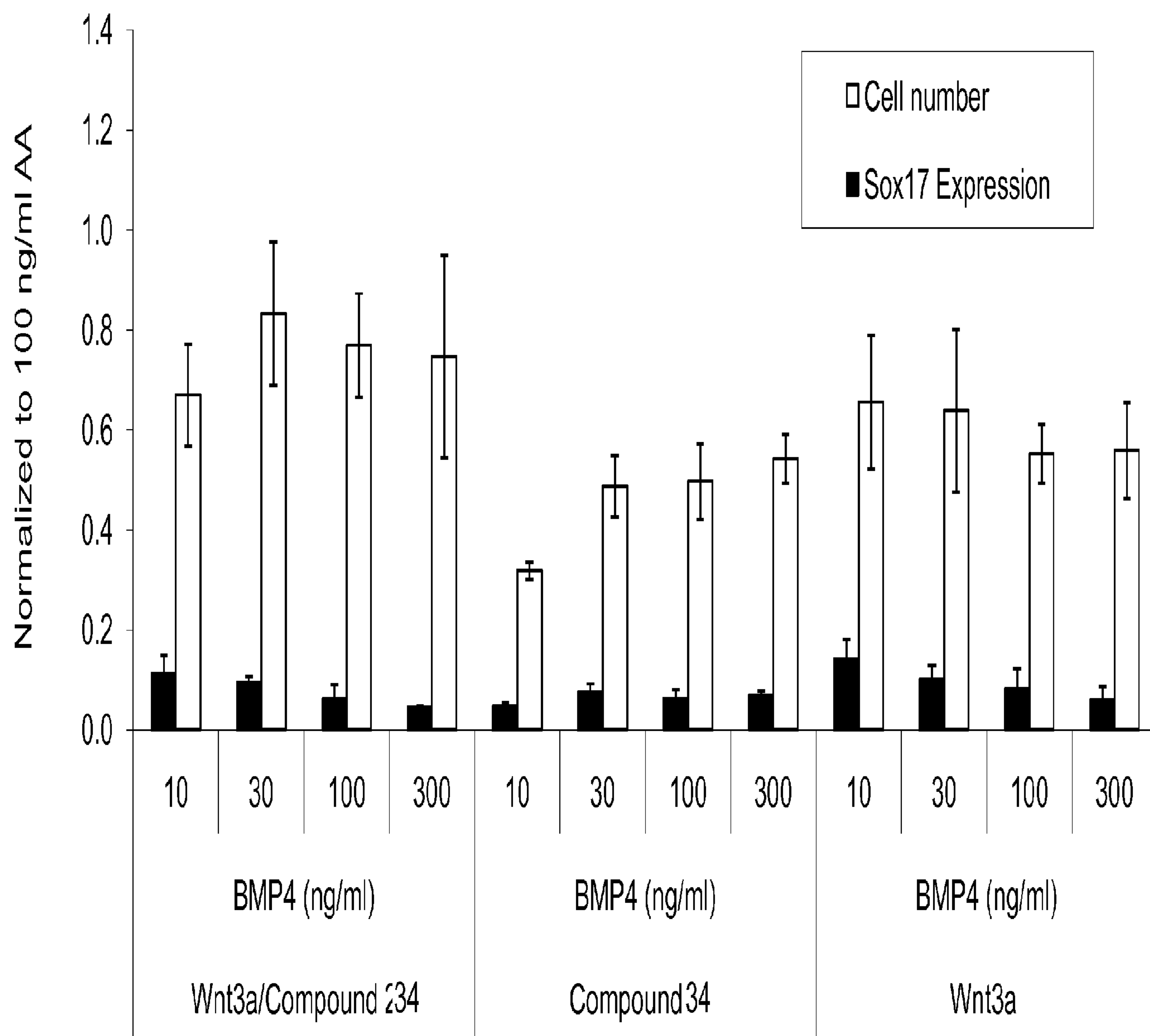


Figure 10

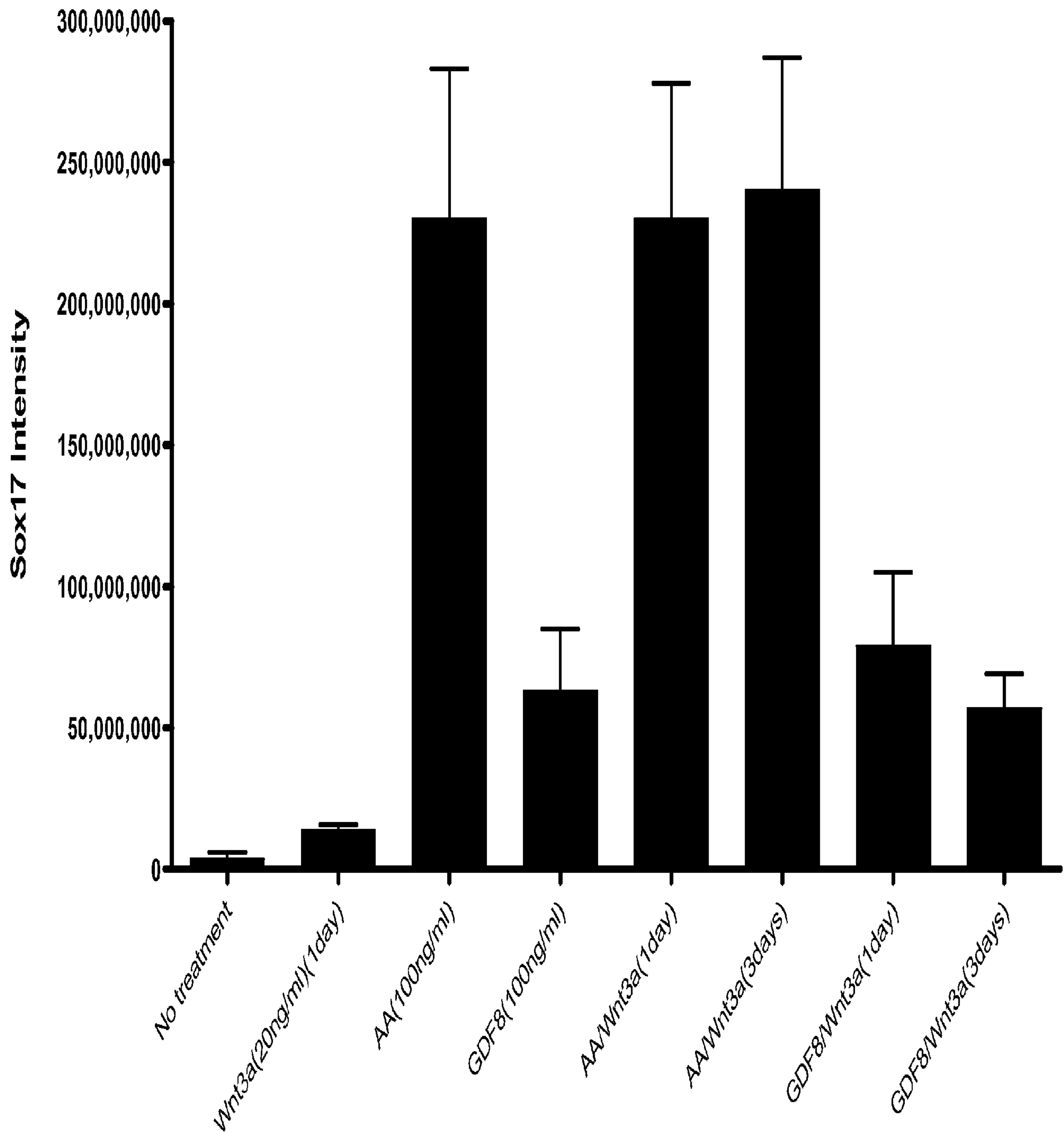


Figure 11A

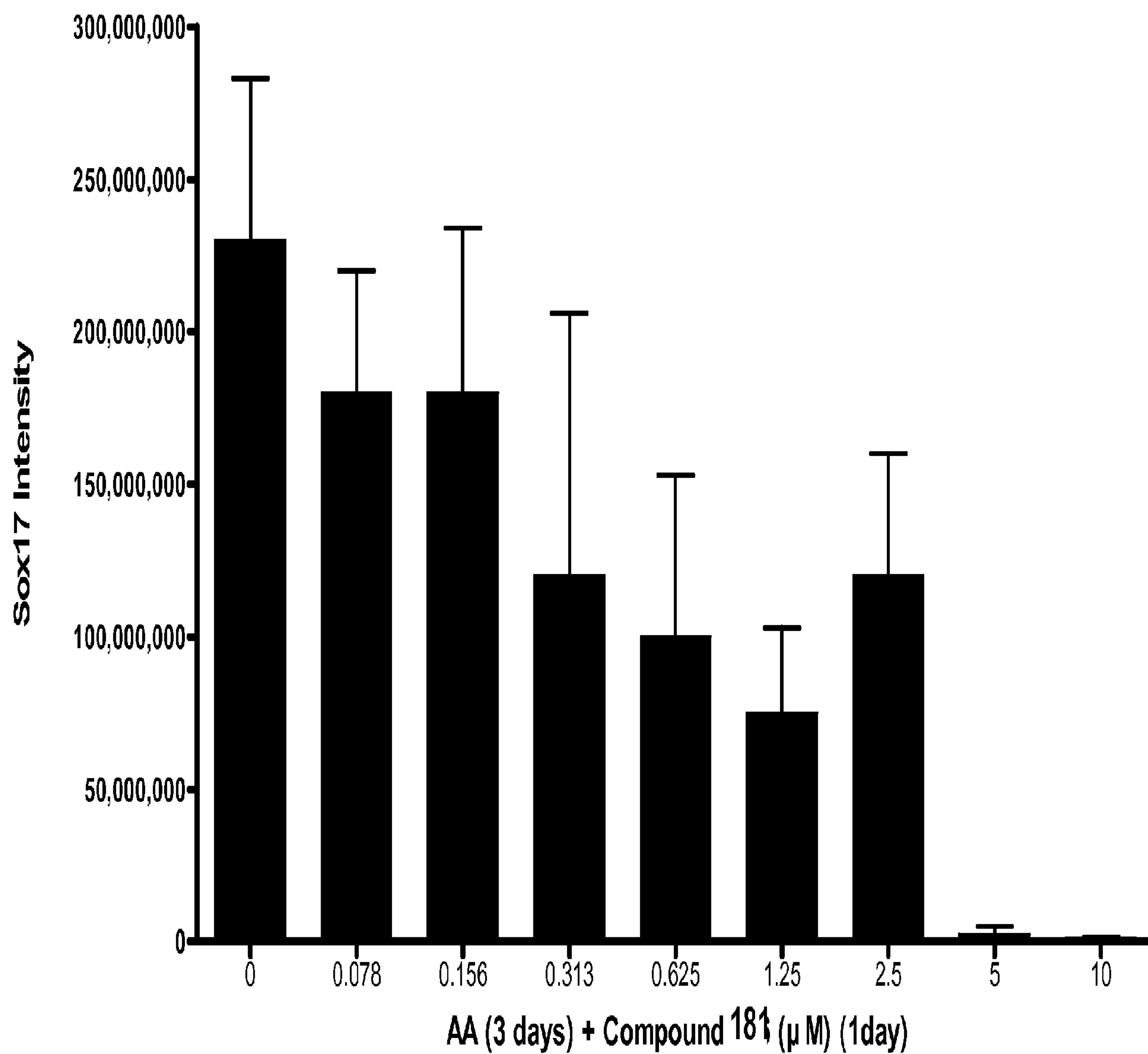


Figure 11B

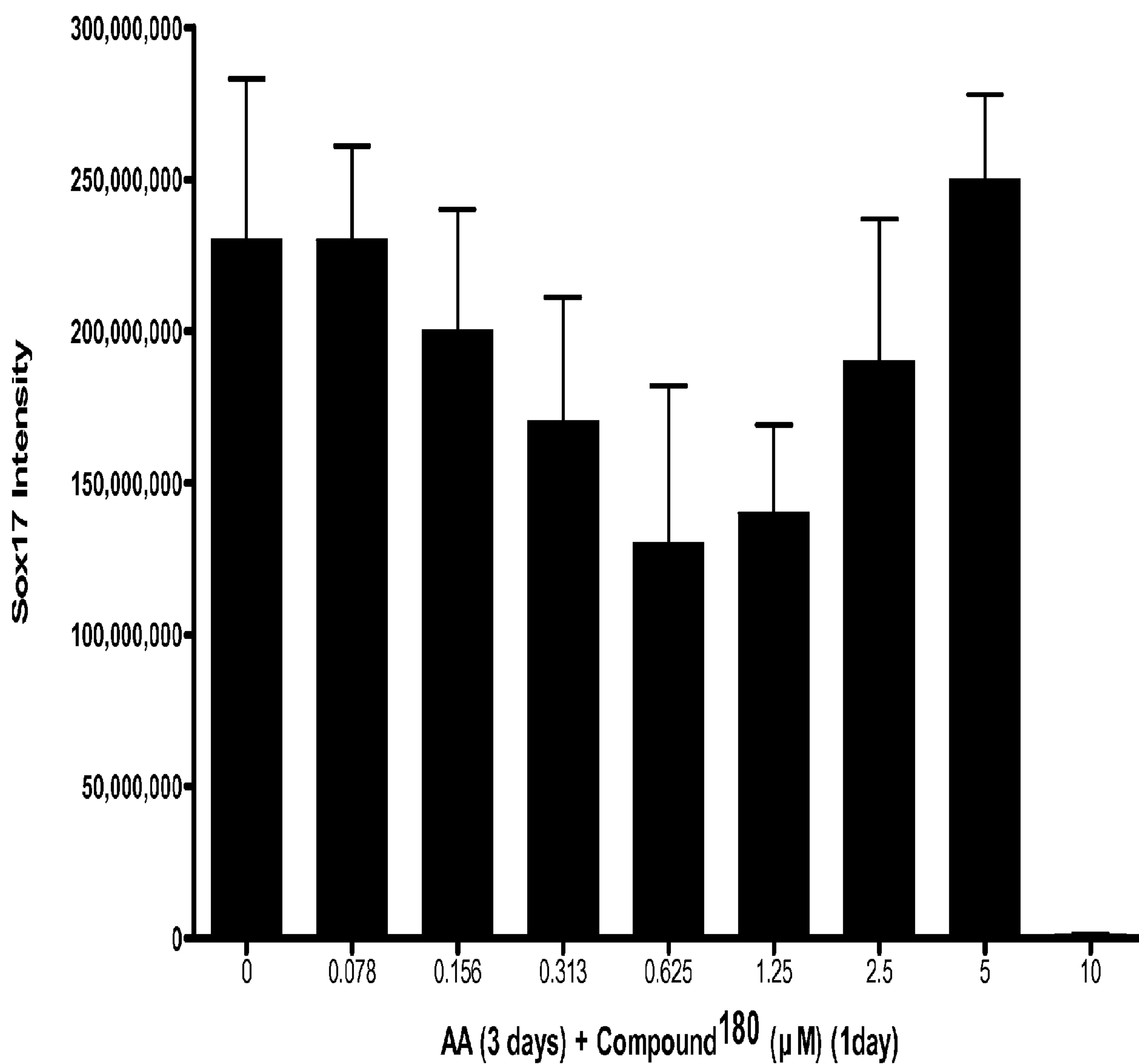


Figure 11C

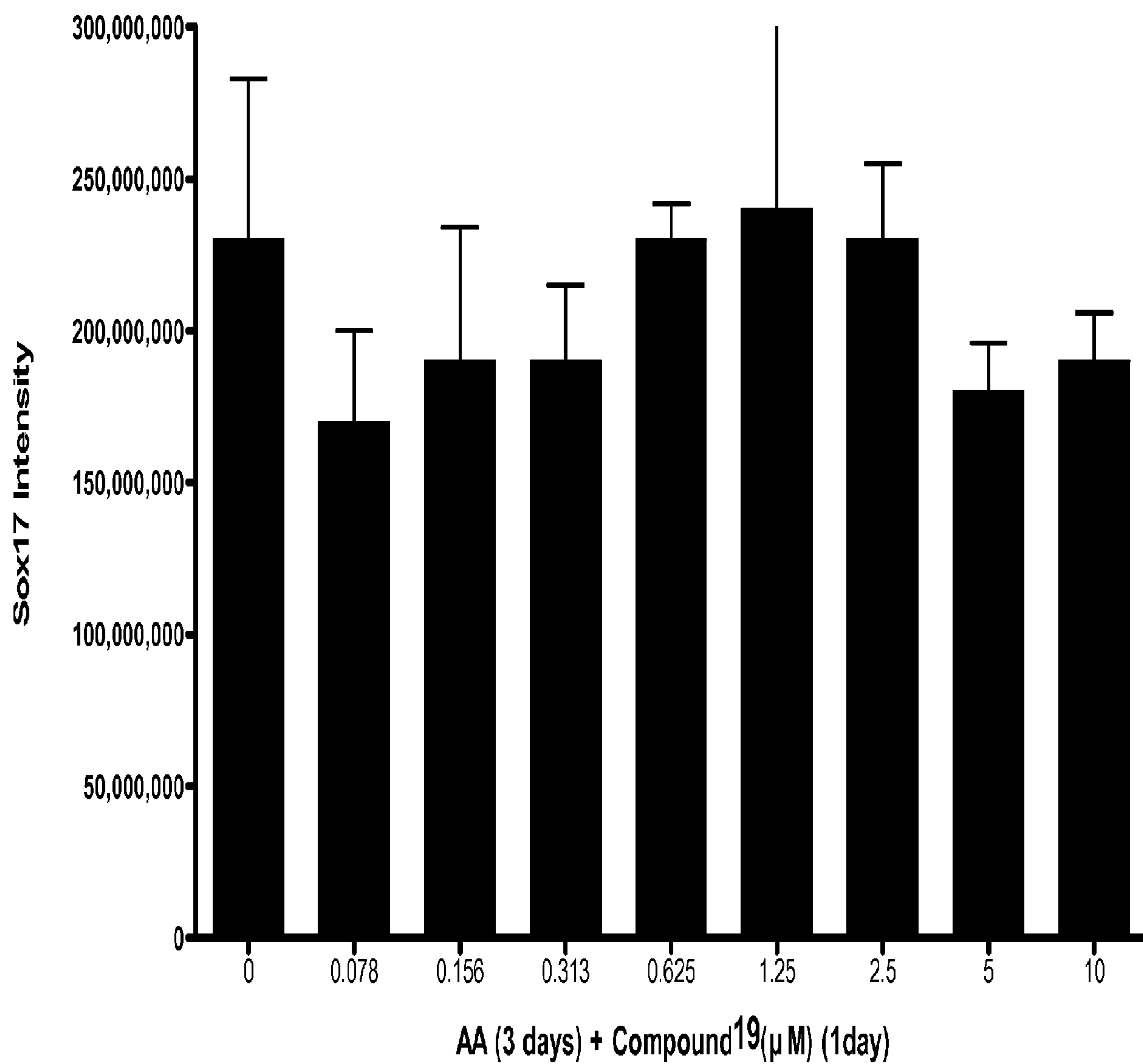


Figure 11D

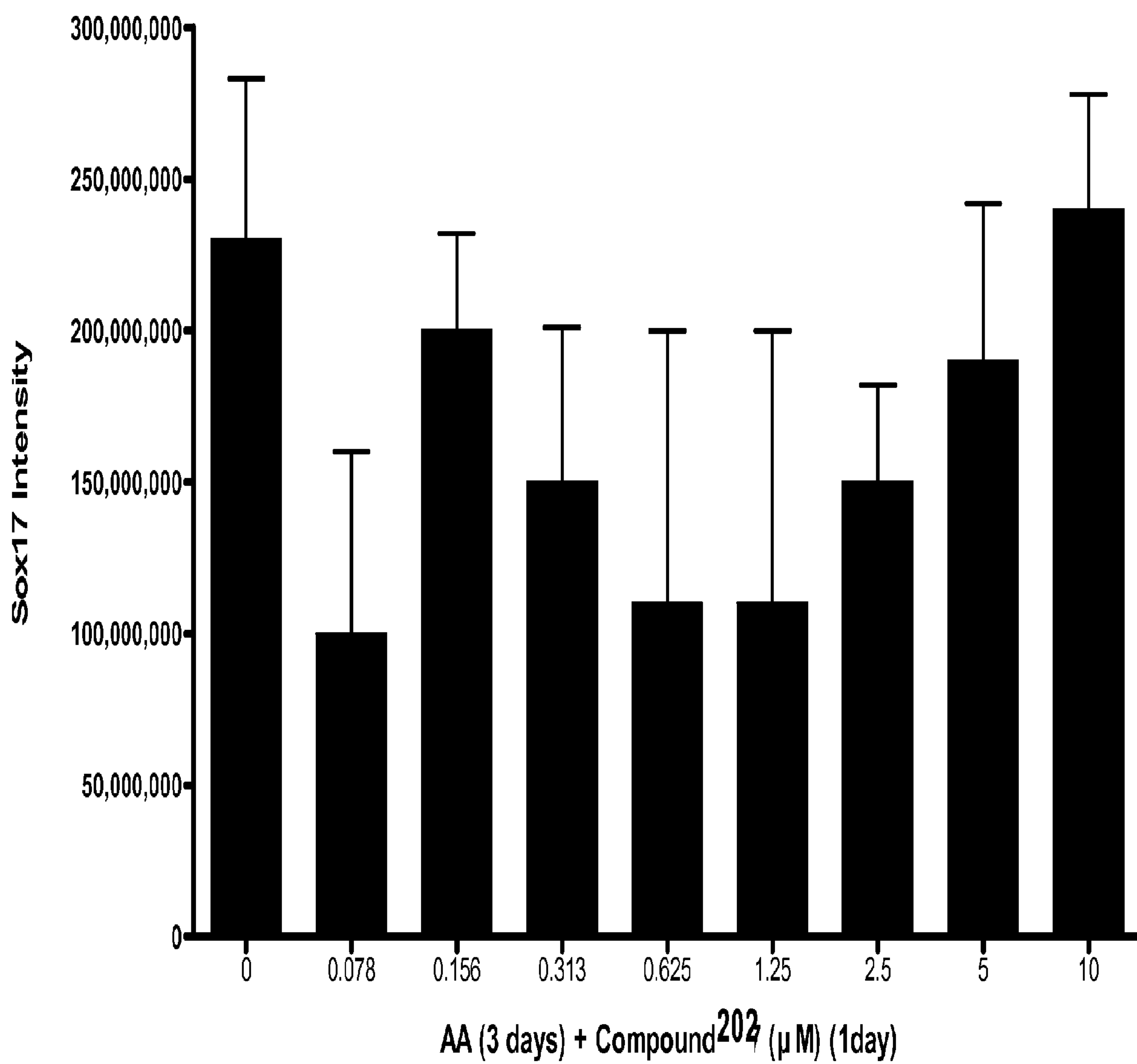


Figure 11E

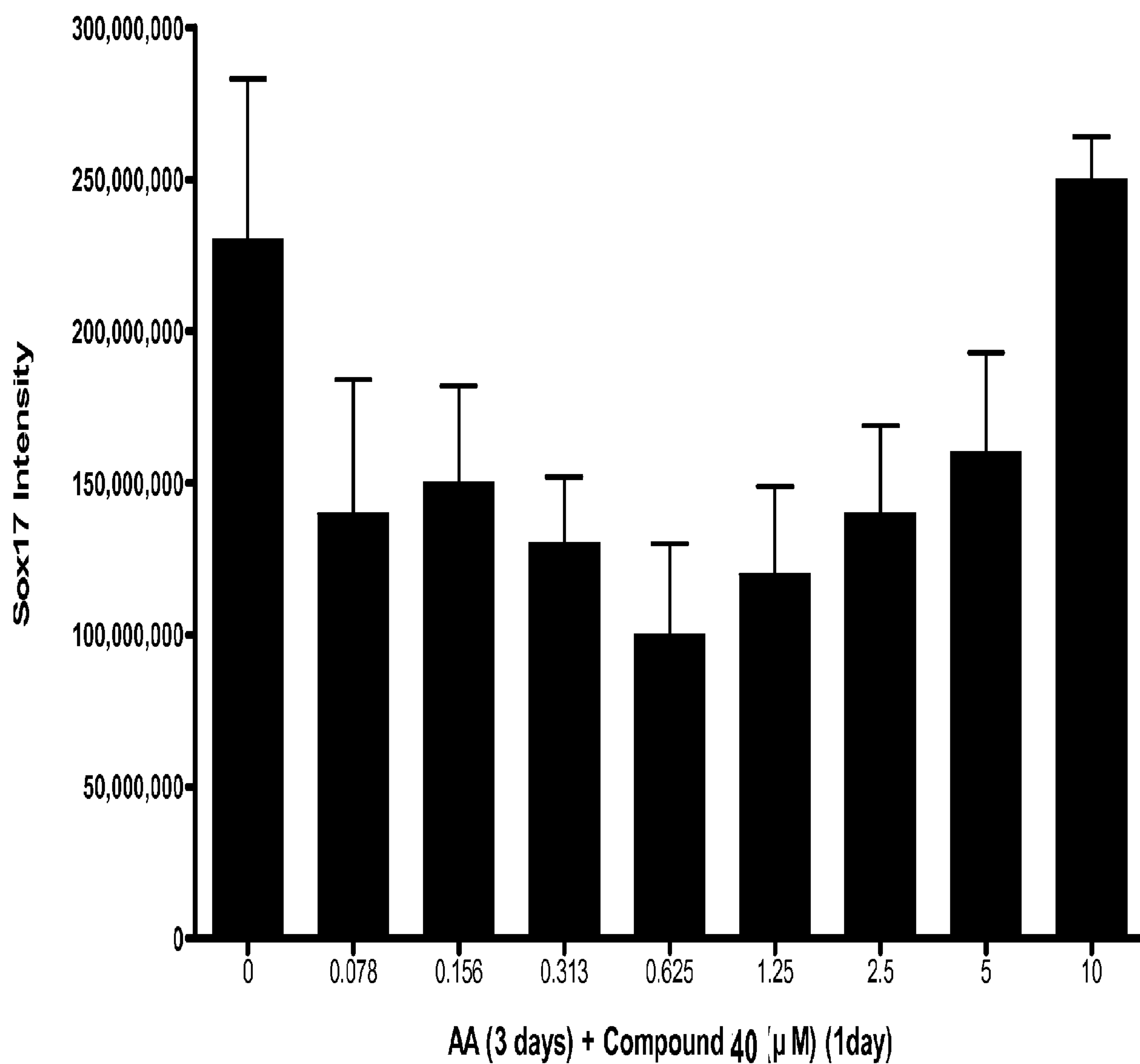


Figure 11F

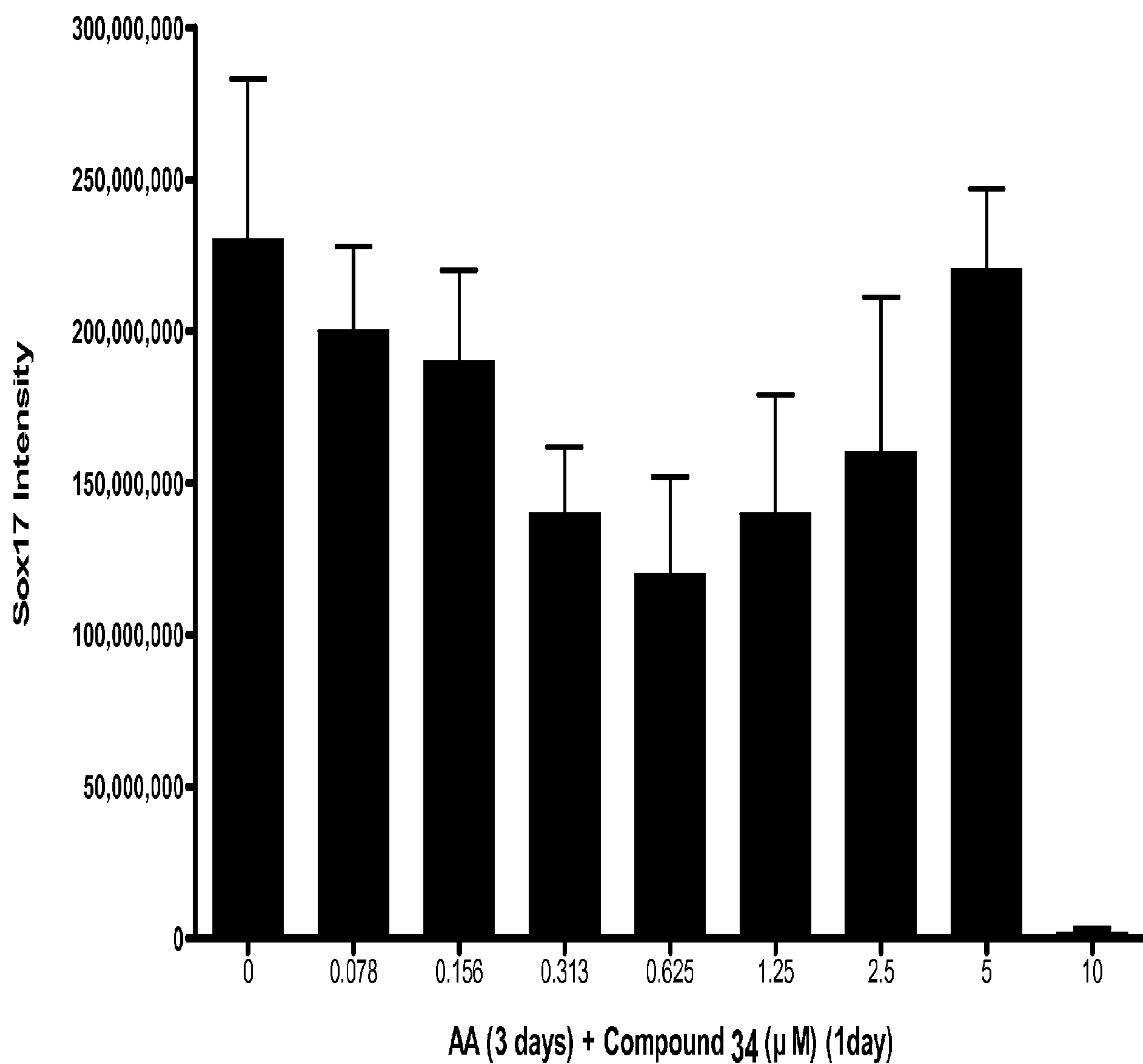


Figure 11G

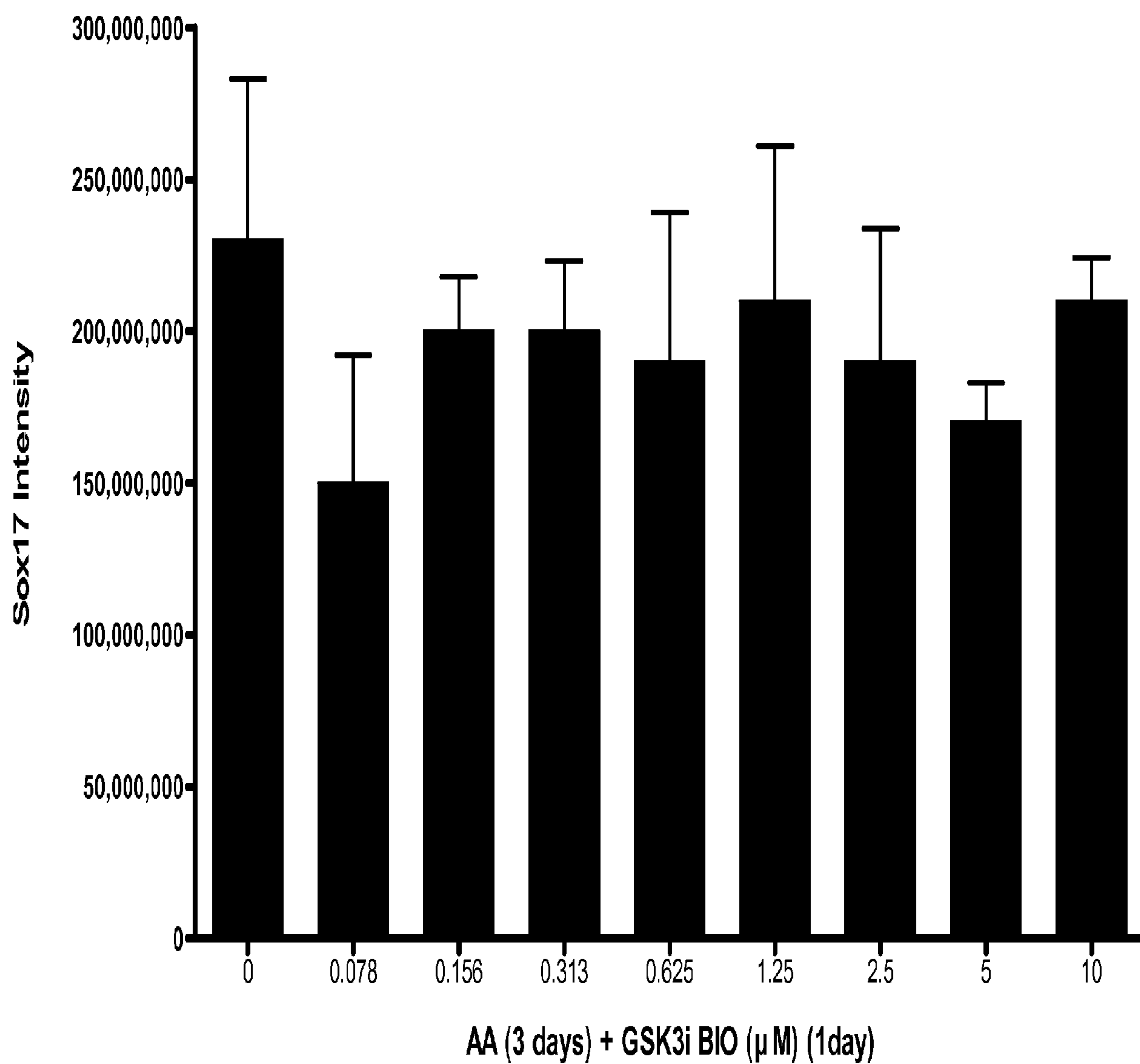


Figure 12A

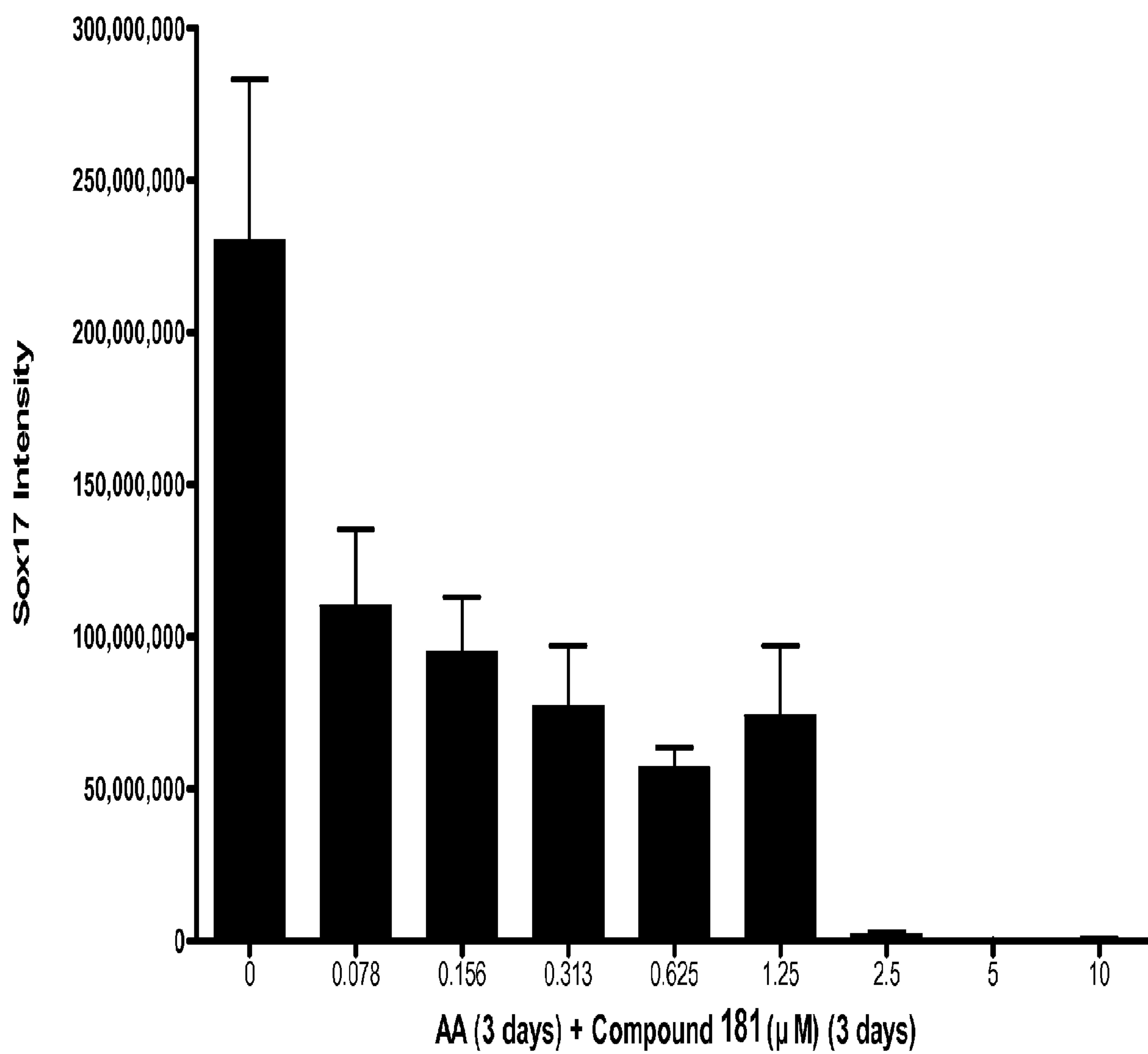


Figure 12B

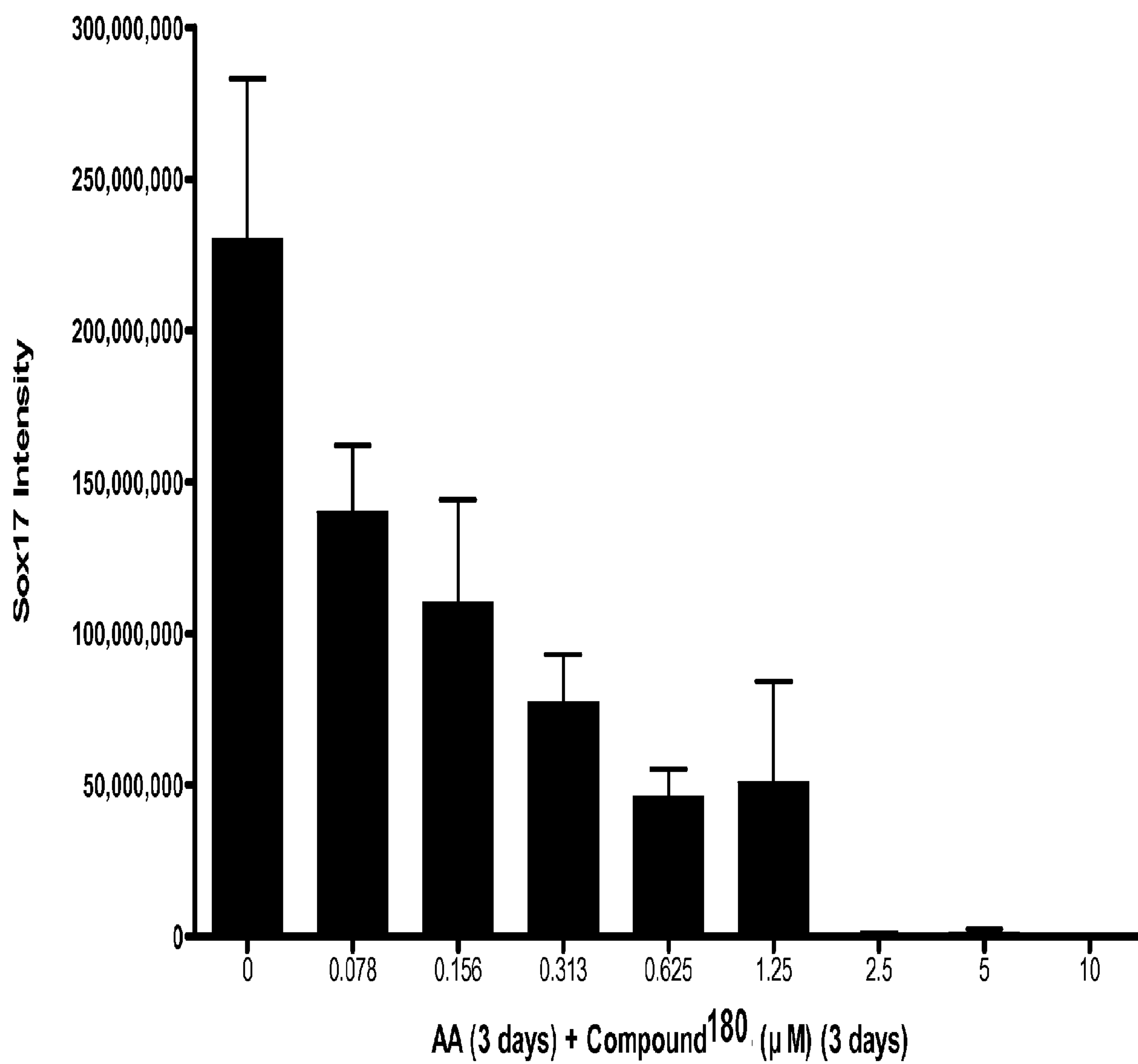


Figure 12C

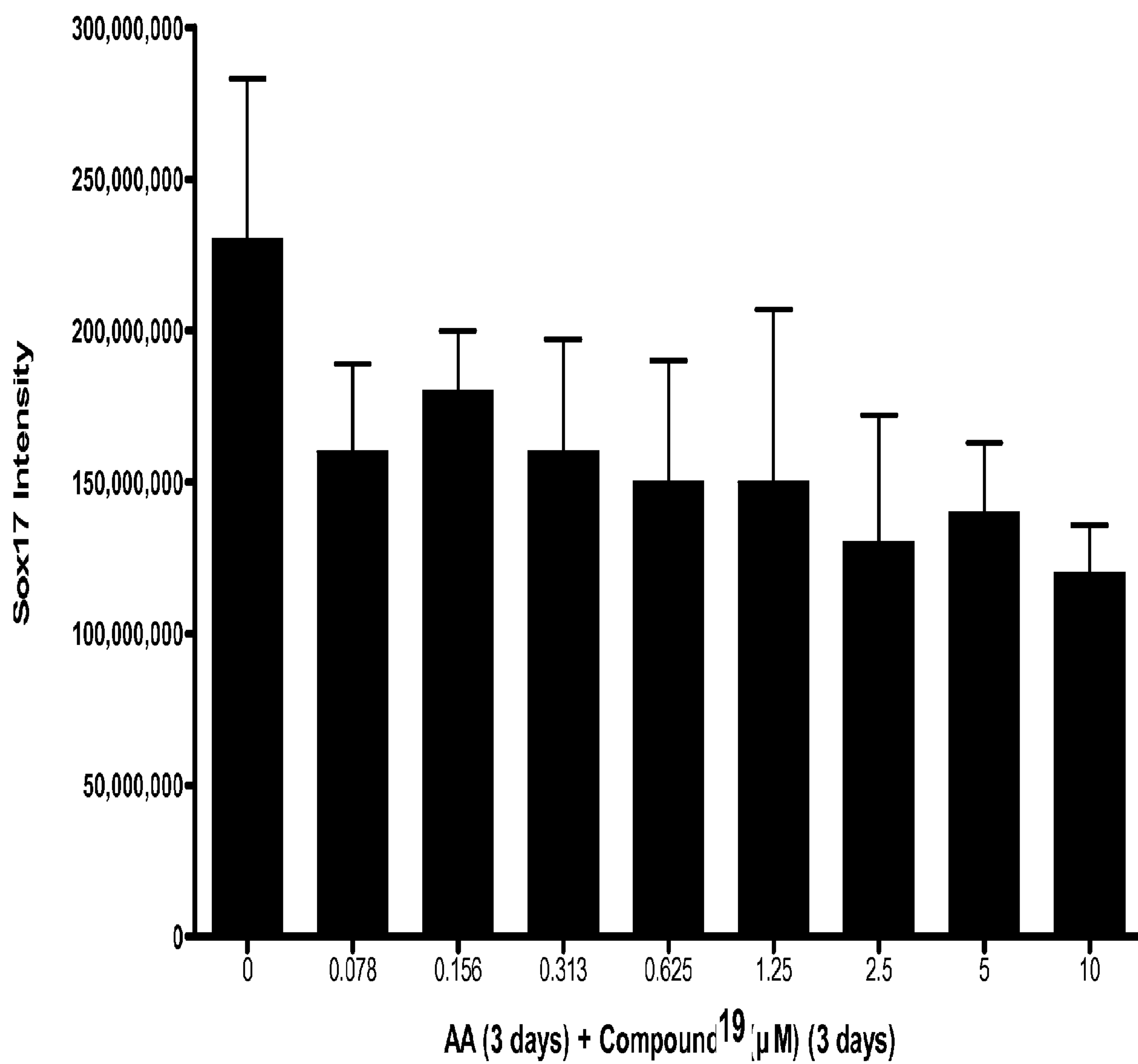


Figure 12D

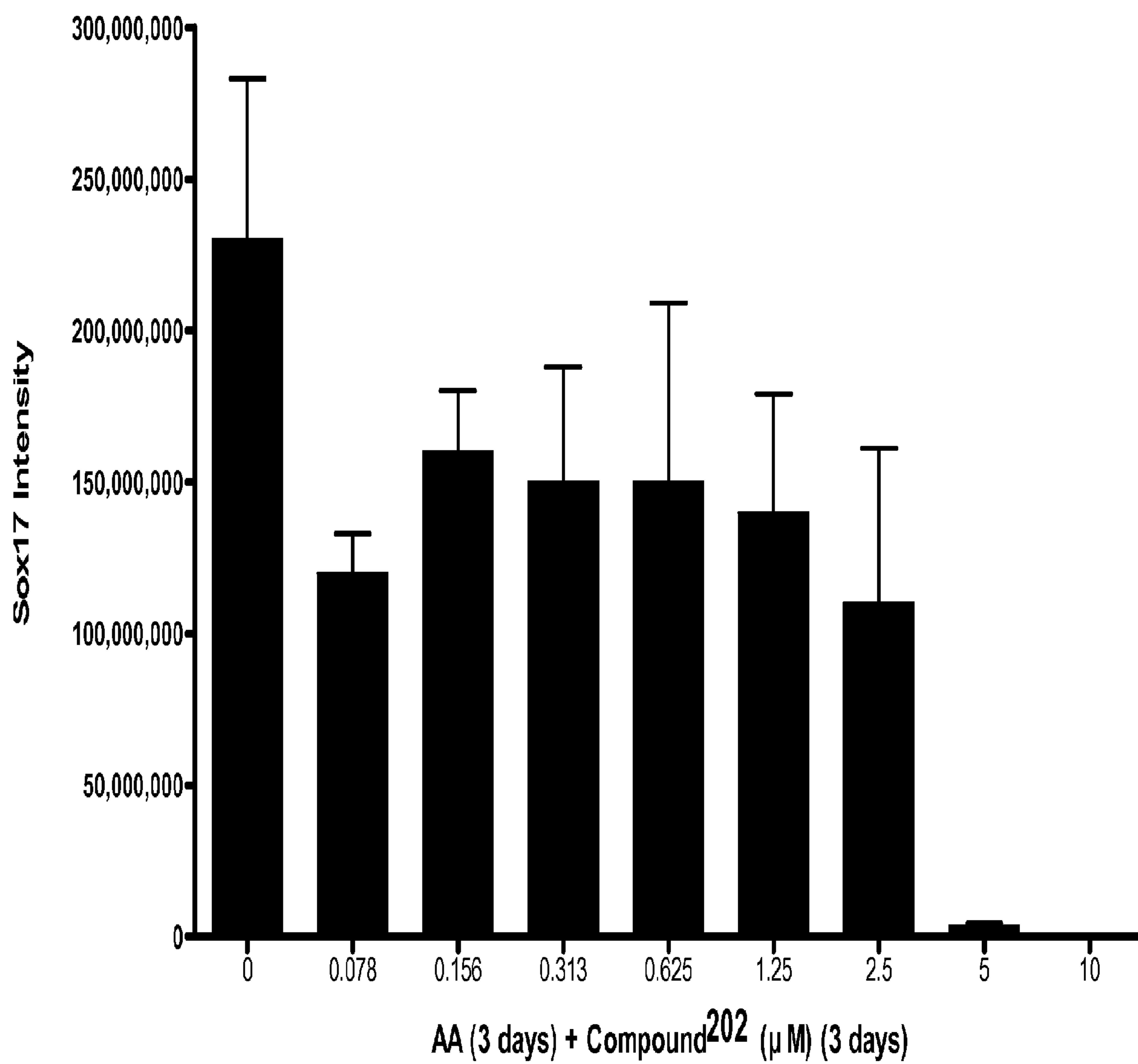


Figure 12E

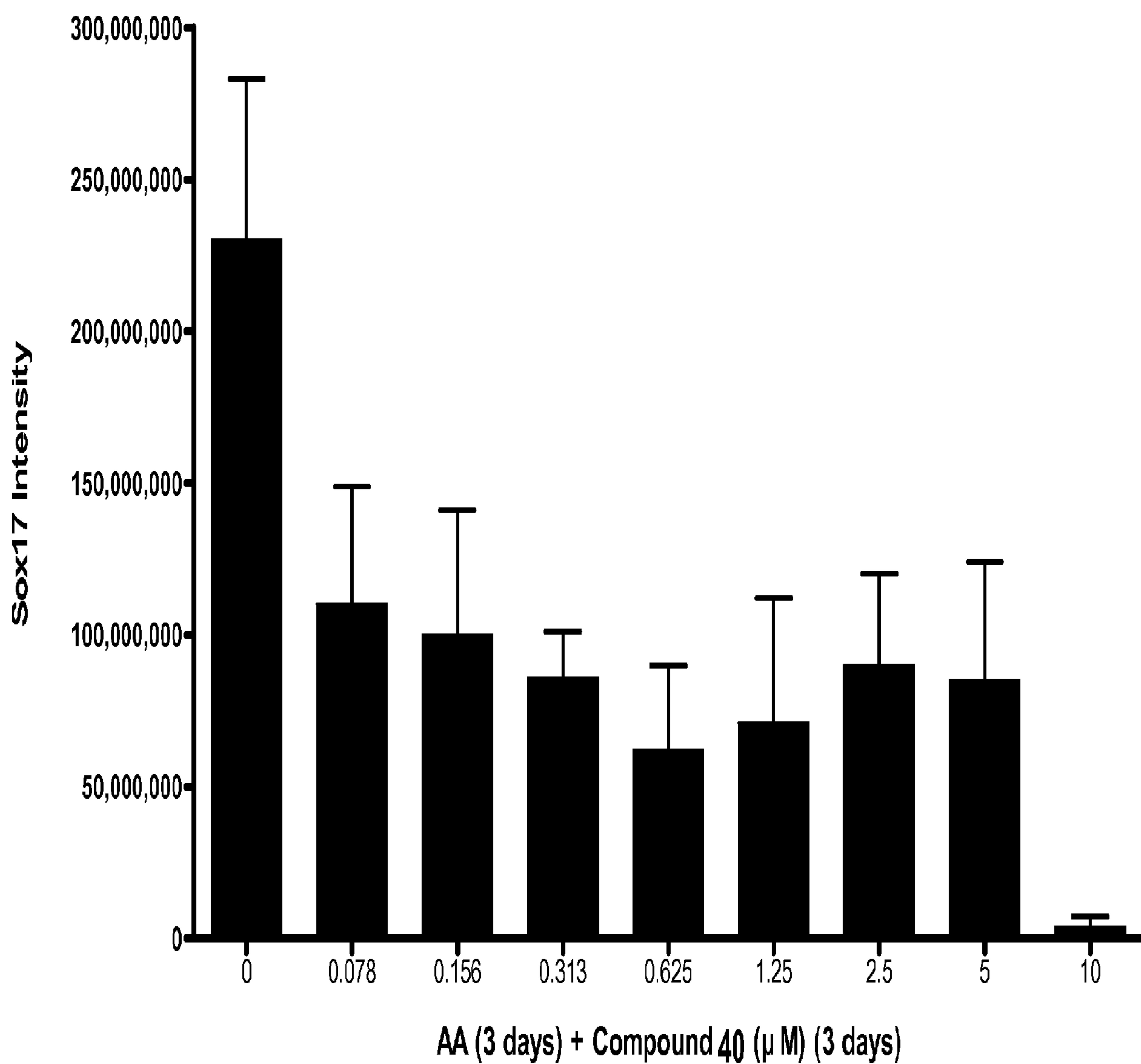


Figure 12F

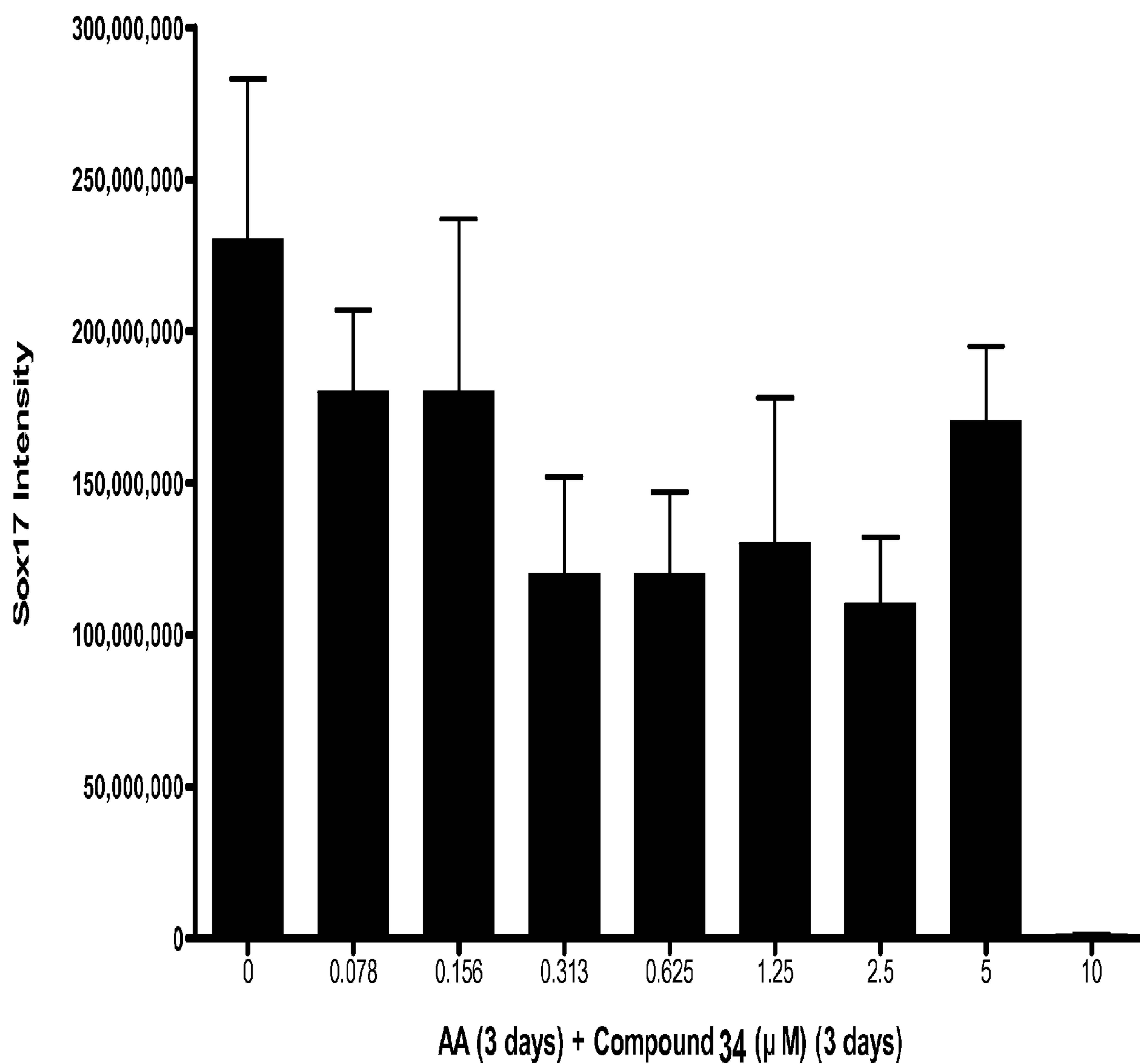


Figure 12G

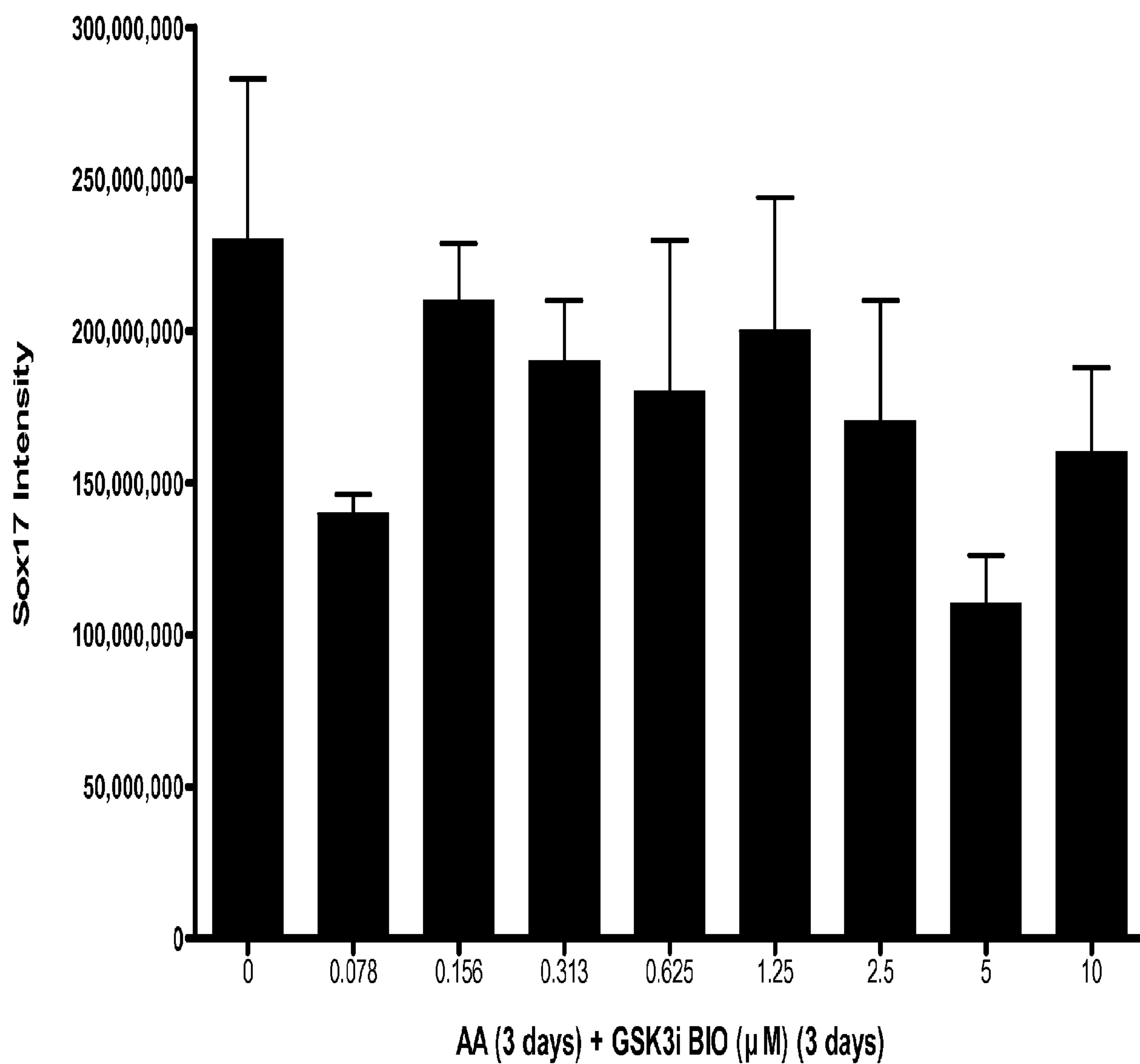


Figure 13A

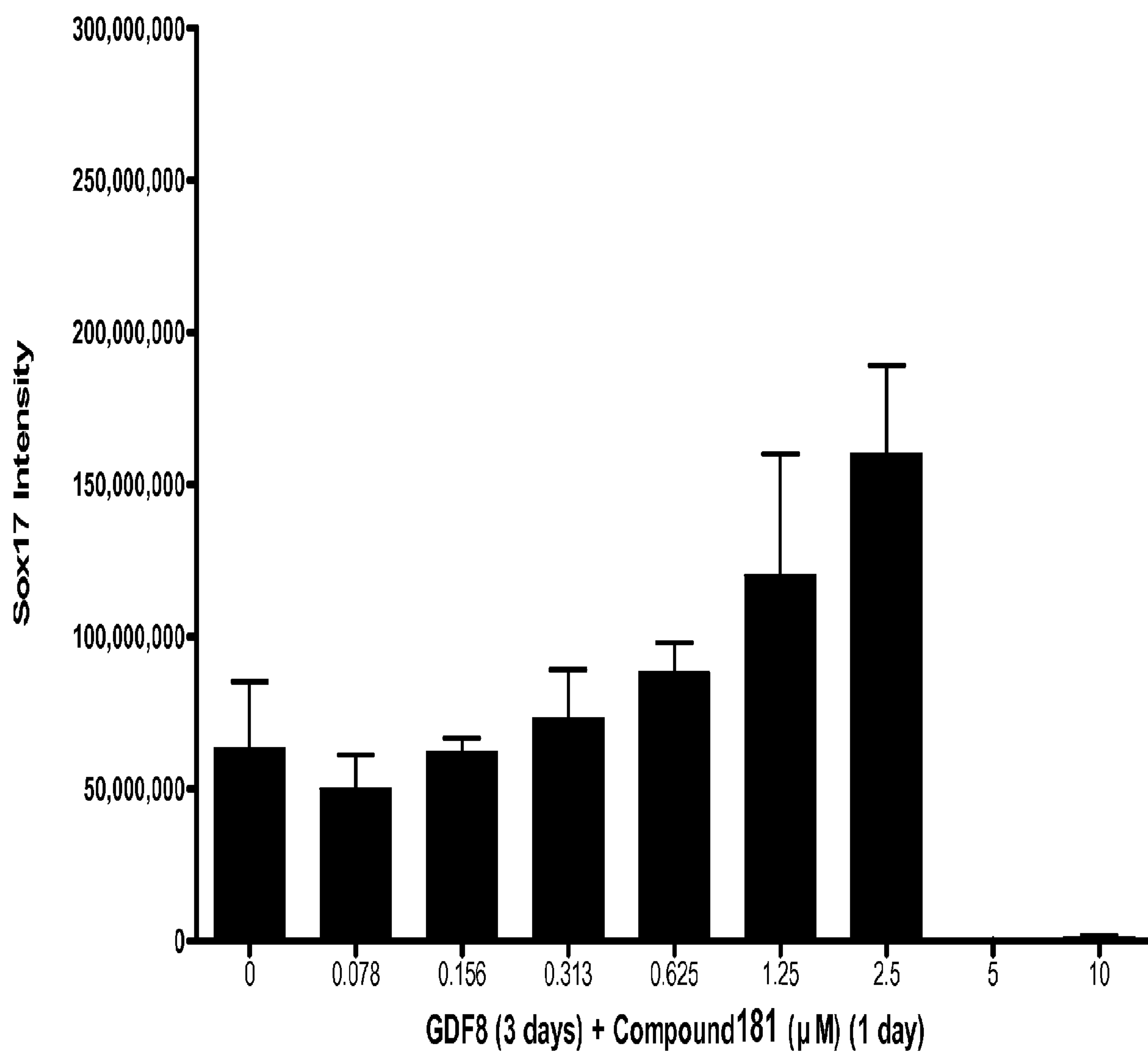


Figure 13B

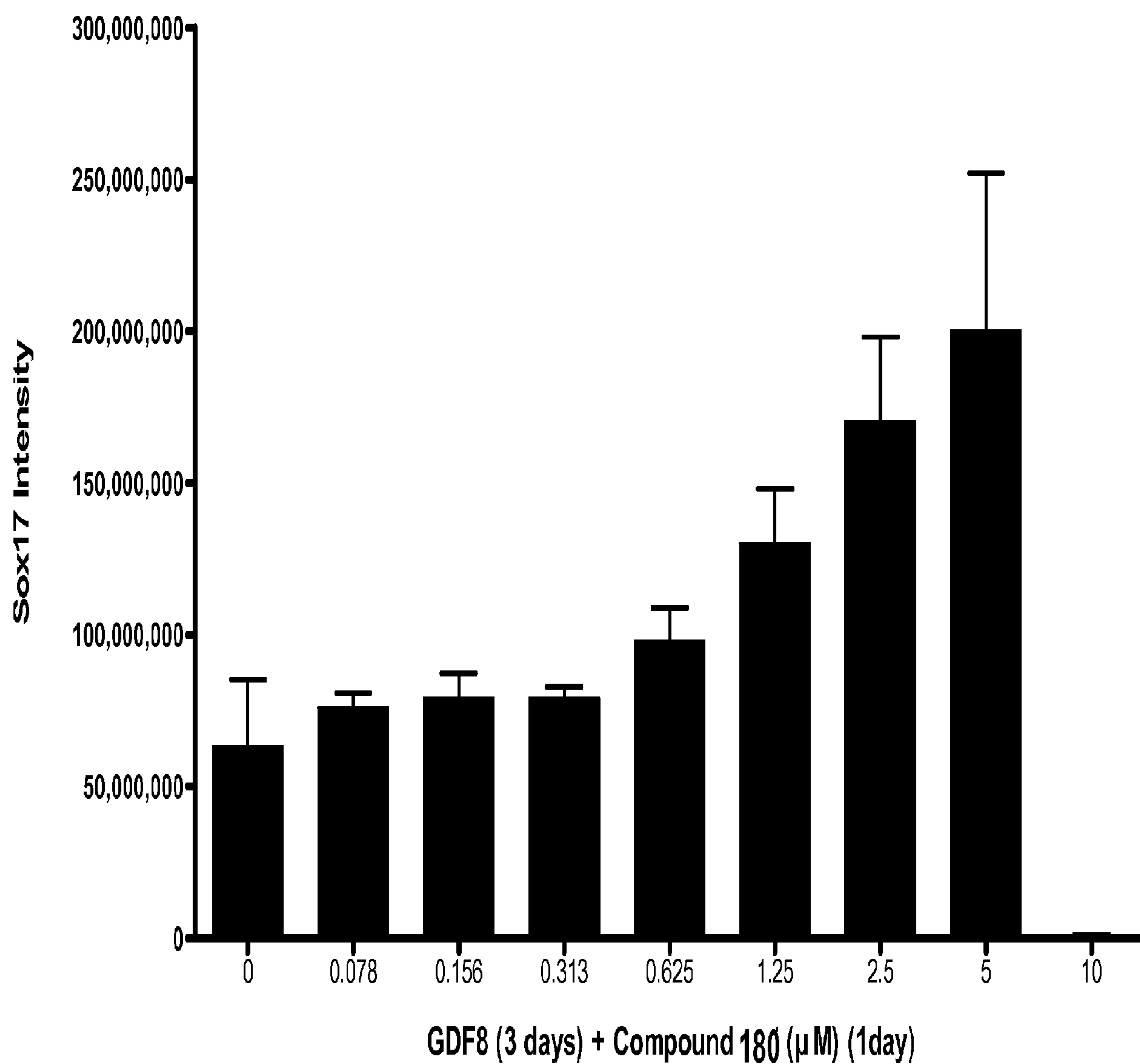


Figure 13C

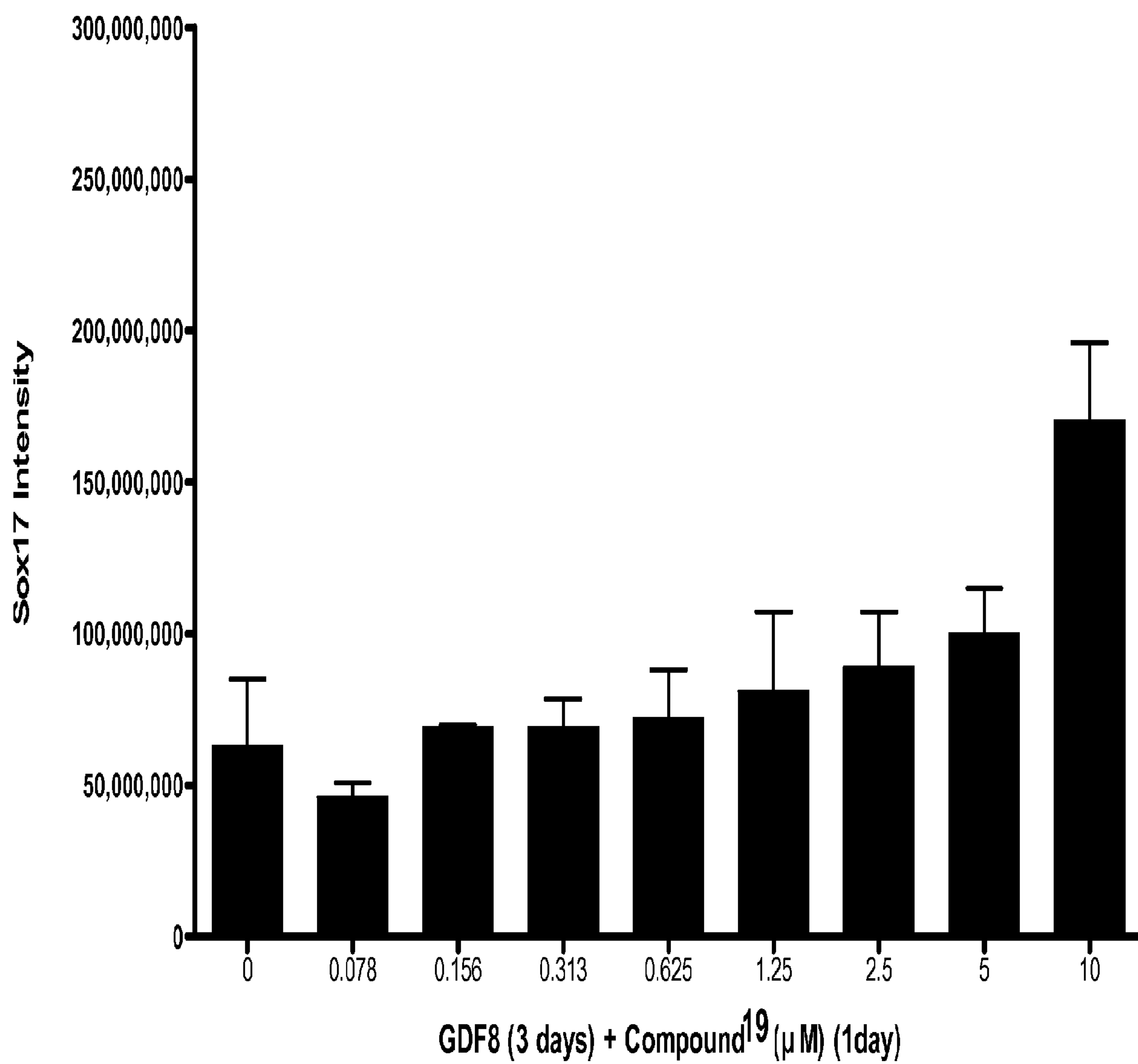


Figure 13D

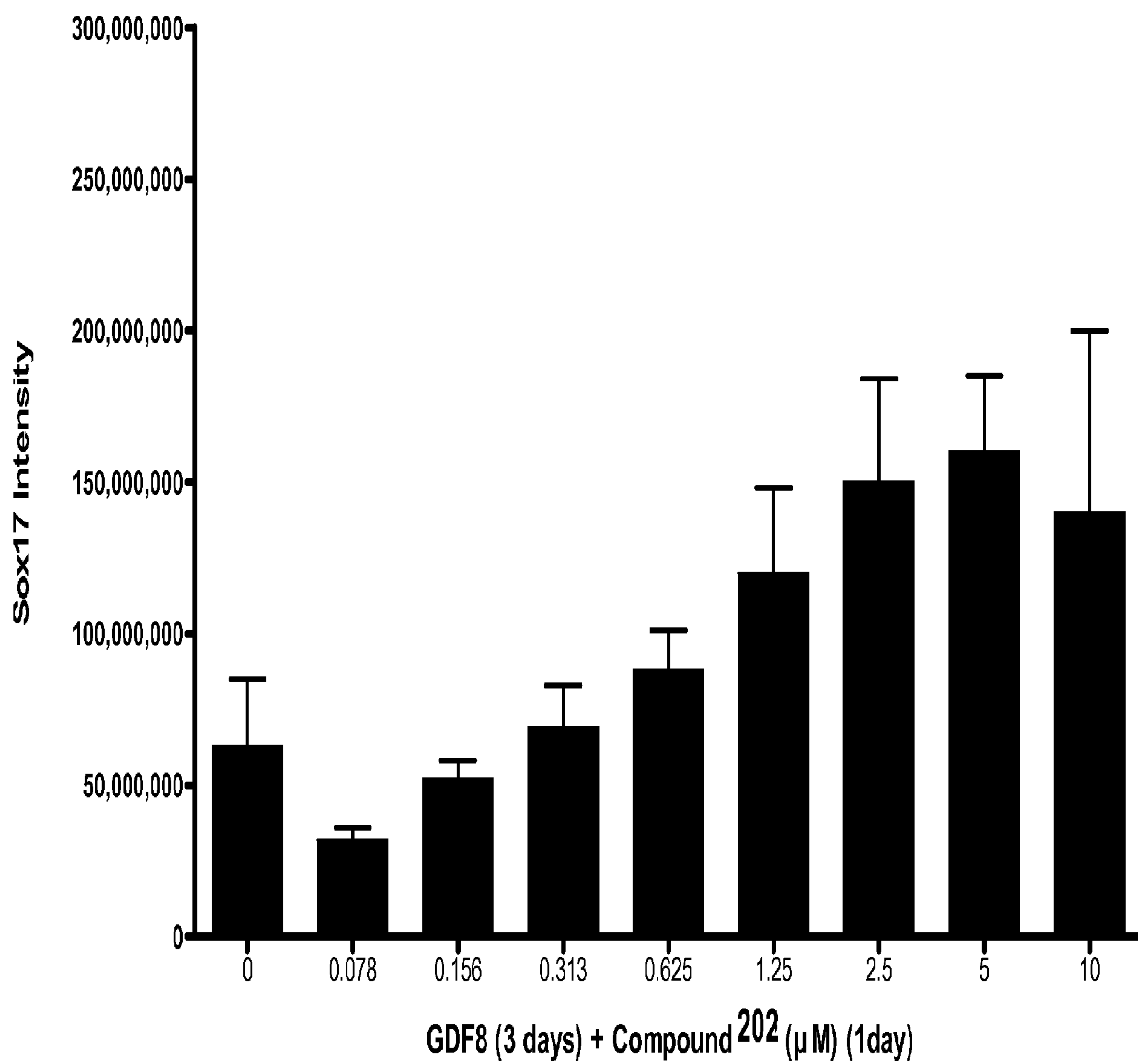


Figure 13E

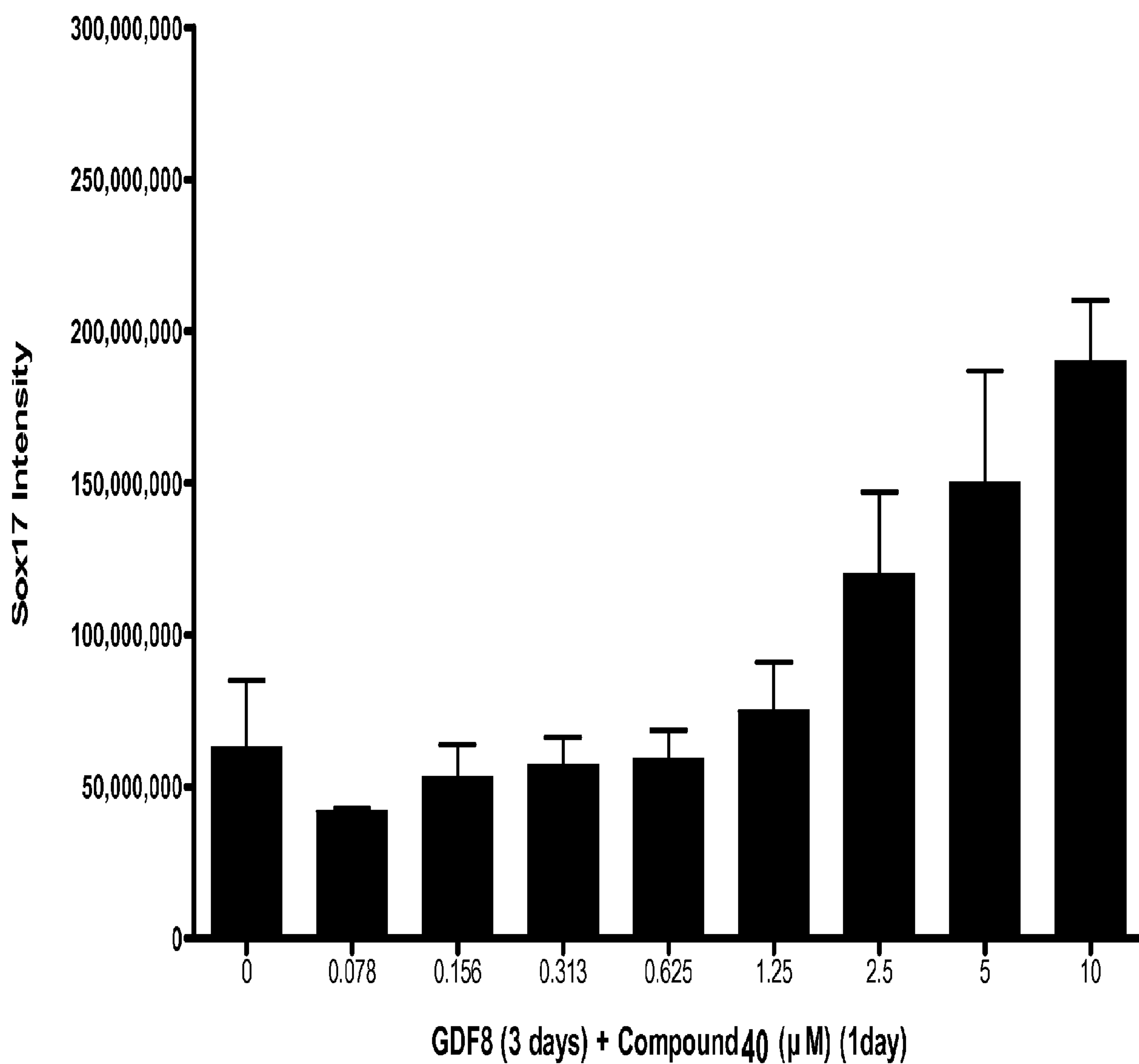


Figure 13F

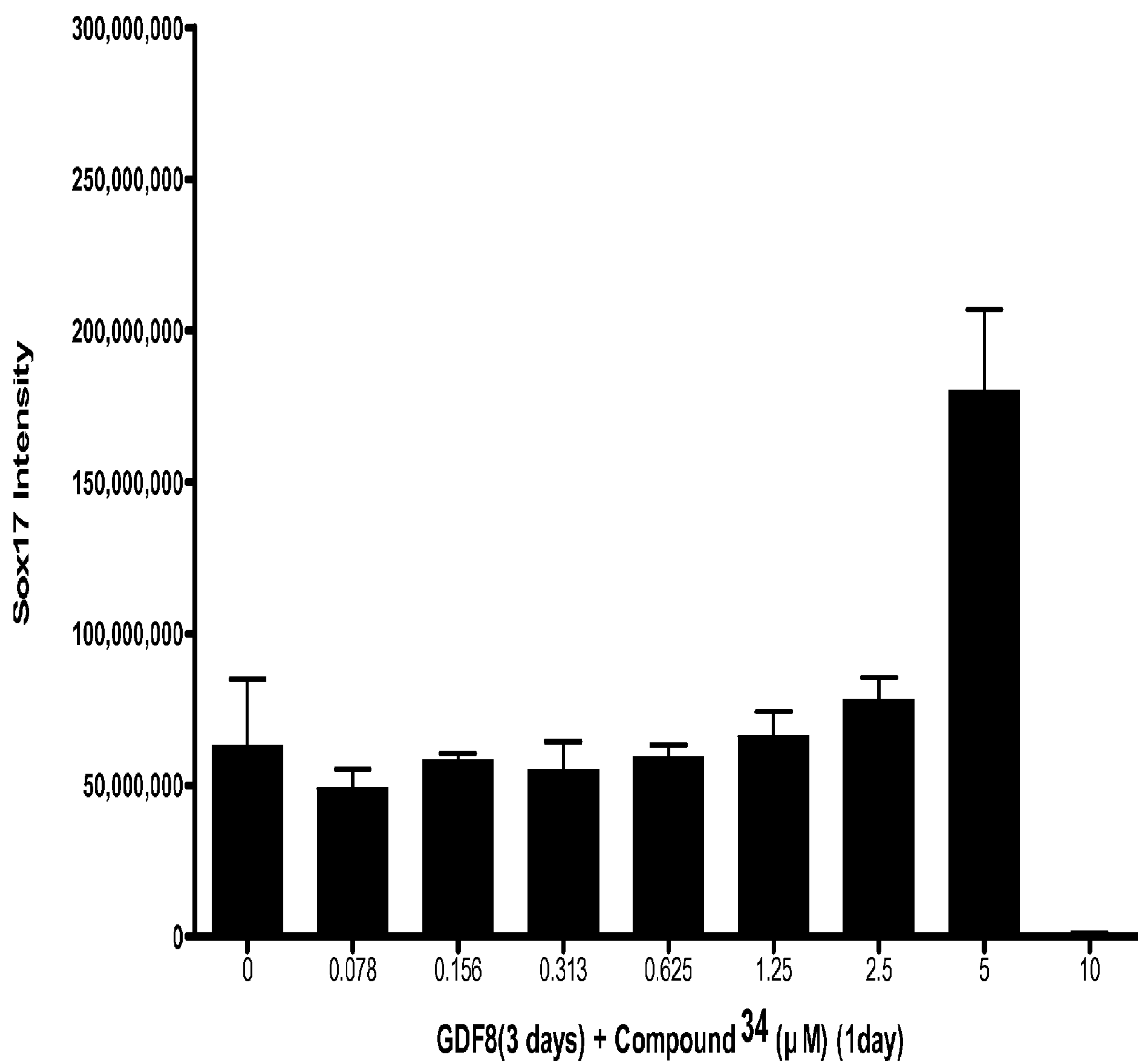


Figure 13G

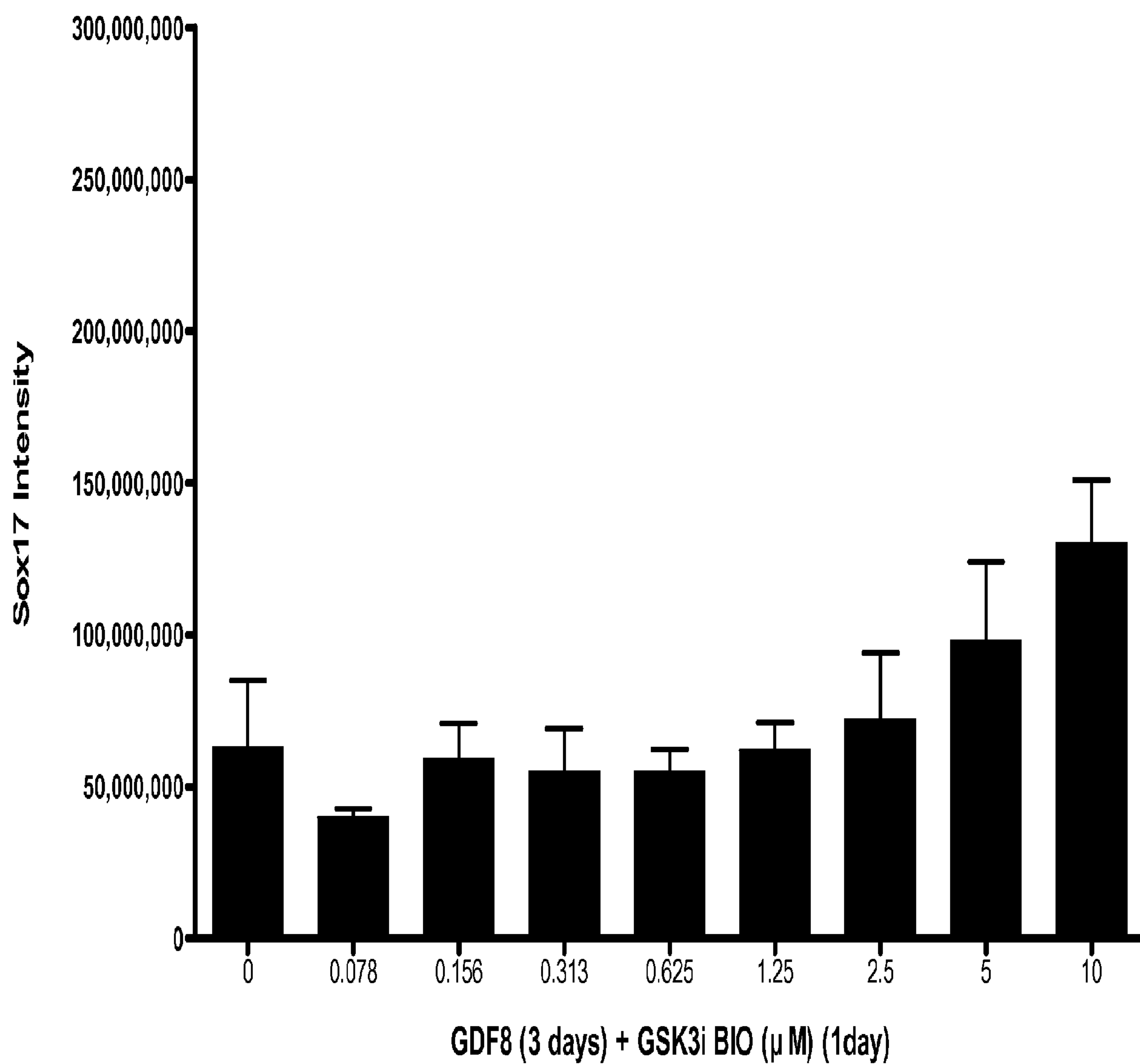


Figure 14A

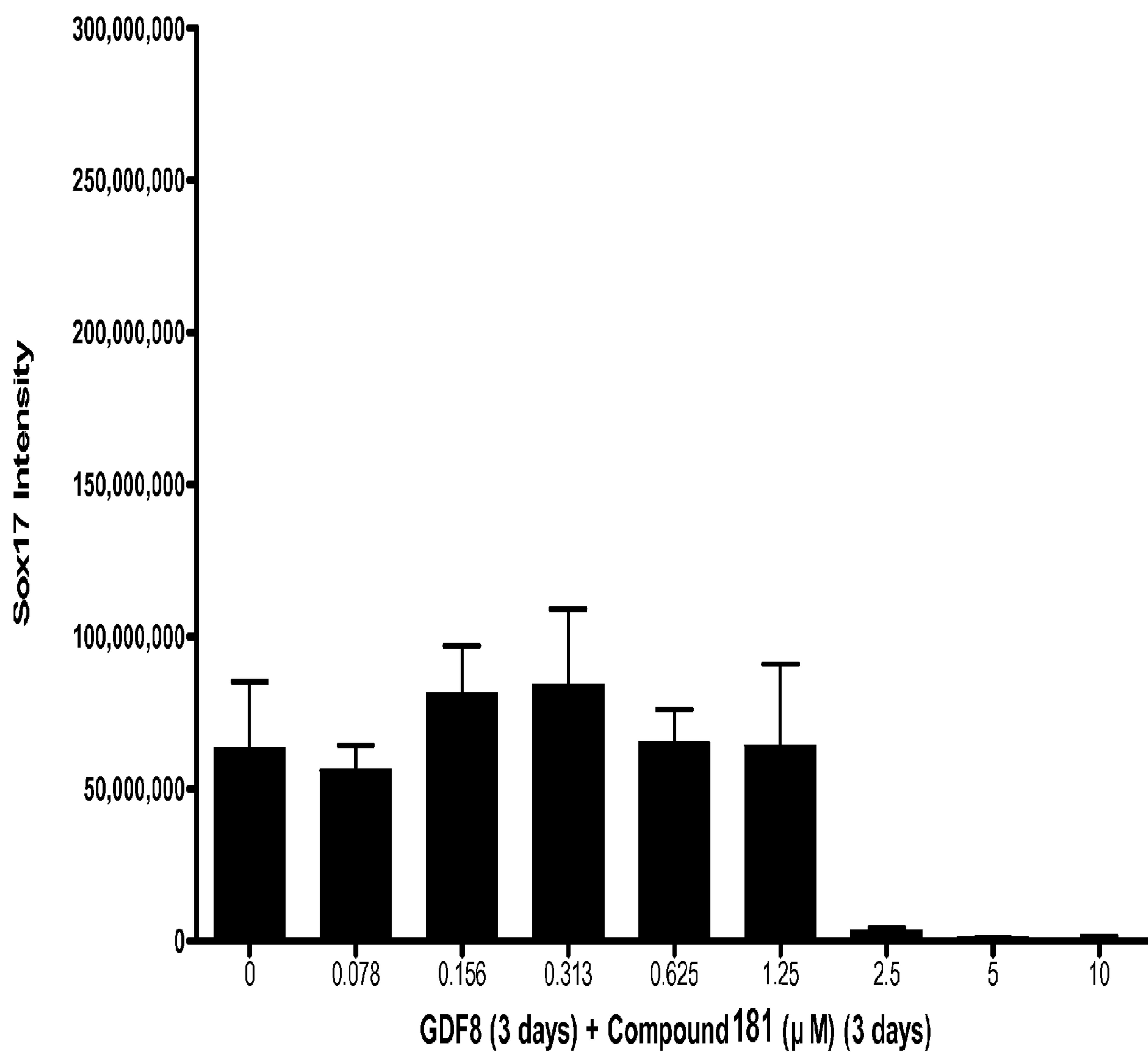


Figure 14B

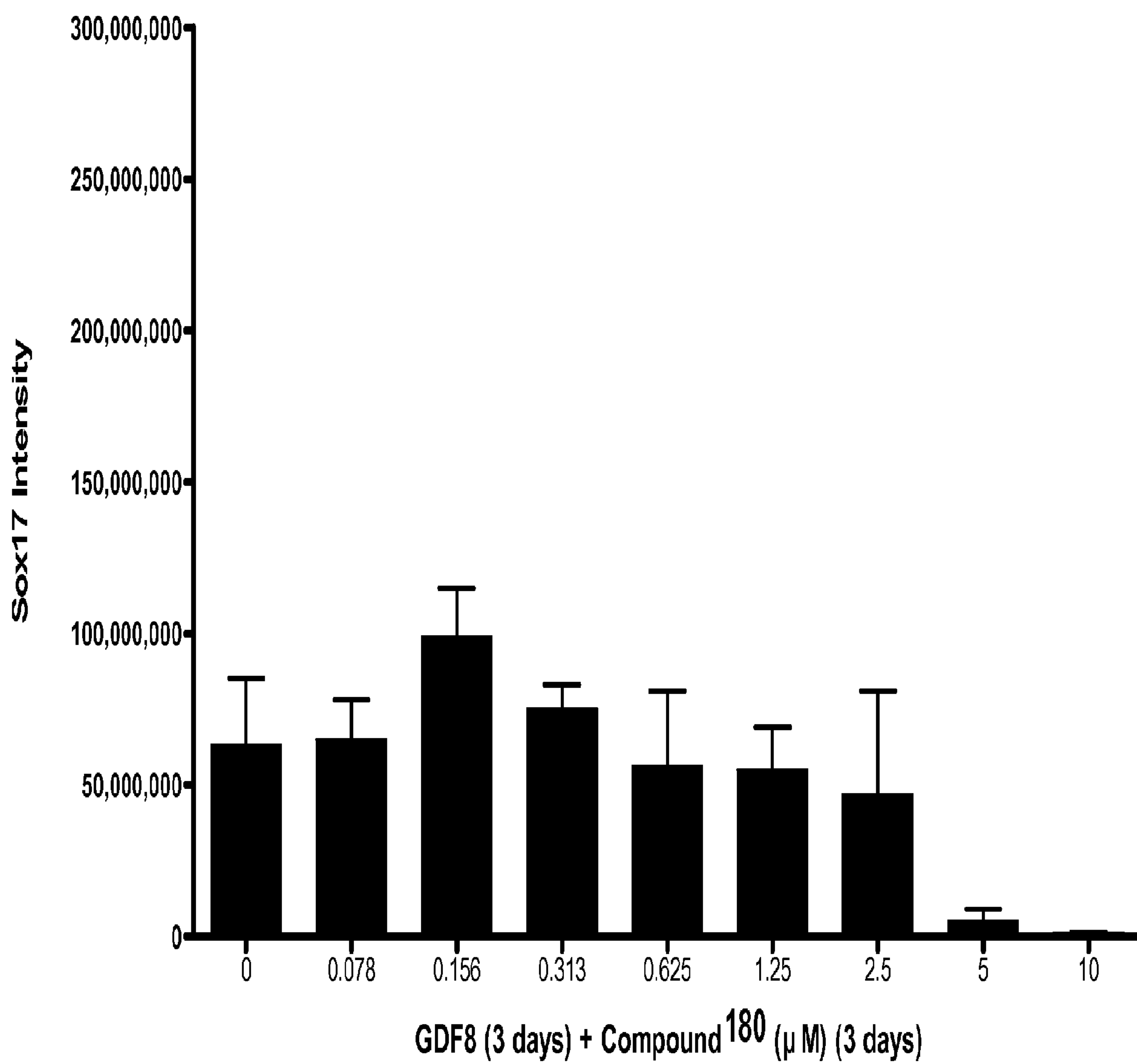


Figure 14C

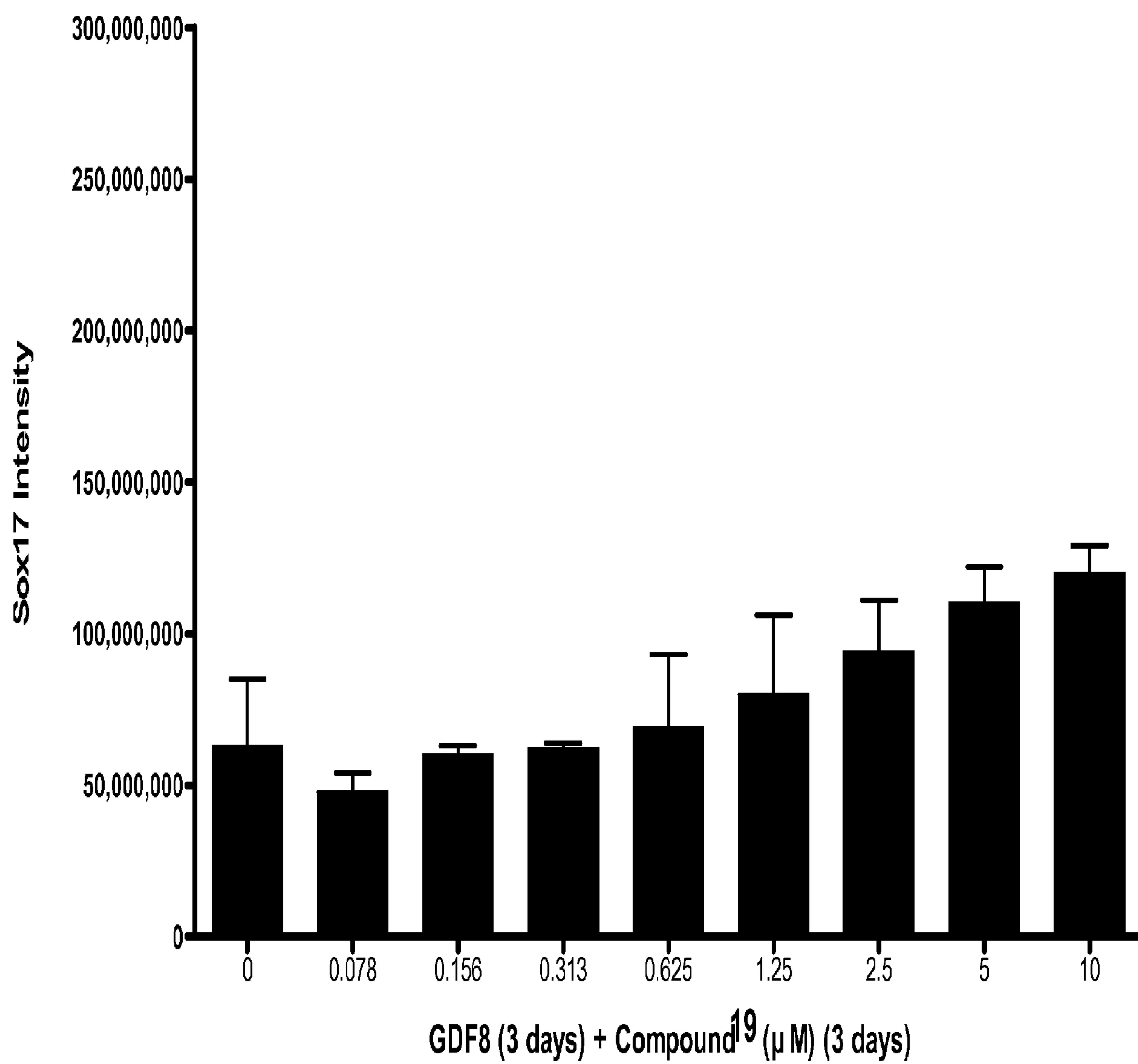


Figure 14D

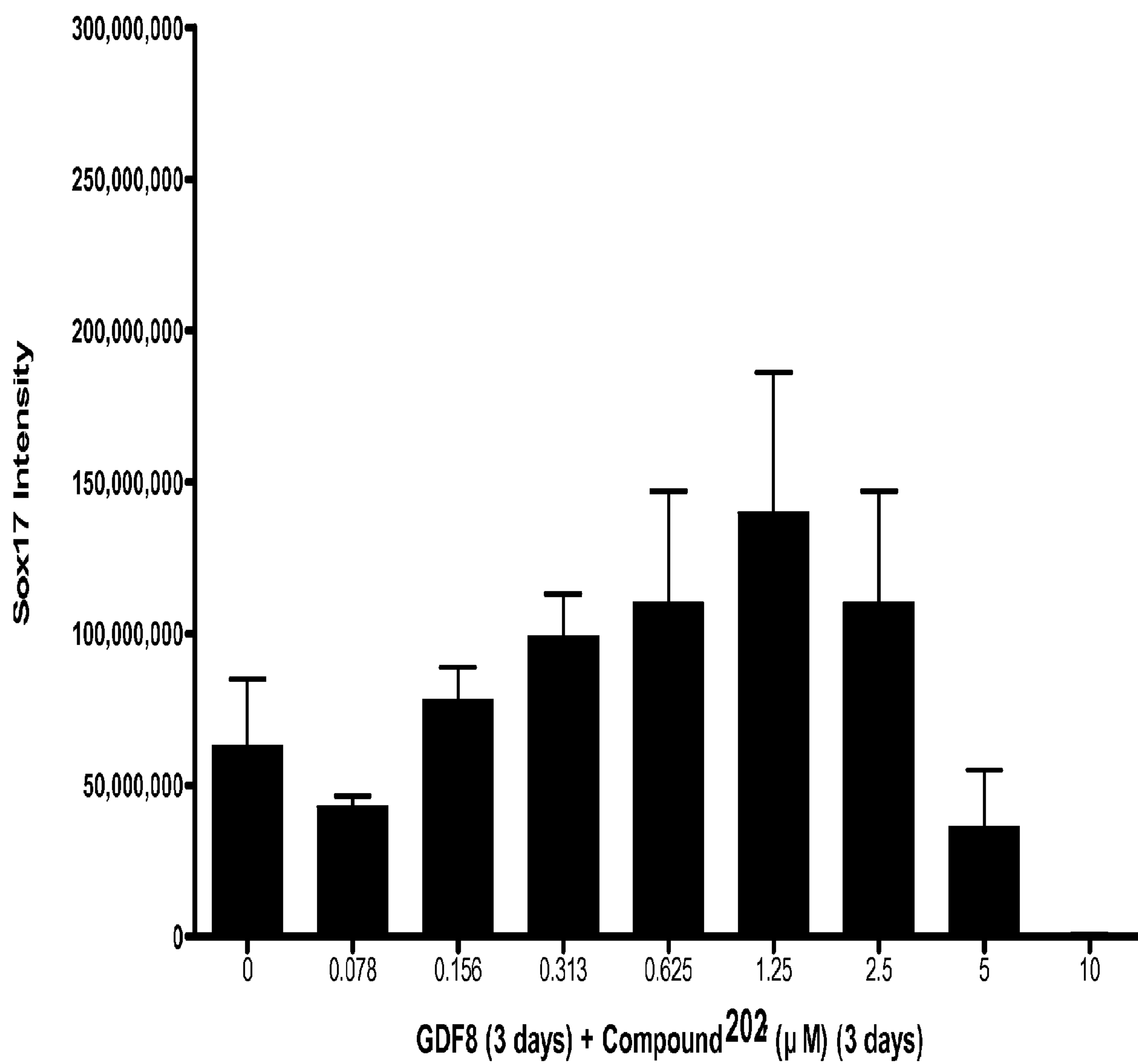


Figure 14E

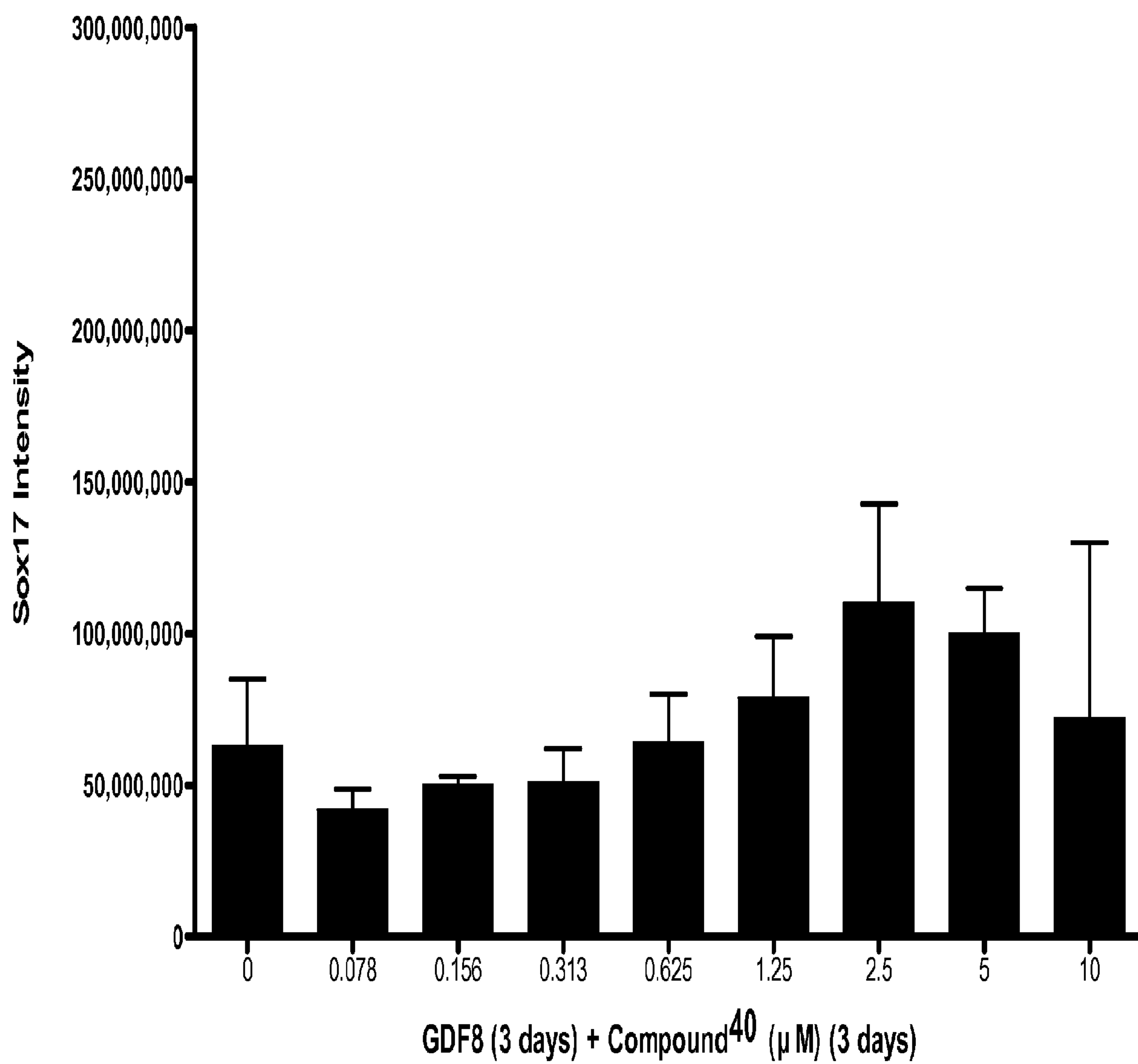


Figure 14F

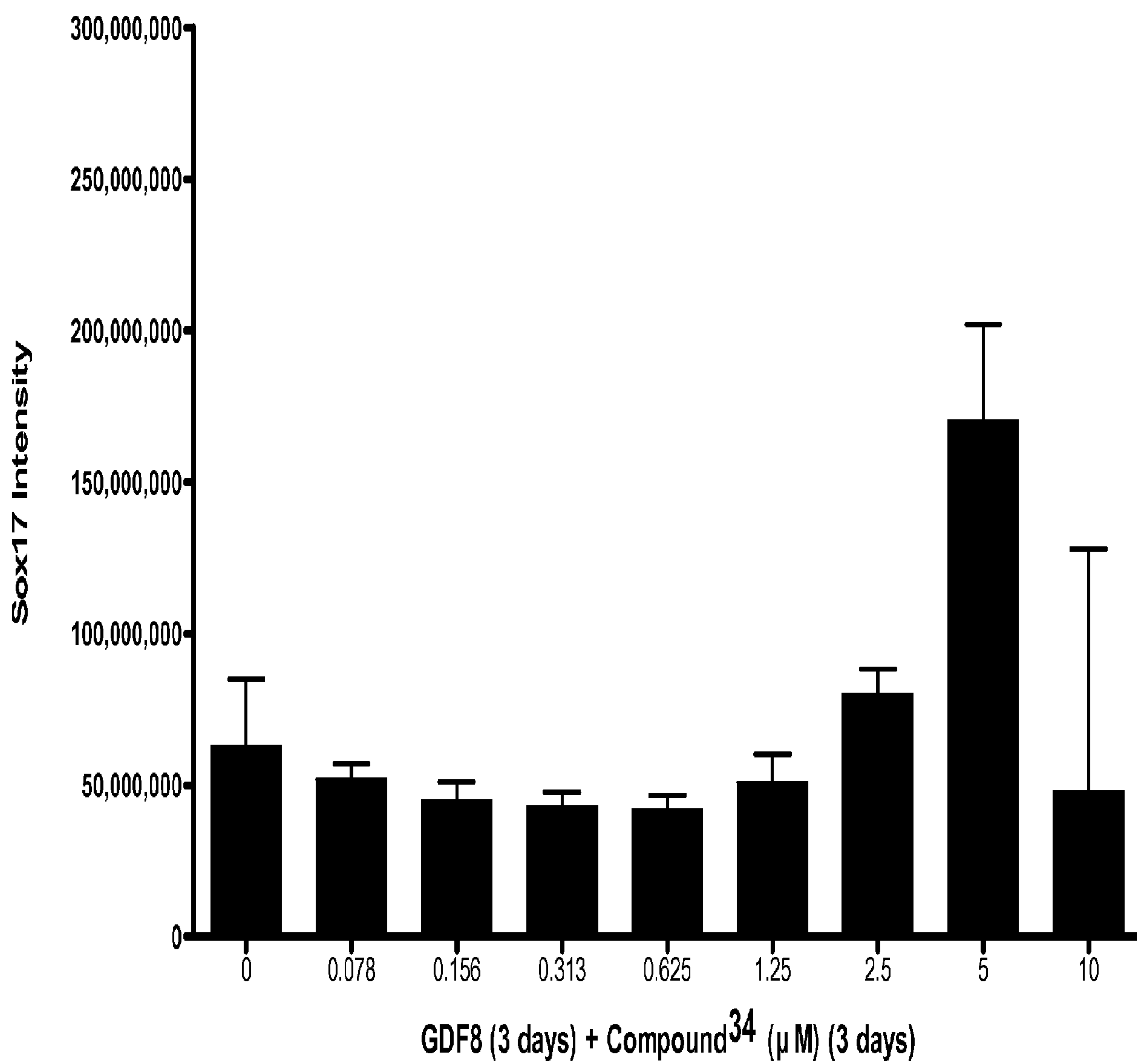


Figure 14G

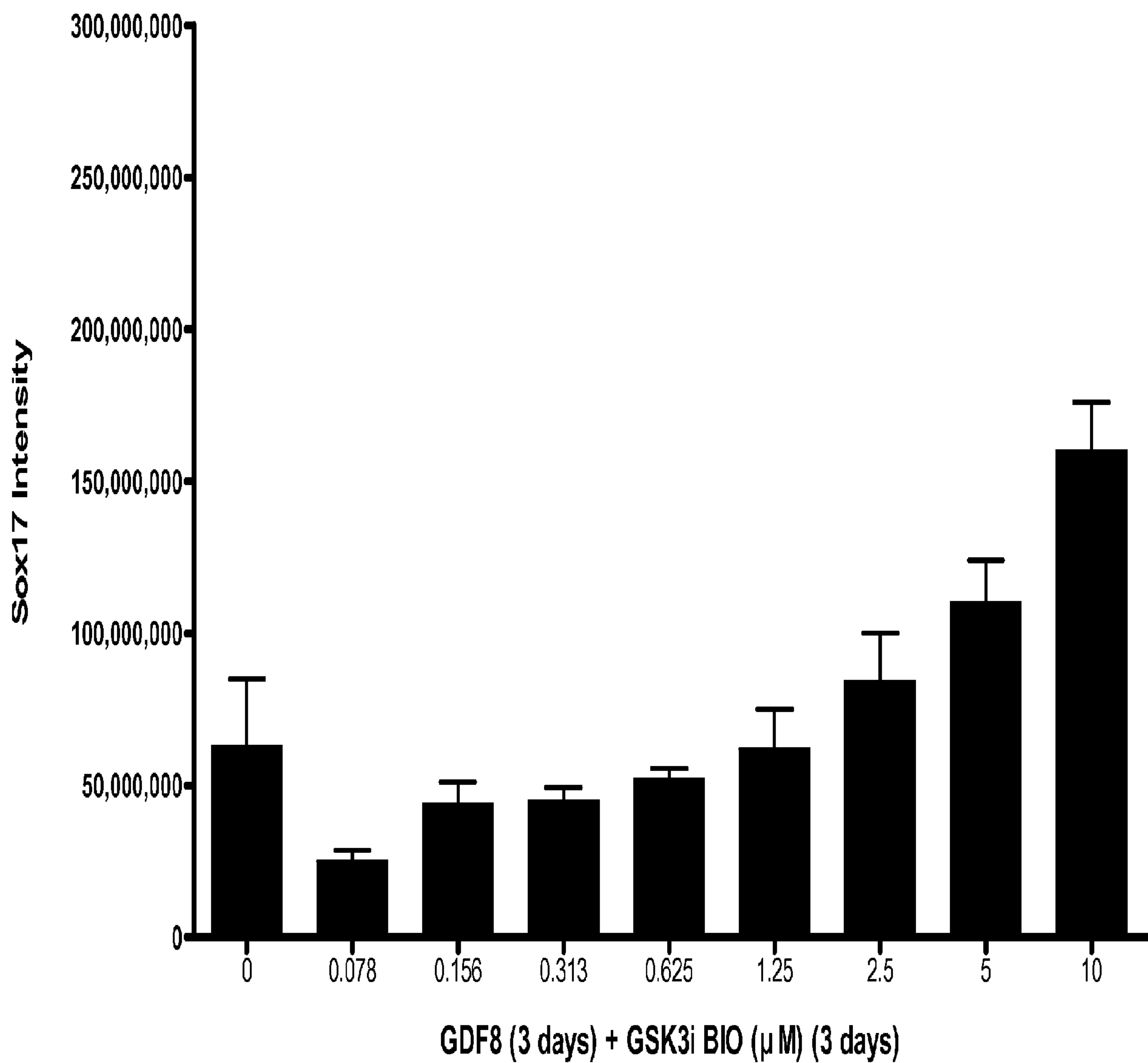


Figure 15

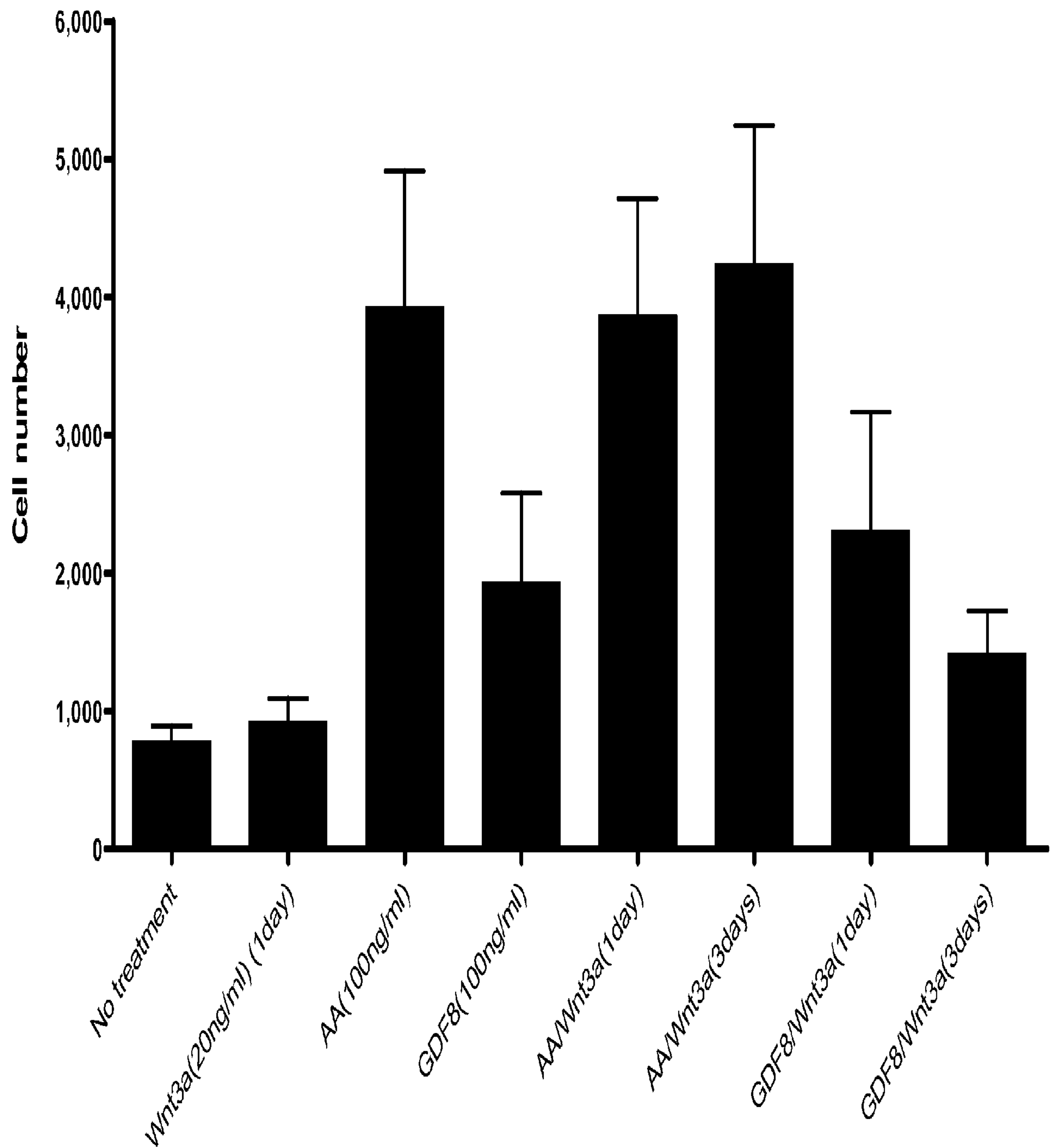


Figure 16A

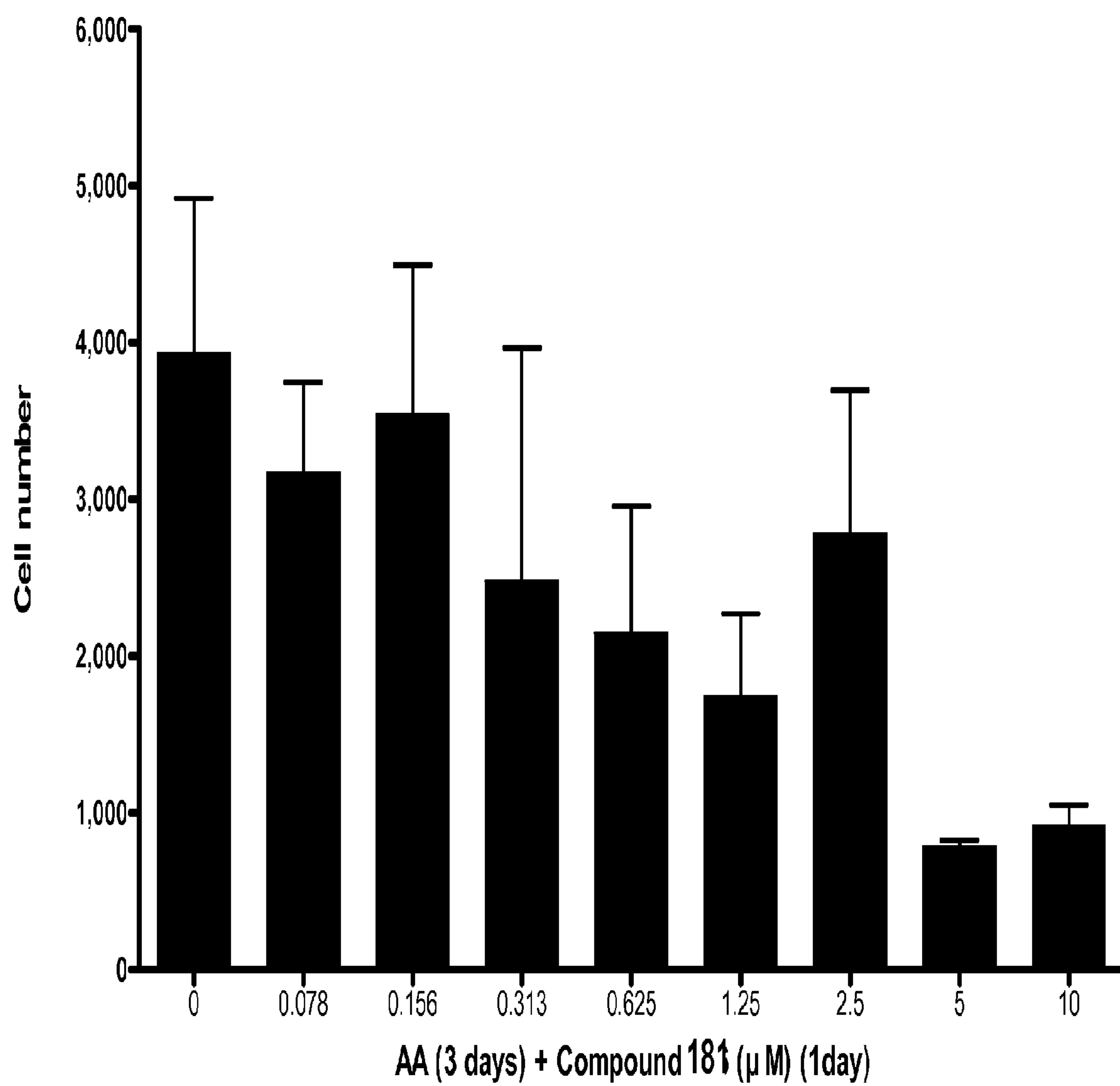


Figure 16B

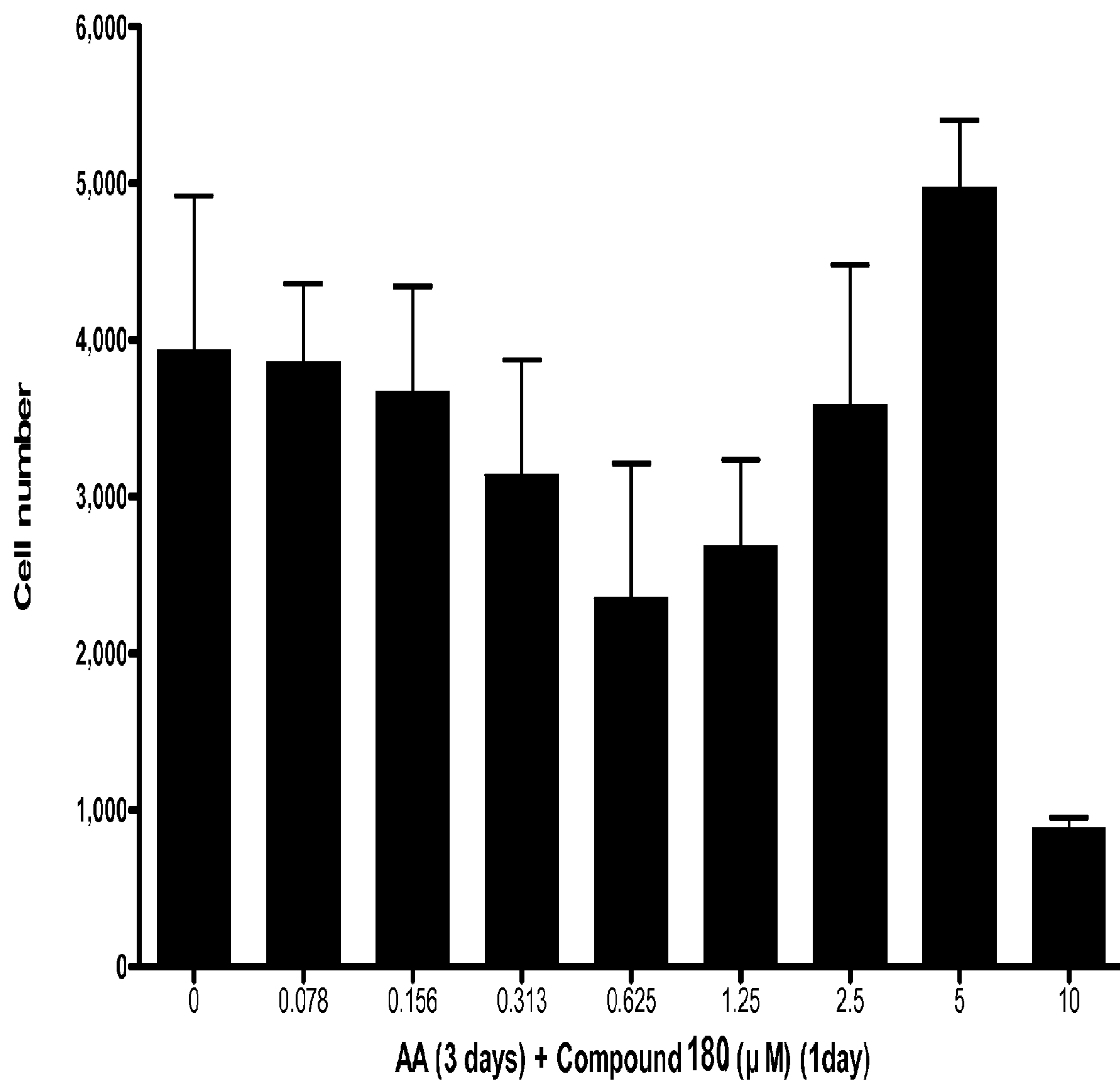


Figure 16C

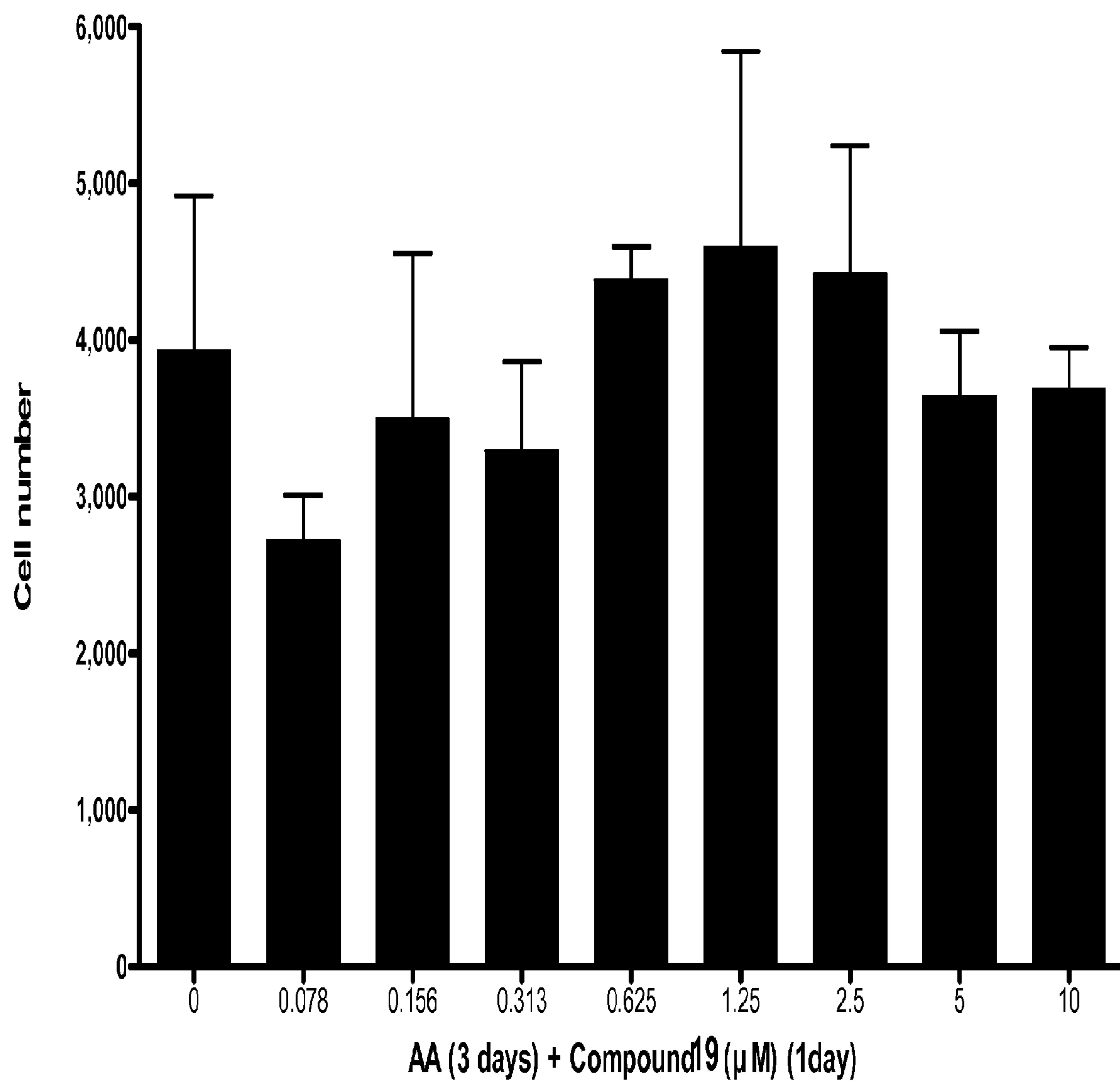


Figure 16D

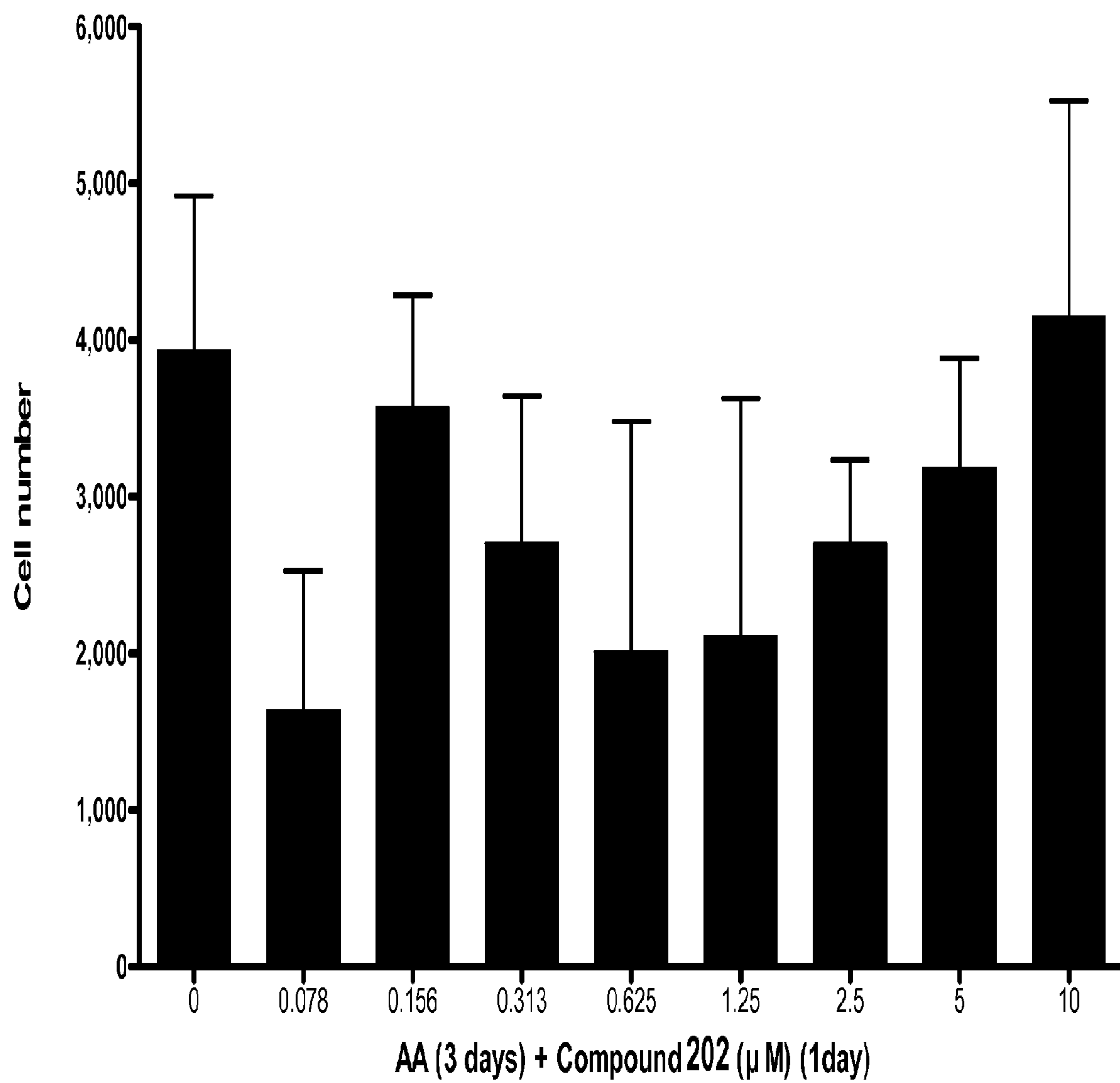


Figure 16E

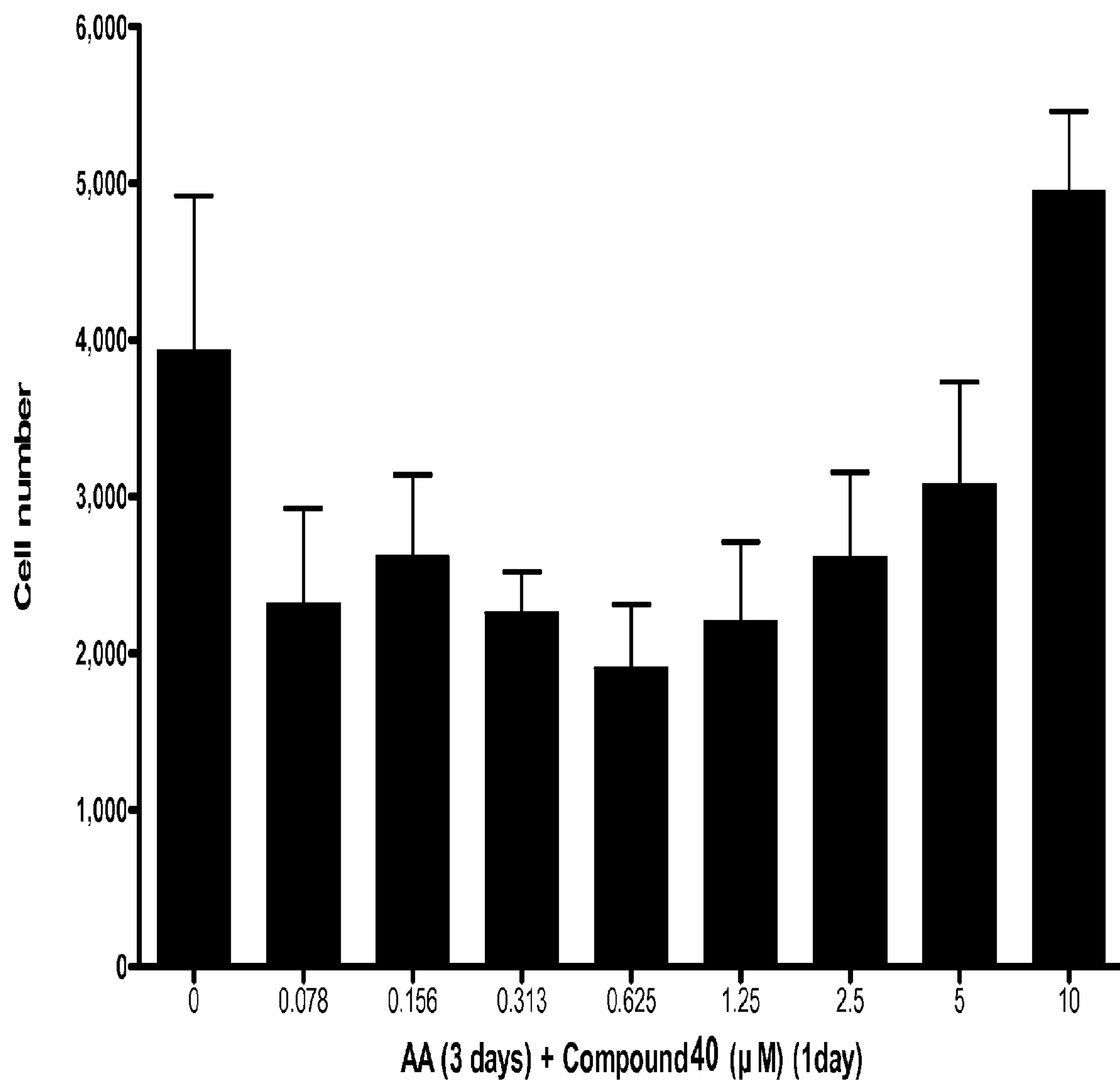


Figure 16F

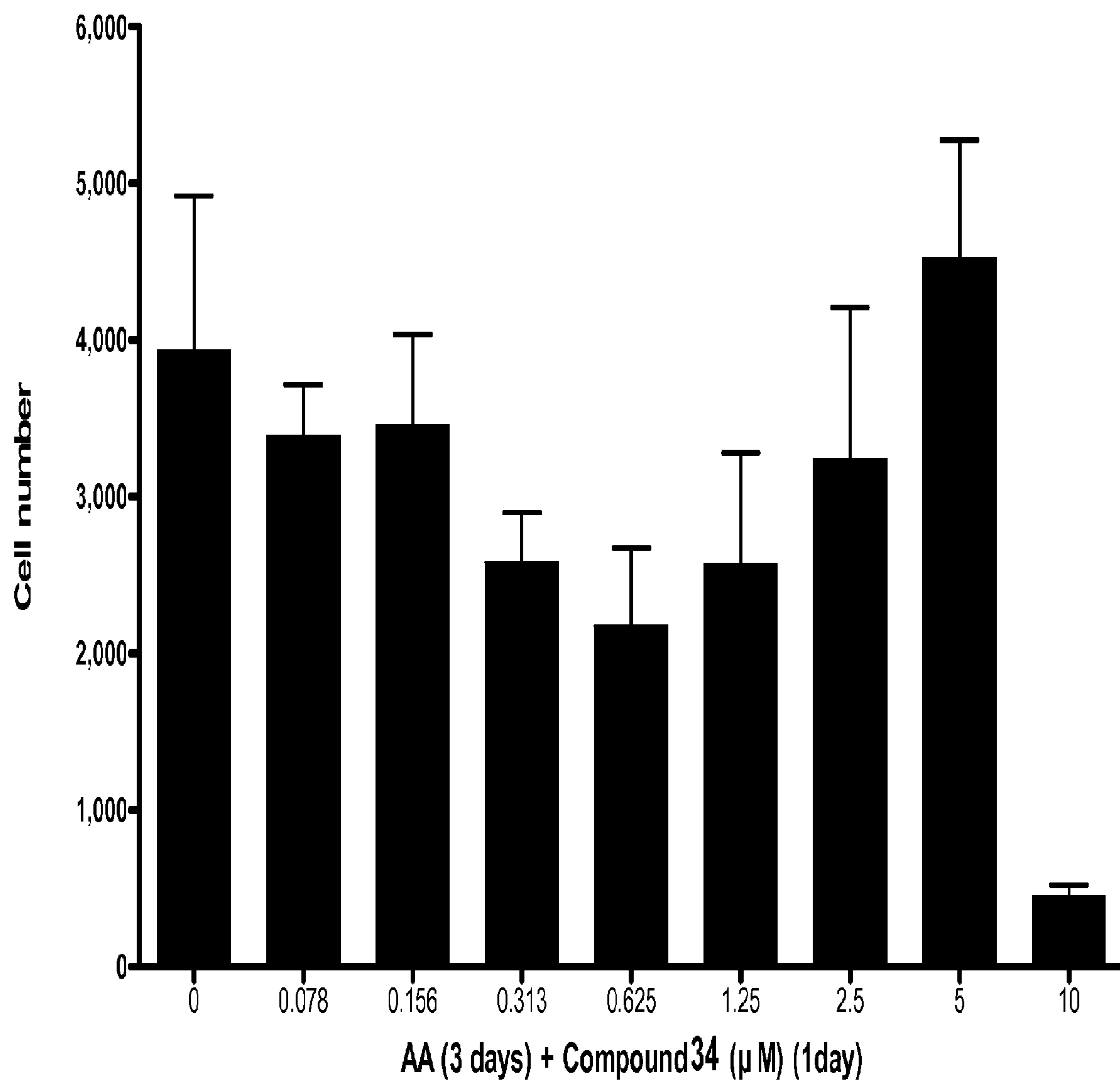


Figure 16G

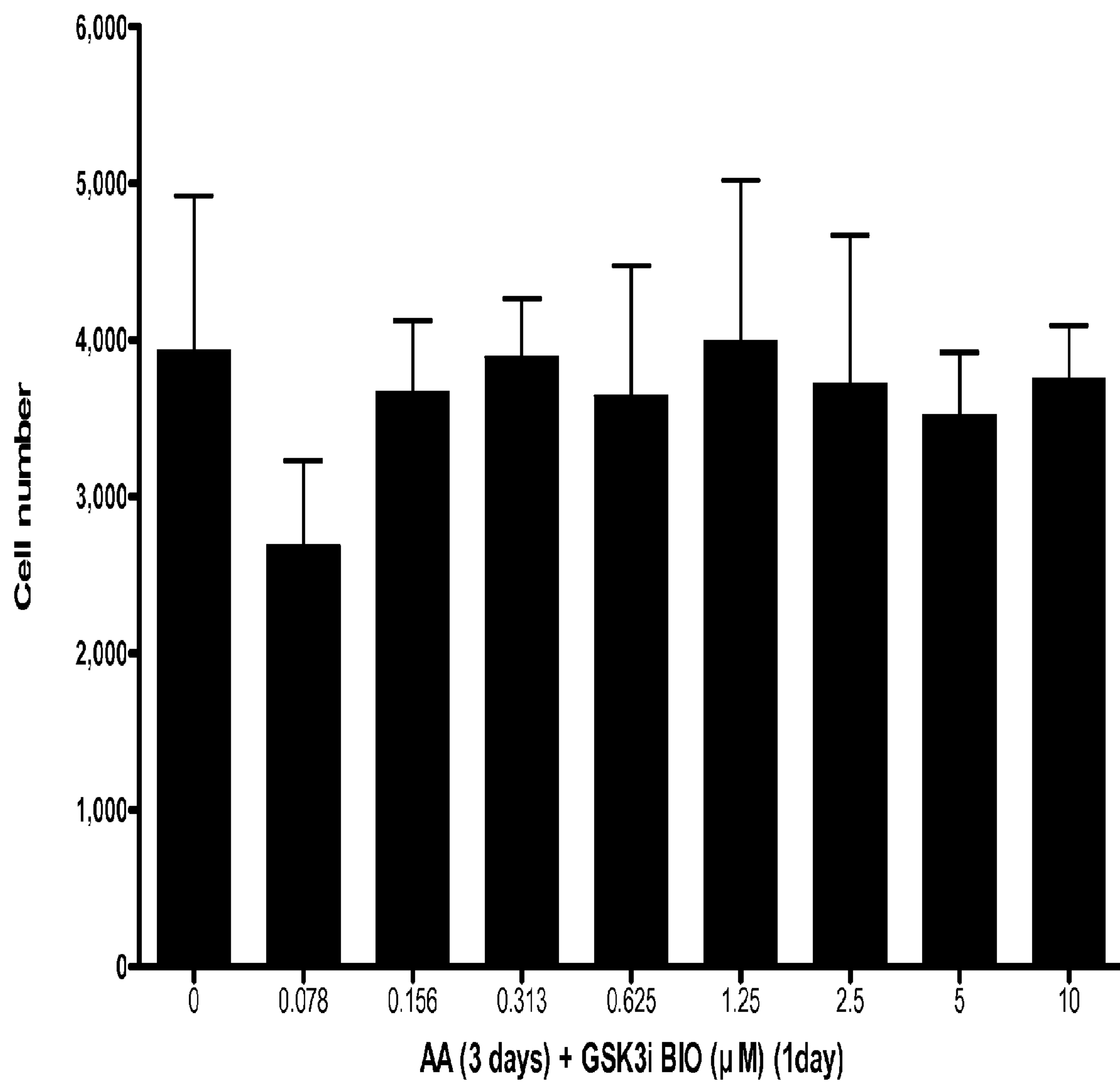


Figure 17A

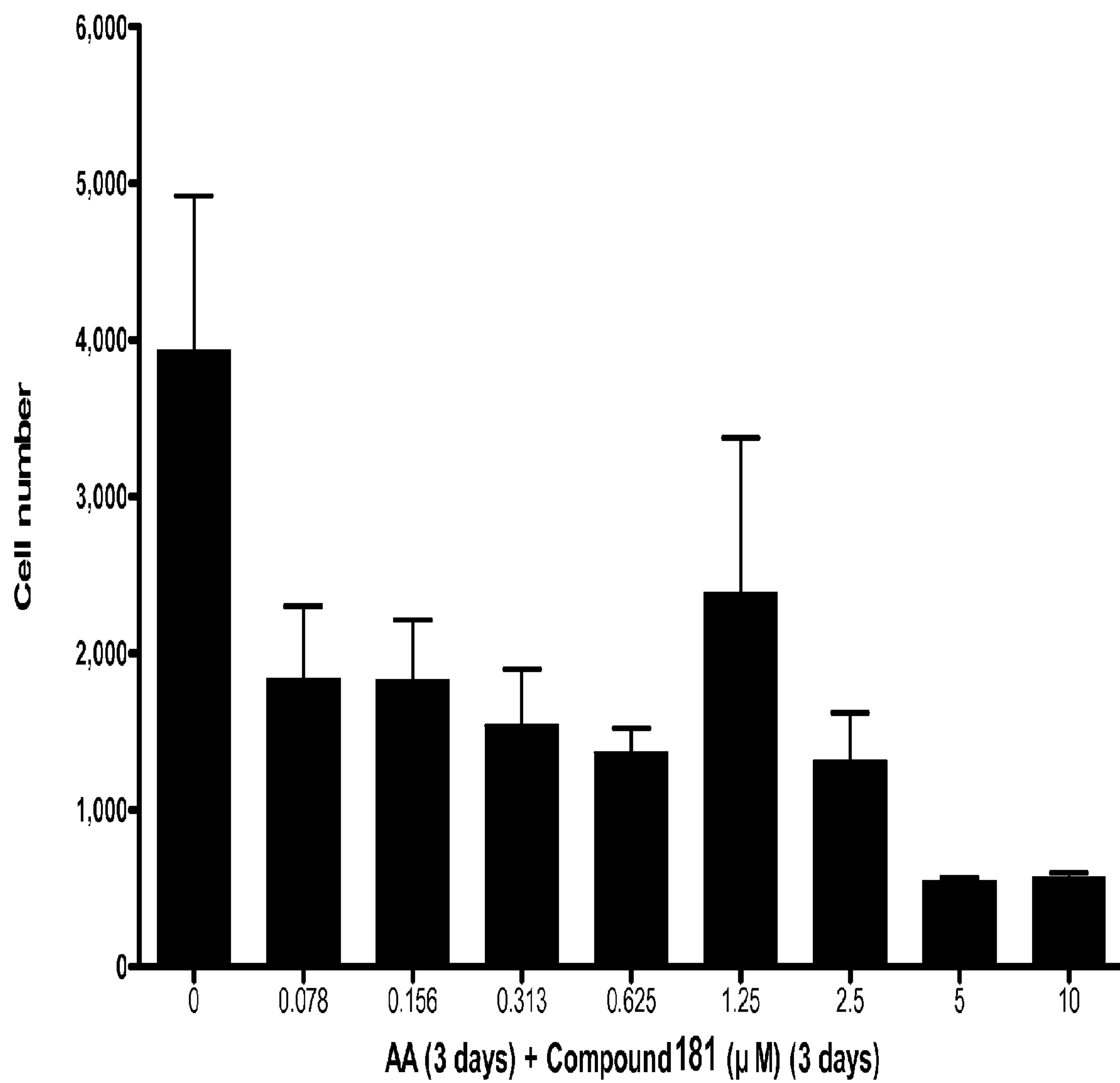


Figure 17B

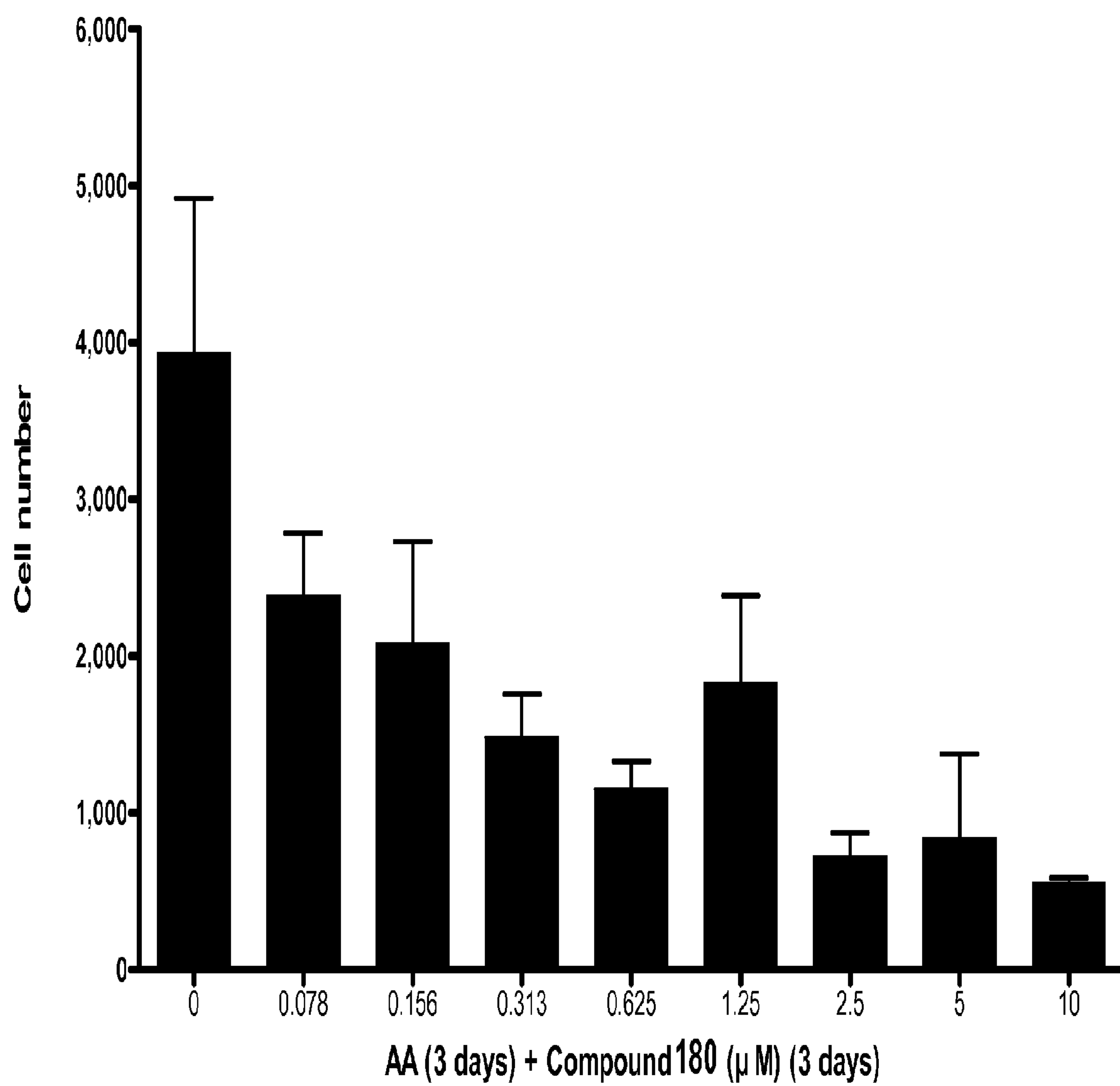


Figure 17C

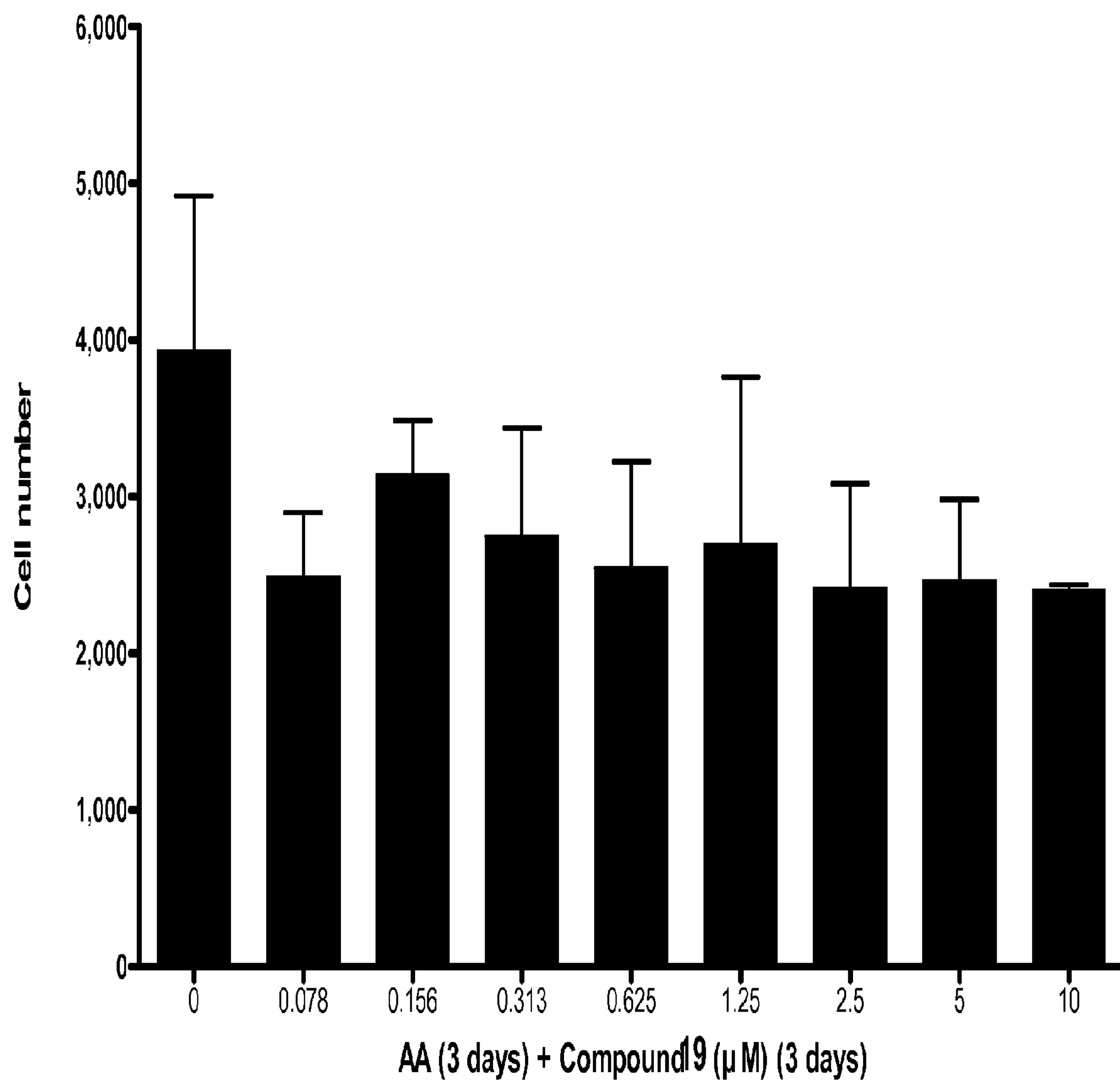


Figure 17D

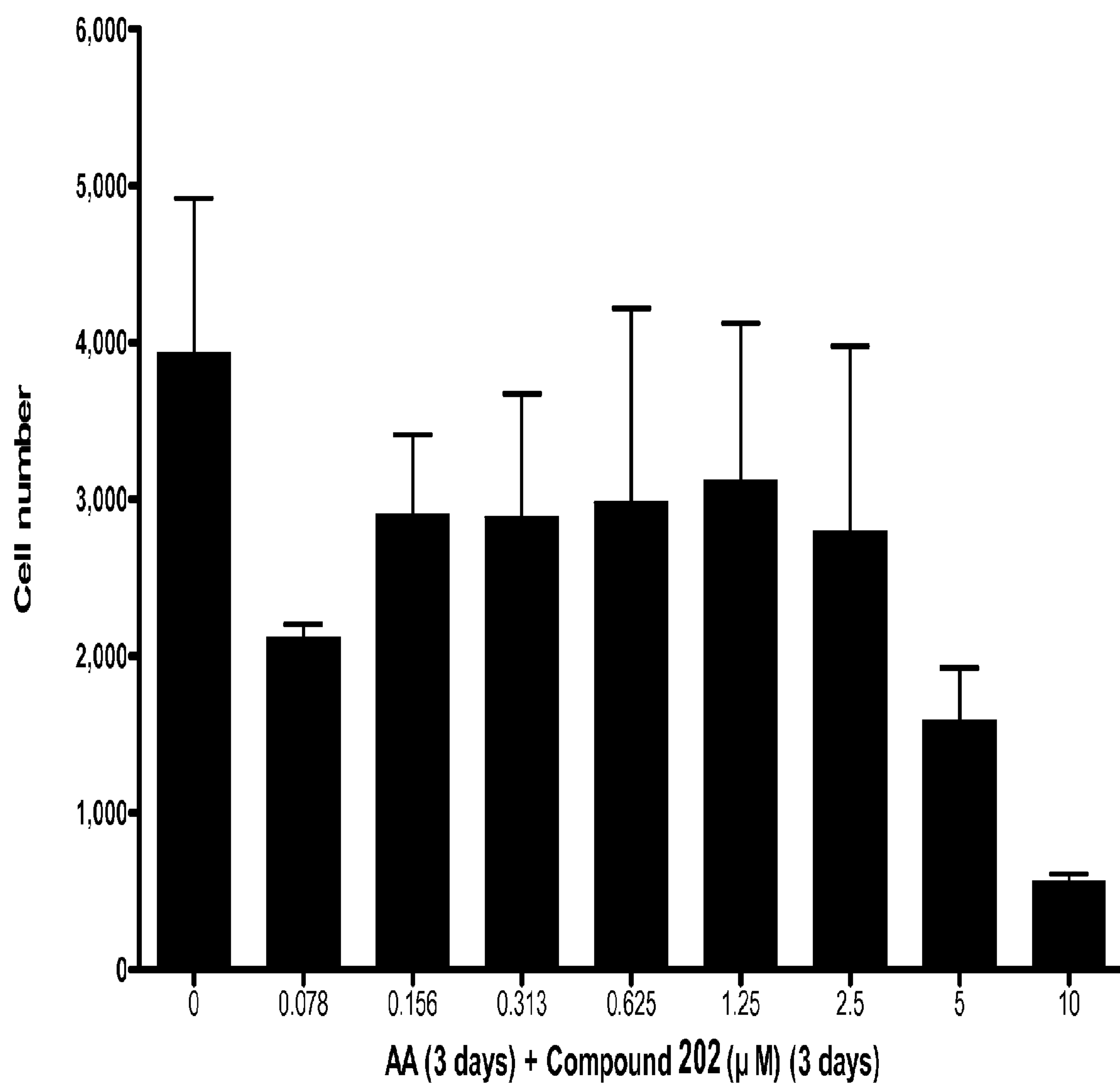


Figure 17E

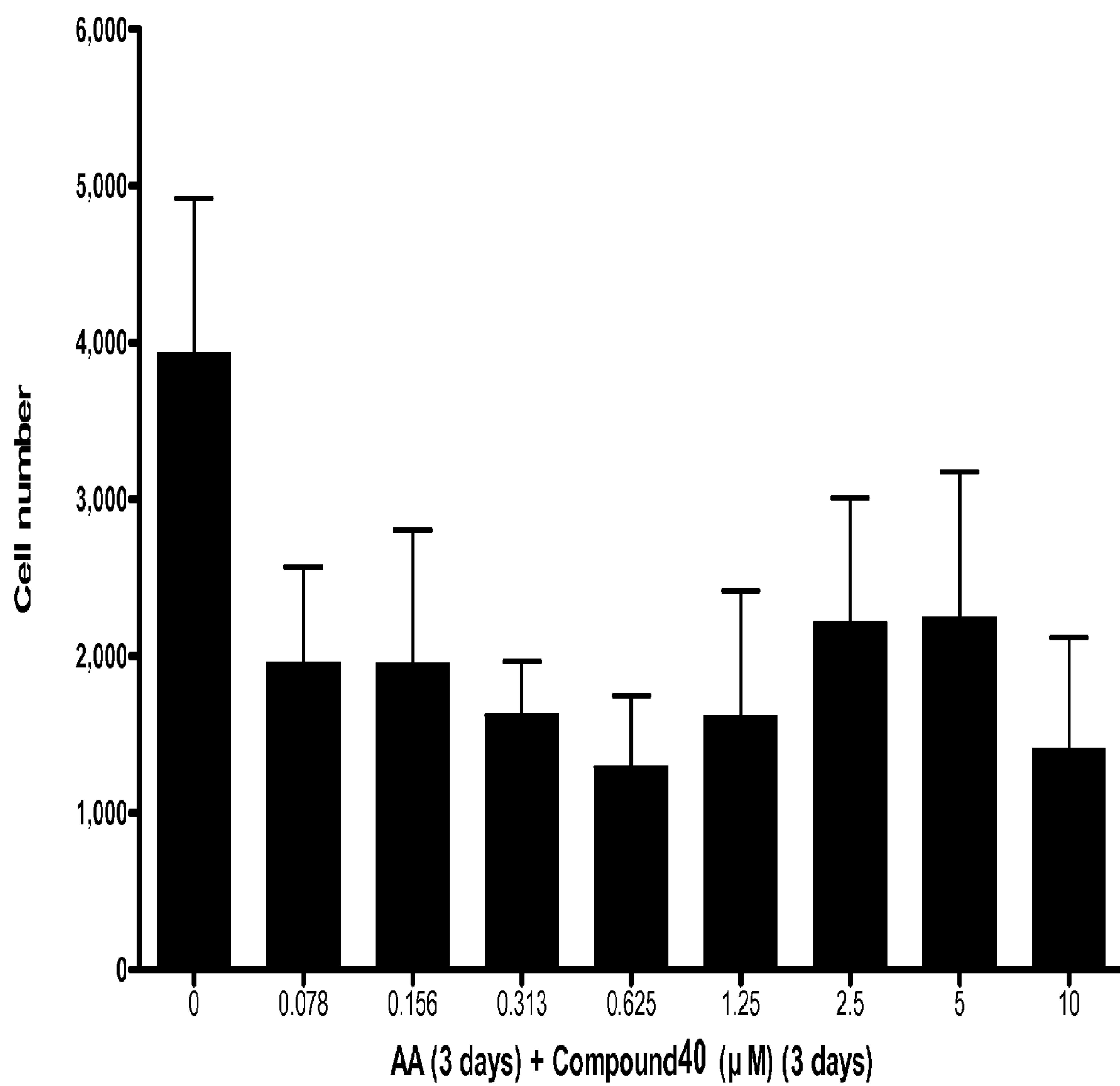


Figure 17F

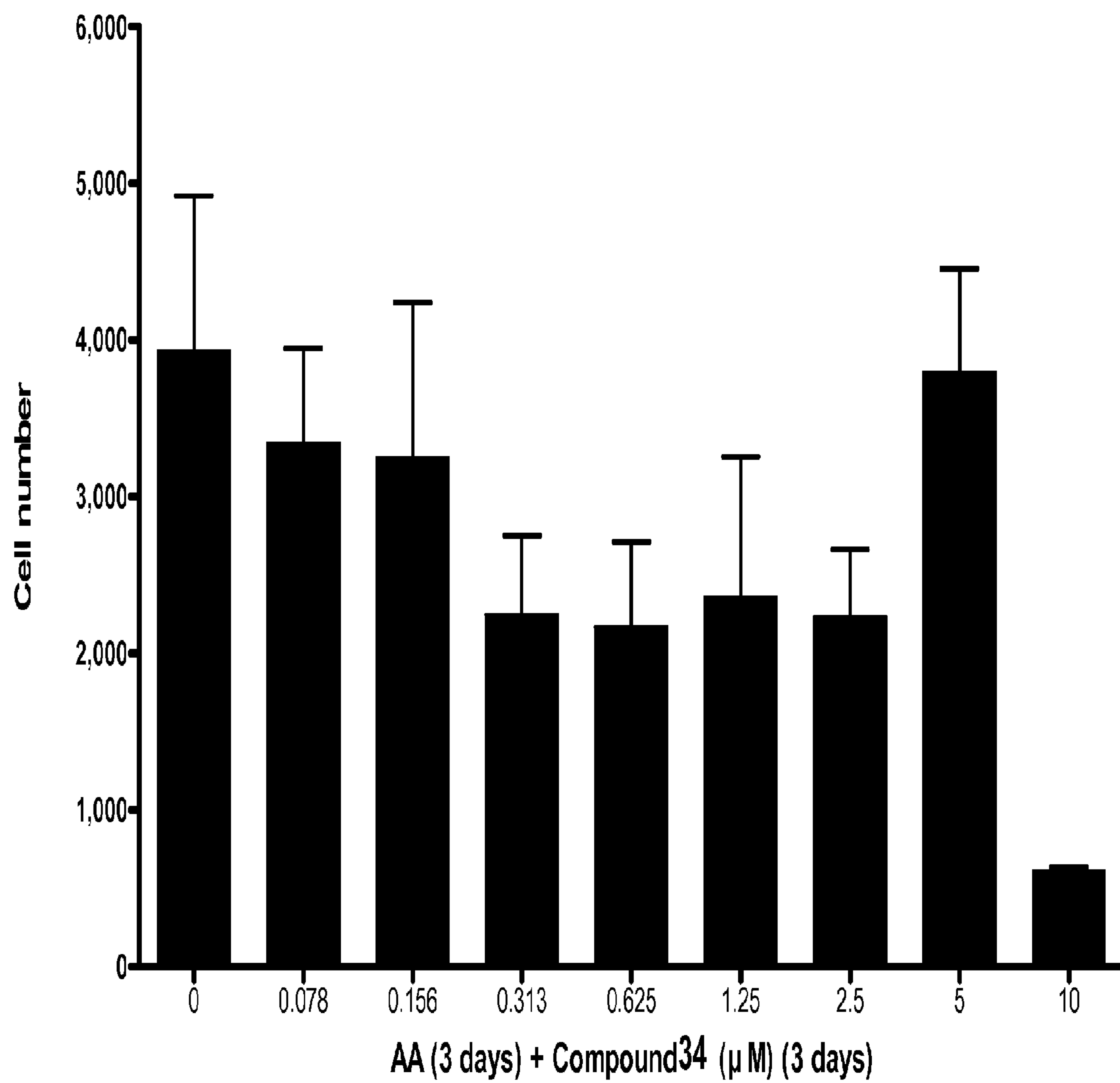


Figure 17G

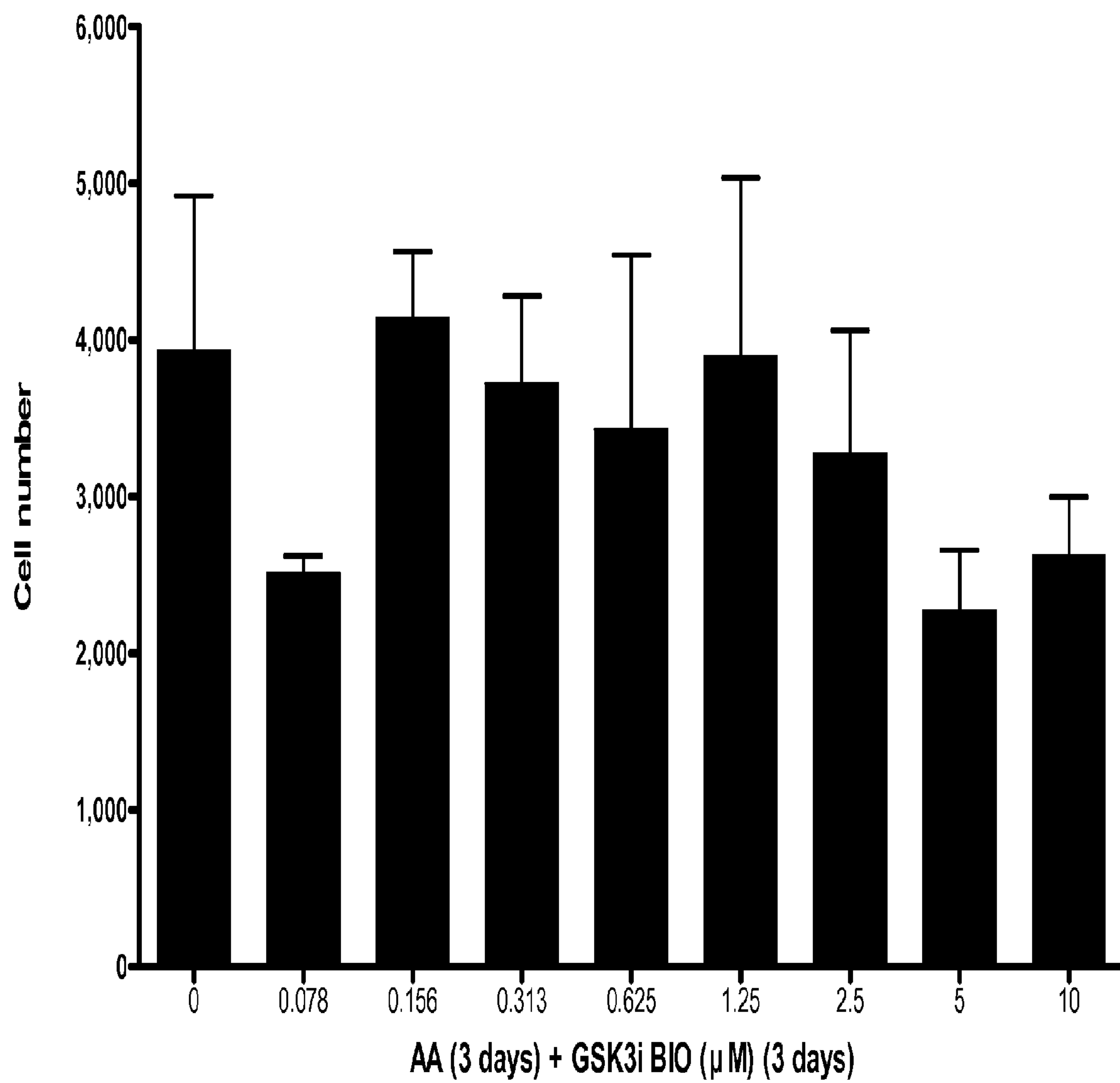


Figure 18A

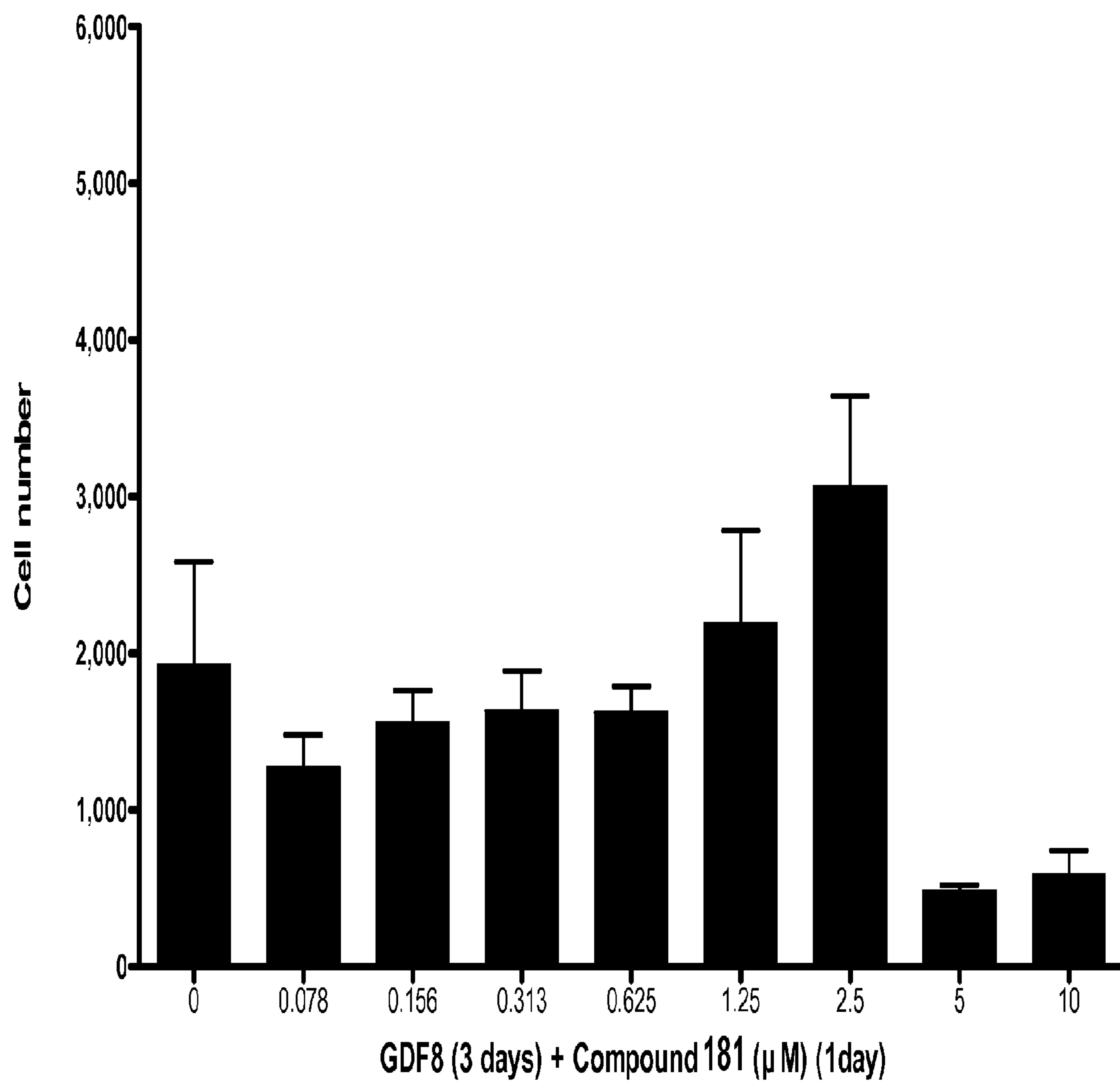


Figure 18B

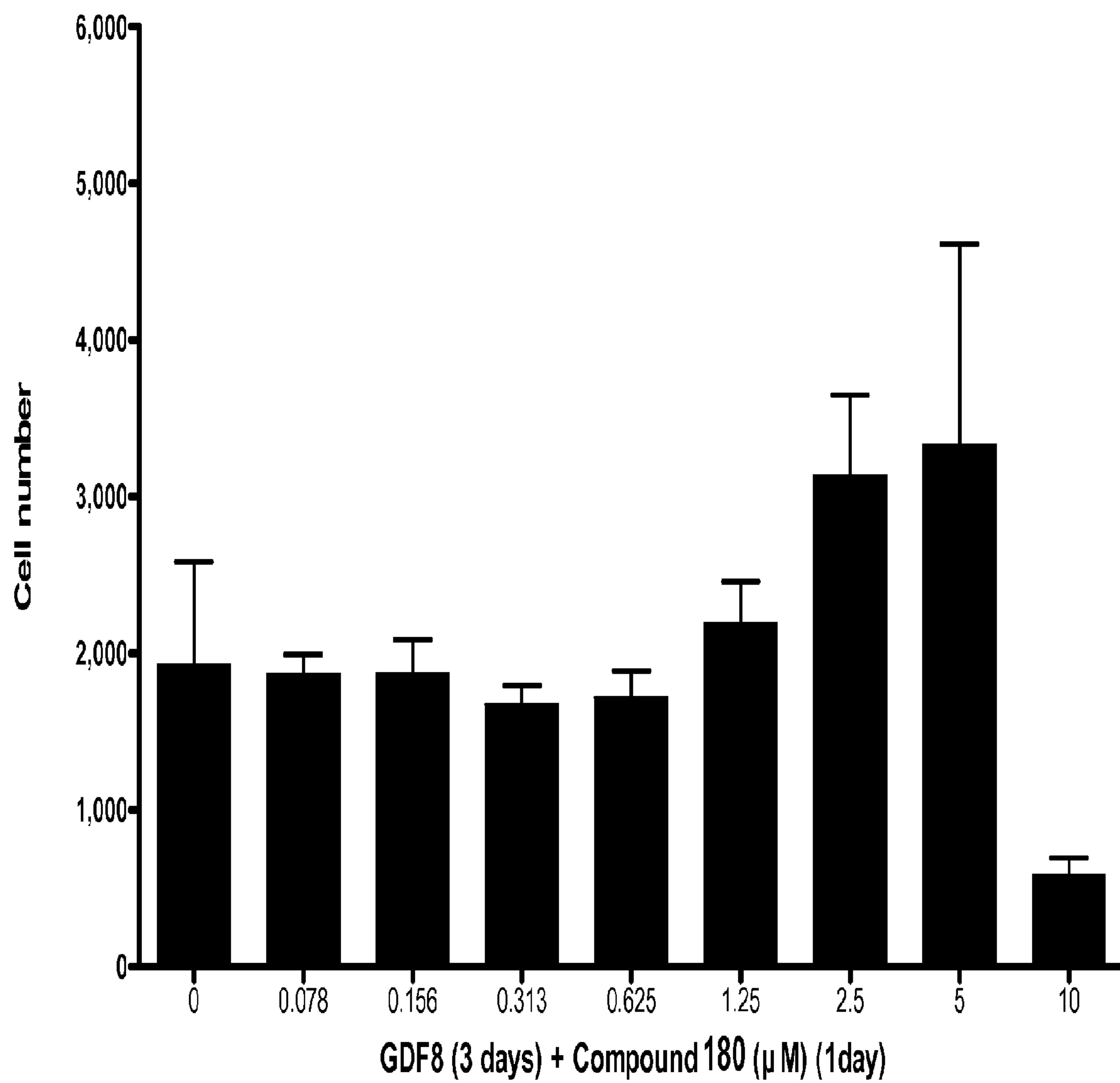


Figure 18C

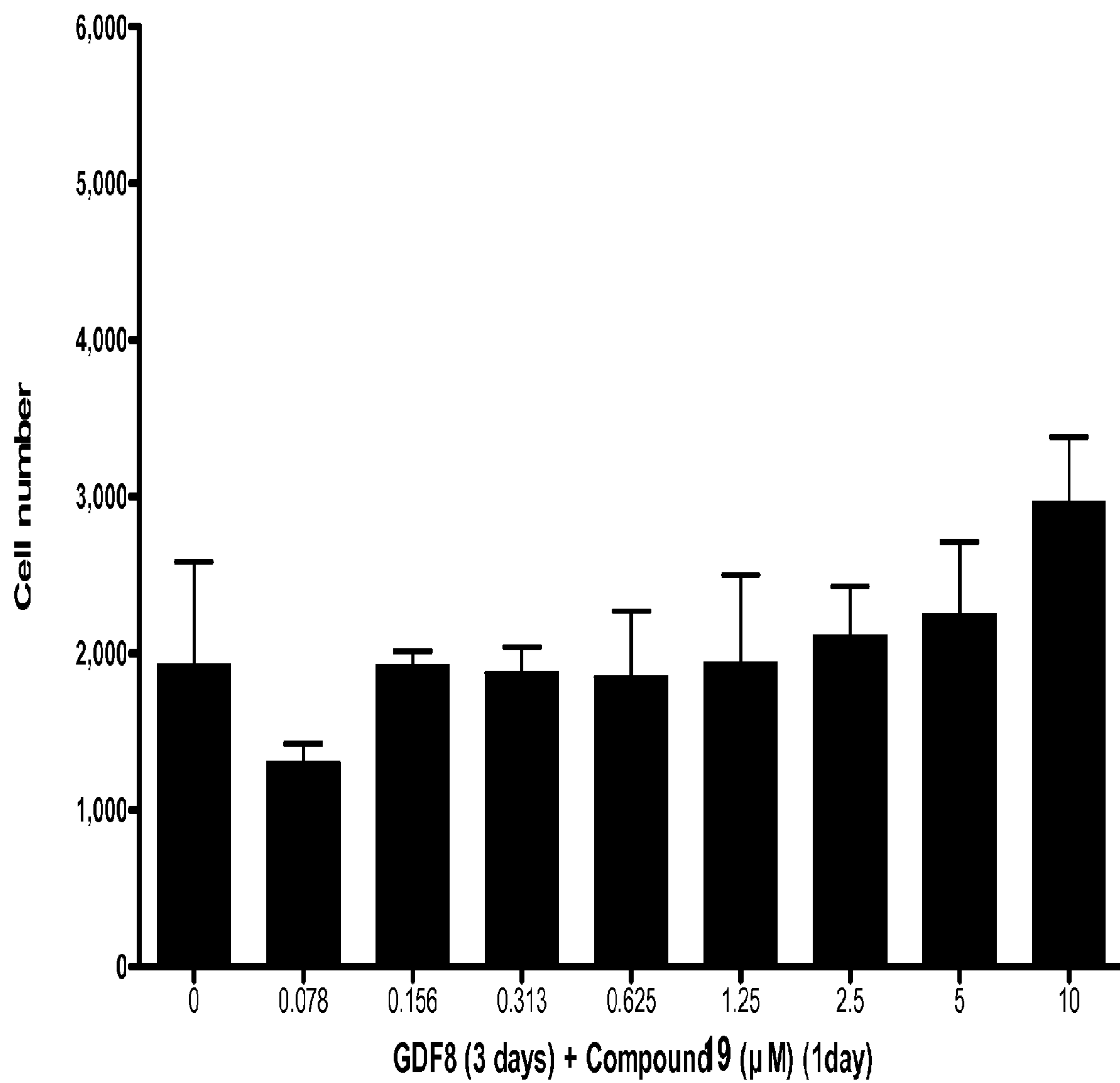


Figure 18D

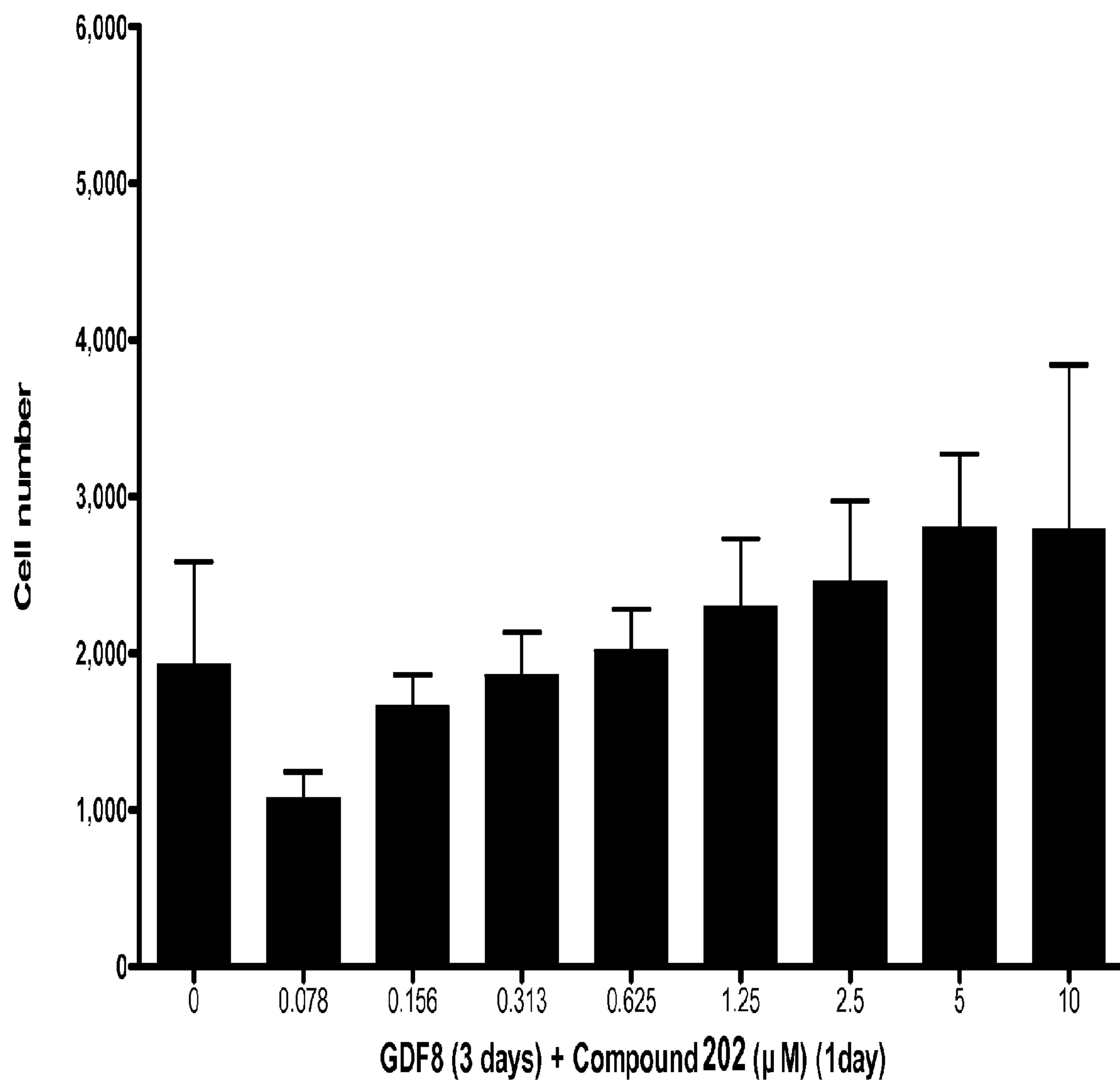


Figure 18E

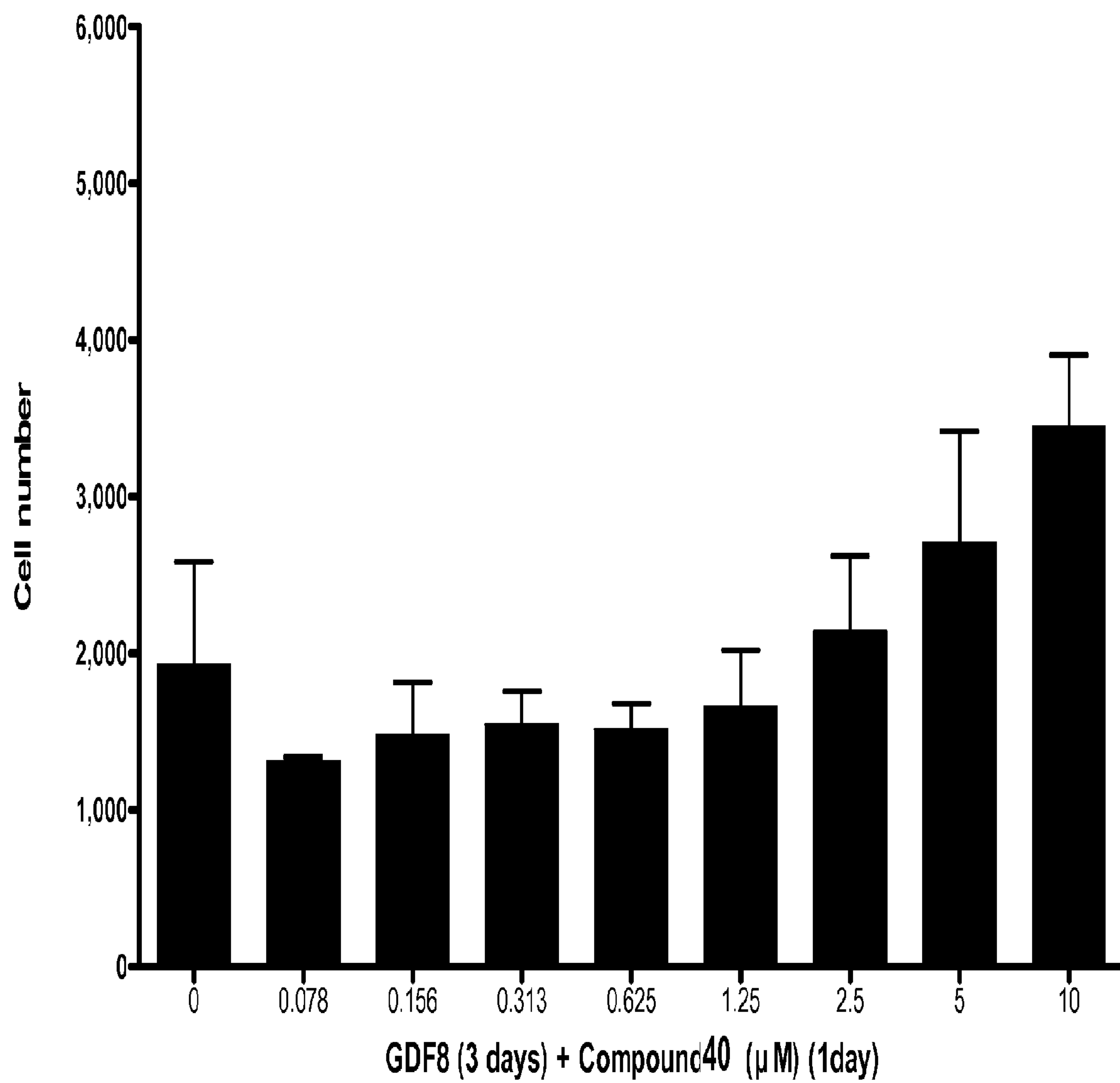


Figure 18F

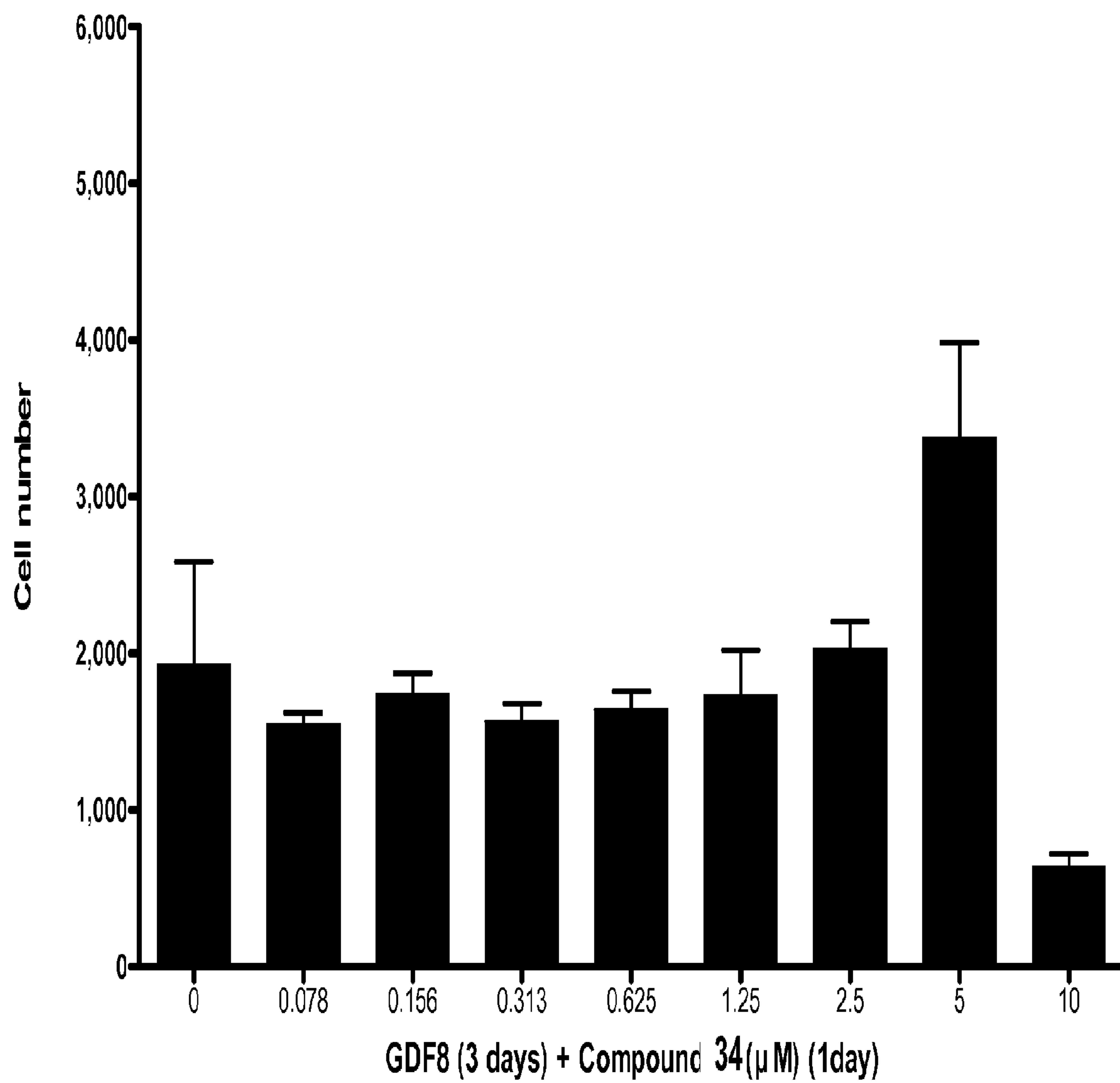


Figure 18G

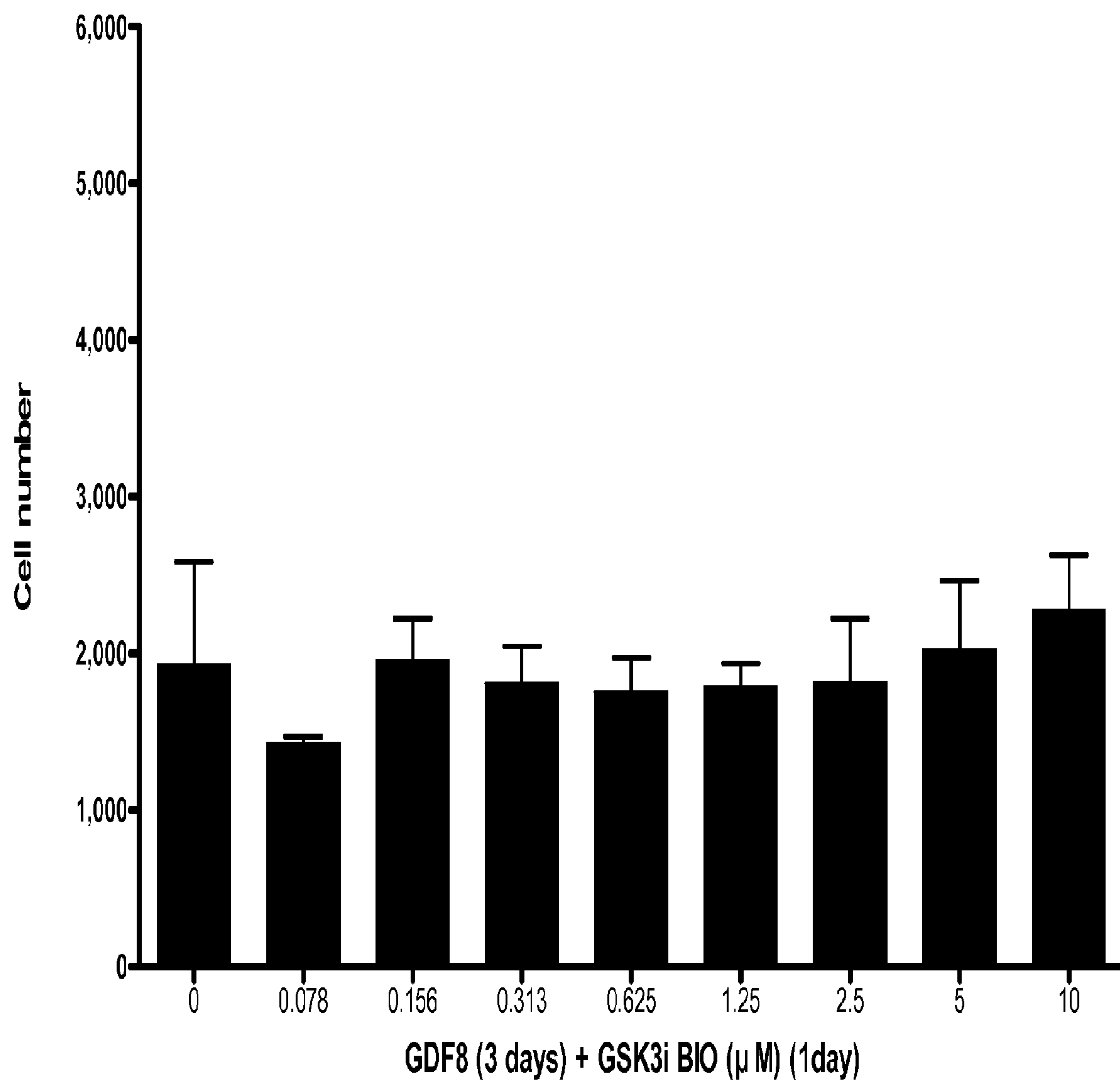


Figure 19A

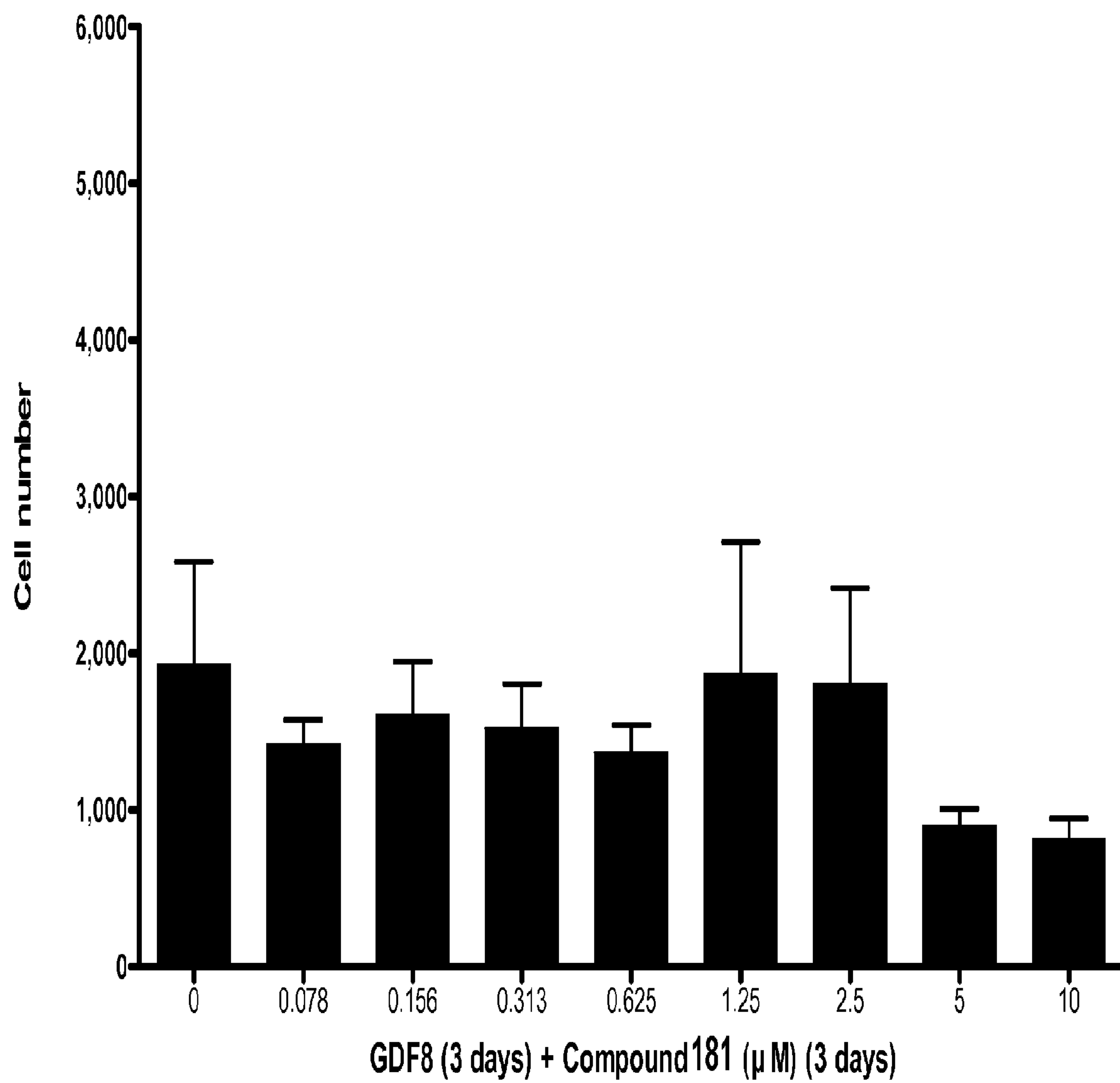


Figure 19B

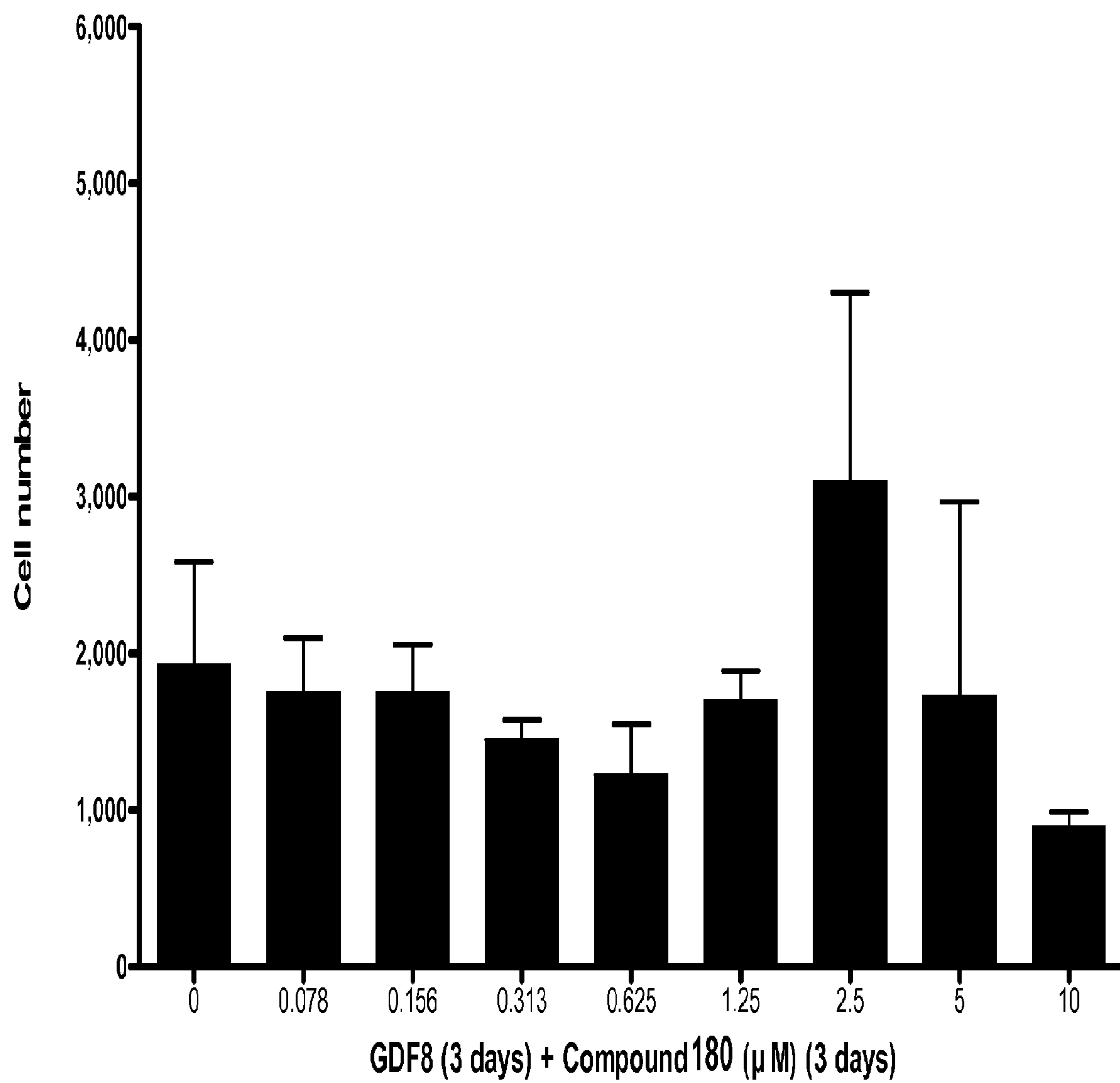


Figure 19C

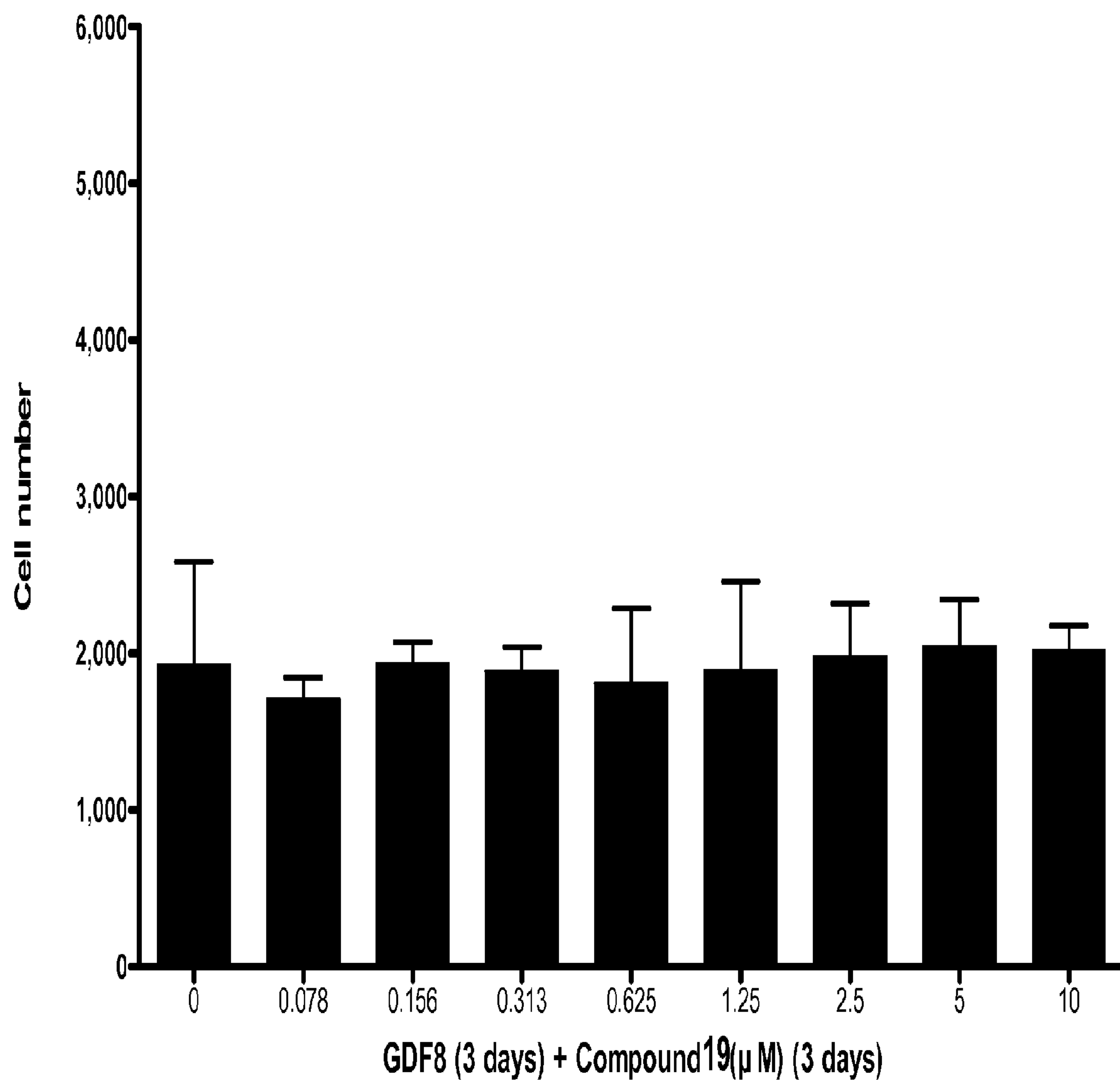


Figure 19D

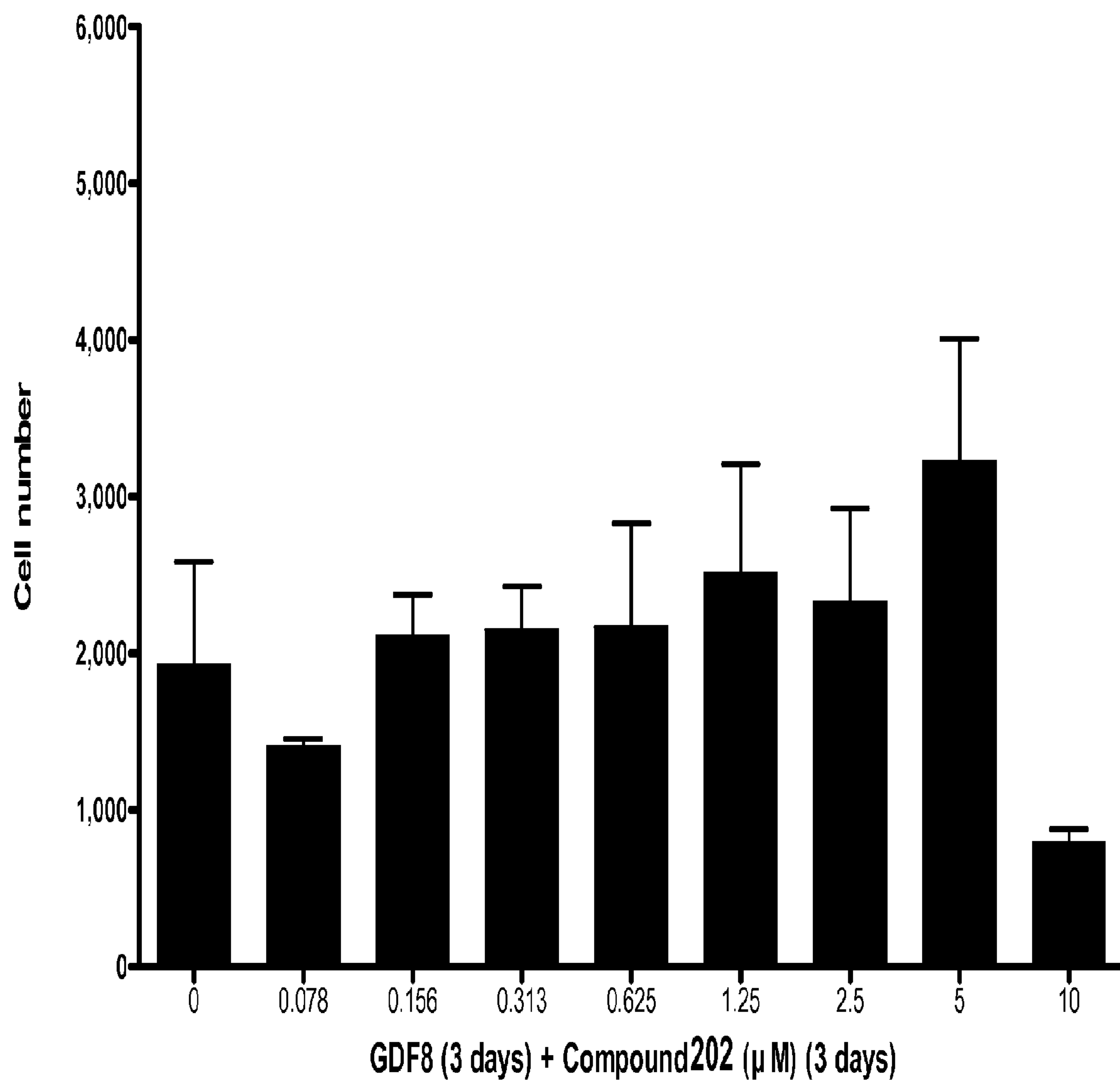


Figure 19E

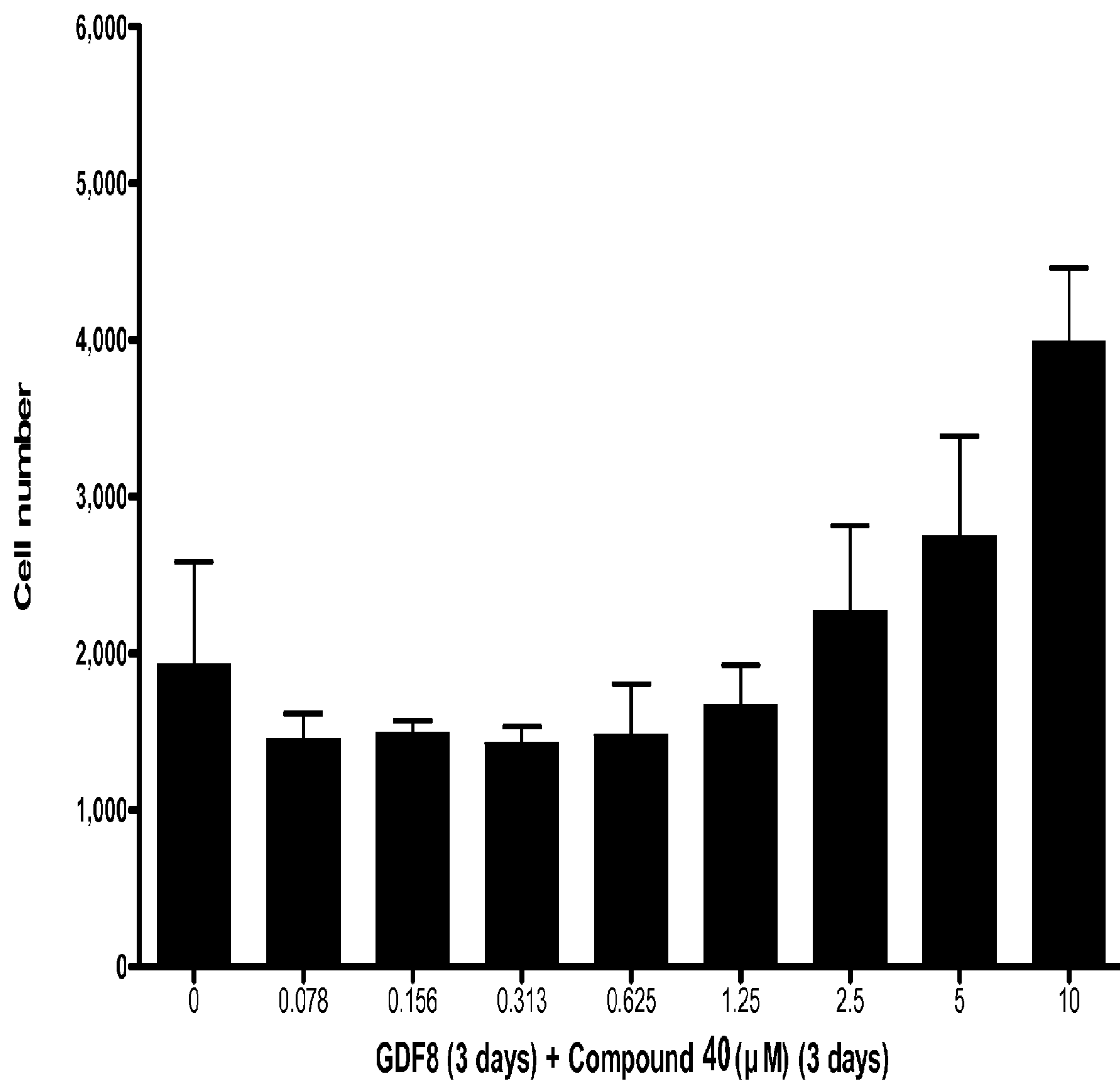


Figure 19F

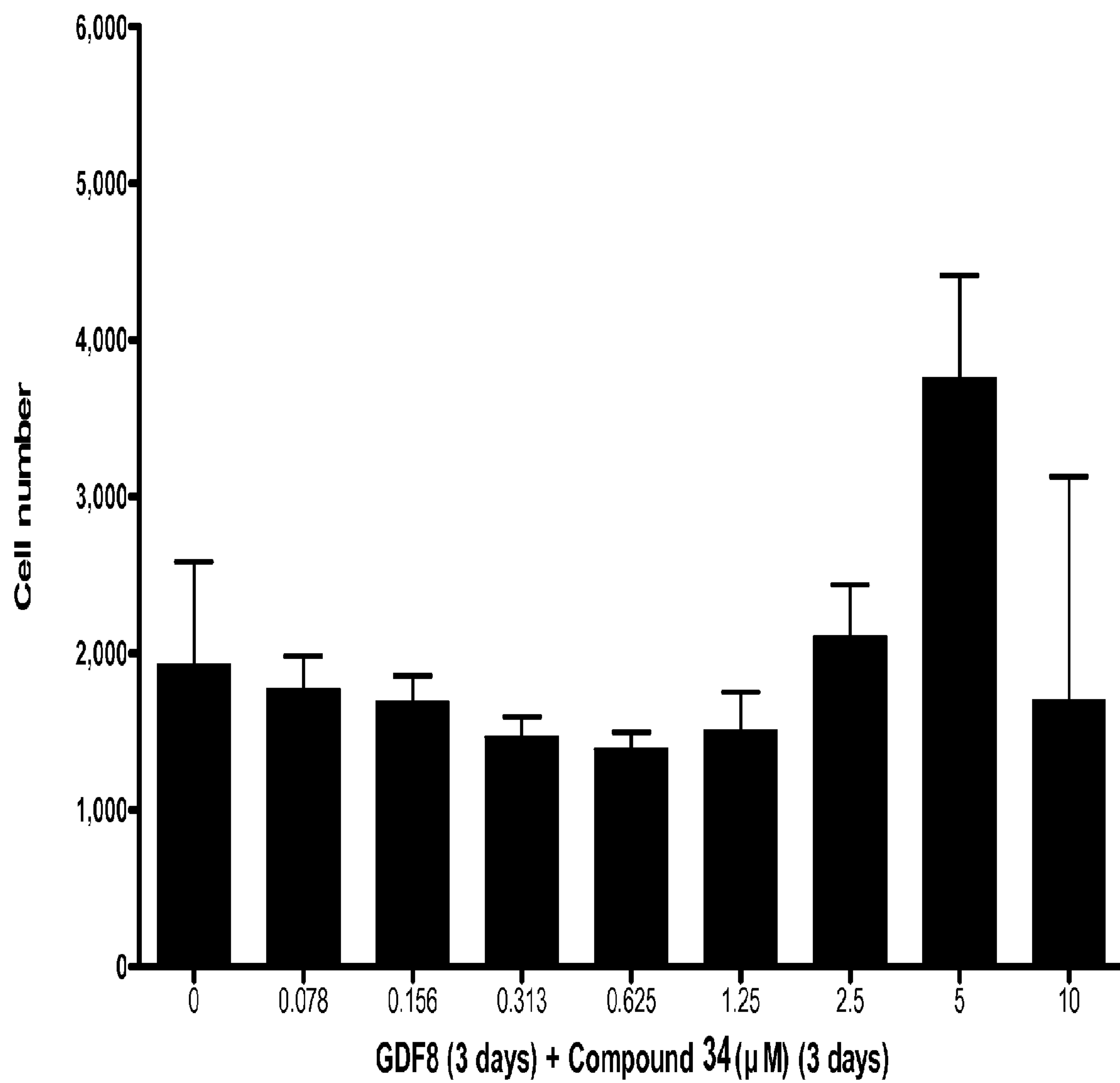


Figure 19G

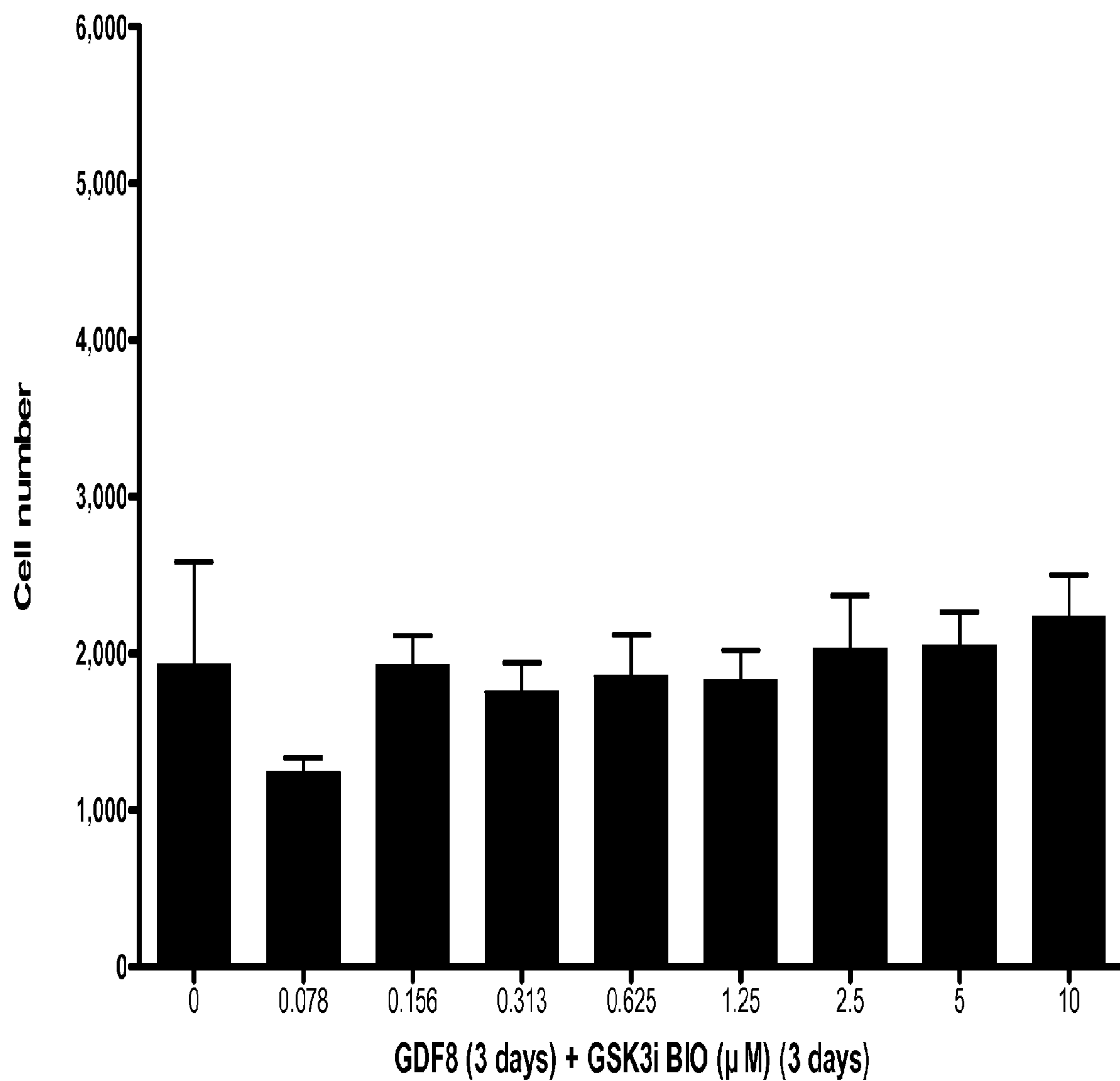


Figure 20A

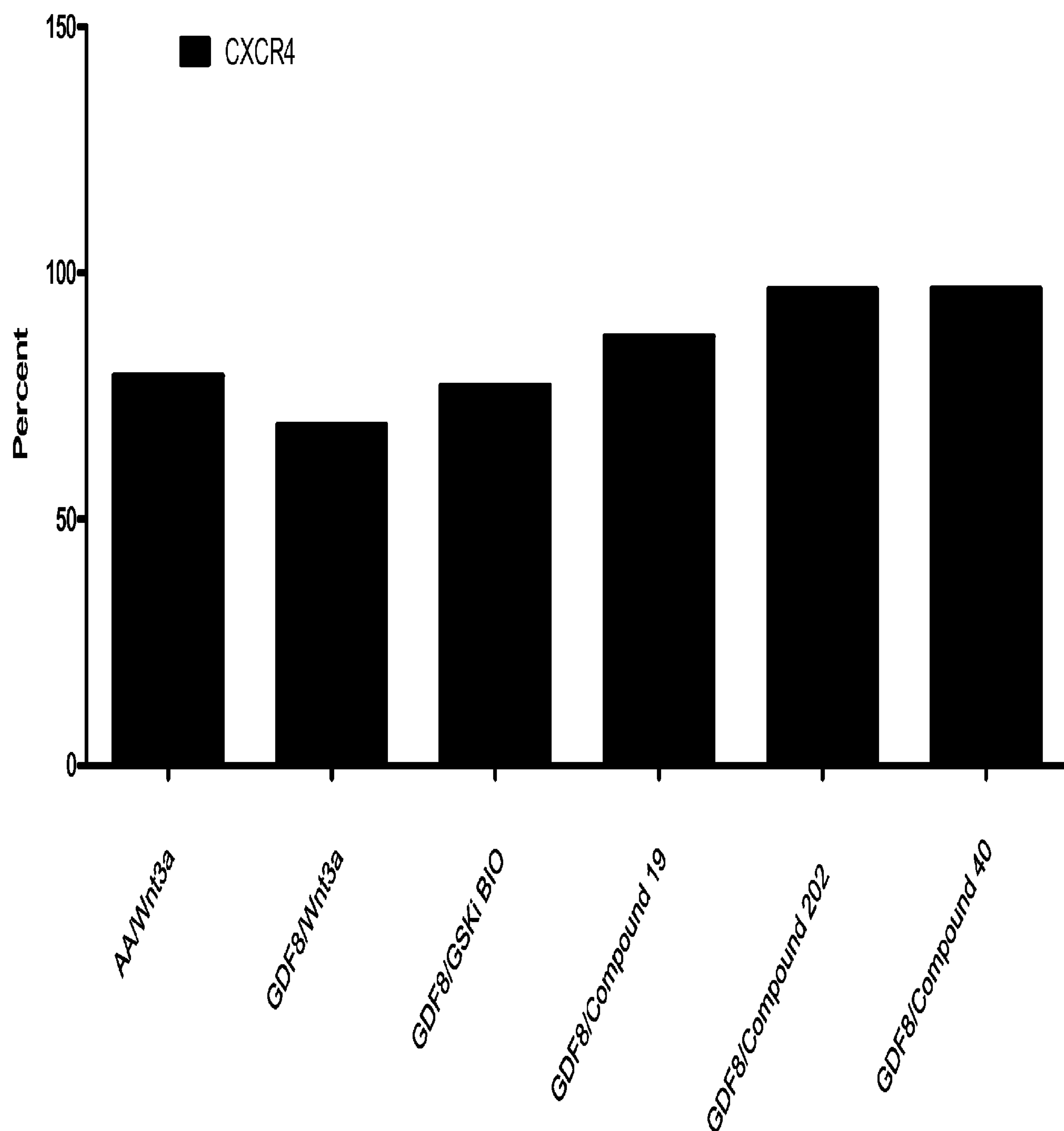


Figure 20B

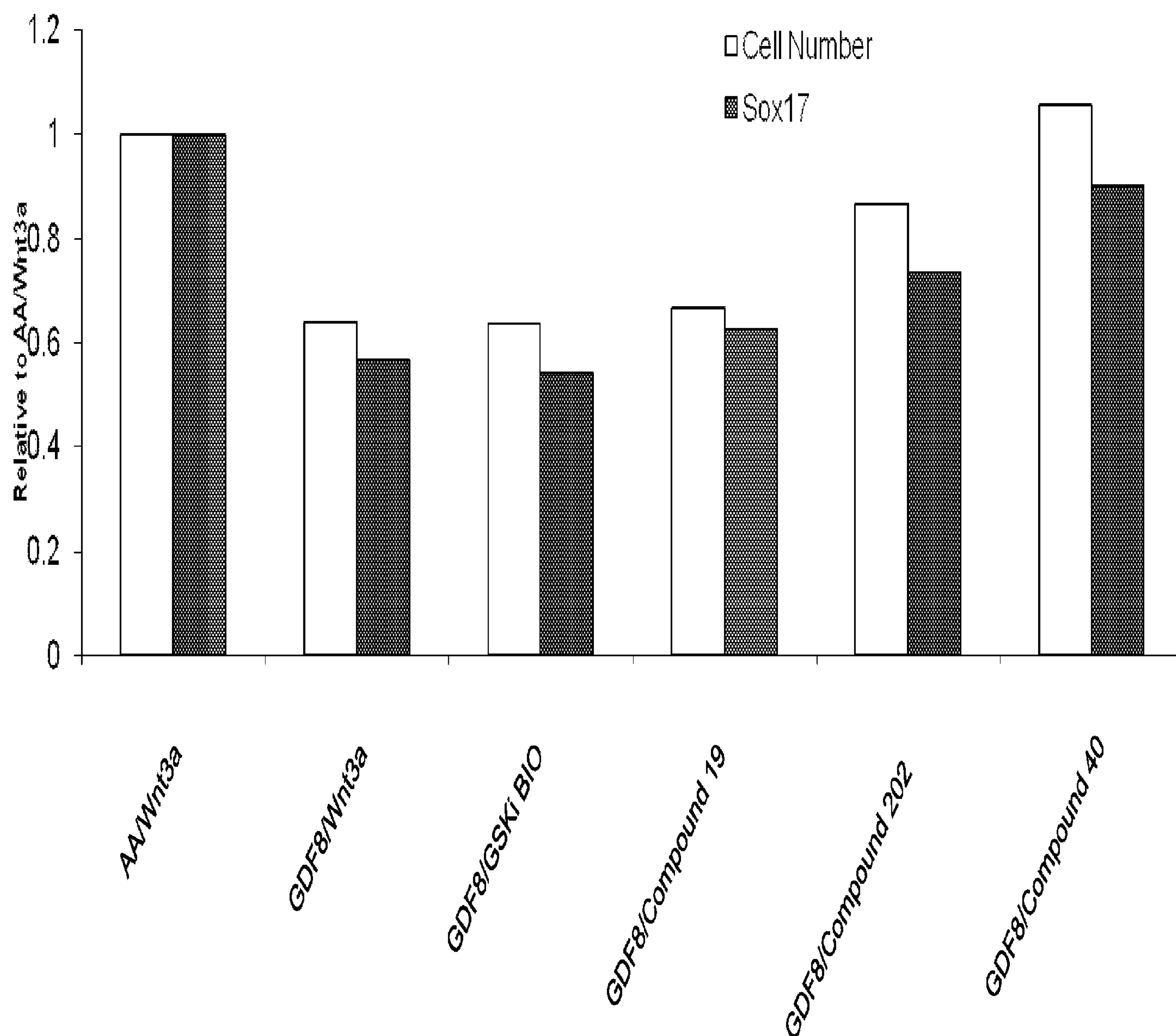


Figure 20C

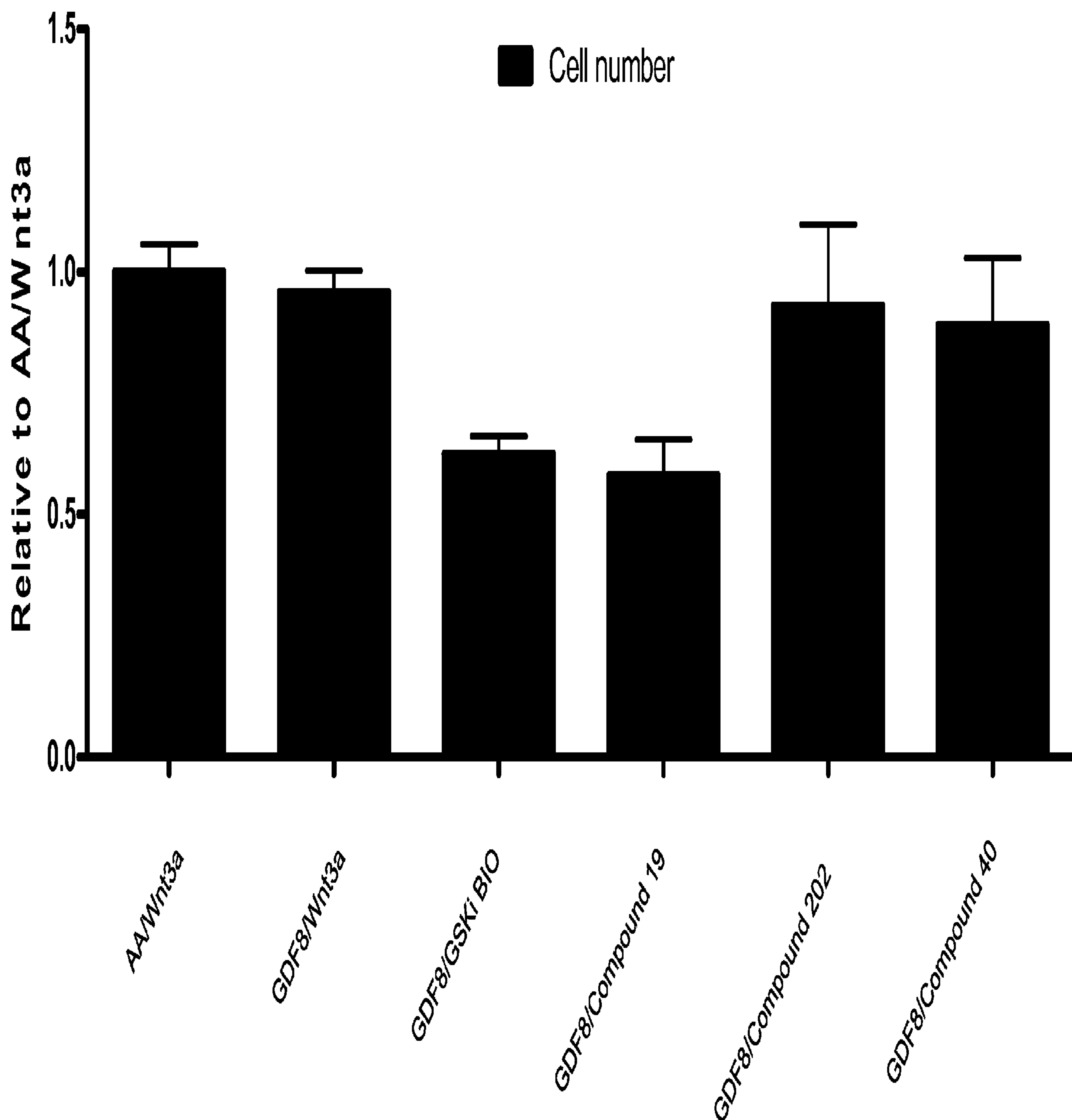


Figure 20D

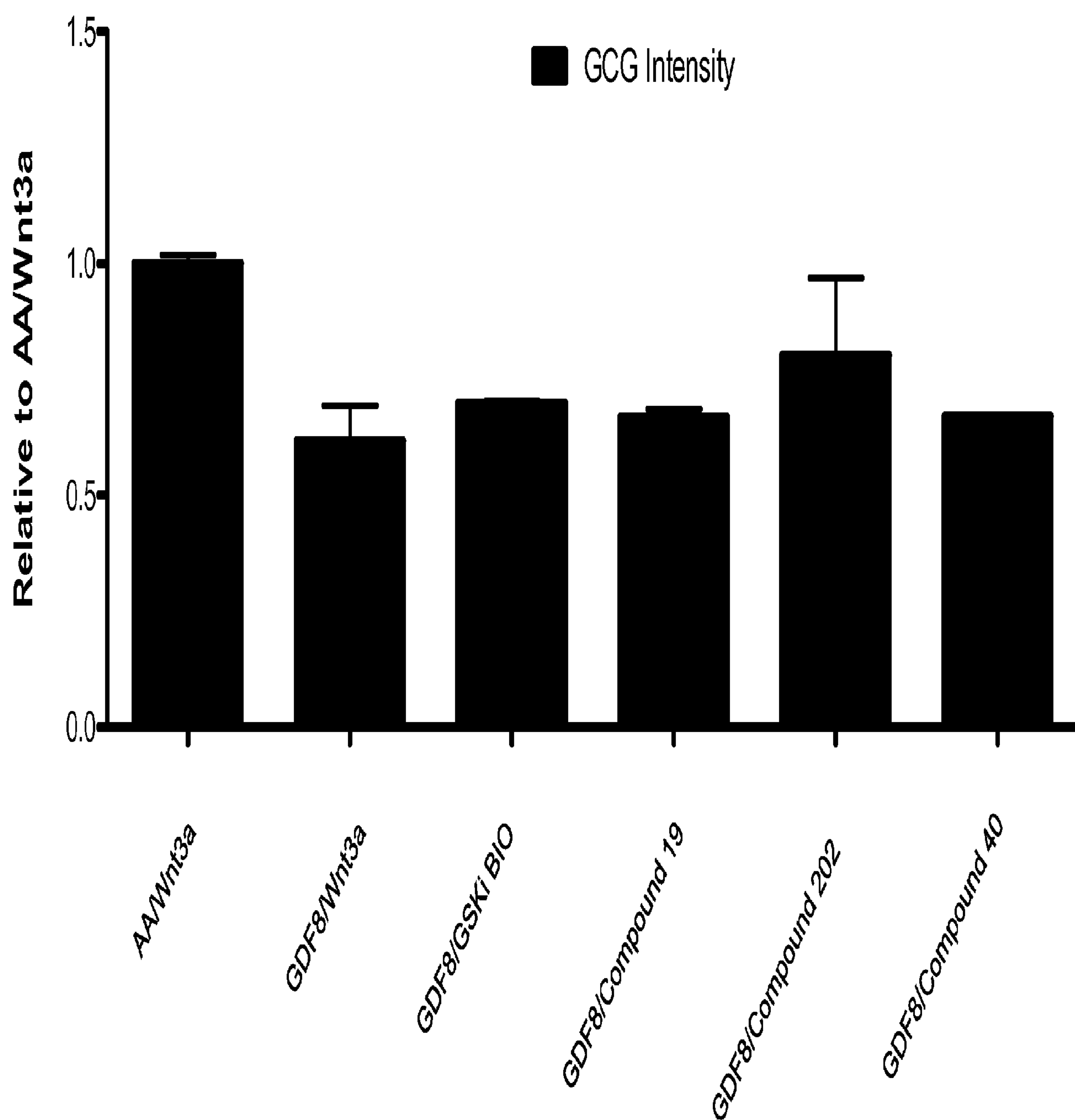


Figure 20E

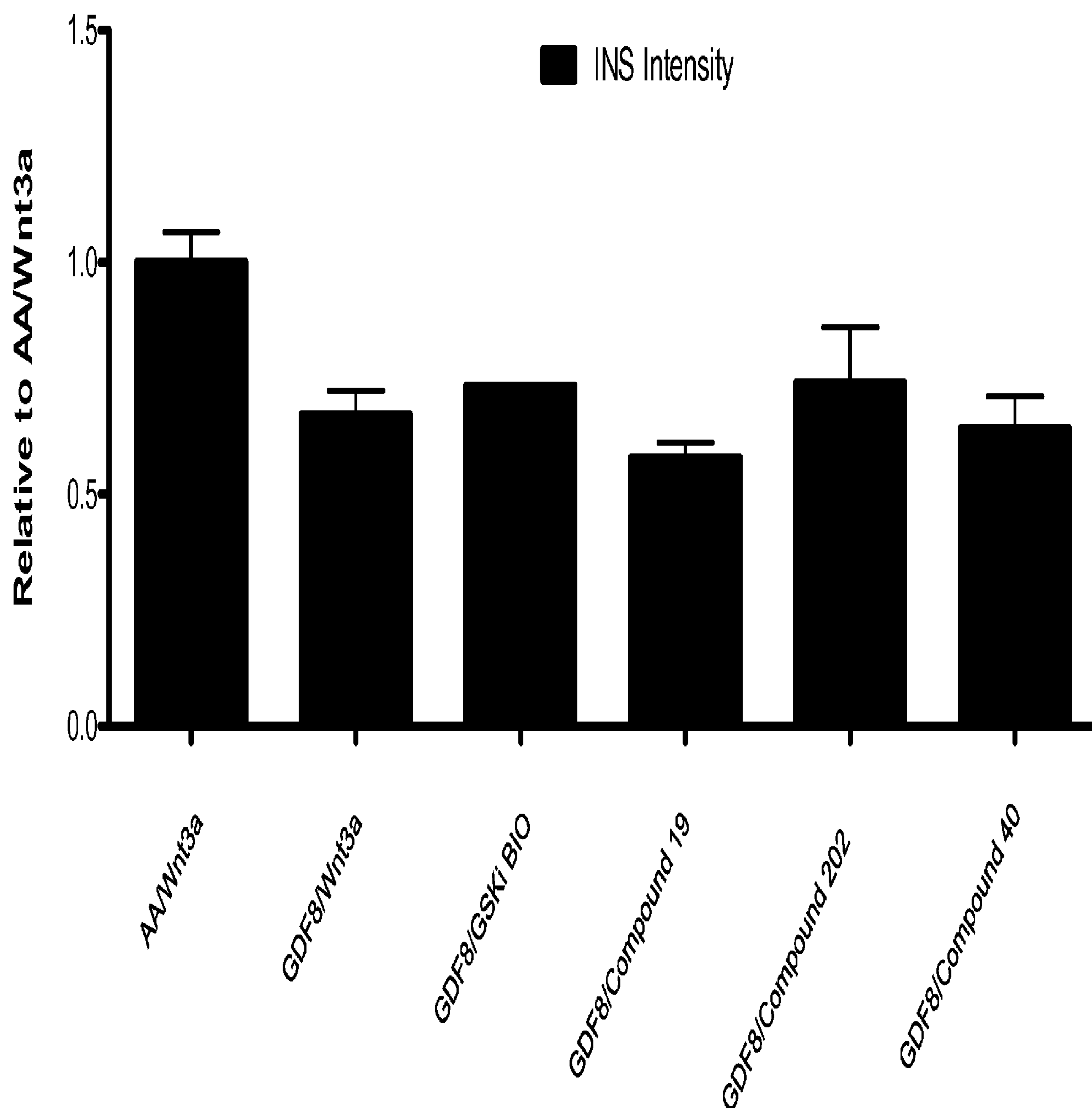


Figure 20F

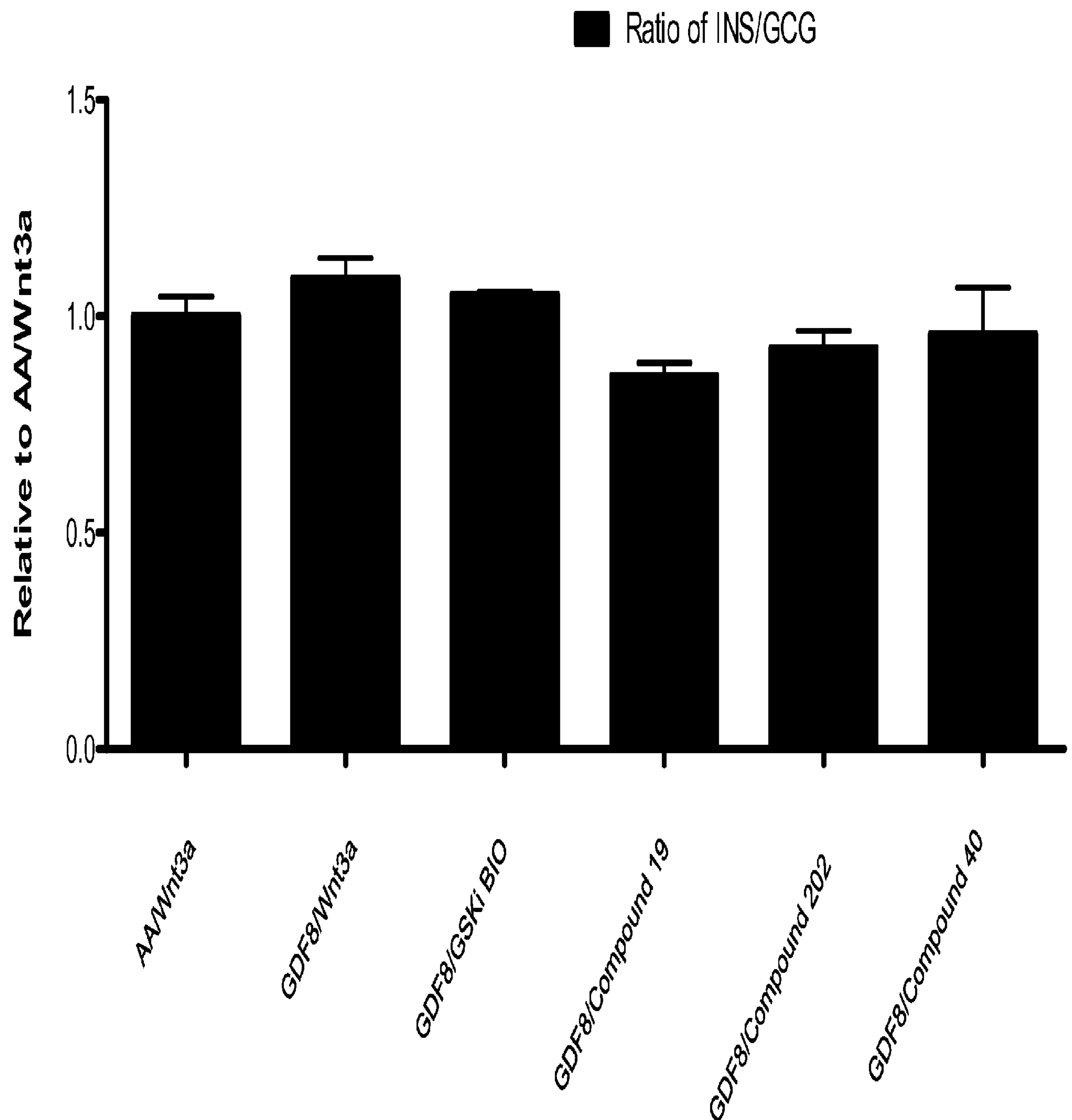


Figure 21A

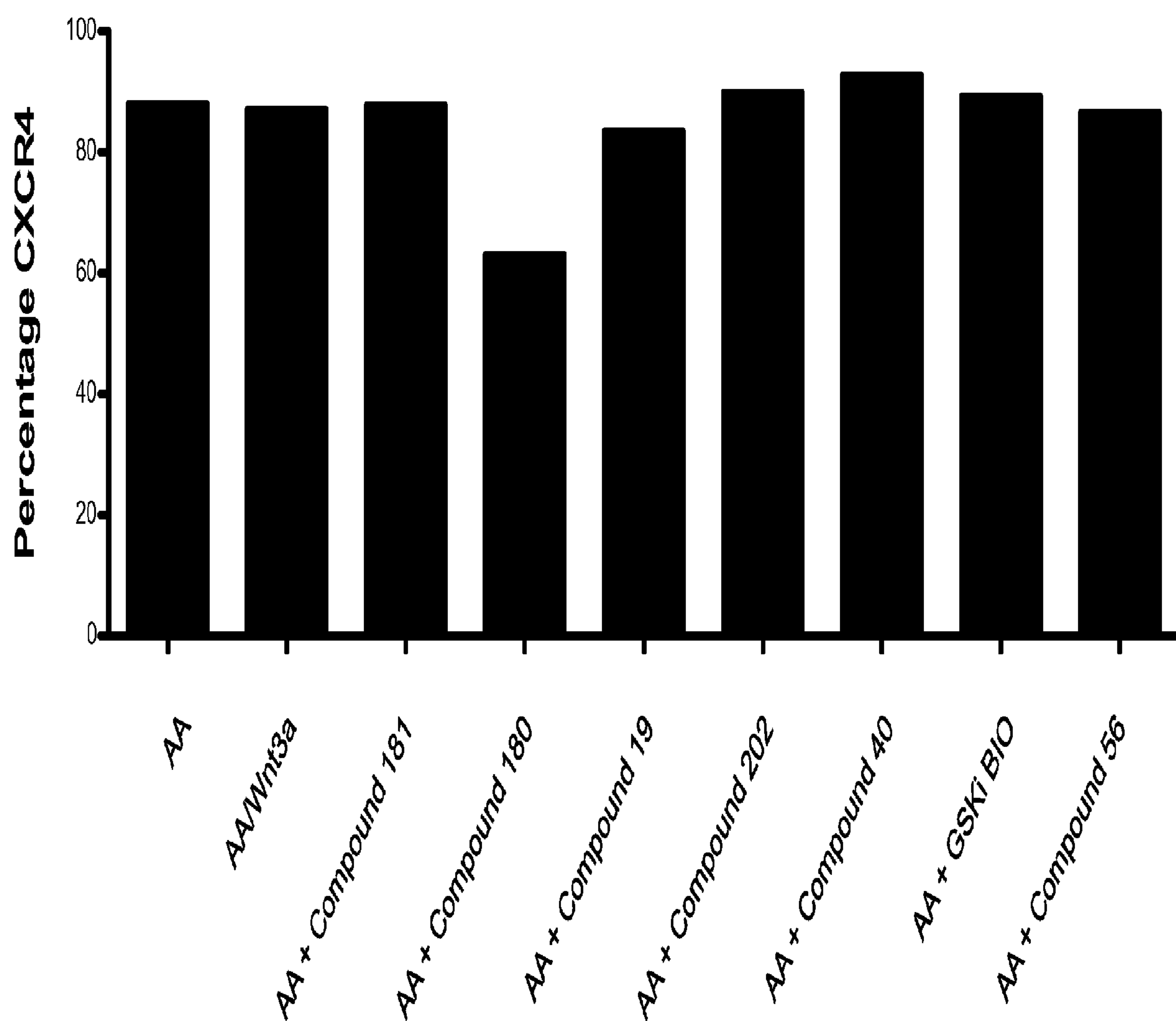


Figure 21B

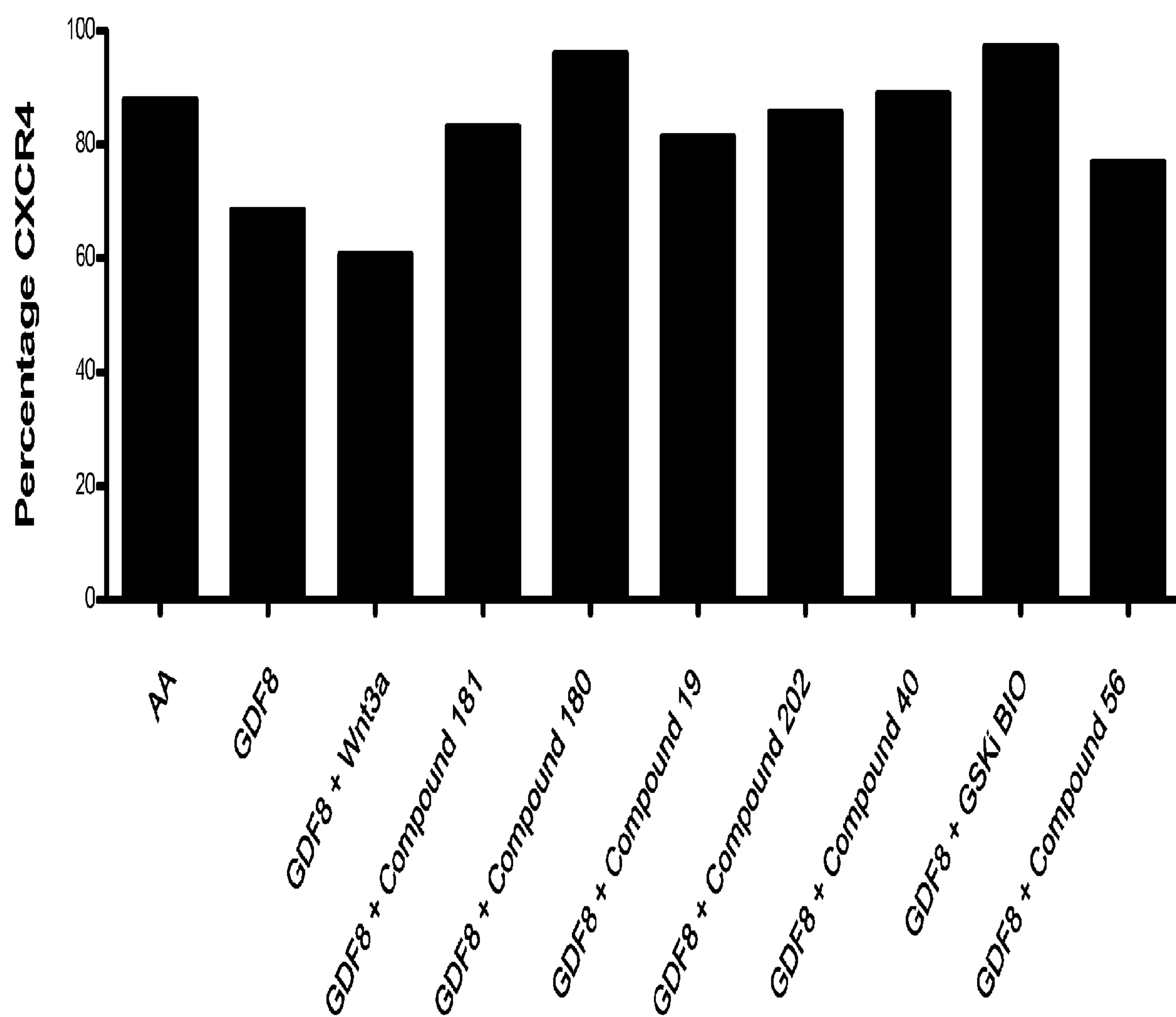
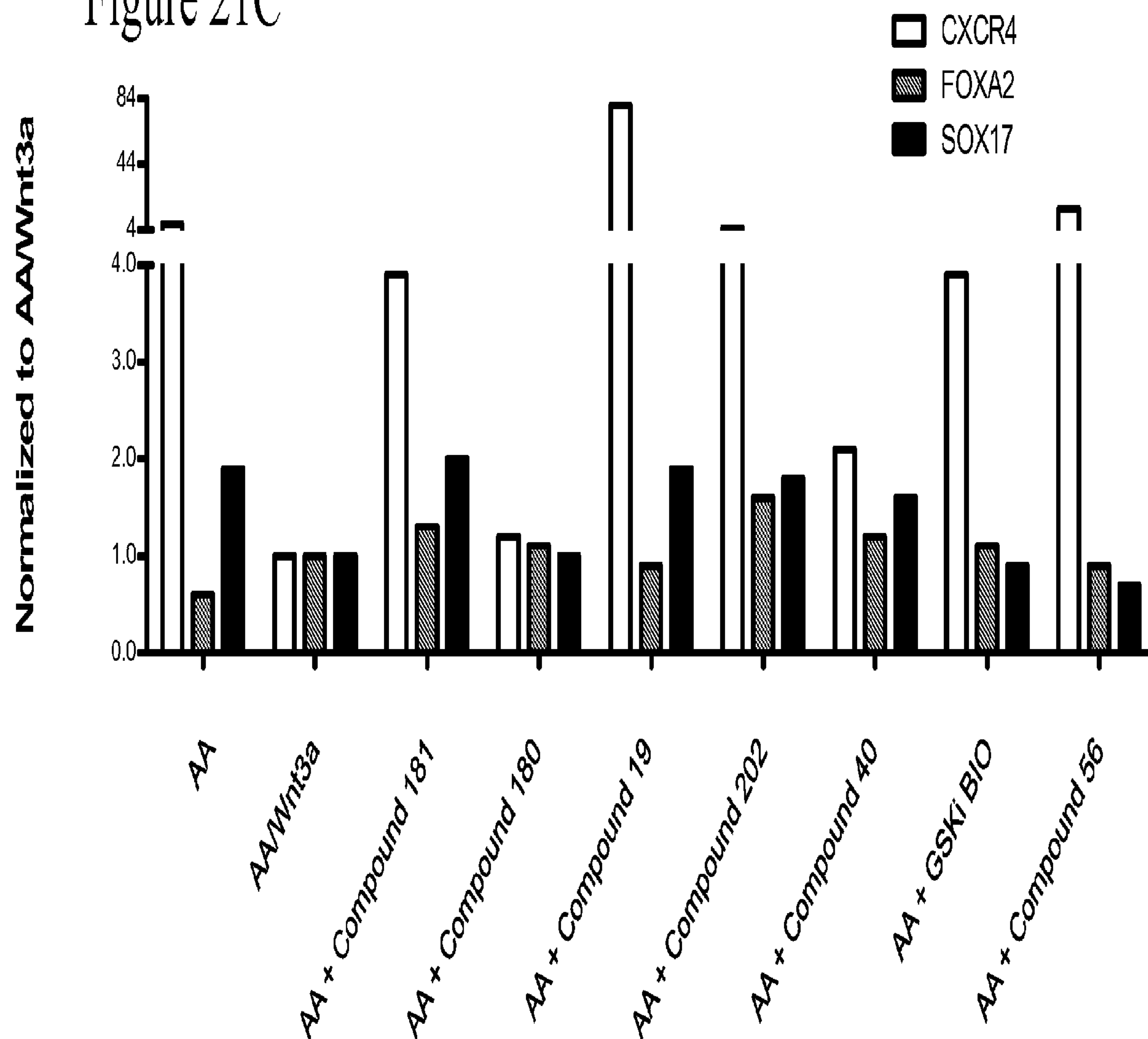
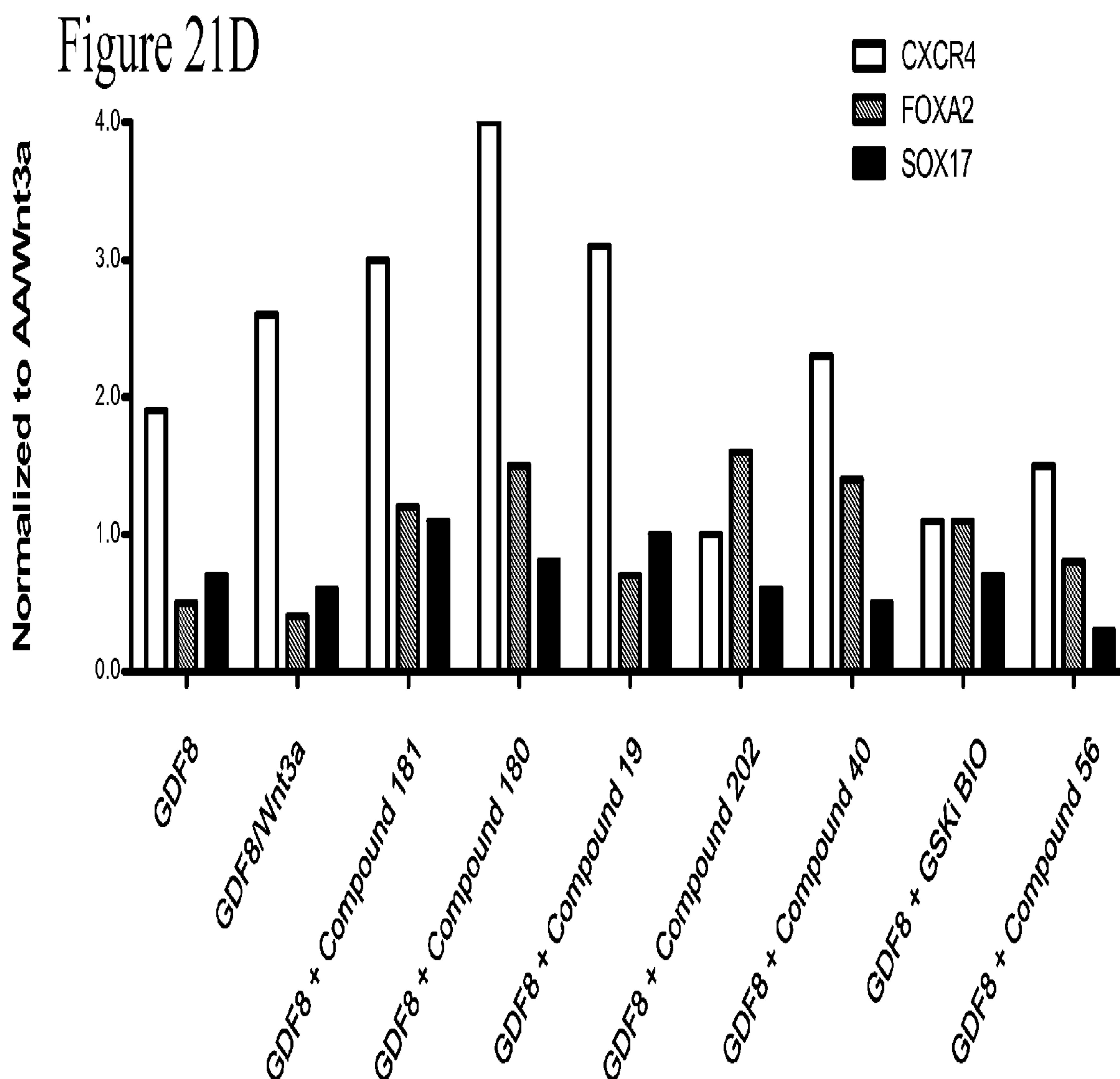


Figure 21C

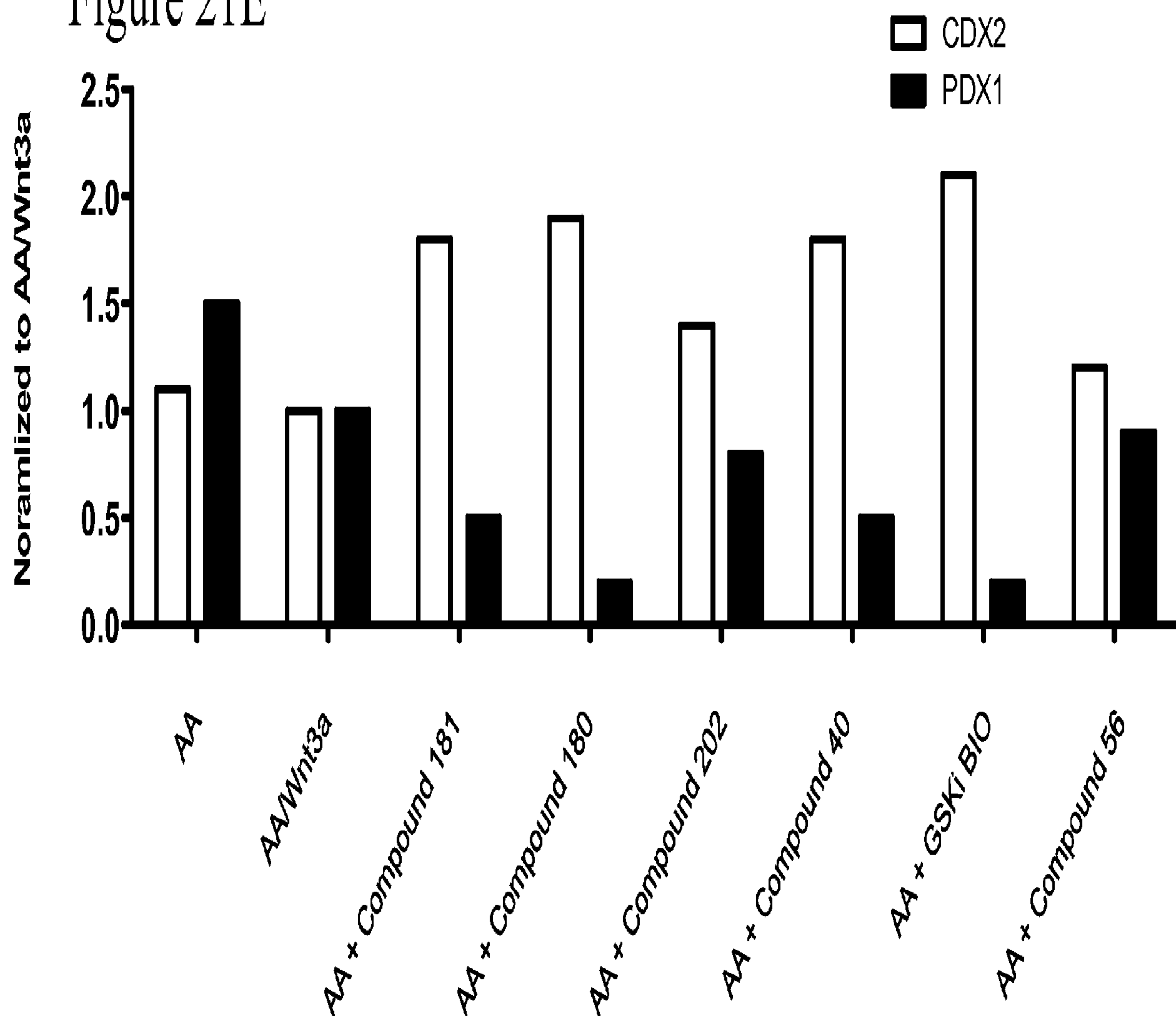


Compound\CT	GAPDH	CXCR4	FOXA2	SOX17
none	19.4	20.4	25.1	21.6
Wnt3a	18.2	22.0	22.9	21.2
Compound 181	20.1	22.0	25.6	22.2
Compound 180	18.4	22.0	24.0	21.4
Compound 19	20.1	17.7	25.6	22.3
Compound 202	20.3	22.0	25.7	22.6
Compound 40	19.9	22.7	25.5	22.3
GSK3i IX(bio)	20.2	22.2	25.8	23.4
Compound 56	19.8	19.6	24.4	23.3



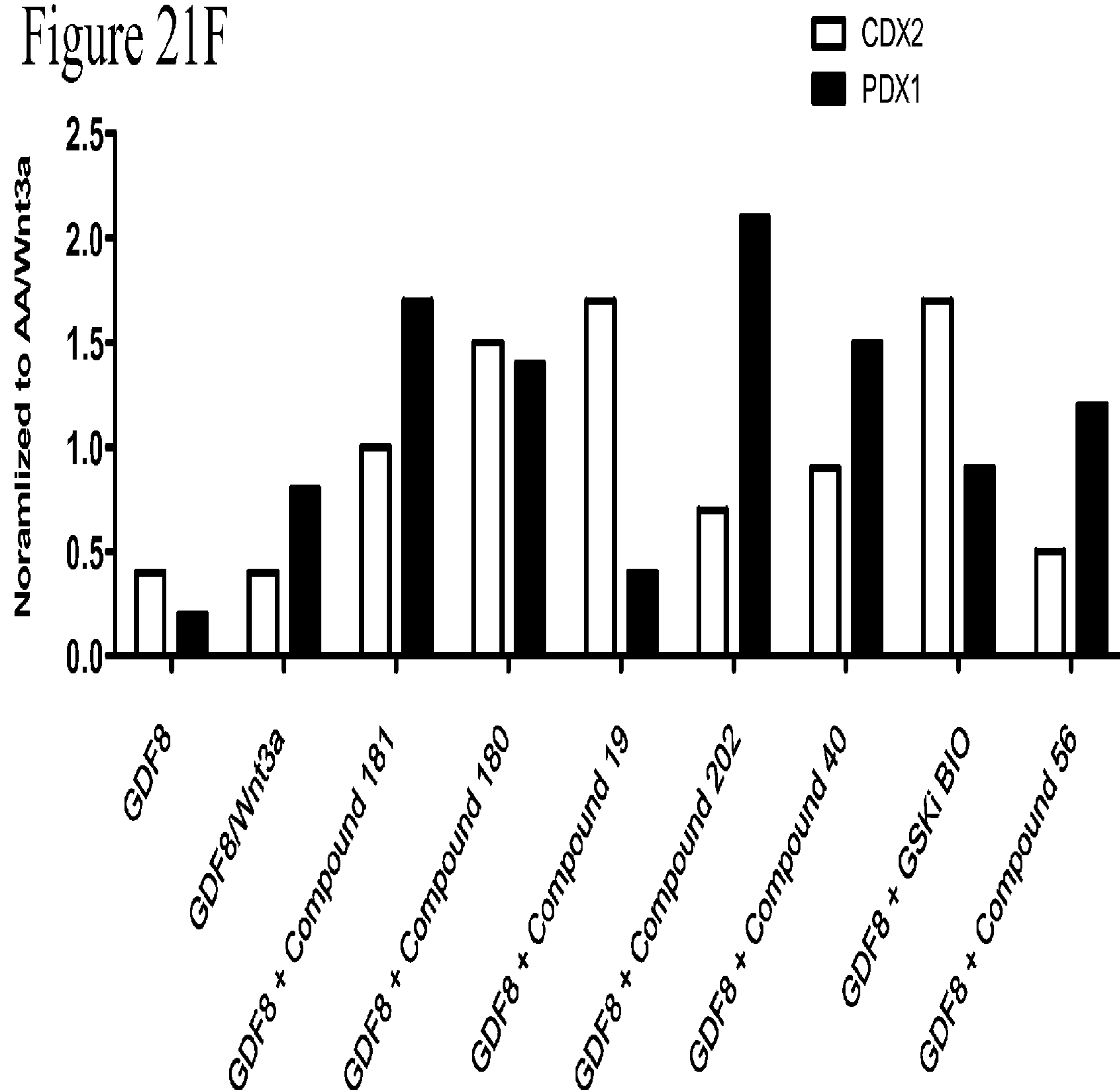
Compound\CT	GAPDH	CXCR4	FOXA2	SOX17
none	21.6	24.6	25.9	25.2
Wnt3a	21.2	23.6	25.7	25.0
Compound 181	20.7	23.0	25.2	23.7
Compound 180	20.9	22.7	25.0	24.2
Compound 19	19.6	21.8	24.2	22.6
Compound 202	18.5	22.4	23.6	22.3
Compound 40	19.7	22.4	24.9	23.7
GSK3i IX(bio)	19.6	23.3	24.7	23.3
Compound 56	19.7	23.0	25.3	24.4

Figure 21E



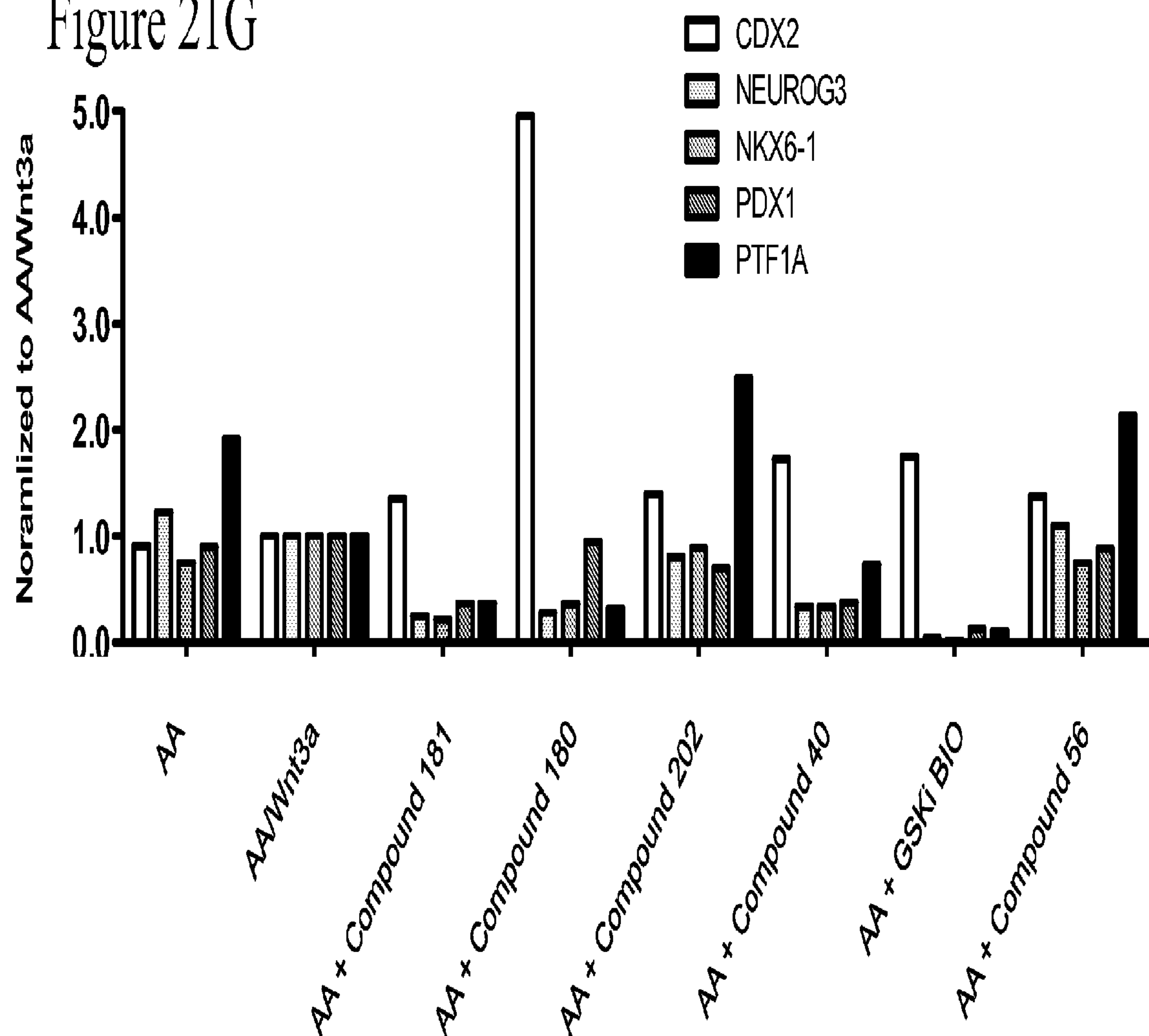
Compound\CT	GAPDH	CDX2	PDX1
none	18.5	22.9	23.3
Wnt3a	18.4	23.0	23.8
Compound 181	18.6	22.3	25.0
Compound 180	18.8	22.5	26.3
Compound 202	18.5	22.5	24.1
Compound 40	18.5	22.2	24.8
GSK3i IX(bio)	18.5	22.0	26.1
Compound 56	18.3	22.7	23.9

Figure 21F



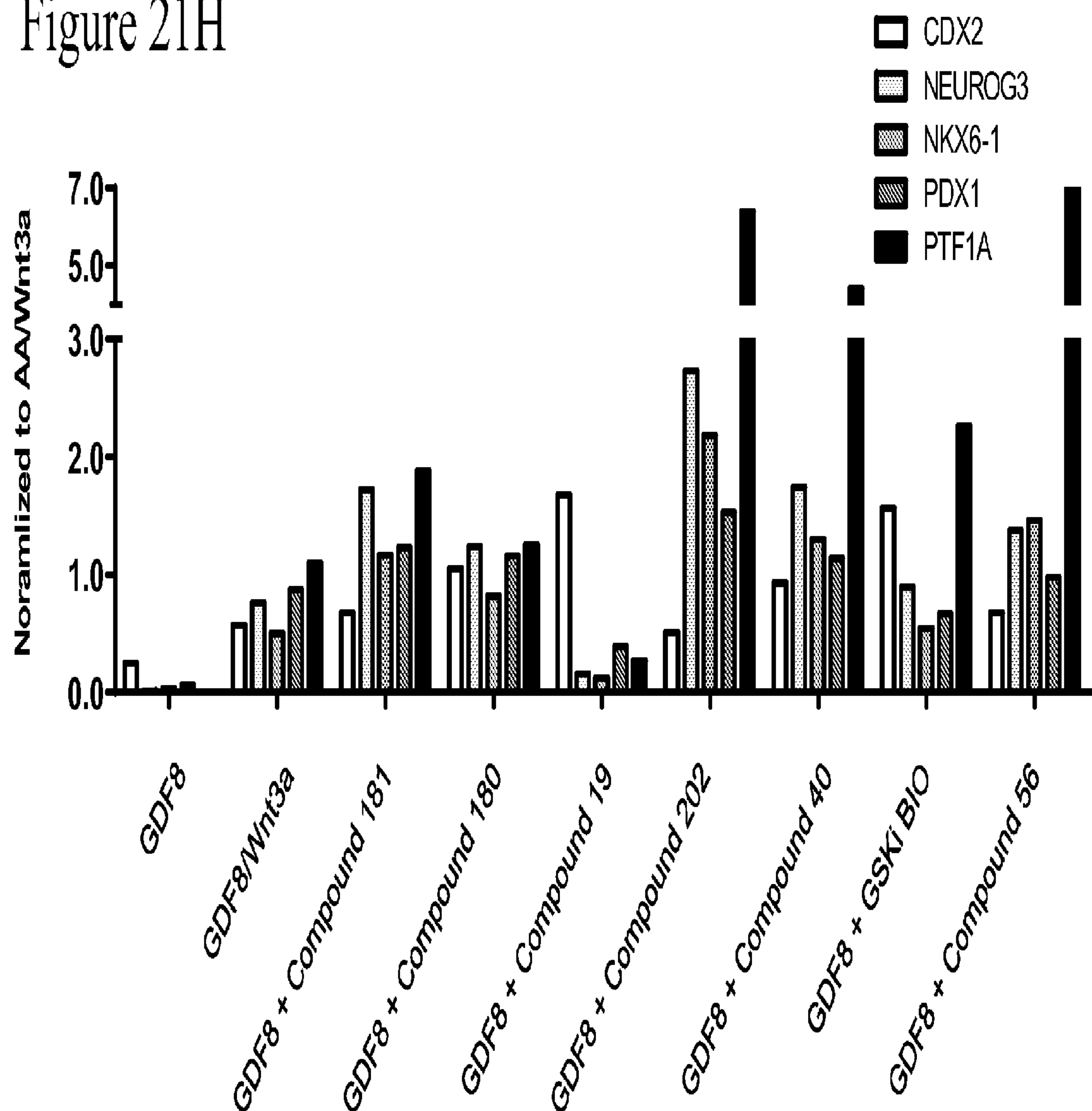
Compound\CT	GAPDH	CDX2	PDX1
none	18.0	23.8	25.5
Wnt3a	17.4	23.1	23.1
Compound 181	18.8	23.4	23.5
Compound 180	18.8	22.8	23.7
Compound 19	18.3	22.0	25.0
Compound 202	18.7	23.8	23.0
Compound 40	18.4	23.0	23.2
GSK3i IX(bio)	18.4	22.2	24.0
Compound 56	18.2	23.8	23.3

Figure 21G

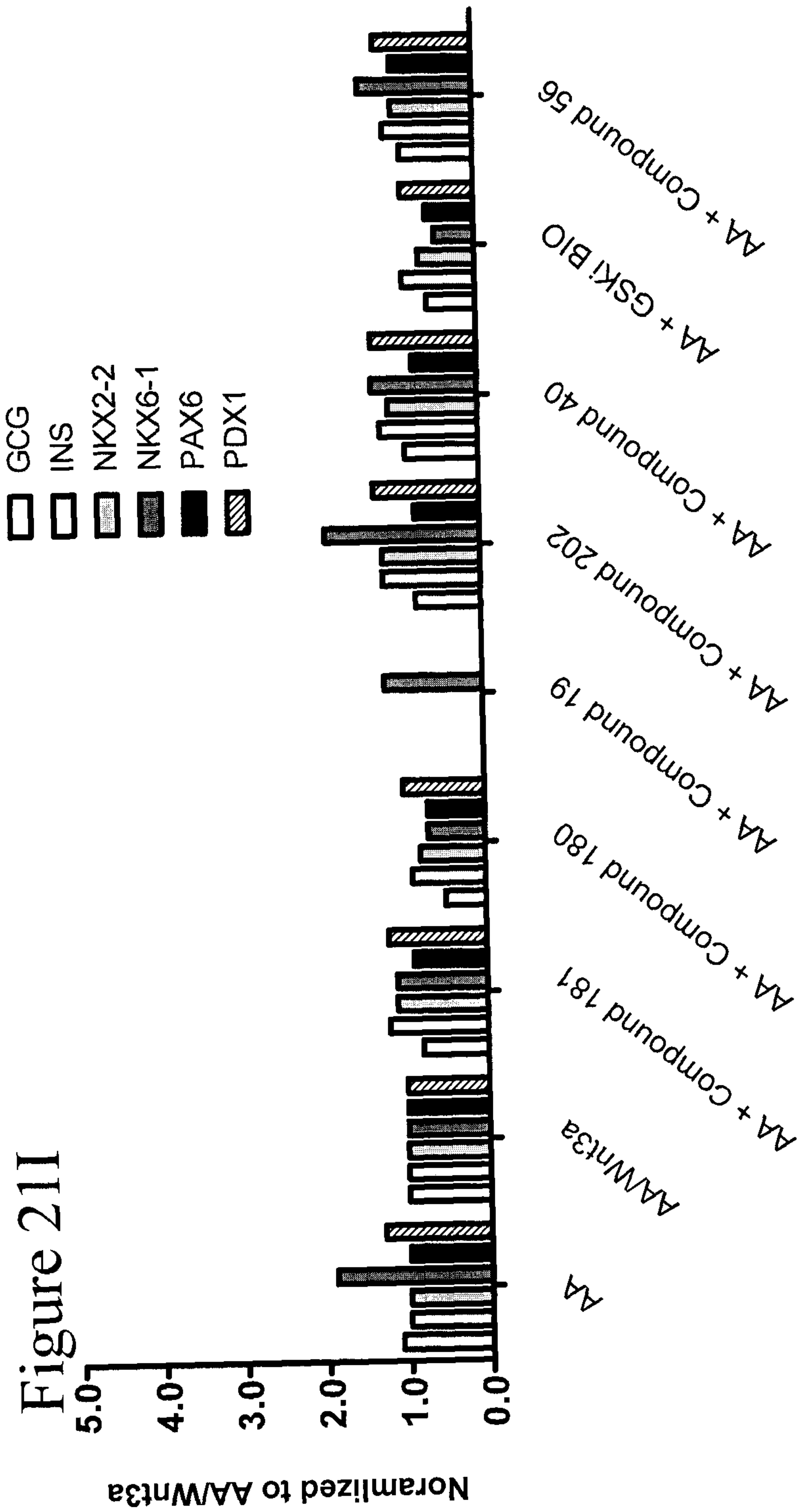


Compound\CT	GAPDH	CDX2	NEUROG3	NKX6-1	PDX1	PTF1A
none	19.0	23.7	23.3	27.4	23.7	29.6
Wnt3a	19.5	24.1	24.1	27.5	24.1	31.1
Compound 181	18.0	22.1	24.6	28.1	24.0	31.0
Compound 180	19.4	21.6	25.8	28.9	24.0	32.6
Compound 202	19.2	23.3	24.1	27.4	24.3	29.5
Compound 40	19.2	22.9	25.3	28.7	25.1	31.2
GSK3i IX(bio)	19.0	22.7	27.7	32.9	26.5	33.7
Compound 56	18.8	22.9	23.3	27.2	23.6	29.3

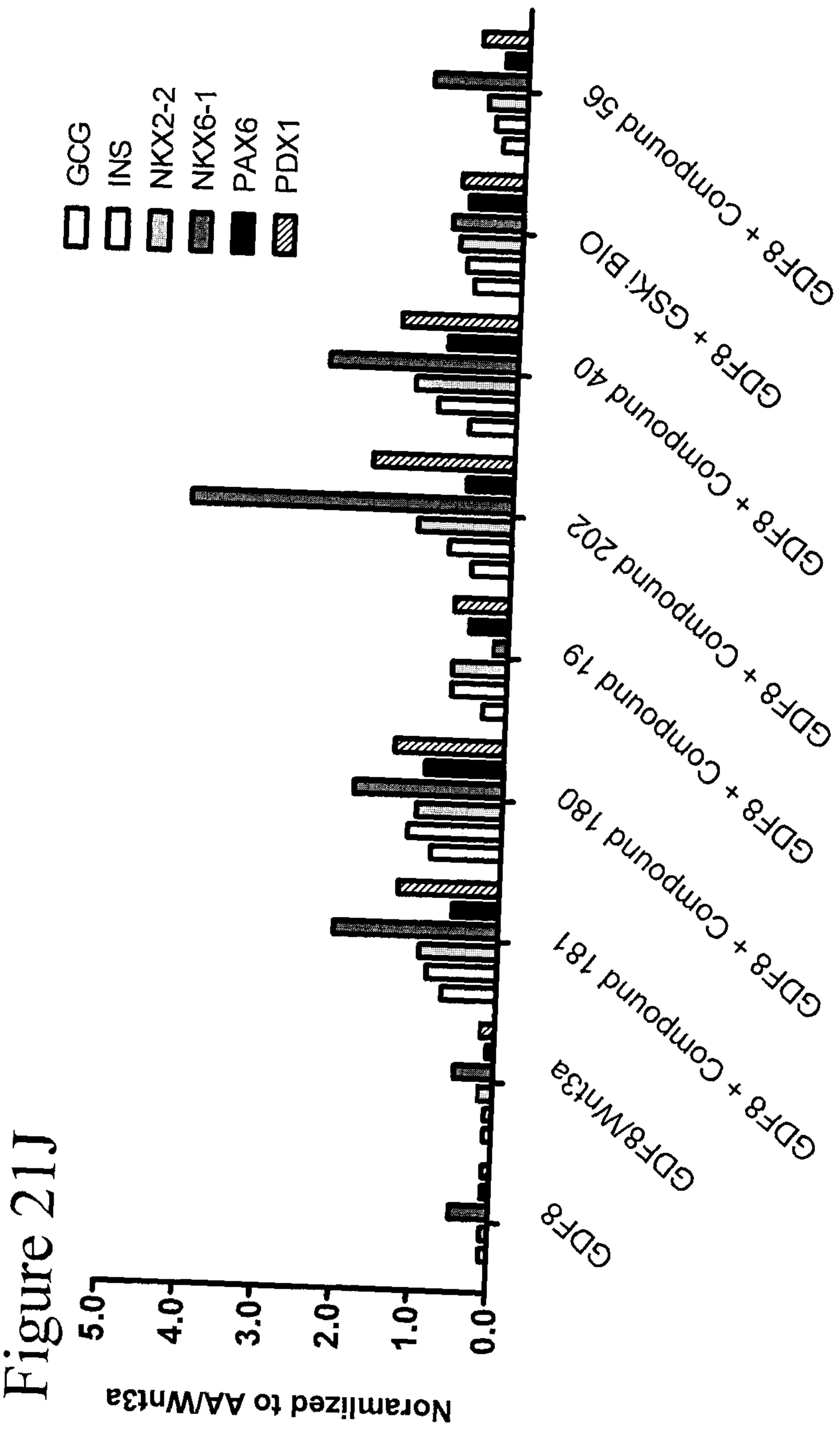
Figure 21H



Compound\CT	GAPDH	CDX2	NEUROG3	NKX6-1	PDX1	PTF1A
none	18.0	24.6	28.7	31.2	26.5	40.0
Wnt3a	19.0	24.4	24.0	28.0	23.8	30.5
Compound 181	18.0	23.1	21.8	25.7	22.3	28.6
Compound 180	19.5	23.9	23.7	27.7	23.8	30.7
Compound 19	19.1	22.9	26.4	30.2	25.1	32.6
Compound 202	19.0	24.5	22.2	25.9	23.0	27.9
Compound 40	19.0	23.6	22.7	26.6	23.4	28.4
GSK3i IX(bio)	19.1	23.0	23.8	27.9	24.2	29.5
Compound 56	18.9	24.0	23.0	26.3	23.5	27.7



Compound\CT	GAPDH	GCG	INS	NKX2-2	NKX6-1	PAX6	PDX1
none	18.3	14.1	14.9	23.7	25.5	24.0	23.2
Wnt3a	18.0	14.0	14.6	23.4	26.1	23.7	23.3
Compound 181	18.0	14.3	14.5	23.3	26.1	24.0	23.1
Compound 180	18.0	14.9	14.7	23.8	26.5	24.3	23.2
Compound 19	17.9	27.0	26.9	28.0	25.8	28.6	29.4
Compound 202	18.6	14.9	15.0	23.7	25.8	24.7	23.6
Compound 40	18.3	14.6	14.7	23.5	26.1	24.4	23.2
GSK3i IX(bio)	18.3	15.1	15.1	24.2	27.5	24.9	23.8
Compound 56	18.2	14.4	14.8	23.6	25.9	24.1	23.3



Compound\CT	GAPDH	GCG	INS	NKX2-2	NKX6-1	PAX6	PDX1
none	17.4	18.1	17.7				
Wnt3a	17.8	17.7	17.5	25.9	26.6	26.6	25.6
Compound 181	18.0	14.5	14.8	25.6	27.0	26.5	25.6
Compound 180	18.0	14.2	14.4	23.4	25.0	24.4	23.0
Compound 19	18.3	16.2	15.6	23.2	25.1	23.8	22.9
Compound 202	18.7	15.7	15.8	24.3	28.6	25.2	24.2
Compound 40	18.1	14.8	14.8	23.8	24.8	25.2	23.2
GSK3i IX(bio)	18.4	15.2	15.5	24.2	25.0	24.1	22.9
Compound 56	18.0	16.0	16.0	24.4	26.7	24.8	24.0

Figure 21K

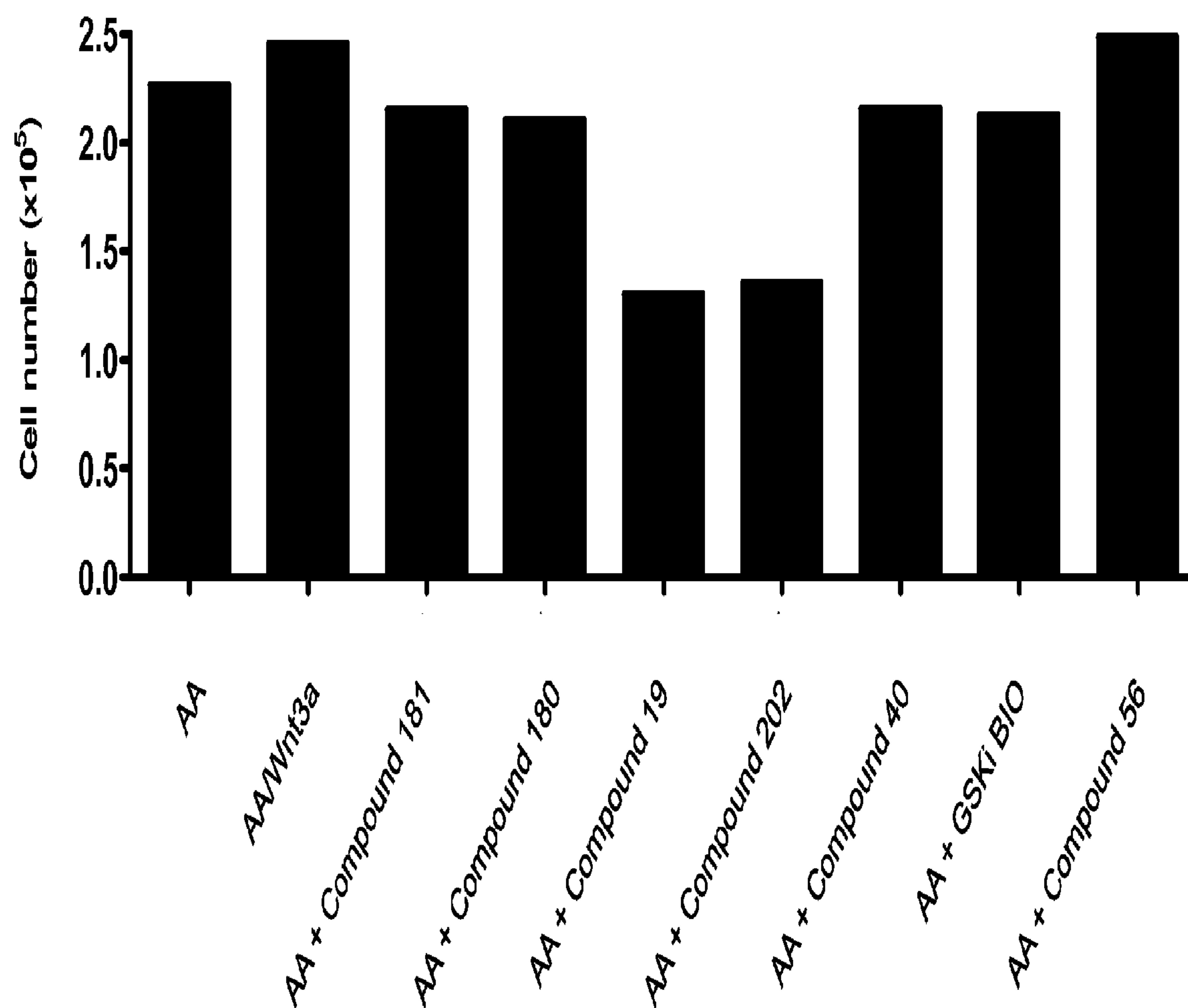


Figure 21L

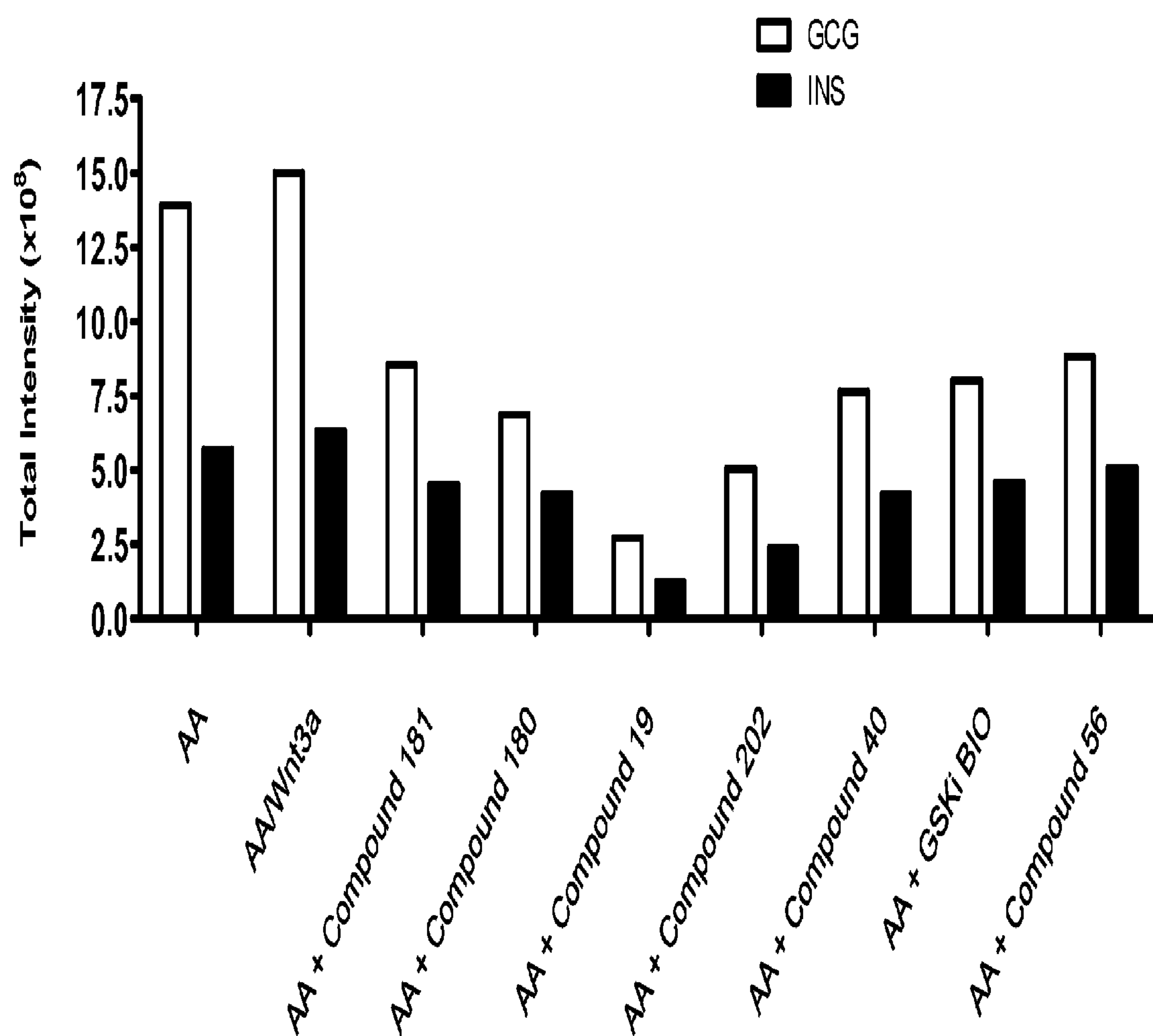


Figure 21M

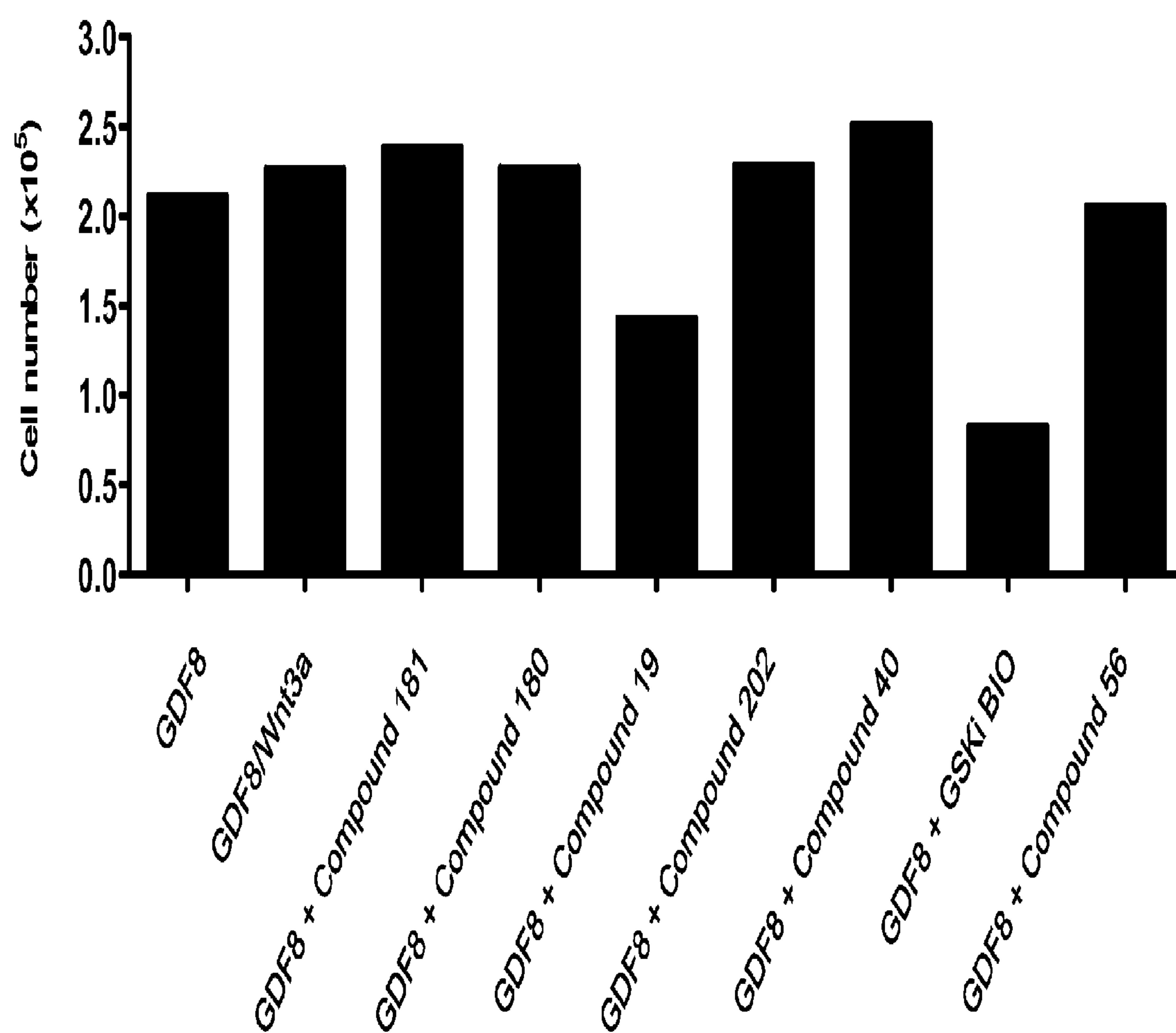


Figure 21N

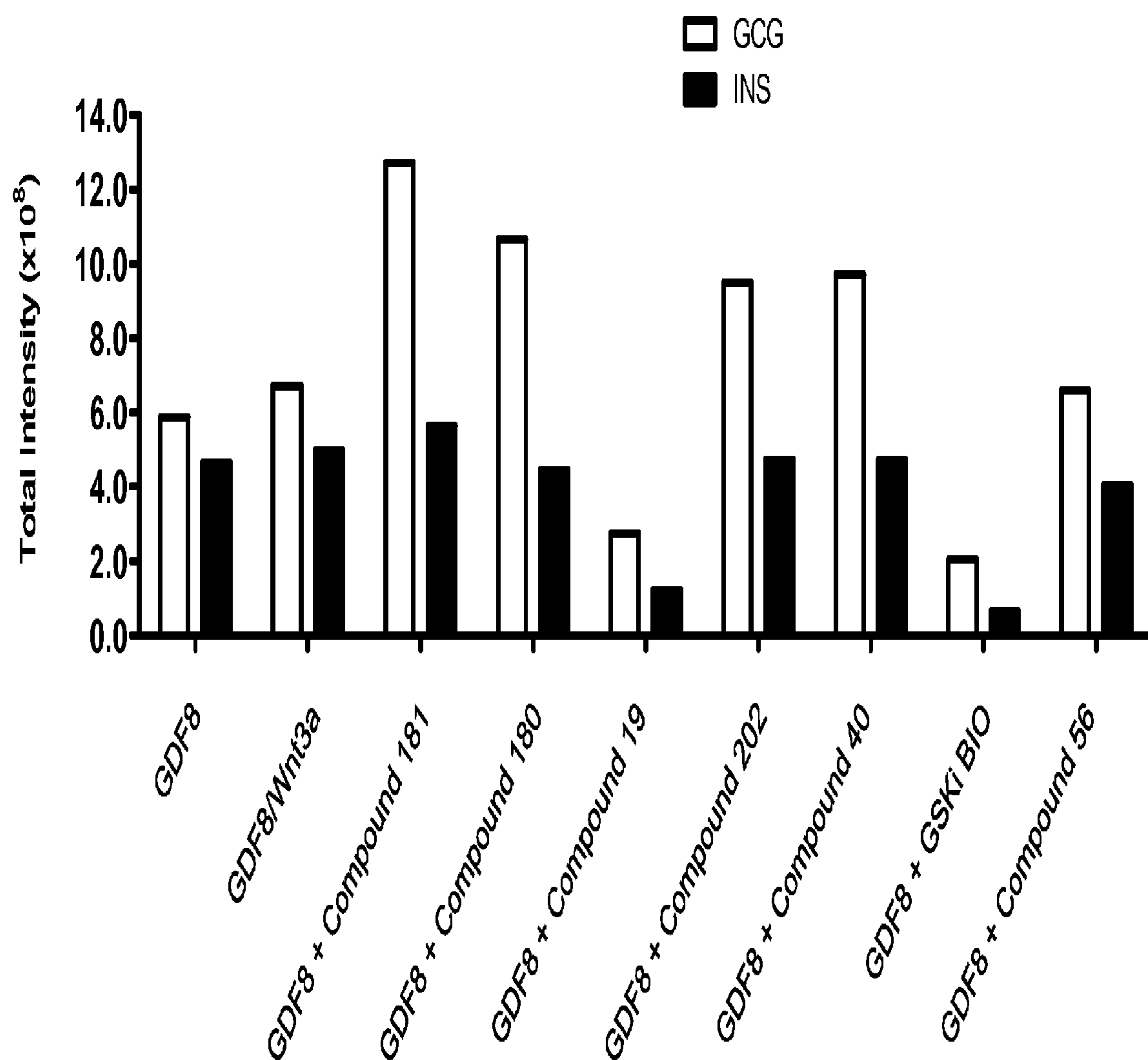


Figure 22A

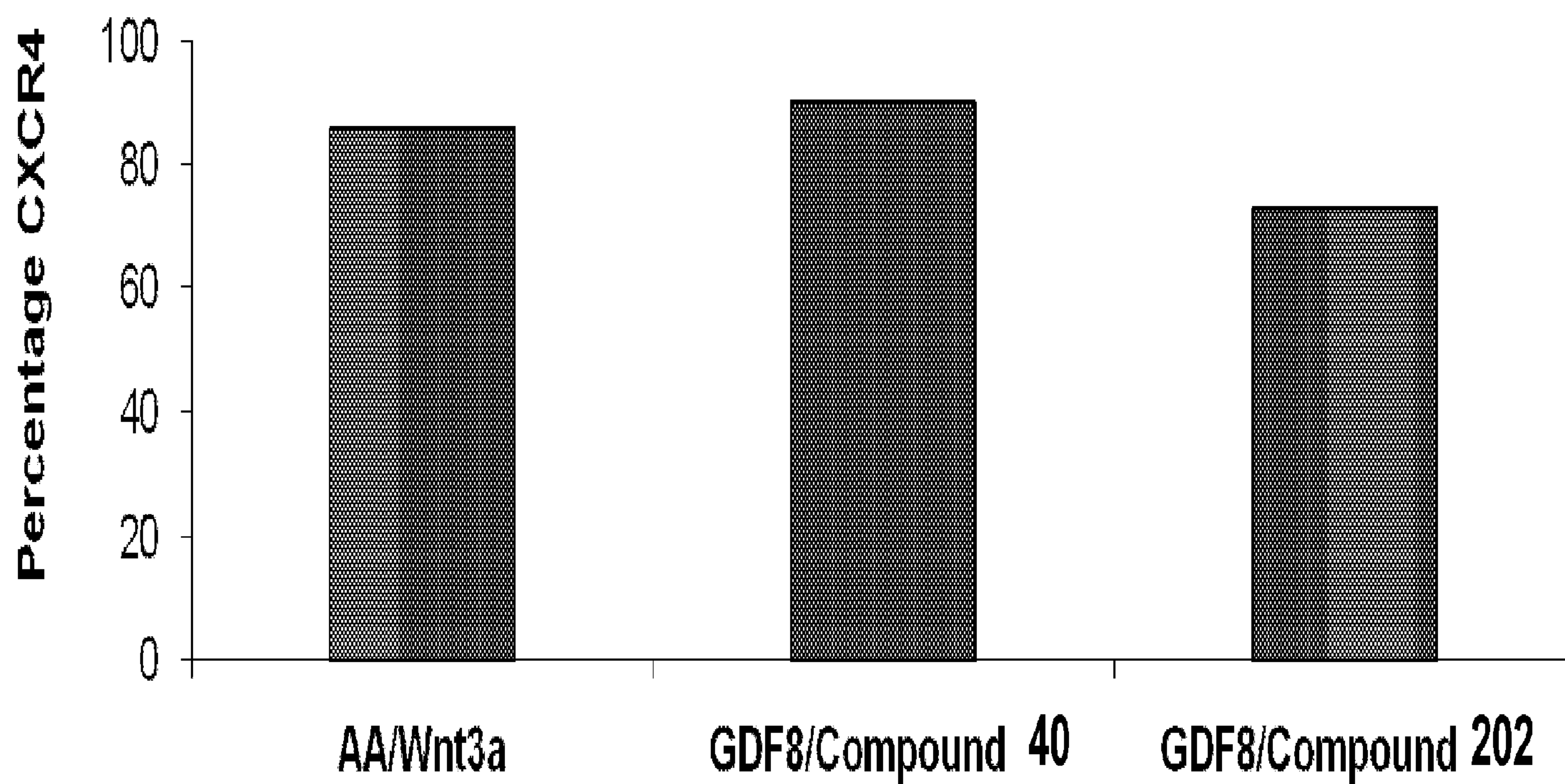


Figure 22B

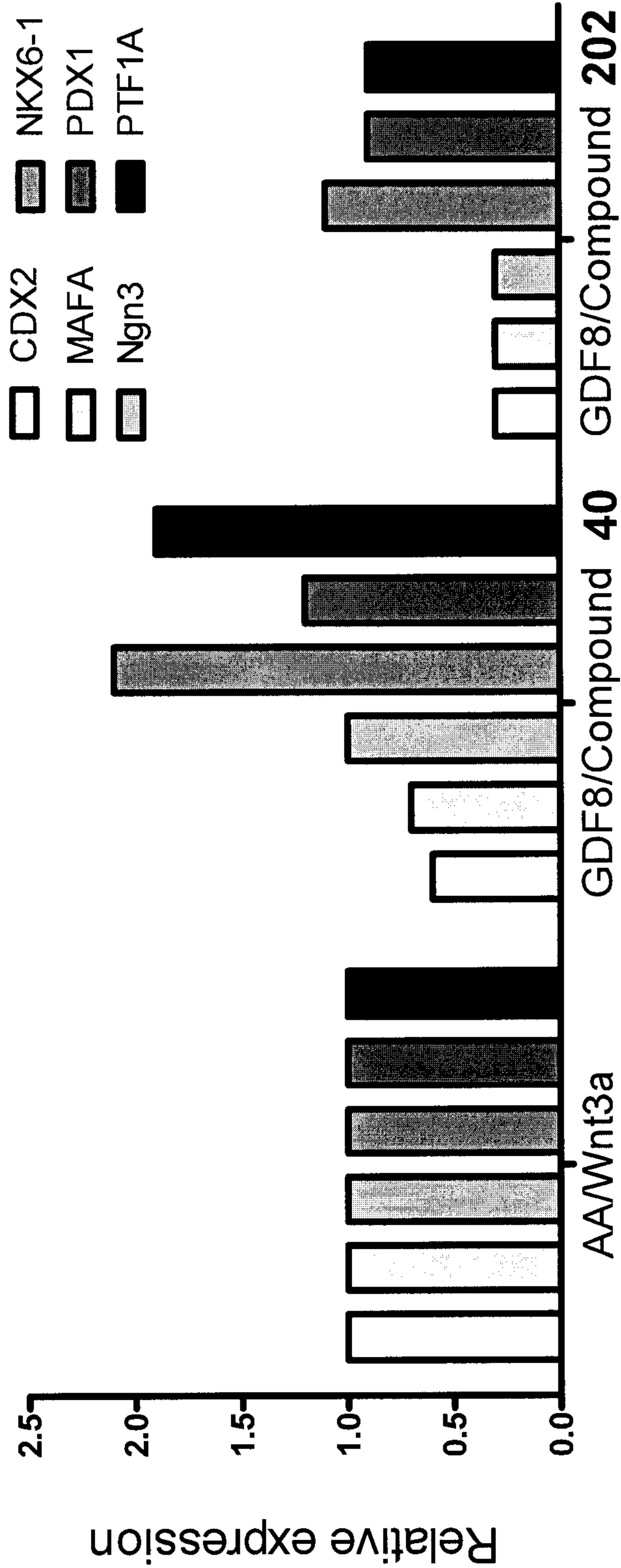


Figure 23A

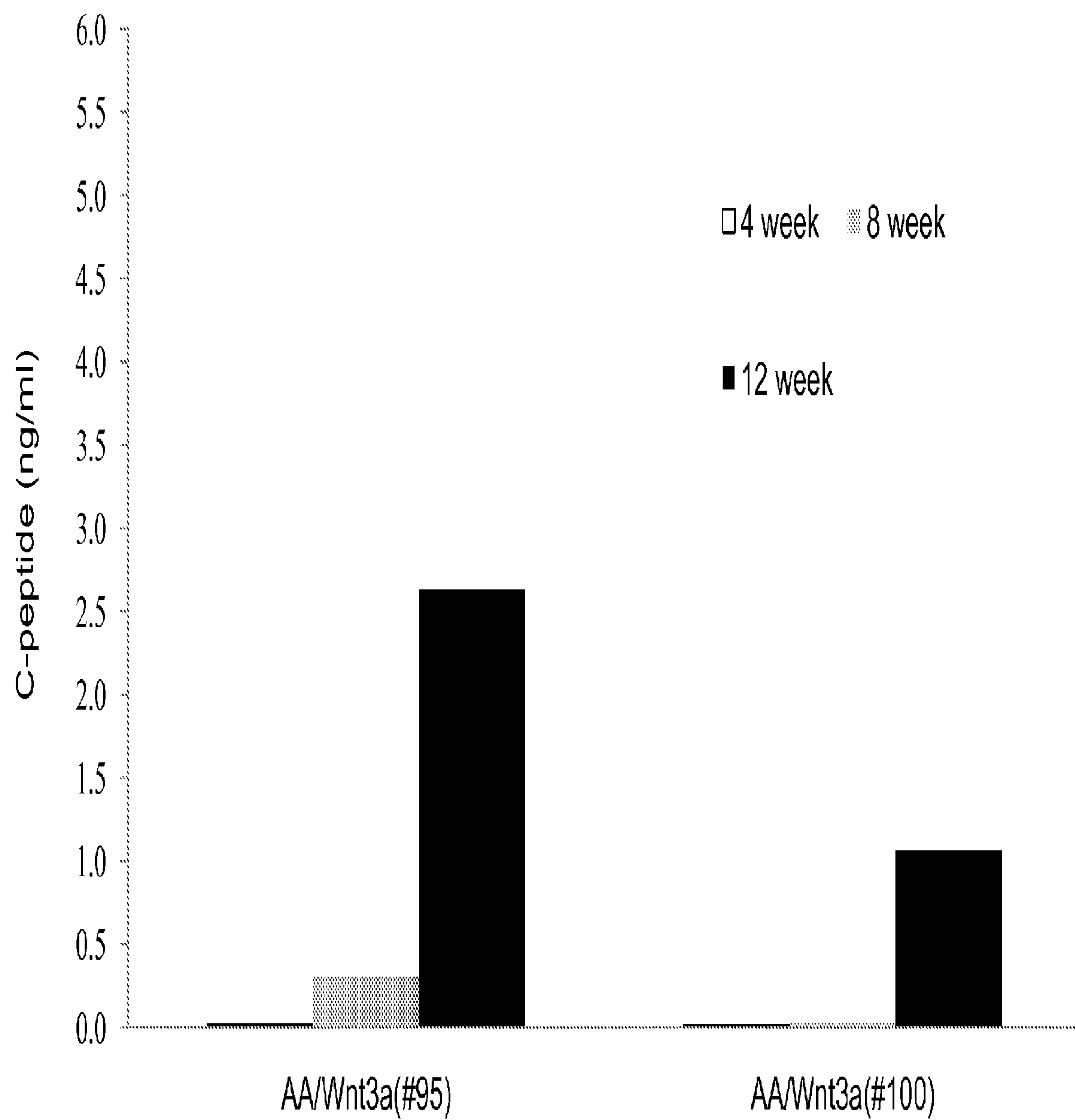


Figure 23B

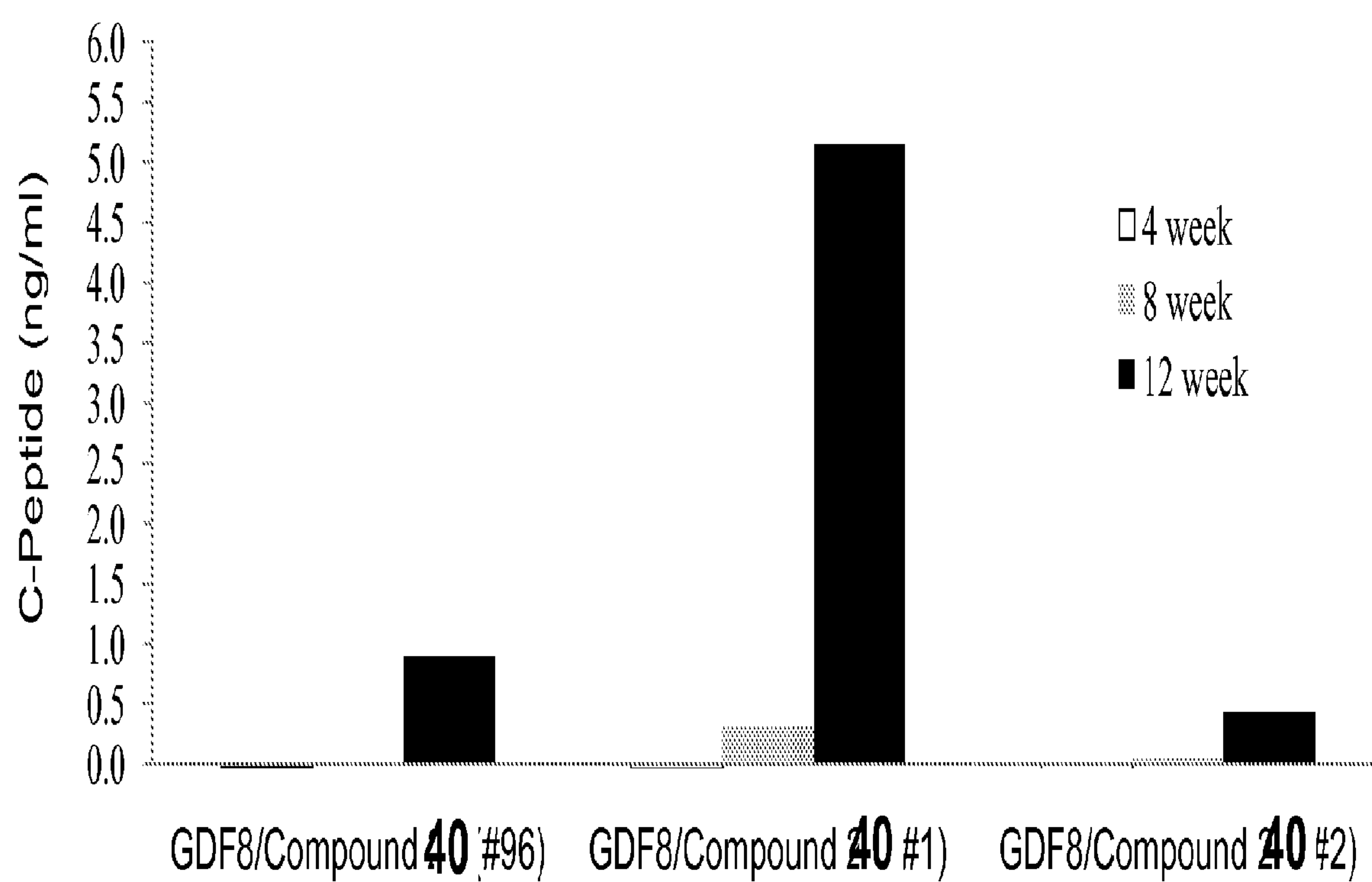


Figure 23C

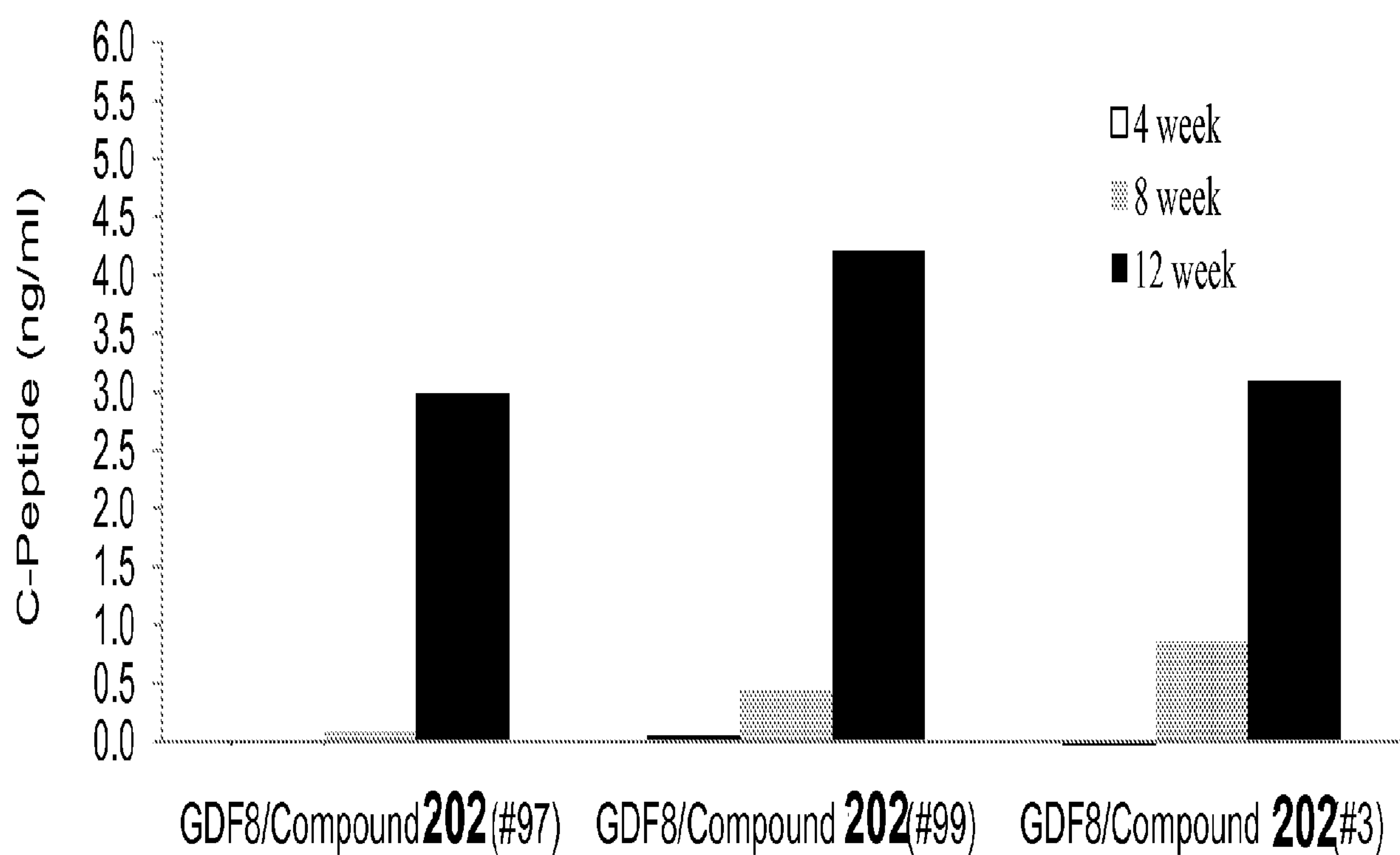


Figure 24A

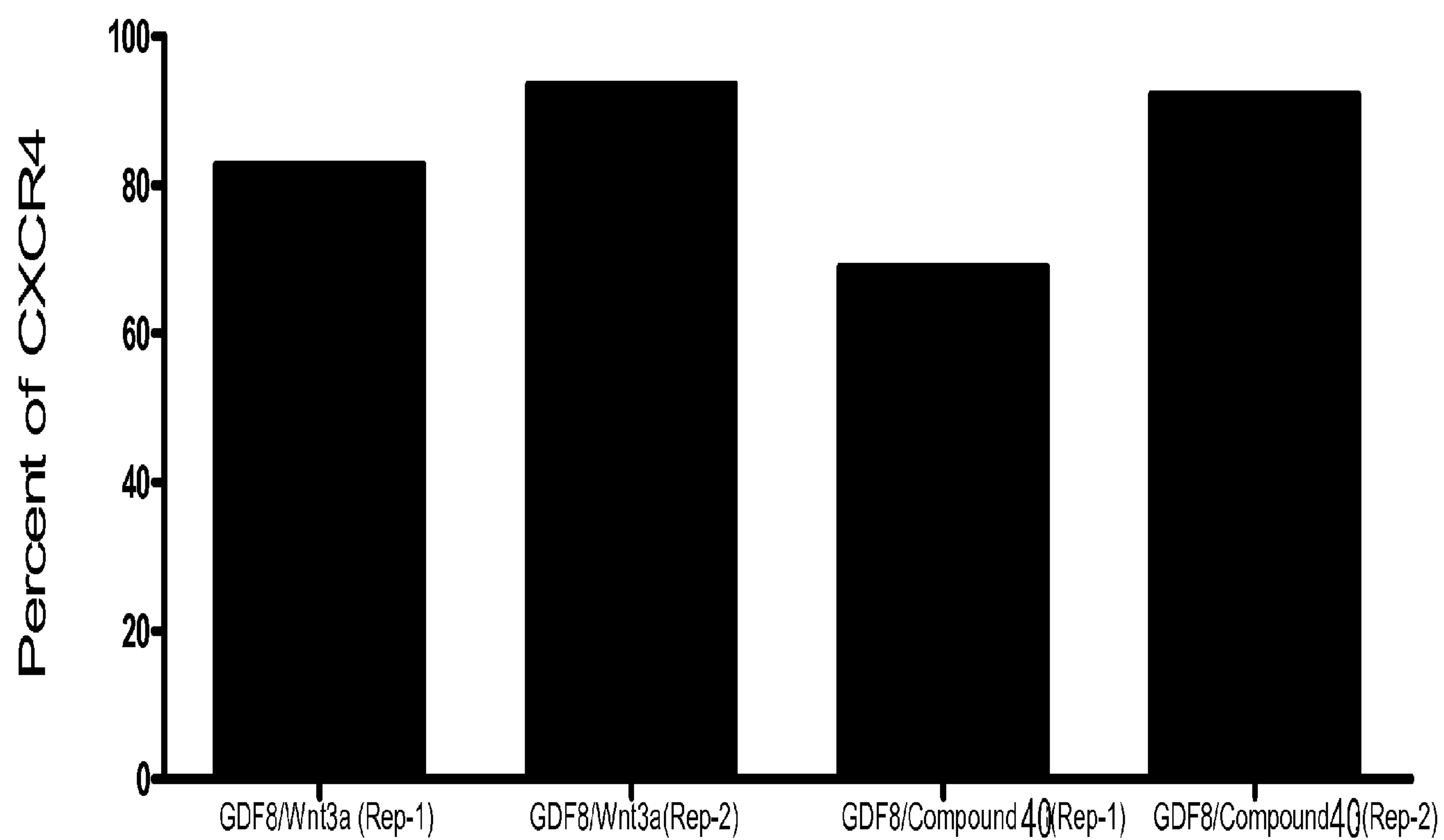
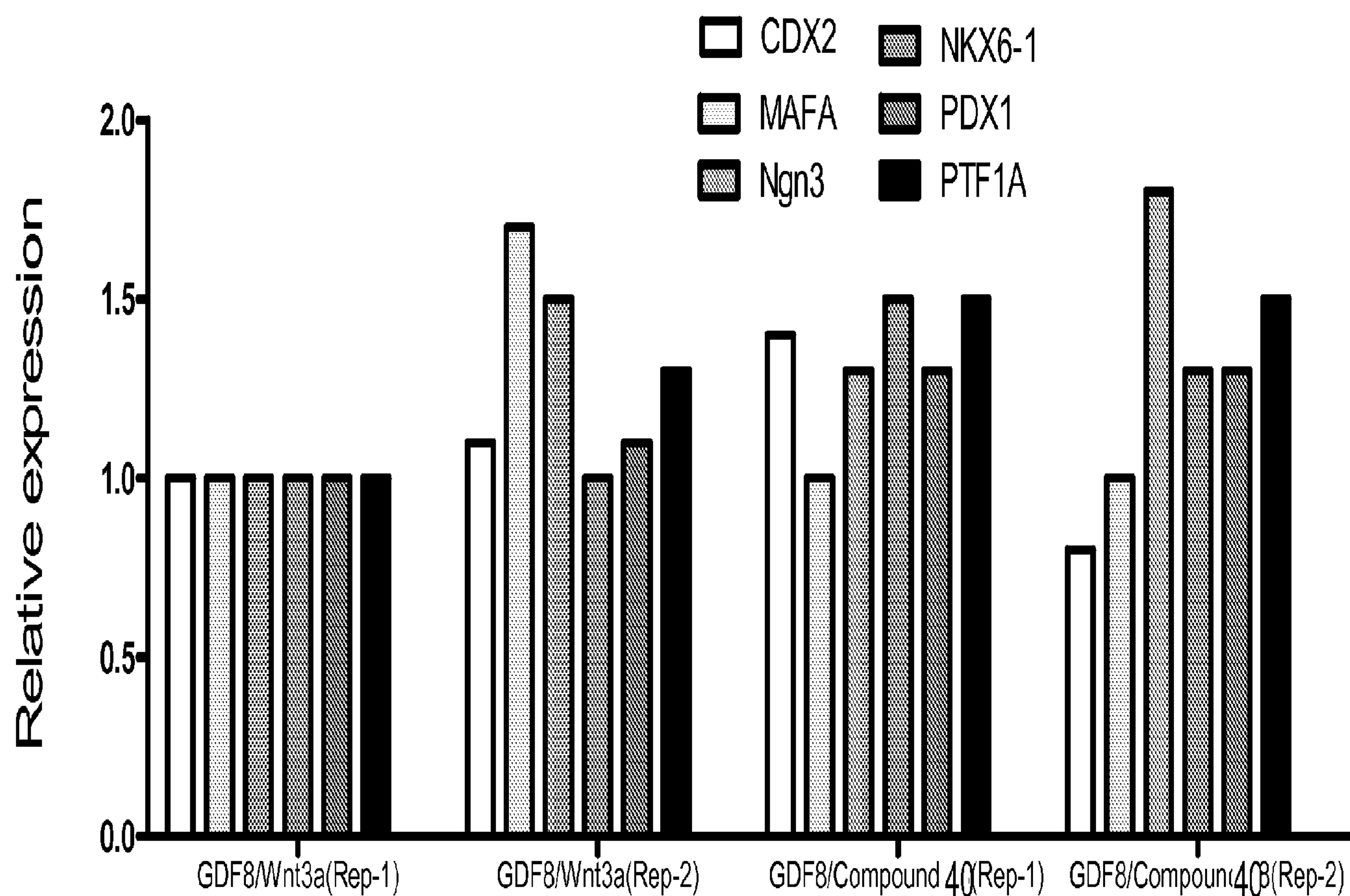


Figure 24B



Treatment\CTs	GAPDH	CDX2	MAFA	NEUROG3	NKX6-1	PDX1	PTF1A
GDF8/Wnt3a (replicate 1)	19.0	24.8	31.0	24.1	24.3	22.5	25.4
GDF8/Wnt3a (replicate 2)	18.6	24.3	29.8	23.1	23.9	22.0	24.7
GDF8/Compound 40 (replicate 1)	19.3	24.6	31.3	23.9	24.0	22.3	25.0
GDF8/Compound 40 (replicate 2)	18.7	24.9	30.6	22.9	23.7	21.8	24.5

Figure 24C

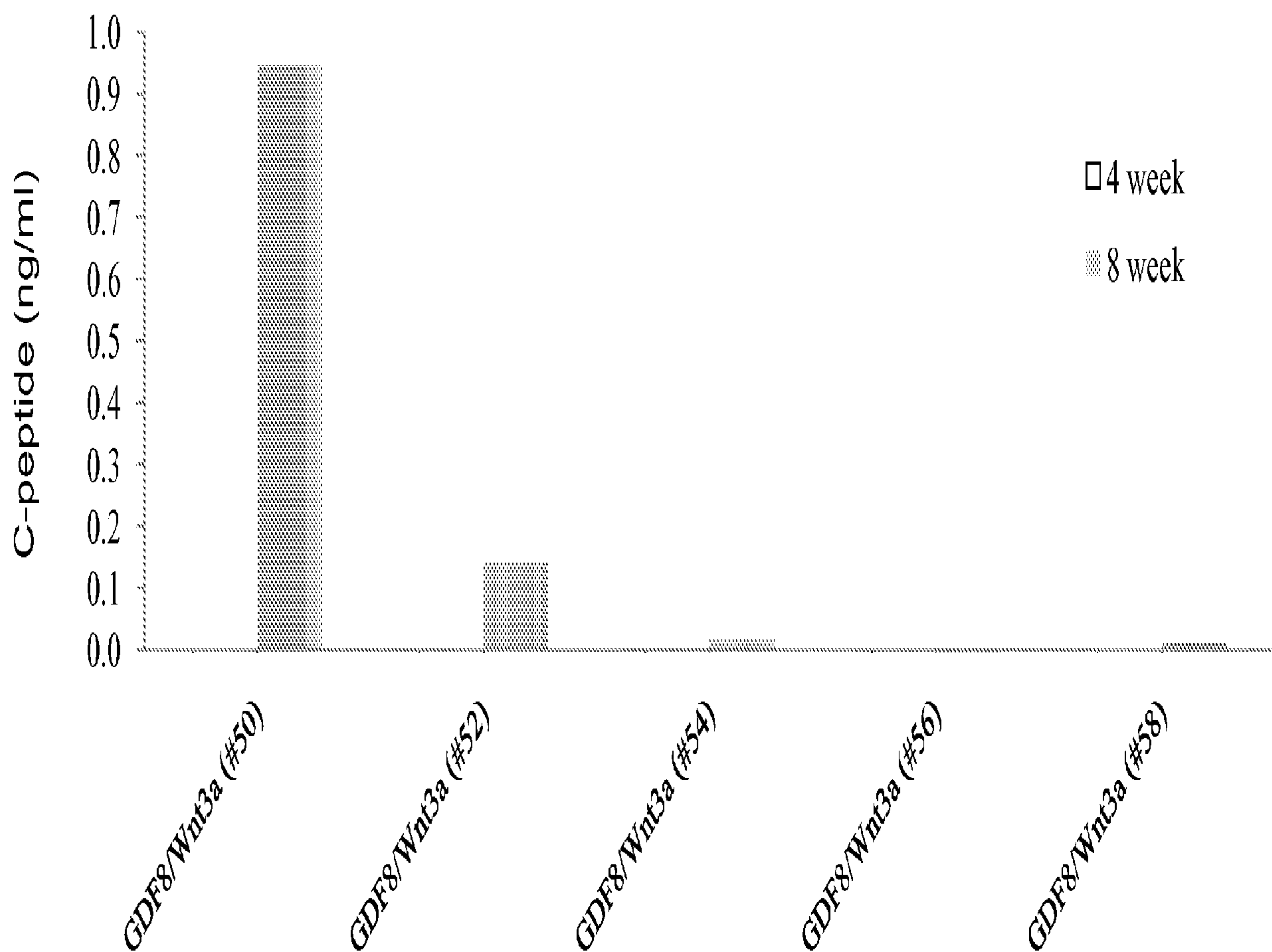


Figure 24D

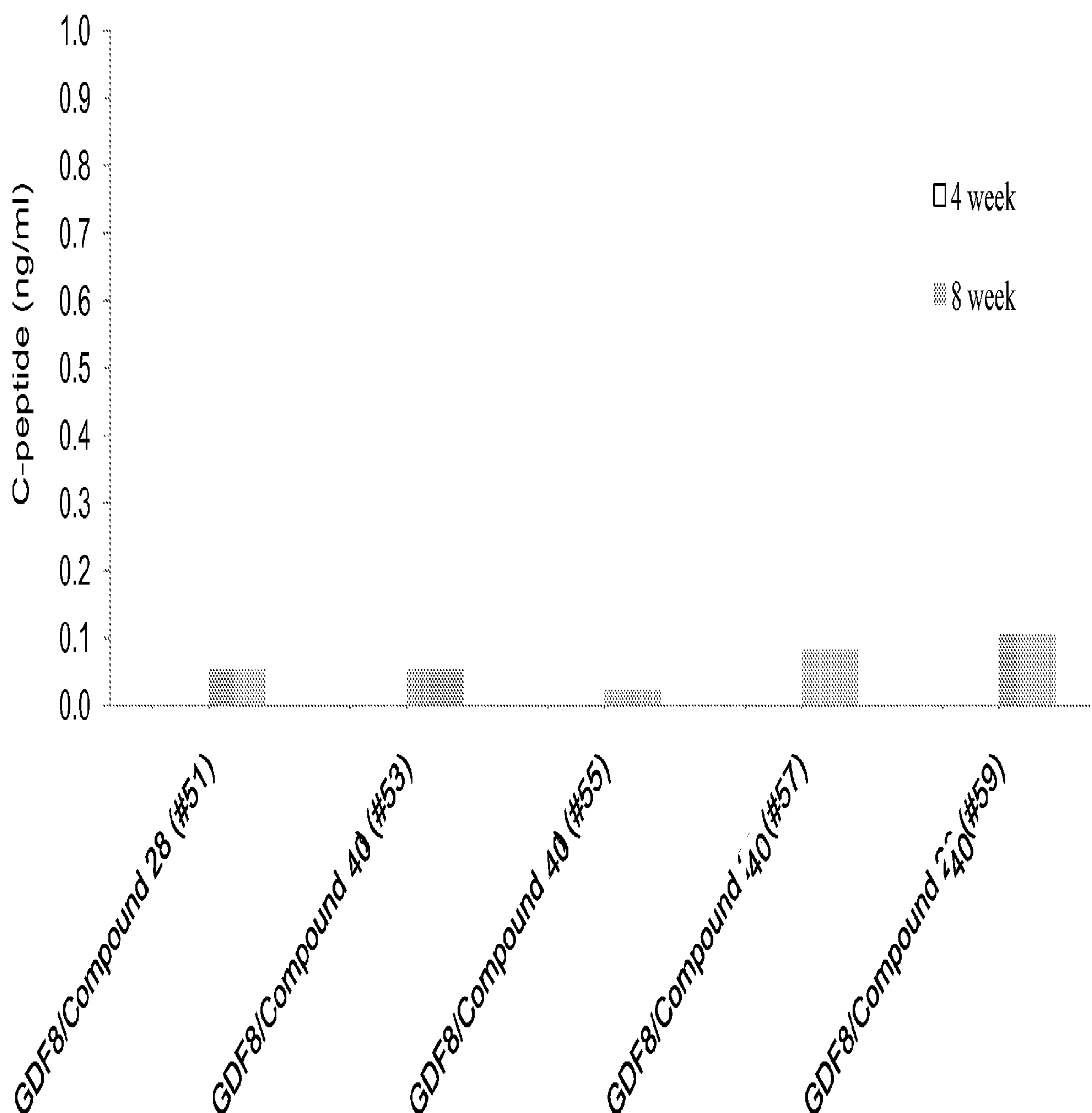


Figure 25A

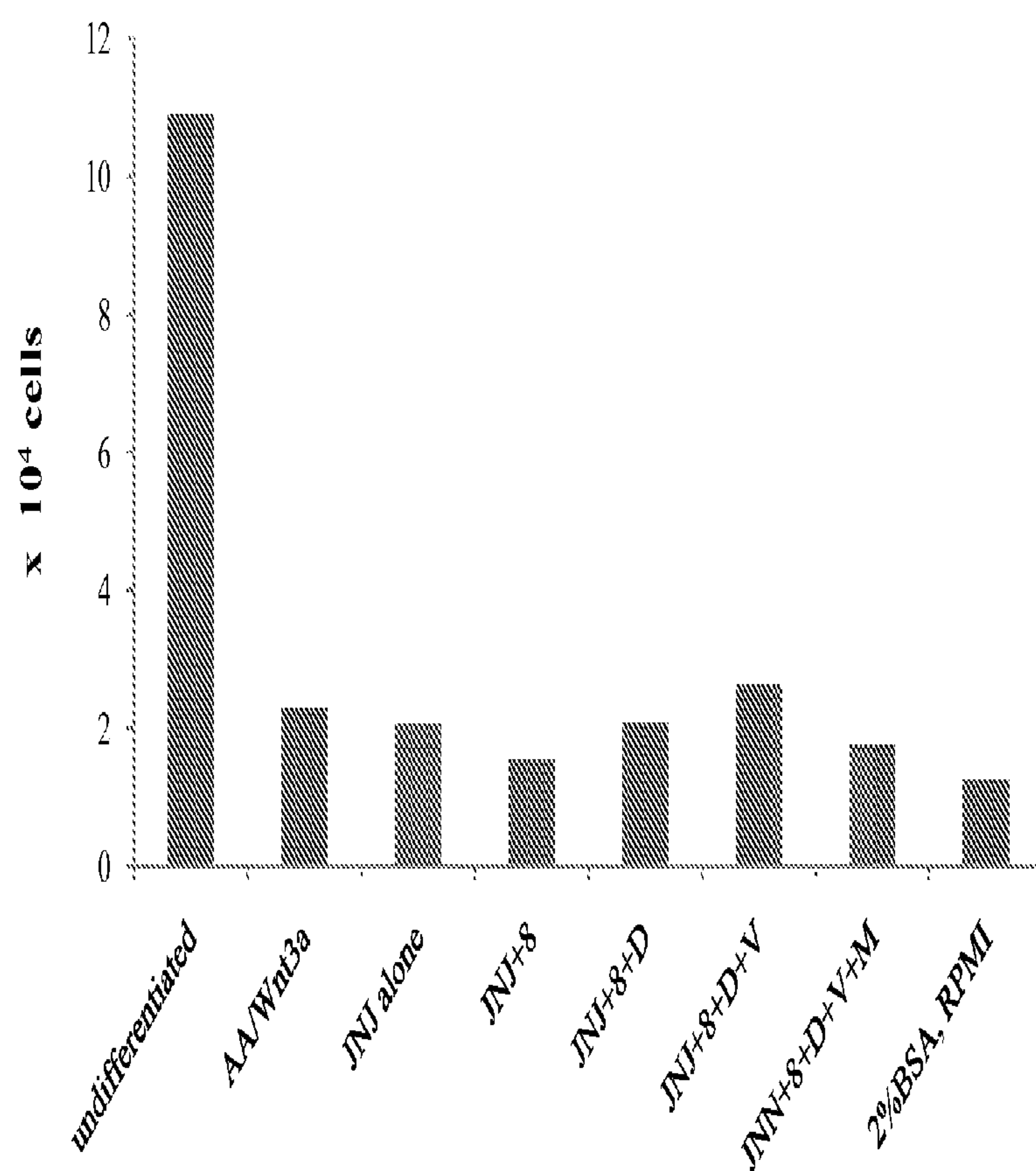


Figure 25B

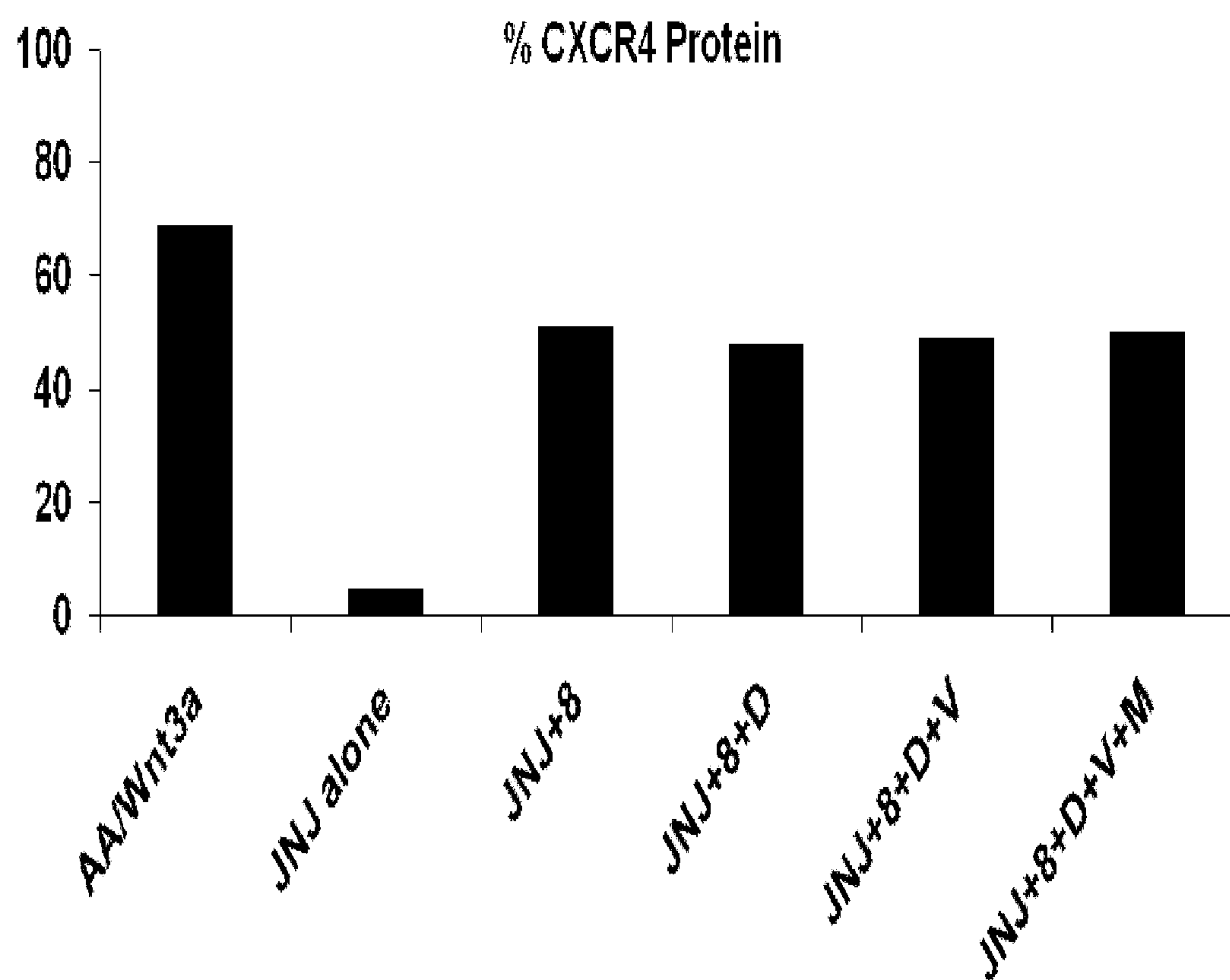


Figure 26A

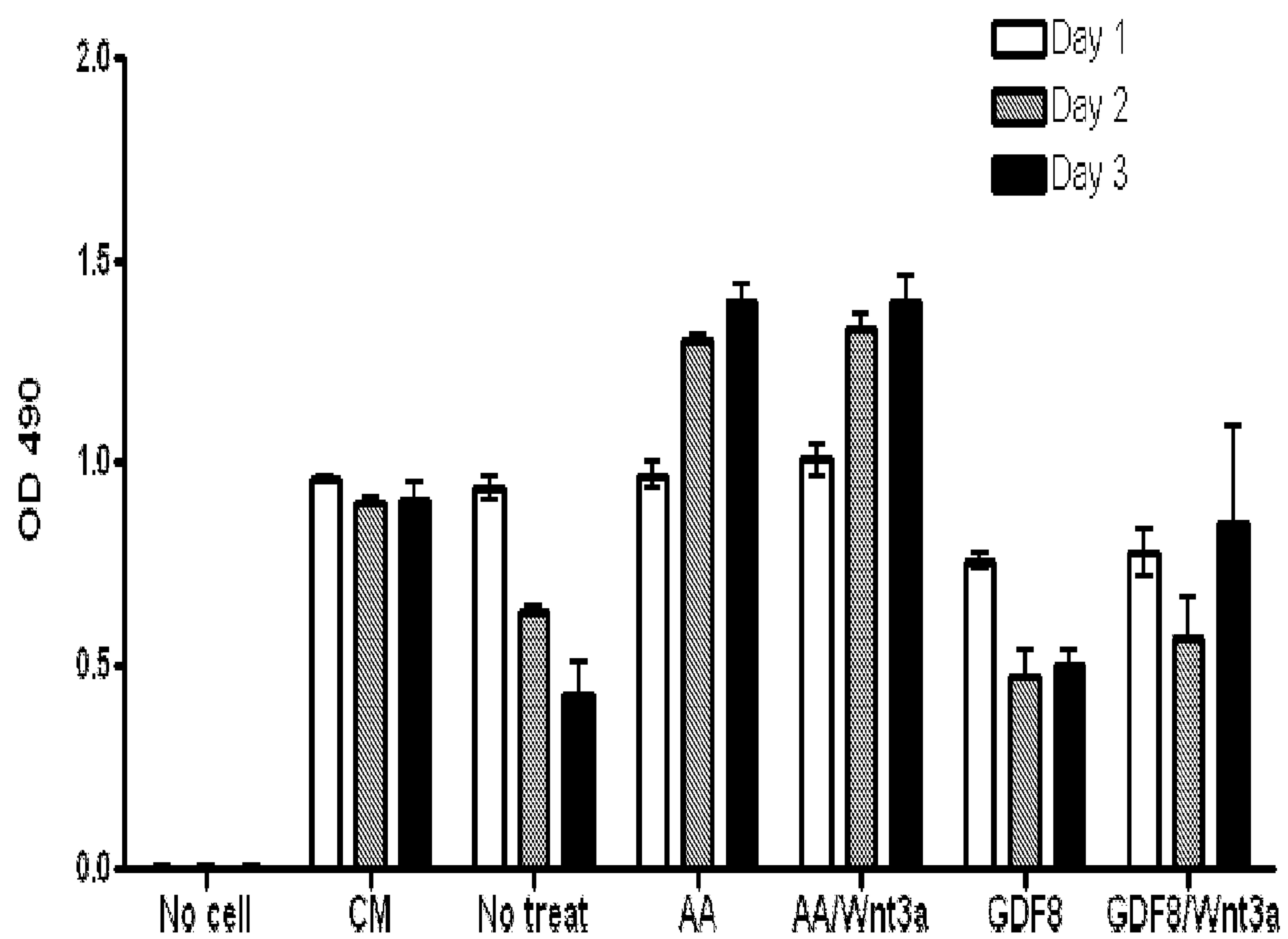


Figure 26B

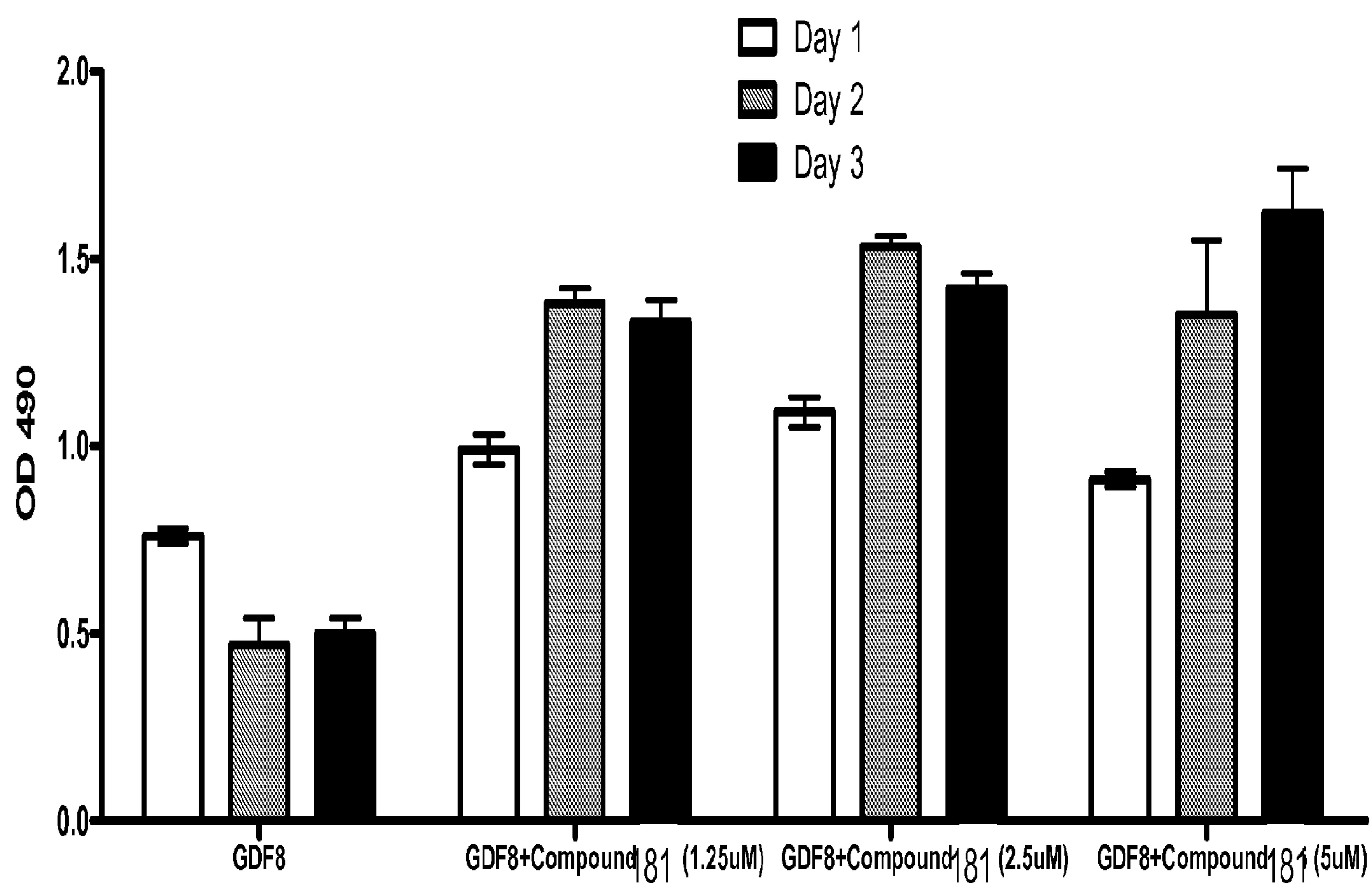


Figure 26C

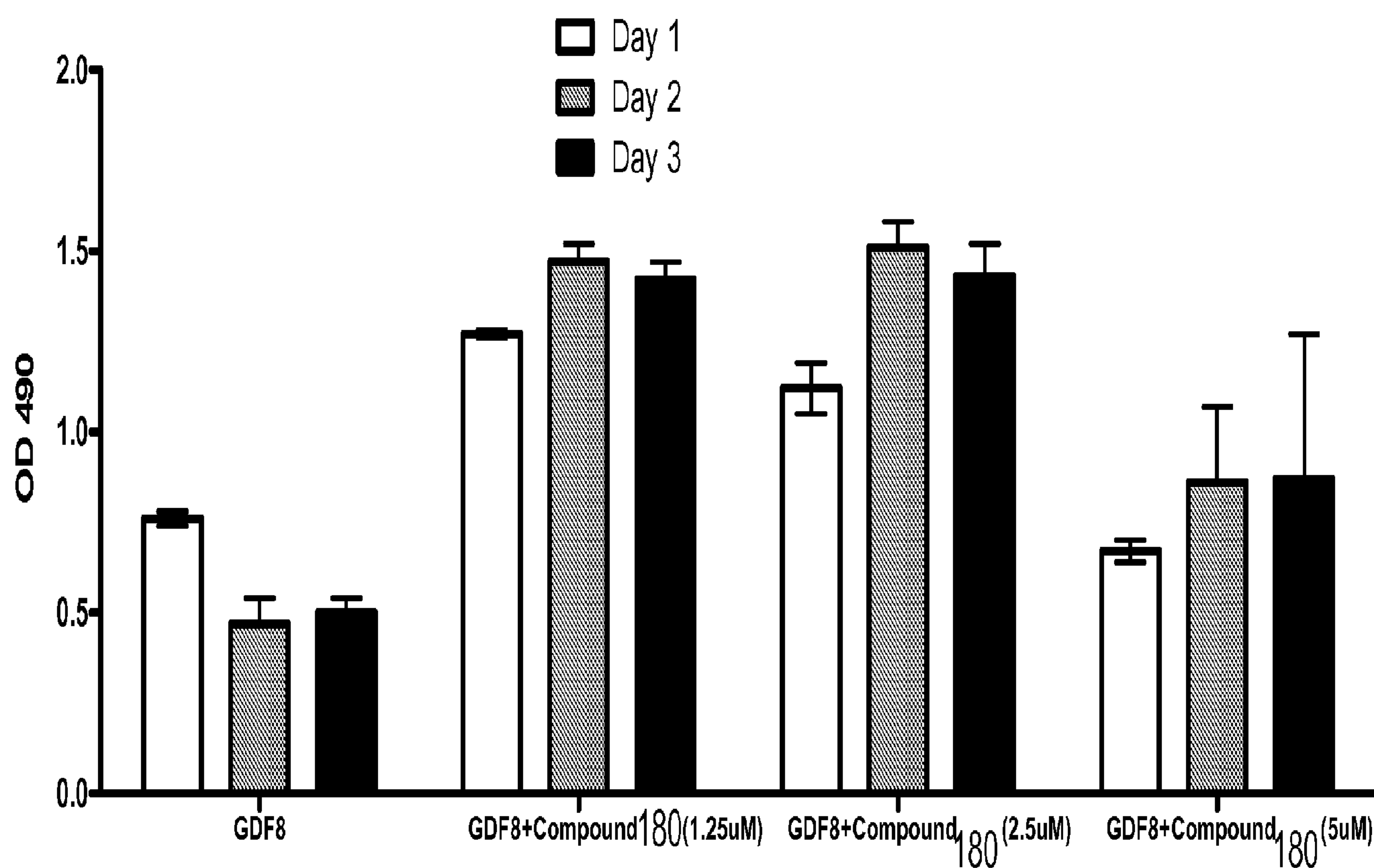


Figure 26D

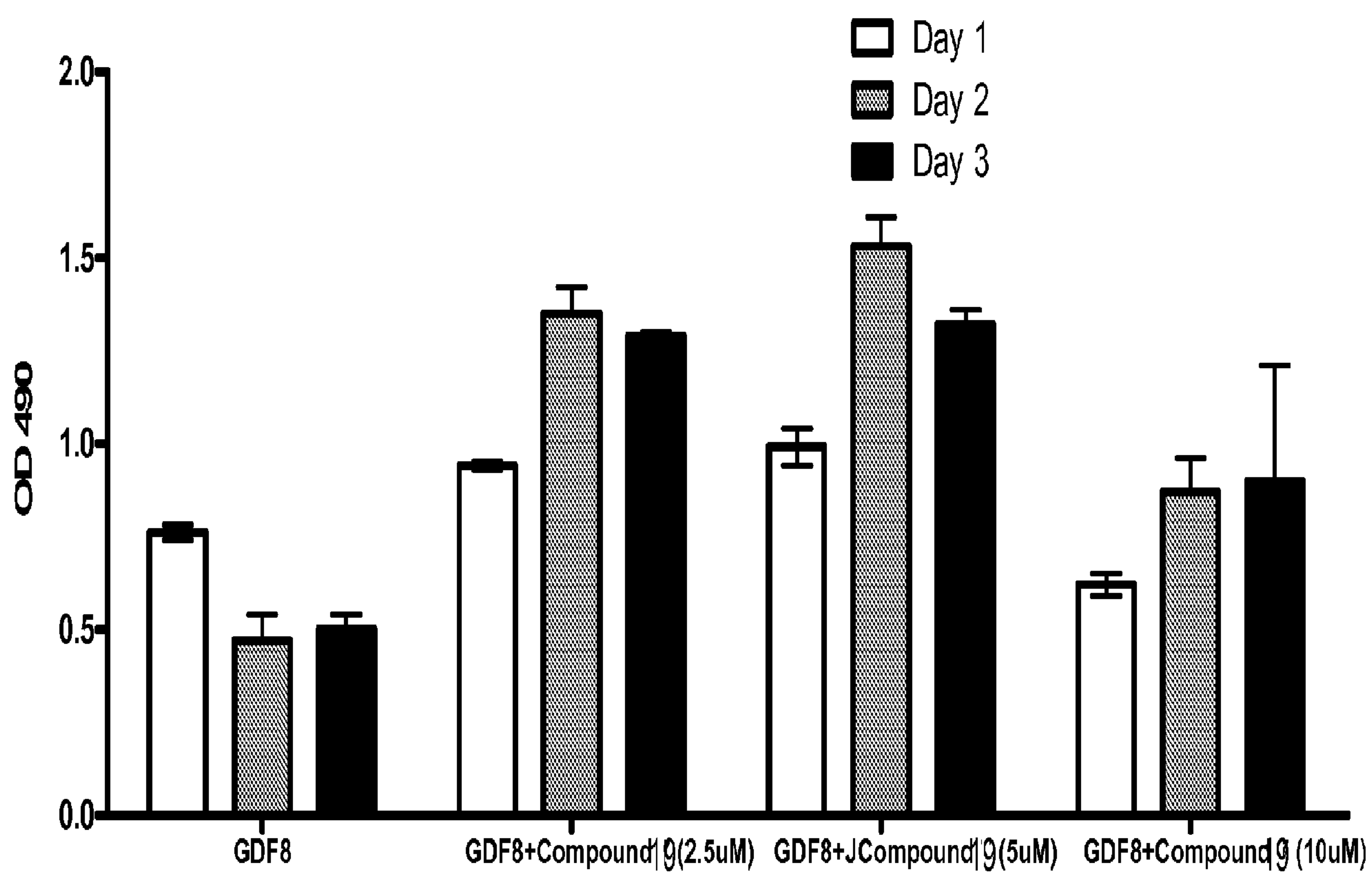


Figure 26E

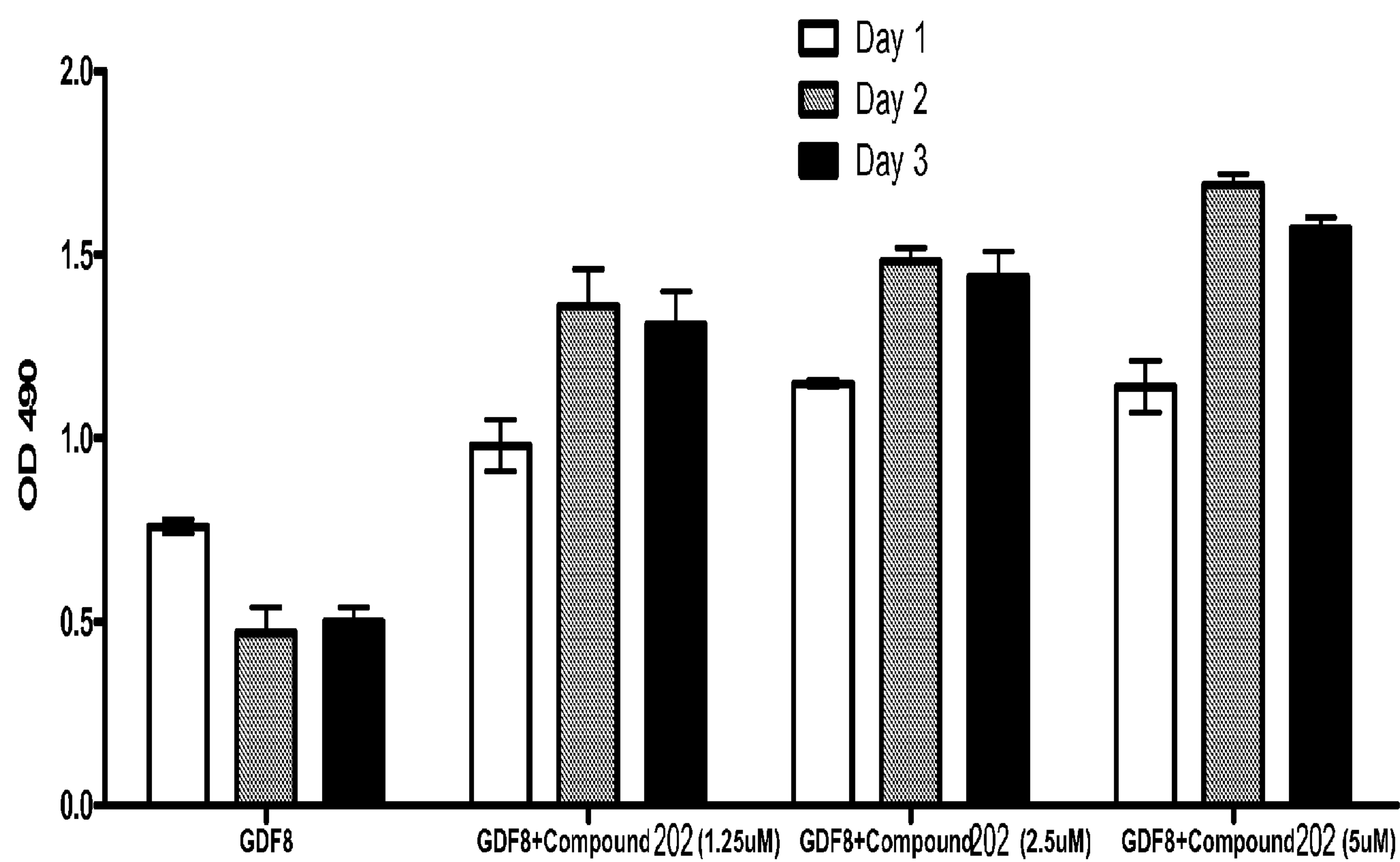


Figure 26F

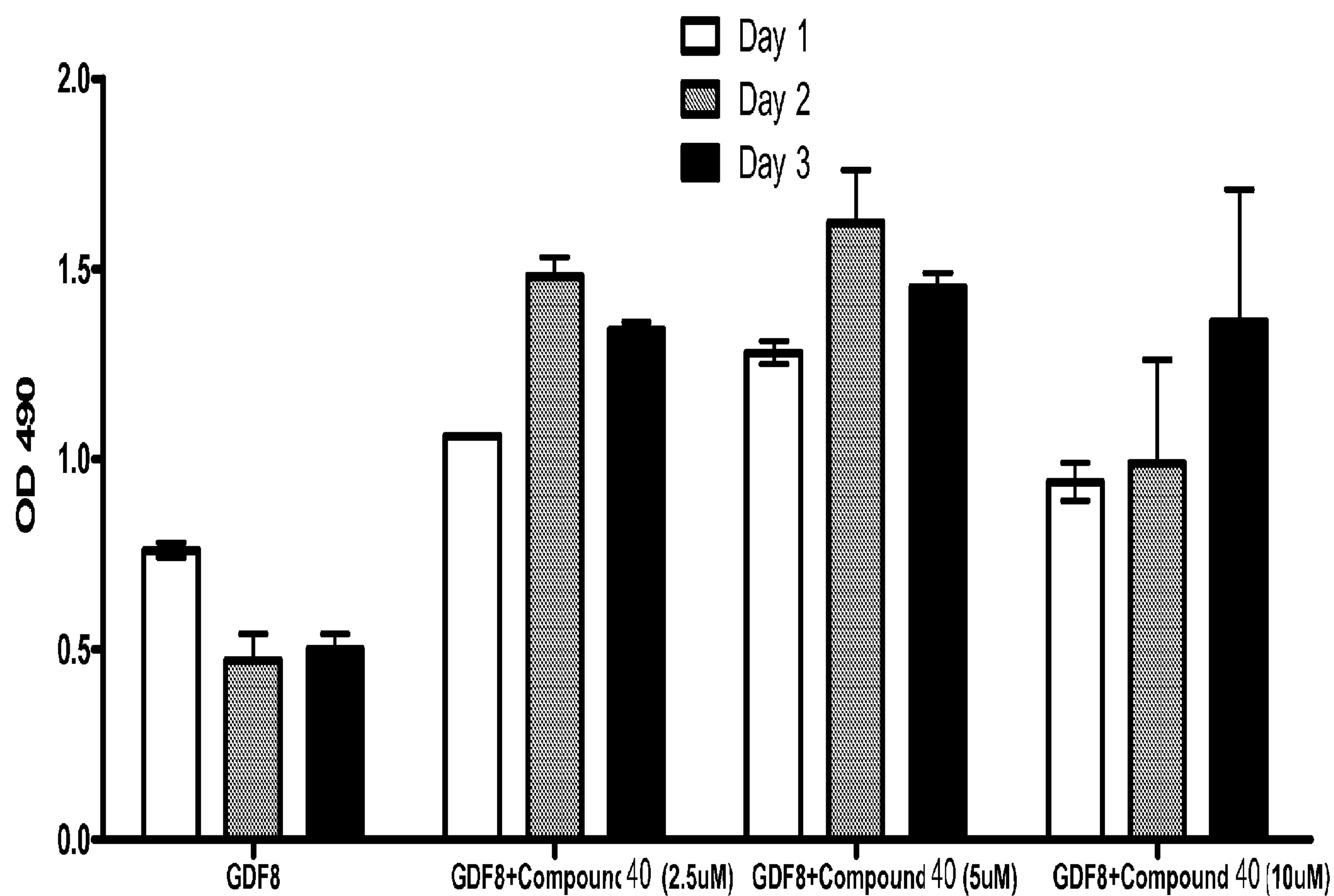


Figure 26G

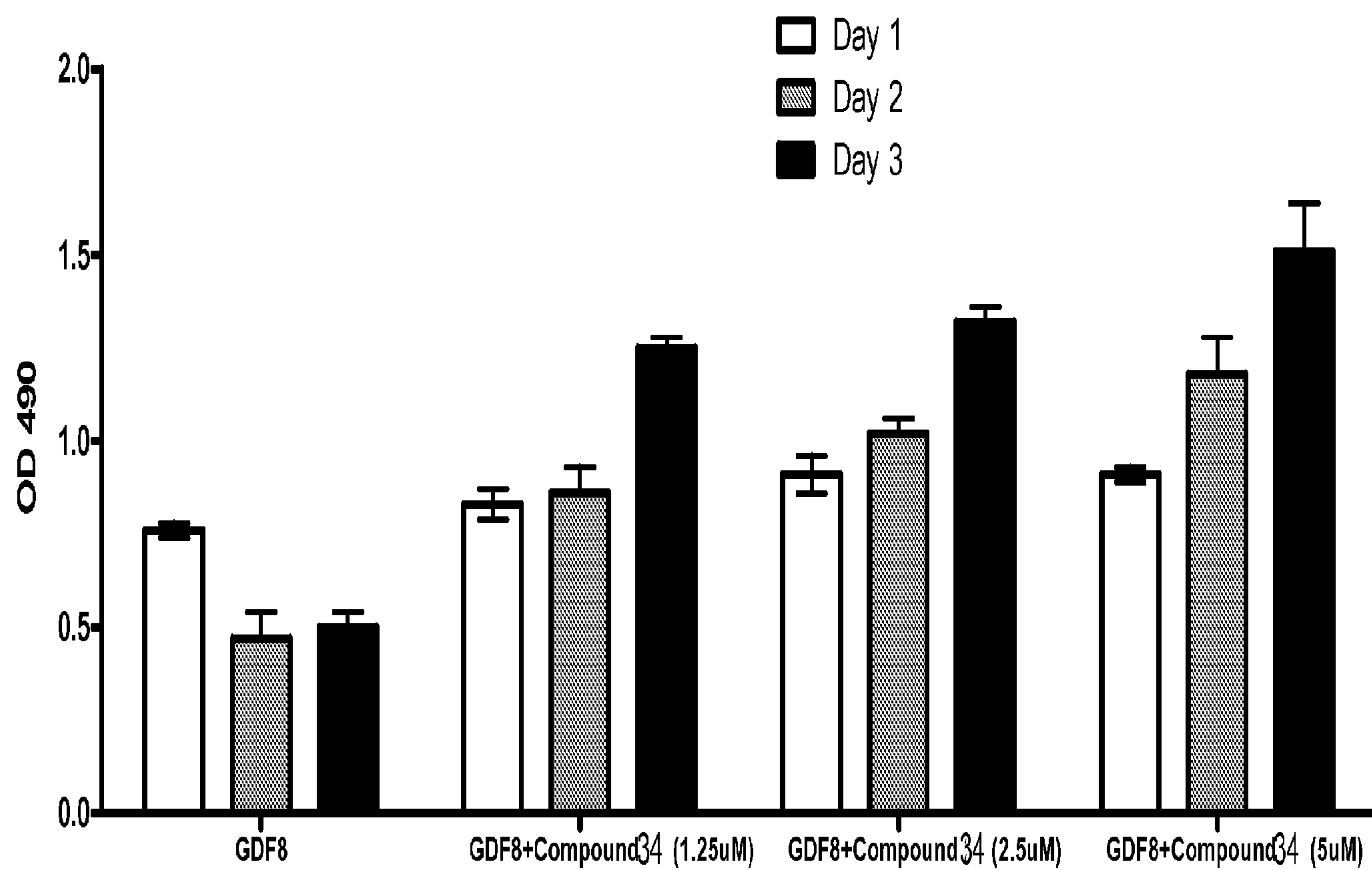


Figure 26H

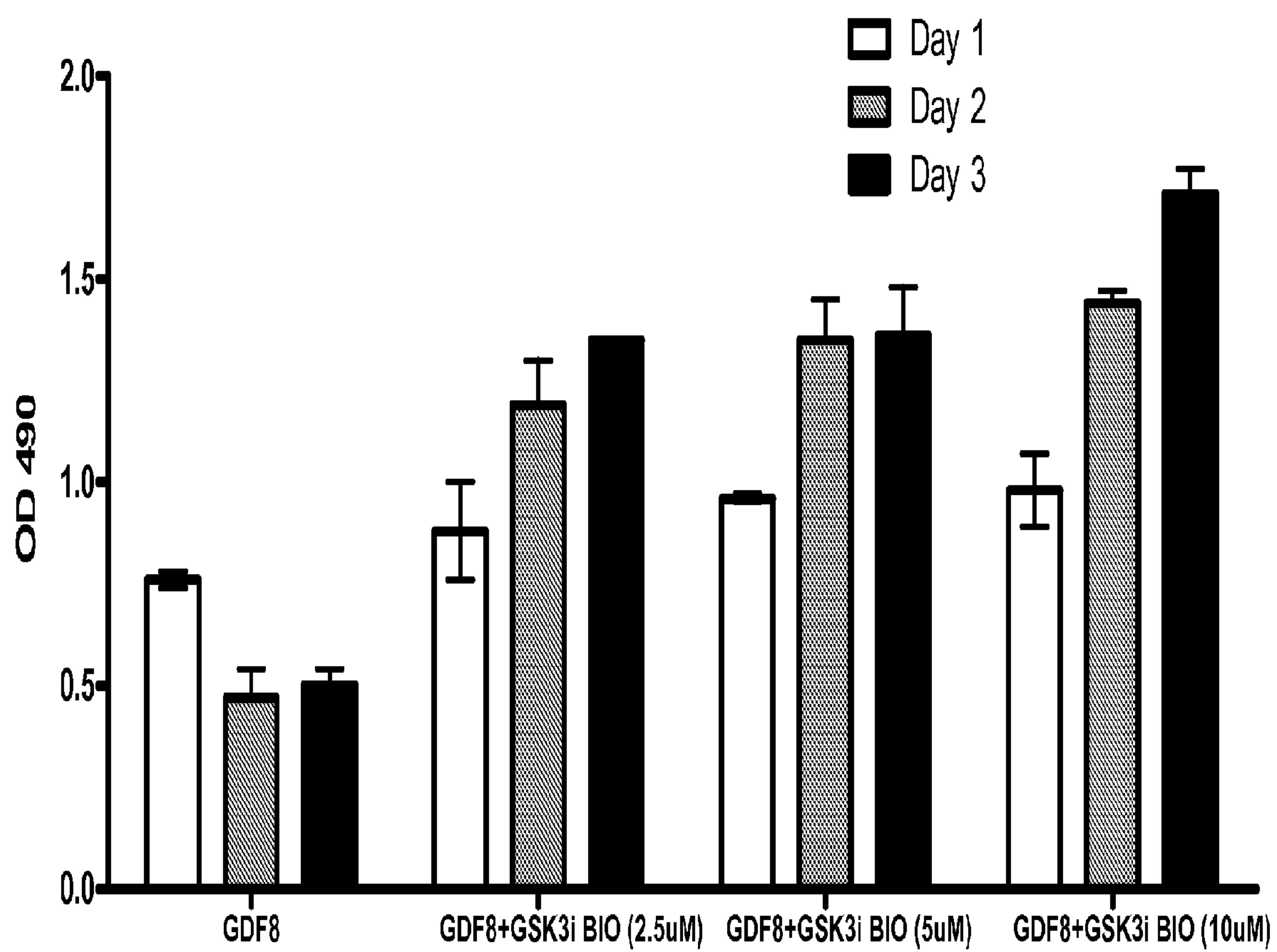
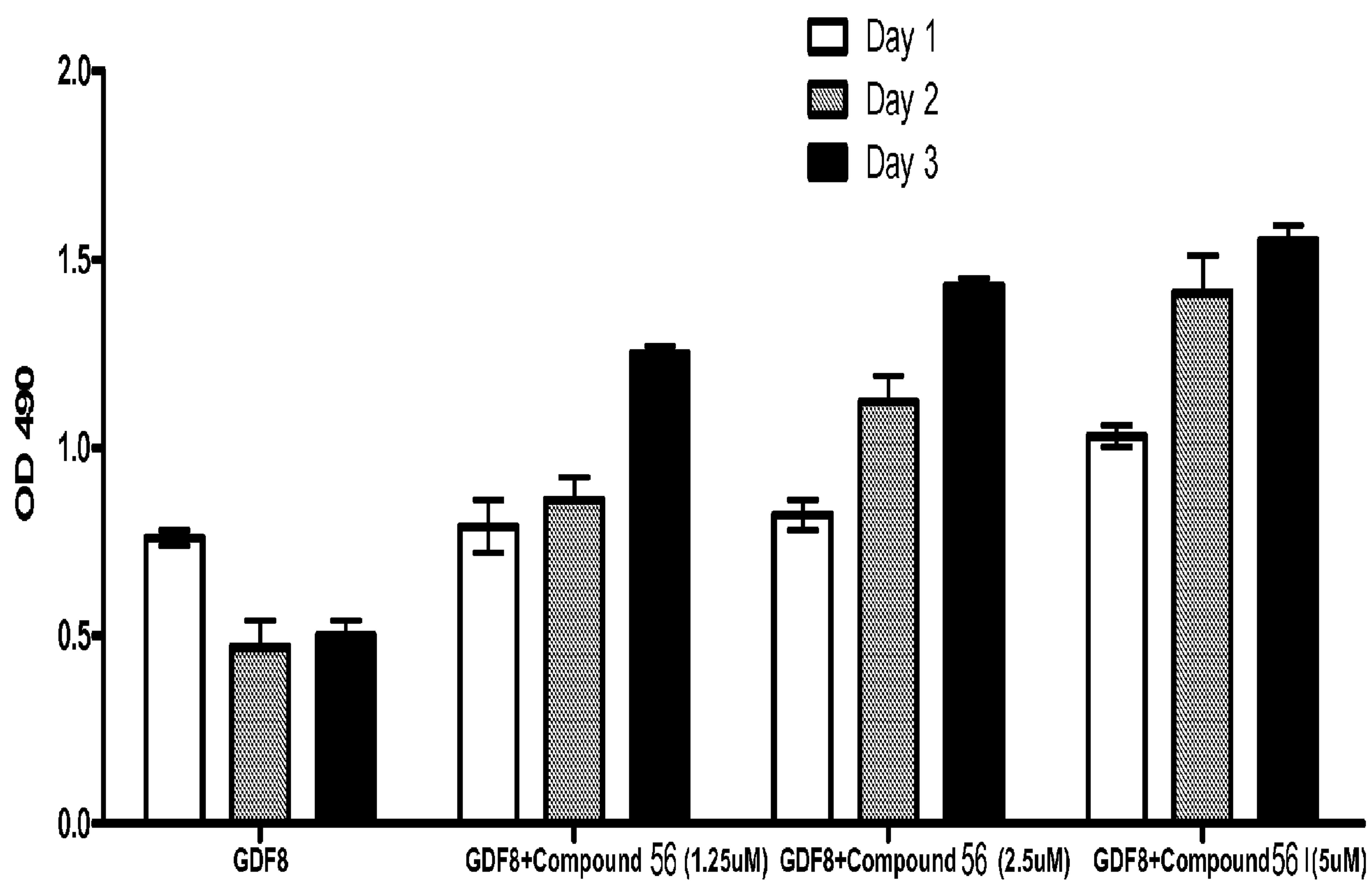


Figure 26I



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Figure 27A

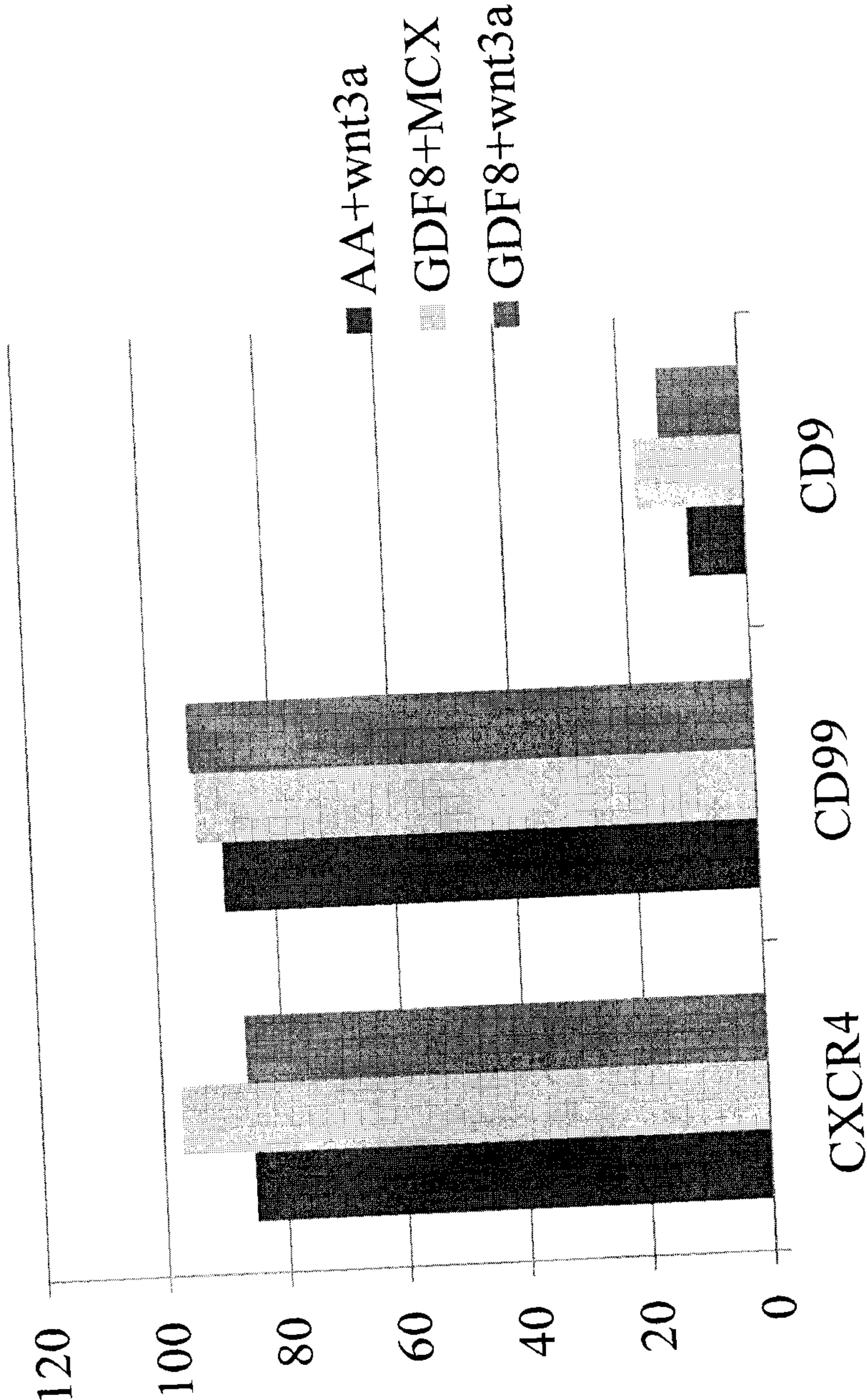


Figure 27B

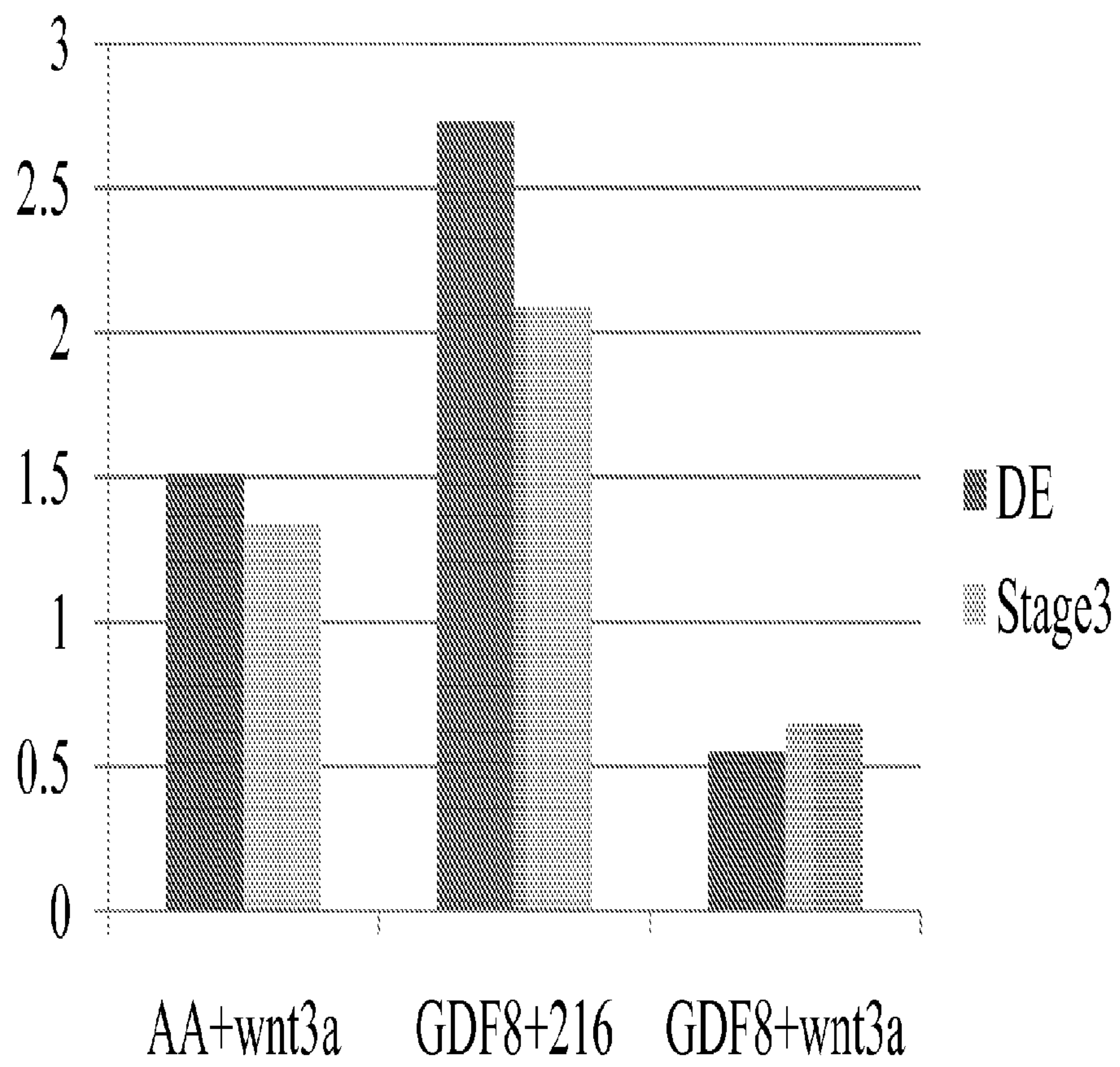


Figure 27C

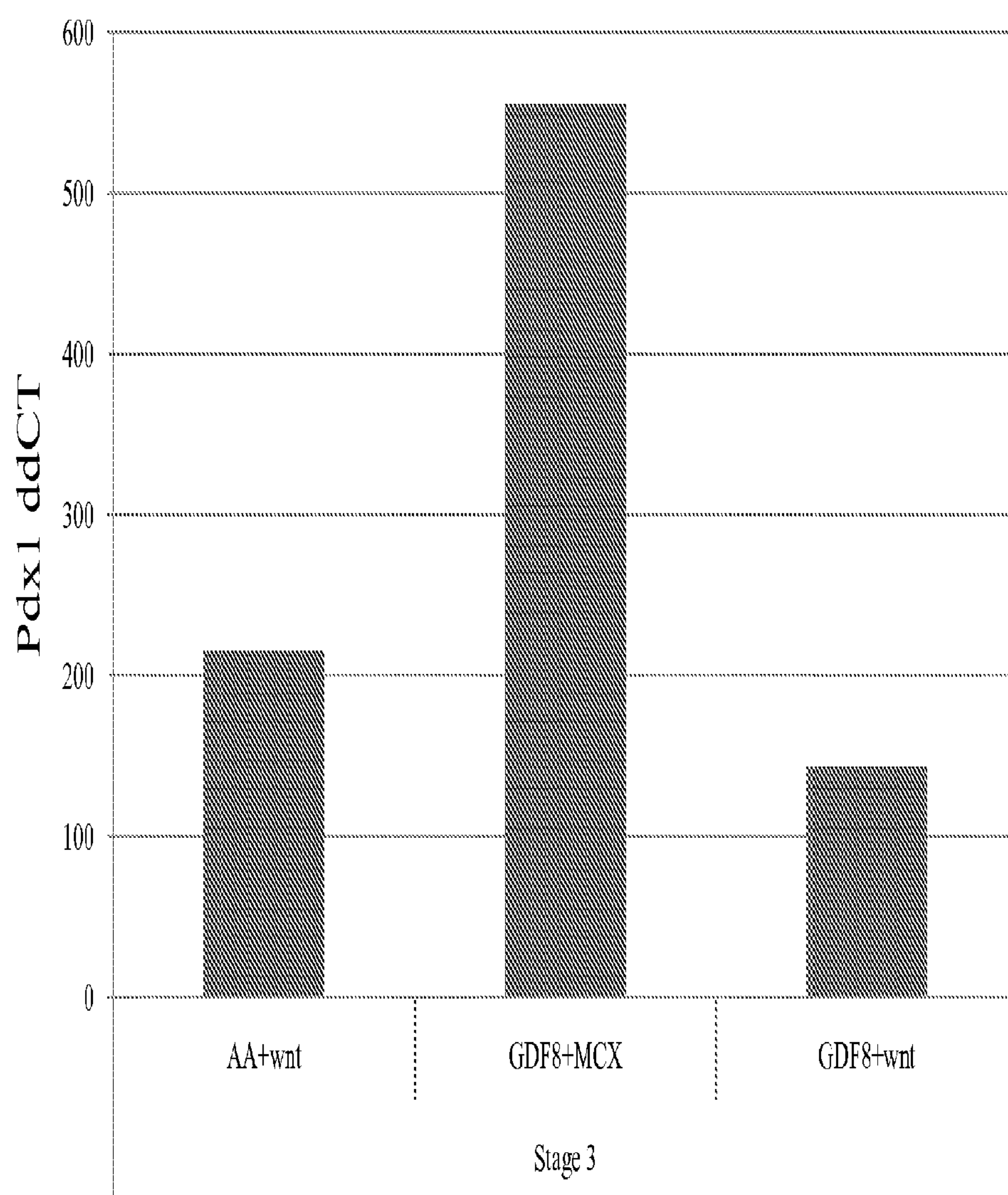


Figure 27D

Stage 3	AA+wnt	GDF8+MCX	GDF8+wnt
Pdx	215	555	142
Cdx2	1614	1567	965
HNF4a	339	210	267

Figure 27E

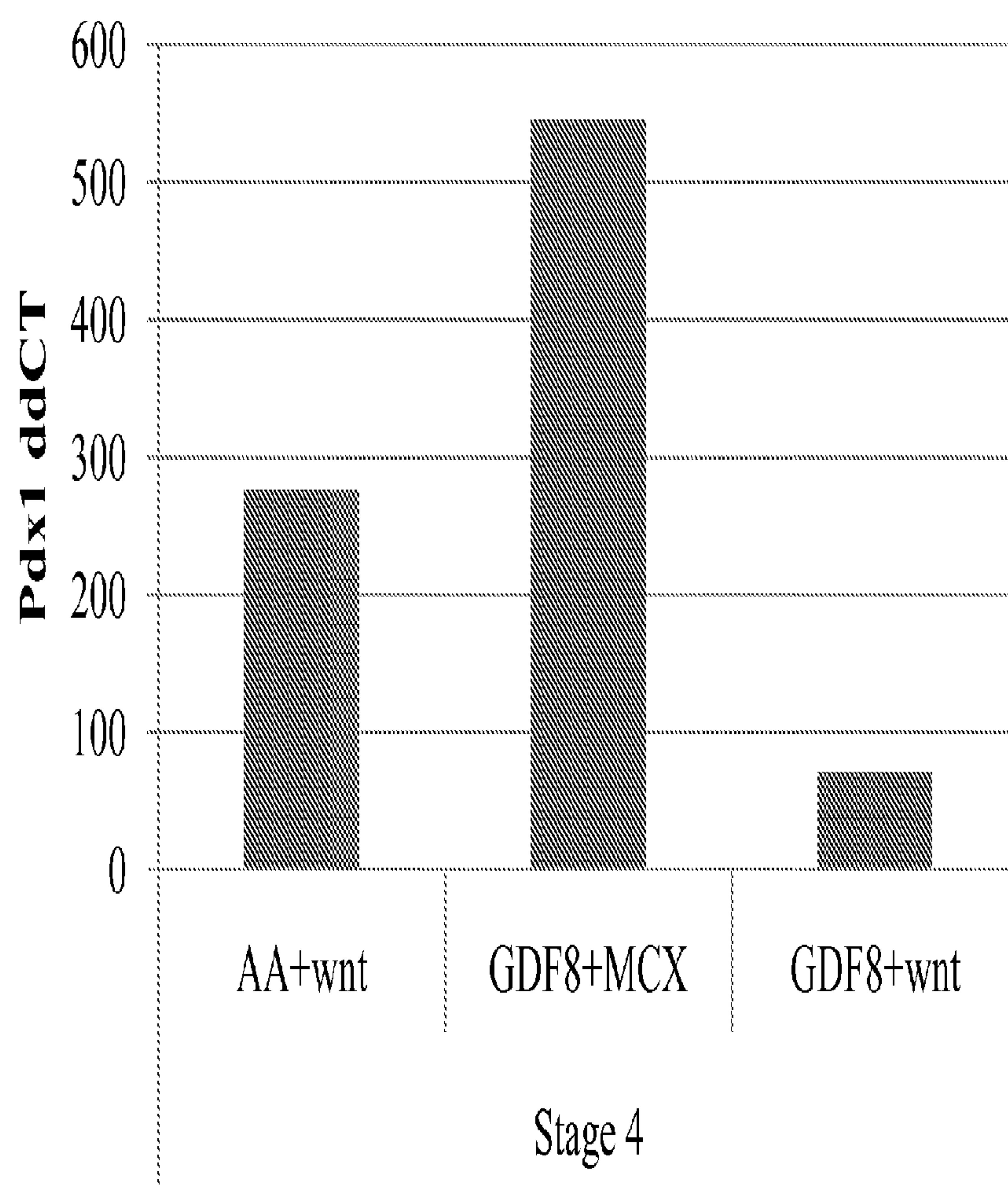


Figure 27F

Stage 4	AA+wnt	GDF8+MCX	GDF8+wnt
Pdx	276	545	71
Cdx2	1971	2496	983
HNF4a	640	426	609

