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(54) Title: PHARMACOLOGICAL TARGETING OF DE NOVO SERINE/GLYCINE SYNTHESIS

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DE NOVO SERINE AND GLYCINE SYNTHESIS

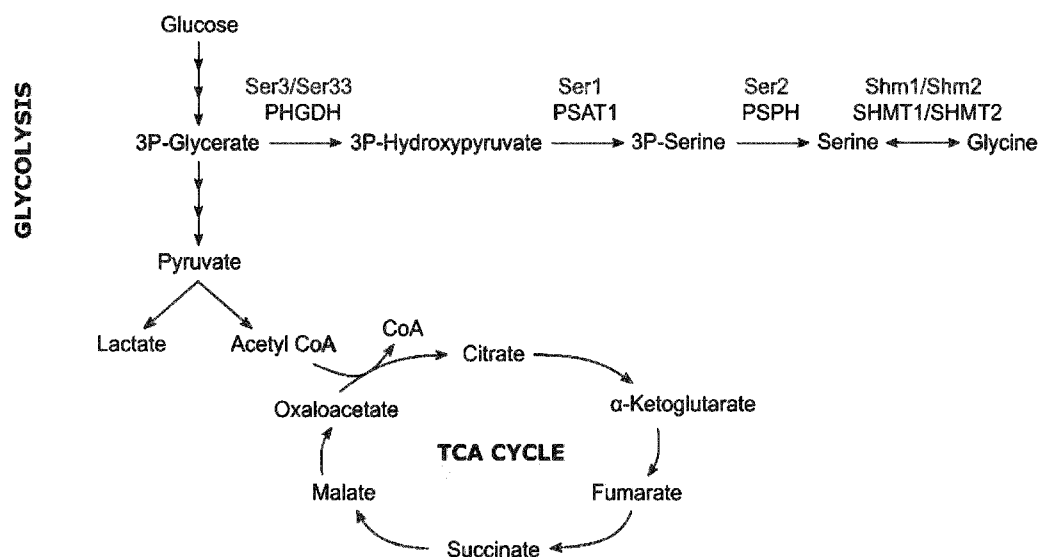


Figure 1

(57) **Abstract:** The invention relates to sertraline for use in the treatment of a cancer with increased serine/glycine synthesis, such as certain types of breast cancer or T-cell acute lymphoblastic leukaemia. Increased serine/glycine synthesis is for example obtained via increased expression of PSPH, PHGDH, PSAT1, SHMT1 or SHMT2.



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PHARMACOLOGICAL TARGETING OF *DE NOVO* SERINE/GLYCINE SYNTHESIS

FIELD OF THE INVENTION

- 5 The invention relates to the treatment of a specific subset of cancers.
The invention further relates to the discovery of a new target of sertraline and its use in cancer treatment.

BACKGROUND OF THE INVENTION

- 10 Particular microbial cells can exist as both an independent planktonic form or as a biofilm, defined as a structured multicellular community, attached to a surface and enclosed within an extracellular matrix. Of all microbial infections in the human body, 80% is biofilm-associated. *Candida albicans*, the major fungal pathogen for humans, typically forms drug-resistant biofilms on biotic surfaces such as the skin,
15 the mouth, the human gastro-intestinal tract and genital area. As *C. albicans* is classified as an opportunistic pathogen, people susceptible to this type of infection are mostly immunocompromised individuals, e.g. HIV patients, elderly people and young children.
- The current treatment of mucosal fungal infections remains a topical azole
20 treatment. Azoles, such as miconazole, inhibit lanosterol 14 α -demethylase, a key-enzyme in the biosynthesis of ergosterol, which is the main fungal sterol. This results in ergosterol depletion, accumulation of toxic ergosterol precursors and consequently growth inhibition. contrast to free-living *C. albicans* cells, the increasing tolerance of biofilm cells to commonly used azoles makes biofilm-
25 associated infections hard to eradicate. Specifically, *C. albicans* biofilm cells are up to thousand-fold more tolerant to azoles than their planktonic counterparts. To obtain more insight in miconazole-induced tolerance pathways in *C. albicans* biofilm cells, an in-depth transcriptome analysis was previously performed [De Cremer et al. (2016) *Sci. Rep.* **6**, 1–14].
- 30 In the field of oncology, rewiring of metabolic processes is one of the ten hallmarks of cancer. In contrast to normal cells, several cancer subtypes have been shown to depend on *de novo* serine synthesis for their proliferation and survival [Locasale (2013) *Nature Rev. Cancer* **13**, 572–583]. The most well-established example is breast cancer, where 6% of samples display copy number gain of the gene
35 encoding phosphoglycerate dehydrogenase (PHGDH), the enzyme catalyzing the first reaction to convert the glycolysis metabolite 3-phospho-glycerate into serine (Figure 1A). Furthermore, 70% of estrogen receptor-negative breast tumors have

increased PHGDH protein levels, and inhibition of PHGDH via RNA interference or PHGDH inhibitors affects both cell proliferation and survival [DeBerardinis (2011) *Cell Metabol.* **14**, 285–286; Locasale et al. (2011) *Nature Genetics* **43**, 869–874; Possemato et al. (2011) *Nature* **476**, 346–350; Pacold et al. (2016) *Nature Chem. Biol.* **12**, 452–458; Mullarky et al. (2016) *Proc. Natl. Acad. Sci.* **113**, 1778–1783]. Subsequent studies showed that, besides PHGDH, expression levels of the mitochondrial enzyme serine hydroxymethyltransferase (SHMT2), converting serine into glycine in the mitochondria, is positively correlated with breast cancer grade [Yin (2015) *OncoTargets and Therapy* **8**, 1069–1074]. Additionally, both isoforms of SHMT are direct target genes of the *MYC* oncogene [Nikiforov et al. (2002) *Mol. Cell. Biol.* **22**, 5793–800]. *MYC* gene amplification appears in about 15.5% of all breast cancer biopsies [Liao & Dickson (2000) *Endocrine-Related Cancer* **7**, 143–164]. *MYC*-driven stimulation of serine synthesis by transcriptional upregulation of all three serine synthetic enzymes is also critical for sustaining survival and rapid proliferation of cancer cells under conditions of nutrient deprivation [Sun et al. (2015) *Cell Res.* **25**, 429–444].

WO2007006799 suggests combination treatments of an alkaloid and sertraline for cancer treatment in general. The compounds have been tested on a leukemia cell line (U937) and a breast cancer cell line (MDA-MB-231). Gwynne et al. (2017) *Oncotarget* **8**, 32101–32116, evaluate various antidepressants on their capacity to inhibit sphere formation of various breast cancer cell lines.

SUMMARY OF THE INVENTION

Sertraline has been suggested as a compound which may provide a synergistic effect with anticancer drugs. In addition, a correlation has been sought between serotonin metabolism in certain cancers and the suggested antitumor effect of sertraline [Gwynne et al. (2017) *Oncotarget* **8**, 32101–32116]. The present invention identifies serine synthesis as a new pathway for cancer treatment by sertraline. This knowledge allows to define a hitherto unidentified group of cancers that may be particularly suitable for treatment with sertraline.

Transcriptome analysis revealed an upregulation of genes involved in *de novo* serine and glycine synthesis (Figure 1A).

Given the similarity between upregulation of serine and glycine synthesis genes in miconazole-stressed *C. albicans* biofilms and the dependency of breast cancer cells on *de novo* serine and glycine synthesis, it was investigated if compounds

sensitizing *C. albicans* biofilms to miconazole, so-called miconazole potentiators, also have *de novo* serine synthesis specific anticancer cell activity.

Genes involved in serine synthesis are overexpressed in drug-tolerant biofilms of the pathogenic fungus *Candida albicans* upon exposure to suboptimal concentrations of the antifungal miconazole. Furthermore, *de novo* serine synthesis has recently gained attention for its role in malignant cell transformation, with a subset of breast cancers being dependent on this process. There are thus conditions in which both lower and higher eukaryotic cells depend on serine synthesis to survive and proliferate and targeting serine synthesis can be a strategy for the treatment of breast cancer as well as for *C. albicans* biofilm-based infections. Screening a library of repurposed drugs for their capacity to potentiate miconazole's antibiofilm activity followed by validation of hits in serine synthesis dependent breast cancer cells identified sertraline, thimerosal and benzalkonium chloride as agents that selectively inhibit proliferation of serine synthesis dependent *Candida albicans* biofilms and breast cancer cells. Sertraline is a clinically used anti-depressant that impaired serine and glycine synthesis in breast cancer cells and whose activity could be rescued by providing cells with extra serine. Most notably, combining sertraline with the mitochondrial inhibitor artemether resulted in an even more pronounced inhibition of *de novo* serine synthesis dependent proliferation, both in cultured cells and in breast cancer xenografts. In conclusion, compounds were identified, including the commonly used anti-depressant sertraline, that inhibit both miconazole-stressed *C. albicans* biofilms and *de novo* serine synthesis dependent breast cancer cells, emphasizing that the upregulation of serine synthesis might be a conserved survival strategy in lower and higher eukaryotes.

The invention relates to antagonists of a serine or glycine synthesis gene for use in the treatment of a cancer with increased serine synthesis, such as PHGDH, PSAT1, PSPH or SHMT1/SHMT2.

Typically such cancer is breast cancer. Examples hereof are breast cancers with a copy number gain of such a serine synthesis gene, estrogen receptor negative breast cancer with increased PHGDH expression, breast cancers with a copy number gain of MYC.

Other cancers suitable in the context of the present invention are melanoma, glioblastoma brain tumors, prostate, testis, ovary, liver, kidney, pancreas, head and neck cancer, lung adenomas, and bladder cancer, wherein increased

expression of PSPH occurs, by PSHP gene duplication or mutations resulting in increased PSPH activity.

Other cancers suitable in the context of the present invention are pediatric T-cell leukemia cancers with RPL10 mutations (such as Arg98Ser mutation).

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The antagonist for use in these treatments is typically sertraline, thimerosal or benzalkonium chloride.

Cancers which can be treated by sertraline can be identified by a method comprising a step of determining increased serine/glycine synthesis. In this context, it was observed that the fraction of labeled serine and glycine that is detected in breast cancer cell lines when providing them with $^{13}\text{C}_6$ -glucose is an indicator of serine/ glycine synthesis levels and that this fractional contribution measure correlates with sertraline sensitivity. Based on these results, cells in which >5% of serine and >3% of glycine derived from $^{13}\text{C}_6$ -labeled glucose upon 48 hrs incubation (fractional contribution serine >5% and glycine >3%) are likely to be sensitive to sertraline.

In this context, the present invention also discloses methods of determining whether a cancer is sensitive for sertraline, comprising the steps of determining in a cancer tissue sample whether serine synthesis is increased as compared with a reference sample.

The invention is further summarized in the following statements.

1. Sertraline for use in the treatment of a breast cancer with increased serine/glycine synthesis.
- 25 2. Sertraline for use in the treatment according to statement 1, wherein the breast cancer has a copy number gain of a serine/glycine synthesis gene selected from the group consisting of PHGDH, PSAT1, PSPH, SHMT1 and SHMT2.
3. Sertraline for use in the treatment according to statement 1 or 2, with increased PHGDH expression or increased SHMT1 or SHMT2 expression.
- 30 4. Sertraline for use in the treatment according to any one of statements 1 to 3, wherein the breast cancer is an estrogen receptor negative cancer with increased PHGDH expression or increased SHMT1 or SHMT2 expression.
5. Sertraline for use in the treatment according to any one of statements 1 to 4, wherein the breast cancer has a copy number gain of MYC.
- 35 6. Sertraline for use in the treatment according to any one of statements 1 to 5, wherein the breast cancer is a triple negative cancer.

7. A method of determining whether a cancer is sensitive for sertraline, comprising the steps of determining in a cancer tissue sample whether serine/glycine synthesis is increased as compared with a reference sample.
8. Sertraline for use in the treatment of a T-cell acute lymphoblastic leukaemia (T-ALL) with increased serine/glycine synthesis
9. Sertraline for use in the treatment according to statement 8, wherein said leukemia is a pediatric T-ALL.
10. Sertraline for use in the treatment according to statement 8 or 9, wherein said leukemia has increased expression of PSPH or PHGDH of at least 1.5 fold higher than normal tissue.
11. Sertraline for use in the treatment according to any one of statements 8 to 10, wherein said leukemia has an RPL10 mutation.
12. Sertraline for use in the treatment according to any one of statements 8 to 11, wherein said RPL10 mutation is R98S.
13. Sertraline for use in the treatment according to statement 11, wherein said RPL10 mutation is selected from the group consisting of I33V, E66G, I70M, I70L R98C, H123P and Q123P.
14. Sertraline for use in the treatment according to any one of statements 8 to 11, wherein said leukemia has a NK2 Homeobox 1 (NKX2-1) mutation
15. Sertraline for use in the treatment according to any one of statement 7 to 14, wherein the leukemia has a copy number gain of a serine/glycine synthesis gene selected from the group consisting of PHGDH, PSAT1, PSPH, SHMT1 and SHMT2.
16. Sertraline for use in the treatment of a cancer with increased serine/glycine synthesis.
17. Sertraline for use in the treatment according to statement 16, wherein said cancer has an increased expression of PSPH (phosphoserine phosphatase), PHGDH (phosphoglycerate dehydrogenase), PSAT1 (phosphoserine aminotransferase), SHMT1 (serine hydroxymethyltransferase2) or SHMT2 (serine hydroxymethyltransferase 2).
18. Sertraline for use in the treatment according to statement 17, wherein the PSPH protein has a Val116Ile mutation.
19. Sertraline for use in the treatment according to statement 16 or 17, wherein the cancer has a copy number gain of a serine/glycine synthesis gene selected from the group consisting of PHGDH, PSAT1, PSPH, SHMT1 and SHMT2.
20. Sertraline for use in the treatment according to any one of statements 16 to 19, wherein said cancer is selected from the group consisting of melanoma, glioblastoma brain tumor, prostate cancer, testis cancer, ovary cancer, liver

cancer, kidney cancer, pancreas cancer, head and neck cancer, lung adenoma, and bladder cancer.

21. Sertraline for use in the treatment according to any one of statements 16 to 20, wherein said cancer is a breast cancer.

5 22. Sertraline for use in the treatment according to statement 20 or 21, wherein said breast cancer has increased SHMT1 and/or SHMT2 expression levels.

23. Sertraline for use in the treatment according to any one of statement 20 to 22, wherein said breast cancer is an estrogen receptor negative cancer.

10 24. Sertraline for use in the treatment according to statement 23, wherein said estrogen receptor negative cancer has an increased PHGDH protein level.

25. Sertraline for use in the treatment according to any one of statements 20 to 24, wherein said breast cancer has a copy number gain of MYC.

26. Sertraline for use in the treatment according to statement 20 to 24, wherein said breast cancer is a triple negative cancer.

15 27. Sertraline for use in the treatment according to any one of statements 16 to 19, wherein said cancer is a T-cell acute lymphoblastic leukaemia (T-ALL).

28. Sertraline for use in the treatment according to statement 27, wherein said leukemia is a pediatric T-ALL.

20 29. Sertraline for use in the treatment according to statement 27 or 28, wherein said leukemia has an RPL10 mutation.

30. Sertraline for use in the treatment according to statement 29, wherein said RPL10 mutation is R98S.

25 31. Sertraline for use in the treatment according to statement 29, wherein said RPL10 mutation is selected from the group consisting of I33V, E66G, I70M, I70L R98C, H123P and Q123P.

32. Sertraline for use in the treatment according to statement 27 or 18, wherein said leukemia has a NKX2-1 rearrangement.

30 33. A combination of sertraline and a further anticancer drug for use in the treatment of a cancer with increased serine/glycine synthesis according to any one of statements 16 to 32.

34. An in vitro method of determining whether a cancer is sensitive for sertraline, comprising the steps of determining in a cancer tissue sample whether serine/glycine synthesis is increased as compared with a reference sample.

35 35. A method of treating in an individual a cancer with increased serine/glycine synthesis, comprising the step of administering to said individual an effective amount of sertraline.

FIGURE LEGENDS**Figure 1. Sertraline is a potentiator of miconazole against *C. albicans* biofilms.**

- A) Overview of glucose metabolism including *de novo* serine and glycine synthesis, as a side chain of glycolysis, and the citric acid (TCA) cycle, downstream of glycolysis. Human (blue) and *C. albicans* (green) serine/glycine synthetic enzymes are shown. PHGDH = phosphoglycerate dehydrogenase; PSAT = phosphoserine aminotransferase; PSPH = phosphoserine phosphatase; SHMT = serine hydroxymethyltransferase.
- 5 B) Serine (left) and glycine (right) levels in the medium ($\mu\text{mol/l}$) of *C. albicans* biofilms treated with DMSO (0.2%) or miconazole (75 μM) for 24 hours. Student's t-test: serine $p = 0.0066$ and glycine $p = 0.0269$.
- C) Relative reductive potential (%), measured with a Cell-Titer Blue (CTB) assay, of *C. albicans* biofilms grown in 6-well plates and treated with DMSO (0.2%) or miconazole (75 μM) for 24 hours. Student's t-test, $p = 0.1550$.
- 15 D) Synergy testing using calcsyn (left) and SyngeryFinder (right). Metabolic activity, measured with a Cell-Titer Blue (CTB) assay, of *C. albicans* biofilms grown in 96-well plates and treated with miconazole (0, 37.5 or 75 μM) and sertraline (0 – 200 μM) for 24 hours, was used as a read-out.
- 20 E) Cell death, measured with a propidium iodide (PI) assay, of *C. albicans* biofilms grown in 96-well plates and treated with DMSO (1.075%), miconazole (75 μM), sertraline (75 μM) or a combination of both. Ordinary two-way ANOVA, Tukey's multiple comparisons test, DMSO vs. miconazole $p = 0.0343$, DMSO vs. sertraline $p = 0.2537$ and DMSO vs. combination $p < 0.0001$.
- 25 The number of biological replicates for each experiment (B-E) was $n \geq 3$. All error bars represent standard deviations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. Miconazole potentiators can selectively inhibit *de novo* serine and glycine synthesis dependent breast cancer.

- 30 A) *In vitro* proliferation of MDA-MB-231 (left) and MDA-MB-468 (right) treated with 0 (DMSO 0.1%), 3 or 5 μM sertraline as determined by real-time monitoring of cell confluence (%). Ordinary one-way ANOVA, Dunnett's multiple comparisons test: MDA-MB-231 3 μM $p = 0.9830$, MDA-MB-231 5 μM $p = 0.0089$, MDA-MB-468 3 μM $p = 0.0010$ and MDA-MB-468 5 μM $p < 0.0001$.
- 35 B) [sheet 4 and 5 of figures] *In vitro* proliferation of MDA-MB-231 (left) and MDA-MB-468 (right) treated with thimerosal, benzalkonium chloride, domiphen bromide or bupropion as determined by real-time monitoring of cell confluence (%).

Ordinary one-way ANOVA, Dunnett's multiple comparisons test: thimerosal MDA-MB-231 2,5 μM $p = 0.0169$, benzalkonium chloride MDA-MB-231 1 μM $p = 0.0008$, benzalkonium chloride MDA-MB-231 2.5 μM $p < 0.0001$, domiphen bromide $p < 0.0001$ and all MDA-MB-468 $p < 0.0001$.

- 5 The number of biological replicates for each experiment (A and B) was $n \geq 3$. All error bars represent standard deviations. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Figure 3. Sertraline reduces the proliferation of MDA-MB-468 breast cancer cells via inhibition of *de novo* serine and glycine synthesis.

- 10 A) Carbon incorporation from $^{13}\text{C}_6$ -glucose into serine and glycine showing that sertraline inhibits proliferation of MDA-MB-468 by decreasing *de novo* serine and glycine synthesis in a dose-dependent manner. Student's t-test: serine M+3 3 μM $p = 0.0026$, serine M+3 5 μM $p = 0.0006$, glycine M+2 3 μM $p = 0.0169$ and glycine M+2 5 μM $p = 0.0232$.
- 15 B) Relative abundance of intracellular serine and glycine (AU/ng protein), originating from both uptake and biosynthesis, of MDA-MB-468 cells treated with 0 (DMSO 0,1%), 3 or 5 μM sertraline for 48 hours. Ordinary one-way ANOVA, Dunnett's multiple comparisons test: serine 3 μM $p = 0.9862$, serine 5 μM $p = 0.8908$, glycine 3 μM $p = 0.0087$ and glycine 5 μM $p = 0.0048$.
- 20 C) Extracellular (medium) serine and glycine levels ($\mu\text{mol/l}$) of MDA-MB-468 cells treated with 0 (DMSO 0,1%), 3 or 5 μM sertraline for 48 hours. Ordinary one-way ANOVA, Dunnett's multiple comparisons test: serine 3 μM $p = 0.9035$, serine 5 μM $p = 0.9532$, glycine 3 μM $p = 0.2621$ and glycine 5 μM $p = 0.0713$.
- D) *In vitro* proliferation of MDA-MB-468 treated with DMSO (0,1%) or sertraline (3 or 5 μM) and cultured in DMEM without serine and glycine (control), with serine (800 μM) or with glycine (800 μM) as determined by real-time monitoring of cell confluence (%). Sertraline 3 μM in media with serine $p = 0.9656$.
- 25 The number of biological replicates for each experiment (A-D) was $n \geq 3$. All error bars represent standard deviations. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.
- 30

Figure 4. Combining artemether and sertraline further reduces *de novo* serine synthesis dependent breast cancer proliferation.

- A) *In vitro* proliferation of MDA-MB-231 (left) and MDA-MB-468 (right) treated with DMSO (0,2%), artemether (40 μM), sertraline (3 μM) or a combination of both compounds as determined by real-time monitoring of cell confluence (%).
- 35 Ordinary two-way ANOVA, Tukey's multiple comparisons test: MDA-MB-231 DMSO vs. artemether $p = 0.8540$, MDA-MB-231 DMSO vs. sertraline $p = 0.4432$, MDA-

MB-231 DMSO vs. combination $p = 0.0597$, MDA-MB-468 artemether vs. combination $p = 0.0008$; MDA-MB-468 sertraline vs. combination $p = 0.0011$.

B) *In vitro* proliferation of MCF7, dependent on serine uptake, (left) and HCC70, dependent on *de novo* serine synthesis, (right) treated with DMSO (0,2%),
 5 artemether (40 μ M), sertraline (3 μ M) or a combination of both compounds as determined by real-time monitoring of cell confluence (%). Ordinary two-way ANOVA, Tukey's multiple comparisons test: MDA-MB-231 DMSO vs. artemether $p = 0.0065$, MDA-MB-231 DMSO vs. sertraline $p = 0.9524$, MDA-MB-231 DMSO vs. combination $p = 0.0508$, MDA-MB-468 DMSO vs. artemether $p = 0.0103$, MDA-MB-468 DMSO vs. sertraline $p < 0.0001$, MDA-MB-468 DMSO vs. combination $p < 0.0001$, MDA-MB-468 artemether vs. combination $p < 0.0001$, MDA-MB-468 sertraline vs. combination $p < 0.0001$

C) [spread over figure sheet 11 and 12] Carbon incorporation from $^{13}\text{C}_6$ -glucose into downstream metabolites showing that both compounds work together in
 15 reducing the proliferation of MDA-MB-468 by decreasing both the amount of labeled TCA cycle metabolites and the flux through *de novo* serine and glycine synthesis. Multiple t-test, Holm-Sidak correction, serine M+3 $p < 0.0001$, glycine M+2 $p = 0.0029$, citrate M+6 $p < 0.0001$, α -ketoglutarate M+5 $p < 0.0001$, fumarate M+4 $p = 0.0008$, succinate M+4 $p = 0.0003$, malate M+4 $p < 0.0001$.

20 D) General set-up of the *in vivo* experiment. Within the same mouse, MDA-MB-231 and MDA-MB-468 cells were subcutaneously injected in the left and right flank respectively (3×10^6 cells/flank).

E) Tumor weight (g) of MDA-MB-231 (left) and MDA-MB-468 (right) mouse xenografts after a treatment with DMSO, sertraline (2,5 mg/kg), artemether (40
 25 mg/kg) or a combination of both compounds for 4 weeks. Kruskal-Wallis test: MDA-MB-231 $p = 0.2151$ and MDA-MB-468 $p = 0.0002$. Mann-Whitney U test: sertraline $p = 0.3615$, artemether $p = 0.0119$, combination $p = 0.0095$.

The number of biological replicates for each experiment (A-C) was $n \geq 3$. All error bars represent standard deviations. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$,
 30 $****p < 0.0001$.

Figure 5. Breast cancer cell lines depend on *de novo* serine and glycine synthesis or on serine uptake.

A) Carbon incorporation from $^{13}\text{C}_6$ -glucose into serine and glycine showing the difference between a breast cancer cell line depending on serine uptake (MDA-MB-
 35 231) and one depending on *de novo* serine and glycine synthesis (MDA-MB-468). Multiple t-test, Holm-Sidak correction: serine $p < 0.0001$ and glycine $p = 0.0005$.

B) *In vitro* proliferation of MDA-MB-231 (left) and MDA-MB-468 (right) in medium (DMEM) with or without serine as determined by real-time monitoring of cell confluence (%). Student's t-test: MDA-MB-231 $p < 0.0001$ and MDA-MB-468 $p = 0.1770$.

- 5 The number of biological replicates for each experiment (A and B) was $n \geq 3$. All error bars represent standard deviations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Artemether decreases mitochondrial activity.

- [spread over figure sheet 15 and 16] Carbon incorporation from $^{13}\text{C}_6$ -glucose into downstream metabolites showing that artemether (40 μM) inhibits proliferation of MDA-MB-468 by decreasing the amount of labeled TCA cycle metabolites. Multiple t-test, Holm-Sidak correction: serine M+3 $p = 0.0031$, glycine M+2 $p = 0.0875$, citrate M+6 $p = 0.0001$, α -ketoglutarate M+5 $p = 0.0030$, fumarate M+4 $p = 0.0015$, succinate M+4 $p = 0.0013$ and malate M+4 $p < 0.0001$. The number of biological replicates was $n \geq 3$. All error bars represent standard deviations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 7. Therapy scheme of in vivo experiment.

Figure 8 : Gene expression analysis defines a general increase in serine synthesis enzymes in T-ALL patient samples.

- 20 Gene expression data of the Meijerink dataset were extracted from the R2 AMC platform presenting 117 pediatric T-ALL (white bars) and 7 normal bone marrow (NBM) control samples (grey bars).

Figure 9 : Sertraline inhibition of the RPL10 R98S enhanced cell survival.

- Absolute cell survival after incubation with sertraline, comparing Ba/F3 RPL10 WT and R98S mutant clones. Data presented show results from an experiment comparing 3 individual RPL10 WT versus 3 RPL10 R98S clones, in which each clone was analyzed in 3 technical replicates. All box-plots show the median and error bars define data distribution.

Figure 10: PSPH mutation analysis in cBioportal across cancers.

- 30 Figure illustrating results of a PSPH mutation search in the cBioportal cancer genomics database. This search identified a hotspot mutation in PSPH, leading to an amino acid change from valine to isoleucine, V116I.

Figure 11. Sertraline does not inhibit *in vitro* PHGDH activity.

- A) PHGDH (= phosphoglycerate dehydrogenase) *in vitro* enzymatic assay, measuring PHGDH activity upon addition of control (DMSO 0.2%), 2.5 or 5 μM sertraline. Values are presented relative to the control (DMSO 0.2%). Results

shown are from two biological replicate, with three technical replicates in each experiment.

B) PHGDH (= phosphoglycerate dehydrogenase) *in vitro* enzymatic assay, measuring PHGDH activity upon addition of control (DMSO 0.2%), 2, 4, 8 or 16 μ M NCT-503 (an established PHGDH inhibitor). Values are presented relative to the control (DMSO 0.2%). Results shown are from at least two biological replicate, with three technical replicates in each experiment.

Figure 12. Structural similarities between the pyrazolopyran plant SHMT inhibitor (A) and sertraline (B).

10 **Figure 13. Sertraline decreases proliferation of MDA-MB-468 cells by inhibition of SHMT.**

A) Glycine mass distribution vector showing normalized metabolite ion counts (A.U./mg protein) of M+0, M+1 and M+2 glycine of MDA-MB-468 cells treated with control (DMSO 0.2%) or 5 μ M sertraline for 48 hours. M+0 and M+1 glycine levels decrease upon sertraline-treatment.

B) Glycine uptake (-) and secretion (+) rates (A.U./cell/h) of MDA-MB-468 cells treated with control (DMSO 0.2%), 3 or 5 μ M sertraline for 72 hours. Ordinary one-way ANOVA, Dunnett's multiple comparisons test: DMSO vs. 3 μ M $p = 0.0844$, DMSO vs. 5 μ M $p = 0.2145$.

20 C) Serine uptake (-) and secretion (+) rates (A.U./cell/h) of MDA-MB-468 cells treated with control (DMSO 0.2%), 3 or 5 μ M sertraline for 72 hours. Ordinary one-way ANOVA, Dunnett's multiple comparisons test: DMSO vs. 3 μ M $p = 0.7872$, DMSO vs. 5 μ M $p = 0.0141$.

25 D) Glucose uptake (-) rates (A.U./cell/h) of MDA-MB-468 cells treated with control (DMSO 0.2%), 3 or 5 μ M sertraline for 72 hours. Ordinary one-way ANOVA, Dunnett's multiple comparisons test: DMSO vs. 3 μ M $p = 0.9313$, DMSO vs. 5 μ M $p = 0.1282$.

30 E) Serine mass distribution vector showing normalized metabolite ion counts (A.U./mg protein) of M+0, M+1, M+2 and M+3 serine of MDA-MB-468 cells treated with control (DMSO 0.2%) or 5 μ M sertraline for 48 hours. M+0, M+1 and M+2 serine levels decrease, while the amount of M+3 serine increases upon sertraline-treatment. Student's t-test, $p = 0.0089$.

35 F) Deuterium labeled M+1 TTP fraction of MDA-MB-468 cells treated with control (DMSO 0.2%) or 5 μ M sertraline for 48 hours. Values are presented relative to the control (DMSO 0.2%).

G) Deuterium label (^2H) incorporation in the downstream metabolite thymidine triphosphate (dTTP) of MDA-MB-468 cells incubated with [2,3,3- ^2H]-serine

(deuterium labeled serine) and treated with control (DMSO 0.2%) or 5 μ M sertraline for 48 hours. Only M+1 dTTP (SHMT2), and no M+2 dTTP (SHMT1), was detected.

The number of biological replicates for each experiment (A-G) was $n \geq 3$. All error bars represent standard deviations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 14. Sertraline acts synergistically with taxanes, such as paclitaxel and docetaxel.

CI-FA plots of drug combinations generated using CompuSyn software. Each dot represents one specific combination of two single concentrations of each drug added to MDA-MB-468 cells. CI values below 1 are considered as synergy, while values above 1 are considered as antagonism. An additive interaction will typically have index values around 1. A) CI-FA plot of paclitaxel-sertraline combinations. B) CI-FA plot of docetaxel-sertraline combinations. C) CI-FA plot of methotrexate-sertraline combinations. D) CI-FA plot of 5-FU-sertraline combinations.

Figure 15. Elevated PSPH expression in the NKX2-1 rearranged T-ALL subgroup.

Violin plot illustrating RNA sequencing based PSPH mRNA expression levels in the LMO2/LYL1, HOXA, TLX3, TLX1, NKX2-1, LMO1/2, TAL1 and TAL2 T-ALL subgroups. Expression levels are indicated as FPKM: Fragments per kilo base per million mapped reads. These analyses were performed on the RNA sequencing dataset published by Liu et al., Nature Genetics, 2017 containing RNA sequencing data from 264 T-ALL patients.

DETAILED DESCRIPTION

Sertraline is the generic name for the compound (1 S-cis)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-N-methyl-1-naphthalenamine. The application equally envisages the use of pharmaceutical salts and structurally related compounds such as disclosed in US4,536,518.

Examples of pharmaceutically acceptable salts of sertraline are the acid addition salts of various mineral and organic acids such as hydrochloric, hydrobromic, hydroiodide, sulfuric, phosphoric, acetic, lactic, maleic, fumaric, citric, tartaric, succinic, and gluconic. Such salts may exist in one or more distinct crystalline forms or polymorphs, as well as in an amorphous state. Several crystalline polymorphs of the hydrochloride salt of sertraline are described in US5,248,699. Accordingly, as used in the claims, the term sertraline comprises the above named salts, related compounds and crystalline polymorphs.

"treatment" in the context of the present invention relates inter alia to a complete or partial disappearance of a cancer tissue, or to the delayed growth of a cancer tissue, a delayed relapse of a cancer, or a delayed metastasis.

Cancer with "increased glycine/serine synthesis" in the context of the present invention, refers to a cancer wherein the ^{13}C stable isotope label of $^{13}\text{C}_6$ -glucose
5 which is administered for 48 hrs to the cells of said cancer is detectable in serine and glycine, in an amount of a fractional contribution of >5% in serine and >3% in glycine.

"increased expression" refers to an expression of a gene in a tumor tissue sample
10 which is at least 1,25, 1,5, 2 or 5 fold higher then that gene in a conotol sample of the normal healthy tissue. Expression levels can be assessed by semi quantitative (e.g. intensity on gel) or quantitative measurements (eg taqman PCR).

PHGDH = phosphoglycerate dehydrogenase (HGNC:8923)

15 PSAT = phosphoserine aminotransferase (HGNC:19129)

PSPH = phosphoserine phosphatase (HGNC:9577)

SHMT = serine hydroxymethyltransferase.

SHMT1 = serine hydroxymethyltransferase (HGNC:10850)

SHMT2 = serine hydroxymethyltransferase. (HGNC:10852)

20

Sertraline is well known as antidepressant and various formulations for oral and parenteral administration are known in the art. The use of intravenous and intramuscular administration is equally envisaged. In specific embodiments sertraline is administered locally to the cancer tissue to be treated.

25

Artemether and sertraline are both drugs that have already been explored in the context of cancer, without knowledge of its target. Beside its use as anti-depressant, sertraline, and other compounds belonging to the class of serotonergic system antagonist, have already been shown to synergize with
30 chemotherapeutics. Moreover, combining sertraline with docetaxel shrinks breast tumor xenografts in immune-compromised mice by inhibiting tumor cell proliferation and inducing their apoptosis [Gwynne et al. (2017) *Oncotarget* **8**, 32101–32116].

Repurposing the serotonergic antagonist (SSRI) sertraline to treat serine synthesis
35 dependent breast cancer will only be possible if the doses that are needed for anticancer effects are not toxic for humans. As anti-depressant, sertraline is given to patients in a dosage ranging from 50 mg to 200 mg a day, resulting in serum

concentrations between 0.065 and 0.54 μ M [Devane et al. (2002) *Clin. Pharmacokinetics* **41**, 1247–1266]. Furthermore, sertraline has a linear pharmacokinetic profile and daily oral intake of a dose of 400 mg, producing a plasma concentration of 0.82 μ M, is well tolerated in patients [Gwynne et al. (2017) *Oncotarget* **8**, 32101–32116; Devane et al. (2002) *Clin. Pharmacokinetics* **41**, 1247–1266]. Despite the fact that doses used in these *in vitro* experiments are higher than the detected serum levels, these concentrations are administered also in other studies in which anticancer activity of sertraline is investigated [Gwynne et al. (2017) *Oncotarget* **8**, 32101–32116]. Furthermore, considering that the average body weight of an adult in Europe is 70.8 kg, sertraline doses vary between 0.7 and 2.8 mg/kg. As mice were treated with 2.5 mg/kg sertraline, this is in the range of the administered doses in humans. Hassell et al. (2016) even used doses of 60 mg/kg in mice and no adverse effects were observed. This suggest that therapeutics doses of this SSRI can be achieved in breast cancer patients. In fact, sertraline, even at low doses, can cause an enormous advantage when combined with commonly used chemotherapeutics, and/or mitochondrial inhibitors such as artemether, in the treatment of breast cancer. This was already shown in the context of breast cancer, referring to sertraline as a compound targeting sphere formation of breast tumor-initiating cells (BTIC) [Gwynne et al. (2017) *Oncotarget* **8**, 32101–32116].

Accordingly, in embodiments of the claimed invention sertraline is administered at a dosis from 0,1 mg/kg, 0,25 mg/kg, 0,5 mg/kg, 0,75 mg/kg or 1 mg/kg up to 1,5 mg/kg, 2 mg/kg, 2,5 mg/kg, 3 mg/kg, 4 mg/kg or 5 mg/kg.

In other embodiments of the claimed invention sertraline is administered at a dosis of from 10 mg, 25 mg, 50 mg up to 100 mg, 150 mg, 200 mg, 250 mg or 500 mg regardless of the weight of the patient.

While normal cells are more reliant on serine and glycine uptake from their environment, and therefore, present very low or even undetectable levels of serine synthesis pathway enzymes, a subset of cancers are dependent on *de novo* serine synthesis for their survival and proliferation [Sun et al. (2015) *Cell Res.* **25**, 429–444]. The increase in serine synthesis in cancer cells can be due to enzyme amplifications, caused by environmental adaptation as hypoxia, and also as a consequence to oncogenes and tumor suppressors that enforce enhanced serine synthesis fluxes [Possemato et al. (2011) *Nature* **476**, 346–350; Ye et al. (2014) *Cancer Discovery* **4**, 1406–1417]. As such, interactions between enzymes belonging to the serine/glycine synthesis pathway and oncogenes as RAS and MYC or tumor suppressors as p53 have been shown to support the serine/glycine flux

in cancer cells [Sun et al. (2015) *Cell Res.* **25**, 429–444; Maddocks et al. (2012) *Nature* **493**, 542–546; Maddocks et al. (2012) *Nature* **493**, 542–546]. While PHGDH amplifications are mainly observed in breast cancer patients, PSPH amplifications are more frequently observed in 8-15% of cancer patients with glioblastoma brain tumors, head and neck cancer, lung adenomas, and bladder cancer (cBioportal). PSPH copy number gains were confirmed in copy number data from 48 in house glioblastoma patients, where evidence was collected for focal amplification of PSPH (amplification peak of 8 genes) that can be independent of EGFR amplifications. Besides amplifications, PSPH mutations can be found at a lower frequency, for which the oncogenic contribution is still unknown (see figure 11). PSPH mutation analysis in cBioportal across cancers, shows a hotspot mutation leading to an amino acid change from valine to isoleucine, V116I which is located in the inner core of the hydrolase domain. All these cancers presenting genetic lesions in serine/glycine synthesis enzymes are expected to be sensitive to sertraline treatment controlled inhibition of serine/glycine synthesis.

Besides the evidence for genetic lesions in cancer cells, other mechanisms of serine/glycine synthesis upregulation have been discovered such as the ribosomal RPL10 R98S mutation [Kampen et al. (2019) *Nat Commun.* **10**, 2542]. RPL10 R98S mutation seems to be just one of the mechanisms by which leukemic cells can increase their serine/glycine synthesis. Serine biosynthesis is also altered in for example Cyclin D3:CDK4/6 complex driven T-ALL [Wang et al. (2017) *Nature* **546**, 426–430]. In addition, it is shown that T-cell lymphoid leukemia cells express higher levels of serine/glycine synthesis enzymes, such as PHGDH and PSPH (as indicated in figure 8), with the subset of T-ALL patients having genomic rearrangements driving overexpression of the transcription factor NKX2-1 (NK2 Homeobox 1) displaying the highest PSPH expression (Figure 15).

Molecular targeting of PSPH in T-ALL cell lines or a primary patient derived xenograft revealed a function for PSPH in sustaining leukemia proliferation in vitro and in vivo at leukemic sites, such as the bone marrow and spleen. These data support that T-ALL is a cancer subgroup expected to be sensitive to the addition of sertraline to current treatment regimens. Of interest, it was noticed that NKX2-1 (NK2 Homeobox 1) rearranged and RPL10 R98s mutant T-ALL patients express the highest PSPH levels, indicating that these subgroups may be most sensitive.

Besides T-ALL and breast cancer, upregulation of serine/glycine synthesis pathway is also known in other types of cancer such as prostate, testis, ovary, brain, liver, kidney and pancreas [Zogg (2014) *J. Oncol.* **2014**, 1–13]. This suggest that expression levels of serine synthesis enzymes, like PHGDH, can serve as a biomarker, since these cell lines are expected to be dependent on serine/glycine synthesis and thus sensitive to sertraline. A short list of the most established cell lines is given in table 1. Herein Sertraline sensitivity was experimentally tested for cell lines MDA-MB-231, MDA-MB-468, MCF7 and HCC70. For all other cell lines in this table, sertraline sensitivity is predicted based on PHGDH expression levels.

10

Table 1 dependency of cell lines on serine/glycine synthesis

Sensitive (serine synthesis dependent)	Insensitive (serine uptake dependent)
BREAST CANCER CELL LINES	BREAST CANCER CELL LINES
MDA-MB-468	MDA-MB-231
MDA-MB-436	MCF-10A
HCC70	MCF-7
Hs-578-T	SW480
BT-20	Sk-BR-3
HCC1599	DU-4475
MT-3	ZR-75-30
HMC-1-8	T-47-D
HCC1395	
	MELANOMA CELL LINES
MELANOMA CELL LINES	GAK
Wm266-3	
Malme	COLON CANCER CELL LINES
SK28	HCT116
	RKO
T-ALL CELL LINES	
ALLSIL	
CCRFCEM	
HPBALL	
HSB2	
JURKAT	
KARPAS45	
KE37	
LOUCY	
MOLT14	
P121CHIKAWA	
PEER	
RPMI8402	
SUPT1	
SUPT13	
TALL1	

Sensitive (serine synthesis dependent)	Insensitive (serine uptake dependent)
PF382	
DND41	
LUNG CANCER CELL LINES	
A549	
H2087	
H1792	
H522	
H1437	

Finally, it is noteworthy that serine/glycine synthesis can crosstalk with other metabolic pathways in tumor-derived cell lines. As the amount of imported serine and glycine is enough to fuel protein synthesis, this indicates different roles of additional serine synthesized from glucose. For example, serine synthesis has already been linked with one-carbon (folate) metabolism and the glycine cleavage system (SOG pathway). Intermediates of this pathway can then again be used as precursors for biosynthetic processes. For instance, methionine is directly required for protein synthesis [Tedeschi et al. (2013) *Cell Death Disease* **4**, e877].

The present invention discloses that sertraline can be used in combination with chemotherapeutics.

Drug combinations have the potential to be more effective than monotherapy, as it reduces the risk of resistance by hitting multiple targets at the same time.

Moreover, toxicity and adverse side effects can be reduced because drugs in combinations can be administered at lower dosages as compared to single agents [Preuer et al. (2018) *Bioinformatics* **34**, 1538–1546]. Therefore, developing synergistic drug combinations is important to improve the efficacy of anticancer treatment.

While normal cells rely on active uptake of serine and glycine metabolites from their environment, it is known that some cancer subtypes are addicted to *de novo* serine synthesis, creating a metabolic independence to support their proliferation and survival [Possemato et al. (2011) *Nature* **476**, 346–350; Kim et al. (2014) *PLoS ONE* **9**, e101004; Amelio et al. (2014) *Trends Biochem. Sci.* **39**, 191–198; Kampen et al. (2019) *Nature Comm.* **10**, 2542]. Selective therapeutic compounds targeting *de novo* serine/glycine synthesis in combination with chemotherapy treatment are an attractive option in order to improve therapy responses and reduce treatment associated toxicity in metabolically addicted cancers.

Being the most frequent cancer in women, triple-negative breast cancers (TNBC) account for approximately 15% of all breast cancers. Due to the absence of any targeted therapies, the standard of care treatment for TNBC consists of chemotherapy, resection, and adjuvant radiotherapy. Even though these patients
5 respond better to chemotherapy than other subtypes, prognosis remains poor and new treatment strategies are desirable. MDA-MB-468 triple negative breast cancer cells are known to be addicted to *de novo* serine/glycine synthesis [Possemato et al. (2011) *Nature* **476**, 346–350; Kim et al. (2014) *PLoS ONE* **9**, e101004].

10 Methotrexate and 5-FU both belong to the class of antimetabolites. 5-FU acts as analogue of uracil whose cytotoxic action has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase. The latter catalyzes *de novo* production of thymidylate for DNA replication and repair. Methotrexate (MTX)
15 is an antimetabolite that disrupts the metabolic pathways requiring one-carbon units supplied by B9 folate vitamins. This antifolate acts as an inhibitor of dihydrofolate reductase (DHFR), a key enzyme of the one-carbon metabolism. THF is known as the general one-carbon unit acceptor and is, together with serine, required for the reaction catalyzed by SHMT. Without THF, SHMT is not able to
20 produce glycine out of serine, because one-carbon units coming from serine cannot be accepted by THF. Consequently, THF is a limiting factor for SHMT activity and thus an active one-carbon metabolism [Newman & Maddocks (2017) *Br. J. Cancer* **116**, 1499–1504]. This supports that MTX and sertraline will, in the end, cause the same effect by lowering the limiting substrate THF or by inhibiting the
25 enzyme (SHMT), respectively, explaining the antagonistic working between both compounds. As sertraline targets SHMT, both enzymes are involved in one-carbon metabolism, which is the central pathway to pyrimidine biosynthesis and is therefore strongly related to cell proliferation. Sertraline, methotrexate and 5-FU all target parts of the same pathway and are therefore not expected to work
30 synergistically.

The molecular mechanism by which taxanes induce cell death is not fully understood, but it has been previously shown that, apart from preventing microtubule depolymerization, they can induce apoptosis by targeting mitochondrial pathways. On the other hand, inhibiting serine into glycine
35 conversion by blocking SHMT activity with sertraline, will also be detrimental for mitochondrial activity as SHMT is extremely important for cellular energy status. As such, both compounds will target the mitochondria via distinct routes. This will

be extremely harmful for rapidly dividing cancer cells that require high amounts of energy and building blocks produced via mitochondrial pathways. Without notice of the underlying mechanism, Hallett *et al.* (2016) already described that docetaxel and sertraline work synergistically. More specifically, they observed therapeutic synergy in female mice transplanted with freshly isolated tumor cells from tumors of the MMTV-Neu (N202) transgenic strain [Hallett *et al.* (2016) *OncoTarget* **7**, 53137–53151].

Genetic events that cause metabolic addiction to *de novo* serine/glycine pathway account for 21% LUAD, 12% GBM, 12% T-ALL, 6% BRCA, and 6% of PROD cancer patients, and numbers will increase with the discovery of additional molecular regulators of this pathway (cBioportal) [Kampen *et al.* (2019) *Nature Comm.* **10**, 2542]. Besides our experimental data, inhibition/downregulation/suppression of serine synthesis enzyme PHGDH has been shown to re-sensitize therapy resistant tumors to anticancer treatment. In particular, PHGDH targeting resulted in re-sensitization towards doxorubicin (TNBC), vemurafenib (melanoma), HIF2 α -antagonist (advanced/metastatic renal cell carcinoma) therapy resistant cancer cells [Zhang & Bai (2016) *Cancer Chemo. Pharmacol.* **78**, 655–659; Ross *et al.* (2017) *Molecular cancer therapeutics* **16**, 1596–1609; Yoshino *et al.* (2017) *Cancer Res.* **77**, 6321–6329]. HIF2 α antagonists have been recently developed as a novel treatment for advanced/metastatic renal cell carcinoma (ccRCC). However, patients develop resistance to these antagonists. PHGDH was found to be upregulated in engineered HIF2 α -deficient tumor cells, mimicking the resistance observed to HIF2 α antagonists. Treatment with a PHGDH inhibitor reduced the growth of HIF2 α -deficient tumor cells *in vivo* and *in vitro* [Yoshino *et al.* (2017) *Cancer Res.* **77**, 6321–6329].

Vemurafenib and dabrafenib are inhibitors of the MAPK pathway that are used to treat unresectable or metastatic melanoma with oncogenic *BRAF* V600E mutations, which accounts for >60% of all melanoma cases [Ross *et al.* (2017) *Molecular cancer therapeutics* **16**, 1596–1609]. Proteomic analysis revealed differential protein expression of serine biosynthetic enzymes PHGDH, PSPH, and PSAT1 upon vemurafenib (*BRAF* inhibitors) treatment in sensitive versus acquired resistant melanoma cells. Ablation of PHGDH via siRNA targeting re-sensitized melanoma cells with acquired resistance to vemurafenib [Ross *et al.* (2017) *Molecular cancer therapeutics* **16**, 1596–1609]. These examples highlight the potential of using serine and glycine synthesis pathway inhibitors in combination with commonly used therapeutic agents such as, HIF2 α antagonists, vemurafenib/dabrafenib, taxanes, doxorubicin and many more combinations with

targeted therapy compounds and chemotherapeutics.

EXAMPLES

Example 1. Material and methods

5 ***C. ALBICANS:***

Yeast strain and chemicals. *C. albicans* strain SC5314 [Fonzi & Irwin (1993) *Genetics* **134**, 717–728] used in this study was grown routinely on YPD (1% yeast extract, 2% peptone (International Medical Products) and 2% glucose (Sigma-Aldrich) agar plates at 30°C. RPMI 1640 medium (pH 7.0) (Sigma-Aldrich) with L-
10 glutamine and without sodium bicarbonate was buffered with MOPS (Sigma-Aldrich). Stock solutions of miconazole (Sigma-Aldrich) and sertraline (Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO) (VWR International).

Serine and glycine medium measurements. A *C. albicans* SC5314 overnight culture, grown in YPD, was diluted to an optical density (OD) of 0.1 (approximately
15 10^6 cells/ml) in RPMI 1640 medium and 2 ml of this suspension was added to the wells of a 6-well plate (Greiner Bio-One). After 1 h of adhesion at 37°C, the medium was aspirated and biofilms were washed with phosphate buffered saline (PBS) to remove non-adherent cells, followed by addition of 2 ml fresh RPMI 1640 medium. Subsequently, the biofilms were allowed to grow for 24 h at 37°C. After
20 washing the biofilms with PBS, miconazole (75 μ M) was added in RPMI, resulting in a DMSO background of 0.2%. Next, biofilms were incubated for an additional 24 h at 37°C. Finally, 1 ml of conditioned medium was collected and serine and glycine were quantified by cation-exchange chromatography on a Biochrom 30 analyzer (Biochrom, Cambourne, UK).

25 **Metabolic activity assay.** Biofilms were grown in 6-well plates and treated in RPMI 1640 medium as described above. After washing with PBS, 2 ml Cell-Titer Blue (CTB; Promega) [O'Brien et al. (2000) *Eur. J. Biochem.* 267(17), 5421–5426], diluted 1/100 in PBS, was added to each well. After 1 h of incubation in the dark at 37°C, fluorescence was measured with a fluorescence spectrometer
30 (Synergy Mx Multimode Microplate Reader; BioTek) at λ_{ex} of 535 nm and a λ_{em} of 590 nm. Finally, percentage of metabolically active biofilm cells was calculated as described in Spincemaille et al. (2014). *Biochim. Biophys. Acta* **1843**, 1207–1215.

Synergy assay. Biofilms were grown in 96-well plates and treated in RPMI 1640
35 medium as described above. After washing with PBS, 100 μ l Cell-Titer Blue (CTB; Promega) [O'Brien et al. (2000) cited above], diluted 1/100 in PBS, was added to each well. After 1 h of incubation in the dark at 37°C, fluorescence was measured

with a fluorescence spectrometer (Synergy Mx Multimode Microplate Reader; BioTek) at λ_{ex} of 535 nm and a λ_{em} of 590 nm. Finally, percentage of metabolically active biofilm cells was calculated as described in Spincemaille et al. (2014) cited above. SynergyFinder and Calcsyn were used to determine synergy
5 between miconazole and sertraline.

Membrane permeability assay. Biofilms were grown in 96-well plates and treated in RPMI 1640 medium as described above. After washing with PBS, propidium iodide staining (Sigma-Aldrich) was performed as previously described (Bink et al. (2012) *J. Infectious Dis.* 206(11), 1790–1797).

10 **BREAST CANCER:**

Cell cultures. MDA-MB-231 and MDA-MB-468 (American Type Culture Collection; ATCC) were cultured in DMEM medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies). MCF7 and HCC70 (American Type Culture Collection; ATCC) were cultured in RPMI 1640 medium supplemented
15 with 10% FBS (Life Technologies).

In vitro proliferation assay. For each cell line, 10.000 cells/well were plated in a 96-well plate (TPP). Cell proliferation was assessed by real-time quantitative live-cell analysis of confluency on an IncuCyte Zoom system (Essen BioScience) using the setting of 4 pictures per well at 2 h intervals. Each experiment was
20 performed in three biological replicates and a minimum of 6 technical replicates were included each time.

¹³C₆-glucose tracer analysis. Labeling experiments were performed in 10% dialyzed serum for 24 h. ¹³C₆-glucose was purchased from Sigma-Aldrich. Metabolites for the subsequent mass spectrometry analysis were prepared by
25 quenching the cells in liquid nitrogen followed by a cold two-phase methanol-water-chloroform extraction [Christen et al. (2016) *Cell Reports* **17**, 837–848; Lorendeau et al. (2017). *Metabolic Engineering* **43**, 187–197]. Phase separation was achieved by centrifugation at 4°C. The methanol-water phase containing polar metabolites was separated and dried using a vacuum concentrator. Dried
30 metabolite samples were stored at –80°C.

Gas chromatography–mass spectrometric analysis. Polar metabolites were derivatized and measured as described before Christen et al. (2016) cited above and Lorendeau et al. (2017) cited above. In brief, polar metabolites were derivatized with 20 mg/ml methoxyamine in pyridine for 90 min at 37°C and
35 subsequently with N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide, with 1% tert-butyldimethylchlorosilane for 60 min at 60°C. Metabolites were measured with a 7890A GC system (Agilent Technologies) combined with a 5975C Inert MS

system (Agilent Technologies). One microliter of samples was injected in splitless mode with an inlet temperature of 270°C onto a DB35MS column. The carrier gas was helium with a flow rate of 1 ml/min. For the measurement of polar metabolites, the GC oven was held at 100°C for 3 min and then ramped to 300°C with a gradient of 2.5°C/min. The MS system was operated under electron impact ionization at 70 eV and a mass range of 100–650 a.m.u. was scanned. Mass distribution vectors were extracted from the raw ion chromatograms using a custom Matlab M-file, which applies consistent integration bounds and baseline correction to each ion. Moreover, a correction was made for naturally occurring isotopes. For metabolite levels, arbitrary units of the metabolites of interest were normalized to an internal standard and protein content.

Amino acid measurements of conditioned medium. 1 ml of conditioned medium was collected and serine and glycine were quantified by cation-exchange chromatography on a Biochrom 30 analyzer (Biochrom, Cambourne, UK).

Xenografts in NOD-SCID/IL2γ^{-/-} (NSG) mice. Animal experiments were approved by the local ethics committee (P262-2015). NSG mice were recently purchased from Charles River laboratories and bred to obtain sufficient animals. 3.10⁶ breast cancer cells were injected subcutaneously in the left (MDA-MB-231) and right (MDA-MB-468) flanks in a 1:1 mixture with Matrigel (Corning). The animals were monitored on a daily basis and sacrificed after 28 days. Mice received treatments on days 7, 9, 11, 13, 15, 20 and 24. Therapy was administered via intra-peritoneal injections at dosages of 2.5 mg/kg sertraline (Sigma-Aldrich) and/or 40 mg/kg artemether (TCI Europe). Control mice were treated with the solvent (DMSO; Merck KGaA).

25

DATA ANALYSIS AND STATISTICAL ANALYSES:

Combination indexes (CI) were calculated with 'CalcuSyn' software based on the Chou-Talalay method [Chou (2010) *Cancer Res.* **70**, 440–447]. All statistical analyses were performed using GraphPad Prism 6 and data were presented as mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 6 and data were presented as mean ± standard deviation (SD). Specific statistical tests used for each experiment are mentioned in detail in the figure legends. Values were considered to be statistically significant when the P value was < 0.05.

35

Computational docking studies

Sertraline was modelled using MOE (chemical computing group) 1 with the MMFF94x force field. The structures of the putative receptors present in the pathway were obtained from the RCSB database² (PHGDH: 5N6C3, PSAT1: 3E774, PSPH: 1NNL5, SHMT1: 1BJ46, SHMT2: 4PRF7). The bioactive conformations were chosen for each receptor (PHGDH, PSPH as monomers and PSAT1, SHMT1, SHMT2 as dimers). The crystal structure of the ts3 human serotonin receptor complexed with sertraline (PDB ID: 6AWO8) was used as a reference for docking scores. All the receptor structures were optimized in MOE using protonate_3D.

Docking was performed using GOLD8 software and active sites were defined by the ligands present in the crystal structure of each receptor. Conformational restraints were applied to the ligand by disallowing the flipping of ring conformations and planar R-NR1R2 groups to ensure the rigidity of sertraline. The ligand was docked 10 times into each receptor and the score was calculated using CHEMPLP scoring function. The complex structures of receptors with highest docking scores (PHGDH and SHMT) were visualized in PYMOL9 to analyze the hydrogen bonding interactions.

Molecular docking was repeated for SHMT in the presence of the PLP co-factor. Furthermore, other known inhibitors/co-factors of SHMT (pyrazolopyran and folic acid) were also docked in the presence of the PLP ligand. The docked conformations of the ligands in SHMT was superimposed using PYMOL to compare the interactions.

PHGDH *in vitro* enzymatic assay

PHGDH enzyme activity upon drug treatment was tested using human PHGDH (BPS bioscience, 71079) and a specific colorimetric PHGDH activity kit (Biovision, K569). The known PHGDH inhibitor, NCT-503, served as a positive control. Human PHGDH enzyme was diluted 5 times in water to reach a concentration of 0.15 mg/ml. Next, 5 µl recombinant PHGDH enzyme and 5 µl of sertraline/NCT-503 (10x stock) was added in one well of a 96-wells plate (flat bottom). Subsequently, 40 µl PHGDH assay buffer (Biovision) and 50 µl PHGDH reaction mix (Biovision), was added. Afterwards, absorbance at 450 nm was measured every 10 min, during at least one hour. In between measurements, the plate was incubated at 37 °C, protected from light.

Deuterated serine tracing

At day 0, a number of 150.000 MDA-MB-468 cells were plated in 2 ml of DMEM culture medium (Gibco 41965, high glucose) in 6-well plates (Greiner Bio-One).

The day after, cells were washed with PBS to get rid of all 'old' medium and 2 ml of fresh tracing medium was added. Specifically, tracing experiments were performed in serine-free DMEM (US Biological life Sciences, D9802-01), supplemented with 4.5 g/l glucose (Sigma-Aldrich), 3.7 g/l sodium bicarbonate (Sigma-Aldrich), 400 µM glycine (Sigma-Aldrich), glutamax (100x, Thermo Fischer Scientific) and 10% dialyzed serum (Thermo Fisher Scientific, A3382001) for 48 hours. [2,3,3-²H]-serine (deuterium labeled serine) was purchased from Sigma-Aldrich. Metabolites for the subsequent mass spectrometry analysis were prepared by quenching the cells in liquid nitrogen followed by a cold two-phase methanol-water-chloroform extraction [Christen et al. (2016) *Cell Rep.* **17**, 837–848; Lorendeau et al. (2017). *Met. Eng.* **43**, 187–197]. Phase separation was achieved by centrifugation at 4 °C. The methanol-water phase containing polar metabolites was separated and dried using a vacuum concentrator. Dried metabolite samples were stored at –80 °C.

15 **Uptake and secretion rates**

At day 0, a number of 150.000 MDA-MB-468 cells were plated in 2 ml of DMEM culture medium (Gibco 41965, high glucose) in 6-well plates (Greiner Bio-One). The day after (day 1), cells were washed with PBS to get rid of all 'old' medium and 2 ml of fresh DMEM medium (Gibco 41965, high glucose) was added. 72 hours later (day 3), medium samples were taken (0.5-1 ml). The cells were counted on day 1 (initial physiology) and after 72 hours (day 3), using an automated cell counter. Medium samples were analyzed by mass spectrometry. HPLC was used for the detection of glucose.

Gas chromatography–mass spectrometry analysis

25 Polar metabolites (amino acids and TCA cycle intermediates) were derivatized and measured as described before [Christen et al. (2016) *Cell Rep.* **17**, 837–848; Lorendeau et al. (2017). *Met. Eng.* **43**, 187–197]. In brief, polar metabolites were derivatized with 20 mg/ml methoxyamine in pyridine for 90 min at 37 °C and subsequently with N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide, with 30 1% tert-butyldimethylchlorosilane for 60 min at 60 °C. Metabolites were measured with a 7890A GC system (Agilent Technologies) combined with a 5975C Inert MS system (Agilent Technologies). One microliter of samples was injected in split mode (ratio 1 to 3) with an inlet temperature of 270 °C onto a DB35MS column. The carrier gas was helium with a flow rate of 1 ml/min. For the measurement of 35 polar metabolites from the deuterated serine labeling experiment, the GC oven was set at 100 °C for 1 min and then increased to 105 °C at 2.5°C/min and with a gradient of 2.5 °C/min finally to 320°C at 22°C/min. The measurement of

metabolites has been performed under electron impact ionization at 70 eV using a selected-ion monitoring (SIM) mode. For the measurement of polar metabolites from the ¹³C₆ glucose labeling experiment, the GC oven was held at 100 °C for 3 min and then ramped to 300 °C with a gradient of 2.5 °C/min. The mass spectrometer system was operated under electron impact ionization at 70 eV and a mass range of 100–650 a.m.u. was scanned. Mass distribution vectors were extracted from the raw ion chromatograms using a custom Matlab M-file, which applies consistent integration bounds and baseline correction to each ion [Young et al. (2008). *Biotechnol. Bioeng.* **99**, 686–699]. Moreover, data were corrected for naturally occurring isotopes [Fernandez et al. (1996) *J. Mass Spectrometry* **31**, 255–262]. For metabolite levels, arbitrary units of the metabolites of interest were normalized to glutaric acid, an internal standard, and protein content.

Liquid chromatography–mass spectrometry analysis

A liquid chromatography, Dionex UltiMate 3000 LC System (Thermo Scientific), coupled to mass spectrometry (MS), a Q Exactive Orbitrap (Thermo Scientific), was used for the separation of polar metabolites. A volume of 15 µl of sample was injected and metabolites were separated on a C18 column (Acquity UPLC HSS T3 1.8µm 2.1x100mm) at a flow rate of 0.25 ml/min, at 40 °C. A gradient was applied for 40 min (solvent A: 0 H₂O, 10 mM Tributyl-Amine, 15 mM acetic acid – solvent B: Methanol) to separate the targeted metabolites (0 min: 0%B, 2 min: 0%B, 7 min: 37%B, 14 min: 41%B, 26 min: 100%B, 30 min: 100%B, 31 min: 0%B; 40 min: 0%B).

The MS operated in full scan in negative mode (m/z range: 70-1050 and 300-800 from 8 to 25 min) using a spray voltage of 4.9 kV, capillary temperature of 320°C, sheath gas at 50.0, auxiliary gas at 10.0. Data was collected using the Xcalibur software (Thermo Scientific) and analyzed with Matlab using the same procedure as described above for the analysis of GC-MS data.

High performance liquid chromatography analysis

Medium samples were analyzed by HPLC (Infinity 1260, Agilent) for the detection of glucose. A volume of 10 µl of medium was injected into the column (BioRad, Aminex HPX-87H, 300 x 7.8 mm, 9 µm particle size). Glucose was eluted at a flow rate of 0.6 ml/min of 5 mM H₂SO₄ in isocratic mode. A multiple wavelength detector with an output voltage of 0.1 V was used to scan the wave lengths from 210 to 550. Peak areas were integrated using the Open Lab software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 and data were presented as mean ± standard deviation (SD). Specific statistical tests used for

each experiment are mentioned in detail in the figure legends. Values were considered to be statistically significant when the P value was < 0.05.

Sertraline in combination with chemotherapeutics

The chemotherapeutics tested were: paclitaxel, docetaxel, methotrexate and 5-fluorouracil (all from Cayman Chemicals, except methotrexate was from Sigma). The lyophilized compounds were stored at -20 °C. Sertraline (Sigma, S6319) was dissolved in DMSO to reach a stock concentration of 14.6 mM. 1000x stock solutions in DMSO were made and stored at 4 °C.

A number of 7500 MDA-MB-468 cells were plated in 100 µl of DMEM culture medium in 96-well plates (TPP 96 well tissue culture plates from Sigma-Aldrich). The chemotherapeutics were added to the cells with a D300e Digital Dispenser (Tecan) the day after plating the cells. Afterwards, sertraline was manually added to each well.

Cell proliferation was assessed by real-time quantitative live-cell imaging analysis of confluency on an IncuCyte Zoom system (Essen BioScience) using the setting of four pictures per well with 24h intervals. This imaging and analysis platform enables automated quantification of cell behavior over time. The system has high definition phase contrast optics and software recognition allowing to mask, quantify and generate time based curves of cellular behavior parameters such as confluency.

With the collected data we calculated the growth rate under various drug conditions. The purpose of measuring the growth rate is to determine the rate of cell number increase in a culture per unit of time. Only the exponential (logarithmic) portions of the resulting growth curves are used for determining the growth rates. To do this, the formula below was used, in which T1 and T2 are two time point within the exponential growth phase. Confluency was used as cell number values.

$$\text{growth rate} \left[\frac{1}{h} \right] = \frac{\text{LN (cell number T1)} - \text{LN (cell number T0)}}{\text{time (T1)} - \text{time (T0)}}$$

30

Obtained growth rates were statistically analyzed with a two-way ANOVA test, using GraphPad Prism 8.1.2 software. Additionally, we corrected for multiple testing using Dunnett's and/or Sidak's multiple correction. Results were considered to be statistically significant if the adjusted p-value was <0.05 (* <0.05, ** <0.01, *** <0.001, **** <0.0001). CompuSyn software was used to

35

calculate the combination index (CI), taking into account the fraction affected (FA) of each combination of drugs.

T-ALL:

- 5 Ba/F3 clones expressing RPL10 WT or R98S clones were generated as described previously [Girardi et al. (2018) *Leukemia* **32(3)**, 809-819]. Cells were analyzed under overgrowth conditions: cells were grown for 48 hrs, followed by addition of 0 or 10 μ M sertraline and incubation for another 48 hrs. After 96 hrs, cell survival was determined by analyzing the number of viable cells / ml based on forward/
10 side scatter plots on a flow cytometer.

Example 2. Sertraline is a potentiator of miconazole against *C. albicans* biofilms.

- Re-analyzing previously obtained transcriptome data of miconazole-induced tolerance pathways in *C. albicans* biofilm cells [De Cremer et al. (2016) *Sci. Rep.*
15 **6**, 1–14] highlighted an upregulation of genes involved in sterol biosynthesis and genes encoding drug efflux pumps. In general, the biofilm transcriptional response to miconazole treatment suggested a broad disturbance of energy metabolism, with a significant upregulation of genes involved in *de novo* serine and glycine synthesis (*SER33*, *SER1*, *SER2* and *SHM2*) (Figure 1A) and one-carbon metabolism
20 (*MET13*, *SAH1*, *GCV1*, *GCV2* and *MIS11*) [De Cremer et al. (2016) *Sci. Rep.* **6**, 1–14]. More evidence was obtained by analyzing conditioned medium of *C. albicans* where treatment with a suboptimal dosage of miconazole (miconazole-stressed) resulted in an increase of serine and glycine levels by 75 and 22 μ M respectively (Figure 1B). The reductive potential, which is a read-out for viability, of
25 miconazole-stressed *C. albicans* biofilm cells was not affected compared to control (Figure 1C). These findings suggest that miconazole-stressed *C. albicans* biofilm cells upregulate their *de novo* serine and glycine synthesis resulting in reduced serine and glycine uptake from the medium.

- In order to tackle the problem of antifungal resistance, repurposing libraries were
30 previously screened for compounds that decrease the metabolic activity of *C. albicans* biofilm cells in the presence of subinhibitory doses of various antifungals, including miconazole.. The serotonin reuptake inhibitor and anti-depressive drug sertraline [Nielsen et al. (2013) *Br. J. Pharmacol.* **170**, 1041–1052] was identified as an antibiofilm potentiator in screens with the antifungals amphotericin B and
35 caspofungin (Unpublished data). Herein the additive effect of sertraline on miconazole-induced *C. albicans* biofilm cells was specifically assessed. Checkerboard titration followed by calculation of combination indexes (CI)

supported synergistic action of sertraline and miconazole (CI<1: between 0.03961 and 0.16268) [Chou (2010) *Cancer Res.* **70**, 440–447] (Figure 1D). Combining these data with another online tool, 'SynergyFinder' [Ianevski et al. (2017) *Bioinformatics* **33**, 2413–2415], showed that 75 µM of miconazole, the concentration frequently used to treat biofilm cells, is most potent when combined with a sertraline dose between 50 µM and 100 µM (Figure 1D). Finally, these data were validated by propidium iodide (PI) staining, a readout for cell death (Figure 1E). While 75 µM of sertraline monotherapy was not affecting cell death and 75 µM of miconazole slightly increased cell death, the combination of both drugs synergistically induced cell death in biofilm cells.

Example 3. Miconazole potentiators can selectively inhibit serine synthesis dependent breast cancers.

As explained above, miconazole-stressed *C. albicans* biofilm cells upregulate their *de novo* serine and glycine synthesis. Synergy between sertraline and miconazole might thus stem from inhibition of serine/ glycine synthesis. 'SwissTargetPrediction', an online tool that predicts the targets of small compounds based on a combination of 2D and 3D similarity with known ligands [Gfeller et al. (2018) *Nucleic Acids Res.* **42**, 32–38], pointed to the sodium- and chloride-dependent glycine transporters 1 and 2 as potential targets of sertraline. In order to further test whether sertraline affects serine/glycine synthesis in different eukaryotic systems, the efficacy of sertraline in inhibiting serine synthesis dependent versus independent triple negative breast cancer cell lines was tested (i.e estrogen receptor-negative, progesterone receptor-negative and HER2-negative). Some breast cancer cell lines depend on serine uptake, whereas others depend on *de novo* serine and glycine synthesis. The latter typically express higher transcript and protein levels of *de novo* serine and glycine synthetic enzymes [Possemato et al. (2011) *Nature* **476**, 346–350; Kim et al. (2014) *PLoS ONE* **9**, e101004], and flux through this metabolic pathway is associated with proliferation and sustained survival [Possemato et al. (2011) *Nature* **476**, 346–350]. Via ¹³C₆-glucose tracing, it was confirmed that serine synthesis dependent MDA-MB-468 cells showed serine M+3 and glycine M+2 contribution from labeled glucose, compared to MDA-MB-231 cells where no labeled carbons originating from glucose were built into serine and glycine (Figure 5A). This indicates that MDA-MB-468 cells are able to endogenously produce serine and glycine, whereas MDA-MB-231 cells are not. As such, serine depletion from the culture media only affected the proliferation of MDA-MB-231 cells (Figure 5B).

The effect of sertraline on *in vitro* proliferation of MDA-MB-231 and MDA-MB-468 breast cancer cells was tested by real-time monitoring of cell confluency. It was confirmed that sertraline doses of 3 μ M and 5 μ M specifically impaired proliferation of MDA-MB-468 cells, whereas MDA-MB-231 cells were not (3 μ M) or hardly (5 μ M) affected (Figure 2A). These results thus suggested that enhancers of miconazole's antibiofilm activity indeed can show specific inhibition of serine dependent breast cancer cells. Besides sertraline, 56 other compounds (Table 2) were also identified as enhancers of miconazole's antibiofilm activity.

10 **Table 2. Miconazole potentiators**

Suloctidil	Triclabendazole	Bosutinib
Isosorbide mononitrate	Tioconazole	Cediranib
Sodium nitroprusside	Butoconazole	AG18 (Tyrphostyn)
Temozolomide	Flucytosine	Ispinesib
Folic acid	BMS-833923	QNZ (EVP4593)
Bupropion	Losartan	P5091
Sulfamethoxypyridazine	Azilsartan	JIB (NSC-693627)
Iodoquinol	Alizarin	NSC-319726
Broxyquinoline	PF-4981517	Acipimox
Clioquinol	Deferasirox	Merbromin
Pyrithione zinc	Orbifloxacin	Phentermine
Benzalkonium chloride	Salinomycin (sodium)	Malathion
Dequalinium chloride	Chloroxine	Dichlorophene
Domiphen bromide	Nitroxoline	Bithionate sodium
Methylbenzethonium chloride	Methenamine	Phenylmercuric acetate
Pyrvinium pamoate	NPC15437	Thimerosal
Piroctone olamine	Staurosporine	Formoterol
Ciclopirox olamine	Warfarin	Hexachlorophene
Oxantel pamoate	Carvedilol	

To select the best candidates for validation in the breast cancer models, this list of 56 agents was filtered based on efficiency of killing miconazole-treated *C. albicans* biofilms, on clinical use, and based on the results from SwissTargetPrediction. As for sertraline, the sodium and chloride dependent glycine transporter was a predicted binding partners for bupropion and benzalkonium chloride, and these agents were therefore retained for validation in the breast cancer lines. Additionally, the serotonin transporter (SLC6A4), the known target of sertraline, was predicted as being a binding partner of thimerosal and therefore this agent was also shortlisted. To examine the potential of the SwissTargetPrediction tool in selecting serine-specific anticancer drugs out of a list

of miconazole potentiators, domiphen bromide was used as a negative control as it does not have any of the above-mentioned transporters as predicted targets but still has high antibiofilm activity in combination with miconazole. Each of the selected compounds was tested for inhibiting proliferation of MDA-MB-231 and MDA-MB-468 breast cancer cells using the same assay as used for sertraline. Thimerosal and benzalkonium chloride also showed serine-specific anticancer activity. Conversely, domiphen bromide had general non serine-specific anticancer activity, while bupropion did not show any anticancer activity, not even at high concentrations (Figure 2B). Overall, three out of four agents that ended up on the shortlist were thus confirmed to have serine-specific anticancer activity.

Example 4. Sertraline decreases the proliferation of MDA-MB-468 breast cancer cells via inhibition of *de novo* serine and glycine synthesis.

The mode of action of sertraline on breast cancer cell lines and sertraline's potential impact on *de novo* serine and glycine synthesis was further investigated. ¹³C₆-glucose tracing of sertraline-treated MDA-MB-468 cells clearly showed decreased levels of serine M+3 and glycine M+2 contribution from labeled glucose, pointing to serine synthesis specific activity of sertraline (Figure 3A). Measurements of intracellular serine and glycine, originating from both biosynthesis and uptake, also revealed that glycine levels significantly decreased in sertraline-treated MDA-MB-468 cells, while serine levels were not changing (Figure 3B). However, serine and glycine concentrations in the cell culture medium were not changing, indicating that these two amino acids are not taken up more by the cells when treated with sertraline (Figure 3C). Additionally, rescue experiments showed that serine supply can completely rescue cellular proliferation impairment associated with 3 µM sertraline, and that extra serine can partially rescue effects of a dose of 5 µM sertraline. Glycine was however unable to rescue proliferation of sertraline-treated MDA-MB-468 cells (Figure 3D). Altogether, these observations further support that sertraline reduces *in vitro* proliferation of MDA-MB-468 breast cancer cells by affecting *de novo* serine synthesis.

Example 5. Combining sertraline and artemether further reduces serine synthesis dependent breast cancer.

Recent work demonstrated that serine deprivation can synergize with compounds targeting mitochondrial function, such as biguanides [Gravel Set al. (2014) *Cancer Res.* **74**, 7521–7534]. As many chemotherapeutics, already used in breast cancer treatment, also inhibit mitochondrial function, combining them with sertraline

might strengthen the anticancer activity. In the biofilm context, the anti-malarial drug artemether was identified as a strong miconazole potentiator [Esu et al. (2014) *Cochrane Database Sytematic Rev.* CD010678]. Interestingly, artemisinin, which belongs to the same structural class of natural plant compounds as artemether, has already been shown to inhibit mitochondrial functioning [Grahame (2016) *Acta Pharm. Sinica B* **6**, 1–19]. As a proof of concept, it was tested if combining artemether and sertraline might enhance the inhibitory effect on cell proliferation of *de novo* serine synthesis dependent breast cancer cells.

First, it was tested if artemether is inhibiting mitochondrial activity in the experimental breast cancer models. Metabolic $^{13}\text{C}_6$ -glucose tracing of artemether-treated MDA-MB-468 cells showed inhibition of serine M+3 contribution from labeled glucose, as was expected for a strong miconazole potentiator (Figure 6). Still, a more distinct inhibitory action of artemether on mitochondrial TCA cycle activity was detected, as evidenced by decreased contribution of labeled glucose into TCA cycle metabolites, especially M+4 compounds (Figure 6). Hence, artemether rather acts as a mild mitochondrial inhibitor than as inhibitor of *de novo* serine and glycine synthesis.

As these data imply that artemether and sertraline affect two strongly correlated metabolic pathways, it was next determined whether combining them is more effective for selective inhibition of *de novo* serine synthesis dependent breast cancer. The combination of suboptimal dosages of monotherapeutics indeed further decreased *in vitro* proliferation of MDA-MB-468 as compared to single drug treatment. Conversely, both single and combination treatment did not affect the MDA-MB-231 cell line (Figure 4A).

To validate the serine-specific anticancer activity of this combination further, sertraline and artemether were tested in an additional pair of breast cancer cell lines. To this end, MCF7 and HCC70 were selected, with MCF7 depending on exogenous serine uptake whereas HCC70 requires *de novo* serine and glycine synthesis [Possemato et al. (2011) *Nature* **476**, 346–350]. In agreement with previous results, sertraline monotherapy affected HCC70 but not MCF7 cells. Moreover, the sertraline–artemether combination further reduced the proliferation of HCC70 as compared to monotherapy, whereas this drug cocktail did not cause biologically relevant effects on MCF7 cell proliferation (Figure 4B). It is therefore concluded that combining artemether and sertraline reduces proliferation of various breast cancer cell lines that depend on *de novo* serine synthesis for proliferation.

Finally, metabolic $^{13}\text{C}_6$ -glucose tracing confirmed the superior effect of the combination, as the contribution of labeled glucose into TCA cycle metabolites was stronger reduced. This implies higher anticancer activity when a known mitochondrial inhibitor is combined with a *de novo* serine and glycine synthesis inhibitor, such as sertraline (Figure 4C).

Example 6. The sertraline-artemether combination inhibits growth of MDA-MB-468 mouse xenografts.

To evaluate the therapeutic potential of these findings more profoundly, the efficacy of sertraline, artemether or a combination of both was tested in an *in vivo* mouse model. To this end, MDA-MB-231 and MDA-MB-468 cell lines were implanted in the opposite flanks of immunodeficient NOD-SCID/IL2 γ ^{-/-} (NSG) recipient mice and treated the animals with DMSO, sertraline, artemether or a combination of the two compounds (Figure 4D and 7). After 4 weeks, only the group of MDA-MB-468 mouse xenografts showed significant differences between treatment groups and artemether significantly reduced MDA-MB-468 tumor growth (Figure 4E). Most notably, the combination of sertraline and artemether caused an even stronger inhibition of MDA-MB-468 tumor formation *in vivo* as compared to monotherapy (Figure 4E). In conclusion, the combination of sertraline and artemether not only reduces *in vitro* proliferation of *de novo* serine synthesis dependent breast cancer cell lines, but is also effective in an *in vivo* mouse model.

Example 7. Sertraline decreases proliferation of MDA-MB-468 cells by inhibition of SHMT.

Computational docking of sertraline to *de novo* serine/glycine synthesis enzymes (Table 3), showed the highest docking scores for PHGDH, SHMT1 and SHMT2. Furthermore, the scores obtained for these enzymes were in the range of the docking score of the human serotonin transporter on sertraline, which served as a reference since this transporter is known to bind sertraline. Specifically, sertraline is predicted to bind in the active site of PHGDH and SHMT1/2. Due to low docking scores and weak predicted interactions, PSPH and PSAT1 were not considered as sertraline binding proteins.

Table 3. Computational docking of sertraline to *de novo* serine/glycine synthesis enzymes.

Enzyme	Docking score
PHGDH	53.56
PSAT1	36.25 – 41.20*
PSPH	20.05
SHMT1	38.80 – 52.37*
SHMT2	16.84 – 46.19*
Human serotonin transporter (=Reference)	60.88

- 5 *Depending on the enzyme, sertraline can bind in different conformations and/or in both allosteric and active sites, giving rise to different docking scores.

The docking studies thus indicated PHGDH and SHMT1/2 as the enzymes with the highest affinity for sertraline. We then performed an *in vitro* enzymatic PHGDH assay to test inhibition of PHGDH by sertraline. In this assay, NCT-503, an established PHGDH inhibitor [Pacold et al. (2016) *Nature chem. biol.* **12**, 452–458], served as positive control. In contrast to NCT-503, sertraline was not able to reduce PHGDH enzymatic activity, excluding PHGDH as sertraline binding protein (Figure 11). As such, we focused on SHMT. Witschel et al. (2013) previously identified a plant pyrazolopyran SHMT inhibitor, which has a number of features in its chemical structure that are also present in sertraline (Figure 12) [EP2858981A1; Witschel et al. (2015) *J. Med. chem.* 58, 3117–3130].

It has been shown that SHMT is a ubiquitous pyridoxal 5'-phosphate- (PLP-) dependent enzyme [EP2858981; Ducker et al. (2017) *Proc. Natl. Acad. Sci.* **114**, 11404–11409]. Therefore, docking scores were determined again, but now with the PLP co-factor inside the binding pocket. As a reference, the folic acid derivative, tetrahydrofolate (THF), and the reported plant SHMT inhibitor with a pyrazolopyran scaffold [EP2858981A1; Ducker et al. (2017) *Proc. Natl. Acad. Sci.* **114**, 11404–11409], were used (Table 4). High docking scores were obtained for sertraline, in the range of the compounds characterized by a pyrazolopyran scaffold. Moreover, sertraline binds in the exact same pocket and has similar interactions with SHMT, namely an hydrogen bond between its -NH group and TYR83.

Table 4. Computational docking of sertraline to SHMT, using both tetrahydrofolate and the pyrazolopyran scaffold as references.

Inhibitor	Docking score
SHMT_sertraline1*	61.49
SHMT_sertraline2*	59.68
THF (=reference, co-factor)	82.76
Pyrazolopyran (= reference, inhibitor scaffold)	66.65

* 1 and 2 refer to the two conformations in which sertraline can bind the SHMT pocket.

To measure cellular SHMT activity, we performed an isotopic tracer analysis in which MDA-MB-468 breast cancer cells were incubated for 48 hours with [2,3,3-²H]-serine (deuterium labeled serine), followed by analysis of ²H incorporation in downstream metabolites glycine and thymidine triphosphate (TTP) [Ducker et al. (2017) *Cell Metabolism* **23**, 1140–1153]. Downstream tracing analysis showed that cells take up the deuterated (M+3 labeled) serine and use this to synthesize glycine with one deuterium label (M+1). The amount of intracellular M+1 glycine is therefore a direct readout for SHMT activity. Sertraline lowered the amounts of M+1 glycine, but also decreased the total amounts of intracellular glycine (Figure 13A). This supports that sertraline has dual action, namely inhibiting SHMT and blocking glycine uptake. The latter has been confirmed by measuring glycine uptake in sertraline-treated MDA-MB-468 breast cancer cells (Figure 13B). As reported in Ducker *et al.* (2017), the strength of blocking both processes is what makes SHMT inhibitors more cytotoxic, explaining sertraline's potent activity to MDA-MB-468 cells [Ducker et al. (2017) *Proc. Natl. Acad. Sci.* **114**, 11404–11409].

In contrast to what may be expected from an SHMT inhibitor, sertraline did not increase the amounts of intracellular serine (Figure 3B). To look closer into this, we determined the impact of sertraline treatment on serine and glucose uptake and secretion rates in MDA-MB-468 cells. When cells were treated with sertraline for 72 hours, they showed a dose dependent reduction of serine uptake or even serine secretion upon the highest 5 μ M dose (Figure 13C). These observations can explain the lack of serine accumulation in these breast cancer cells. Whether these effects might be due to direct inhibition of serine uptake by sertraline or a sensory feedback mechanism of pyruvate kinase (PKM2), an important regulator of the glycolytic flux, is unknown [Chaneton et al. (2012) *Nature* **491**, 458–462]. Furthermore, sertraline decreased the glucose uptake (Figure 13D), explaining the

reduction in M+3 carbon labeled serine directly made out of ^{13}C -labeled glucose (Figure 3A). Focusing on the serine mass distribution vector of sertraline-treated MDA-MB-468 cells, after incubation with deuterated serine for 48 hours, again implies inhibition of SHMT supported by decreased M0, M+1 and M+2 serine, which can be generated via SHMT activity or glucose synthesis. On the contrary, a higher completely deuterated M+3 serine fraction arises as these cells cannot process it anymore due to an SHMT block (Figure 13E). Taken together, these results suggest that sertraline inhibits SHMT activity, and as a consequence cells will no longer take up serine and reduce their *de novo* serine synthesis.

To distinguish mitochondrial SHMT2 from cytosolic SHMT1 activity, we next evaluated deuterated serine incorporation into dTTP. While cytosolic SHMT1 will produce M+2 TTP, mitochondrial SHMT2 will generate M+1 TTP via transfer of one-carbon (1C) units from the mitochondria to the cytosol and/or interchange between $\text{CH}_2\text{-THF}$ and CHO-THF [Ducker et al. (2017) *Cell Metabolism* **23**, 1140–1153 ; Gao et al. (2018) *Cell Reports* **22**, 3507–3520]. In proliferating MDA-MB-468 breast cancer cells, only M+1 TTP was detected (Figure 13F), supporting that these cells process their 1C units in the mitochondria via SHMT2. Additionally, the TTP fraction with one deuterium label was decreased by sertraline treatment (Figure 13G). Again, these results support that sertraline inhibits thymidine synthesis from mitochondrial serine metabolism by blocking SHMT2 activity.

Example 8. Sertraline acts synergistically with taxanes, such as paclitaxel and docetaxel.

To investigate the potency of sertraline in drug combinations, we tested a concentration range of sertraline combined with different dosages of one of the following chemotherapeutic agents: paclitaxel, docetaxel, methotrexate and 5-fluorouracil (5-FU). MDA-MB-468 cell proliferation was assessed by cell confluency analysis on a live cell imaging system and these results were used to calculate combination index (CI) values, taking into account the fraction affected (FA) of each combination of drugs. CI values below 1 are considered as synergy, while values above 1 are considered as antagonism. An additive interaction will typically have index values around 1.

In Figure 14, the CI values, linked to a combination of two specific concentrations of each drug, are shown for all four chemotherapeutics tested. Overall, sertraline acted synergistic with both taxanes paclitaxel and docetaxel, while the interaction with methotrexate and 5-FU was rather additive or even antagonistic. Specifically, most of the CI values of sertraline in combination with both taxanes (Figure 14A

and 14B), were below one, indicating that these combinations had a synergistic effect on the MDA-MB-468 cell line. Moreover, most combinations showed relatively high FA values (around or above 0.5), which supports the hypothesis that sertraline addition to treatment with taxanes may be beneficial to metabolically addicted cancers. In contrast, most CI values of sertraline in combination with methotrexate and 5-FU cluster around 1 (Figure 14C and 14D). These compounds mainly showed monotherapy efficacy.

Example 9

We found that the recurrent somatic hotspot mutation at RPL10 (ribosomal protein L10) R98S affecting 8% of the pediatric T-cell leukemia (T-ALL) cases strongly induces PSPH protein expression. An overall increase in PSPH transcription and on top of that an induction of PSPH translational efficiency (ribosome footprinting) in RPL10 R98S cells lead to the accumulation of PSPH, which forces increased levels of labelled $^{13}\text{C}_6$ -Glucose into *de novo* serine/glycine synthesis [Kampen et al. (2019) cited above]. RPL10 R98S cells are more resistant to therapeutic targeting, due to their RPL10 R98S mutant ribosomal preference for BCL-2 translation as a survival factor [Wang et al. (2017) *Nature* **546**, 426–430]. Nevertheless, it was shown that RPL10 R98S mutant cells present an increased sensitivity towards sertraline treatment, suggesting that these cells are reliant on serine/glycine synthesis (see figure 9). This figure shows absolute cell survival after 96h of incubation with sertraline, comparing Ba/F3 RPL10 WT and R98S mutant clones n=3.

CLAIMS

1. Sertraline for use in the treatment of a cancer with increased serine/glycine synthesis.
- 5 2. Sertraline for use in the treatment according to claim 1, wherein said cancer has an increased expression of PSPH (phosphoserine phosphatase), PHGDH (phosphoglycerate dehydrogenase), PSAT1 (phosphoserine amino-transferase), SHMT1 (serine hydroxymethyltransferase2) or SHMT2 (serine hydroxymethyltransferase 2).
- 10 3. Sertraline for use in the treatment according to claim 2, wherein the PSPH protein has a Val116Ile mutation.
- 15 4. Sertraline for use in the treatment according to claim 1 or 2, wherein the cancer has a copy number gain of a serine/glycine synthesis gene selected from the group consisting of PHGDH, PSAT1, PSPH, SHMT1 and SHMT2.
- 20 5. Sertraline for use in the treatment according to any one of claims 1 to 4, wherein said cancer is selected from the group consisting of melanoma, glioblastoma brain tumor, prostate cancer, testis cancer, ovary cancer, liver cancer, kidney cancer, pancreas cancer, head and neck cancer, lung adenoma, and bladder cancer.
- 25 6. Sertraline for use in the treatment according to any one of claims 1 to 5, wherein said cancer is a breast cancer.
7. Sertraline for use in the treatment according to claim 5 or 6, wherein said breast cancer has increased SHMIT1 and/or SHMT2 expression levels.
- 30 8. Sertraline for use in the treatment according to any one of claim 5 to 7, wherein said breast cancer is an estrogen receptor negative cancer.
9. Sertraline for use in the treatment according to claim 8, wherein said estrogen receptor negative cancer has an increased PHGDH protein level.
- 35

10. Sertraline for use in the treatment according to any one of claims 5 to 9, wherein said breast cancer has a copy number gain of MYC.
- 5 11. Sertraline for use in the treatment according to claim 5 to 10, wherein said breast cancer is a triple negative cancer.
12. Sertraline for use in the treatment according to any one of claims 1 to 4, wherein said cancer is a T-cell acute lymphoblastic leukaemia (T-ALL).
- 10 13. Sertraline for use in the treatment according to claim 12, wherein said leukemia is a pediatric T-ALL.
14. Sertraline for use in the treatment according to claim 12 or 13, wherein said leukemia has an RPL10 mutation.
- 15 15. Sertraline for use in the treatment according to claim 14, wherein said RPL10 mutation is R98S.
16. Sertraline for use in the treatment according to claim 14, wherein said
20 RPL10 mutation is selected from the group consisting of I33V, E66G, I70M, I70L R98C, H123P and Q123P.
17. Sertraline for use in the treatment according to claim 12 or 13, wherein said leukemia has a NKX2-1 rearrangement.
- 25 18. A combination of sertraline and a further anticancer drug for use in the treatment of a cancer with increased serine/glycine synthesis according to any one of claims 1 to 17.
- 30 19. A method of treating in an individual a cancer with increased serine/glycine synthesis, comprising the step of administering to said individual an effective amount of sertraline.
- 35 20. An in vitro method of determining whether a cancer is sensitive for sertraline, comprising the steps of determining in a cancer tissue sample whether serine/glycine synthesis is increased as compared with a reference sample.

A

DE NOVO SERINE AND GLYCINE SYNTHESIS

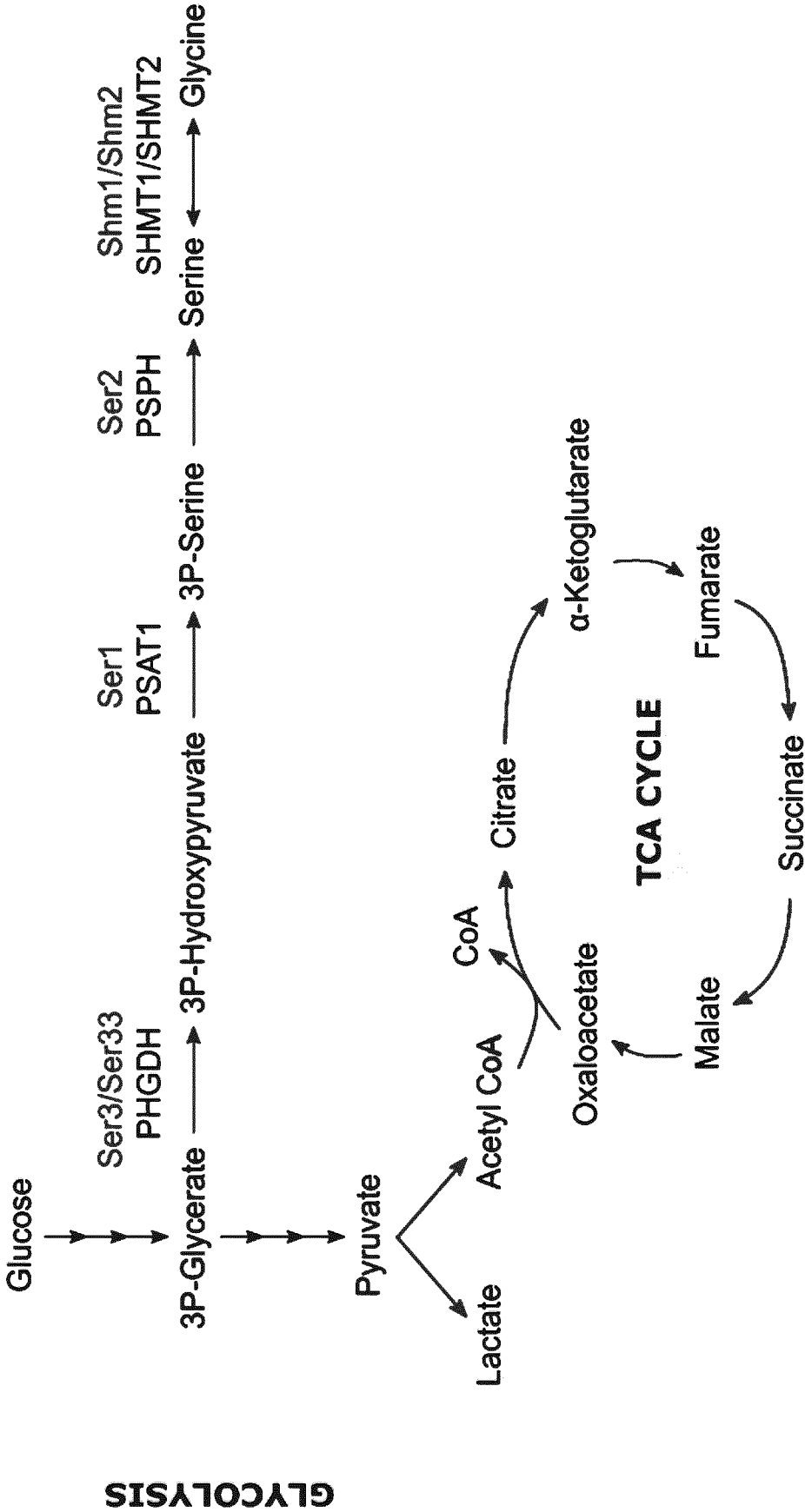
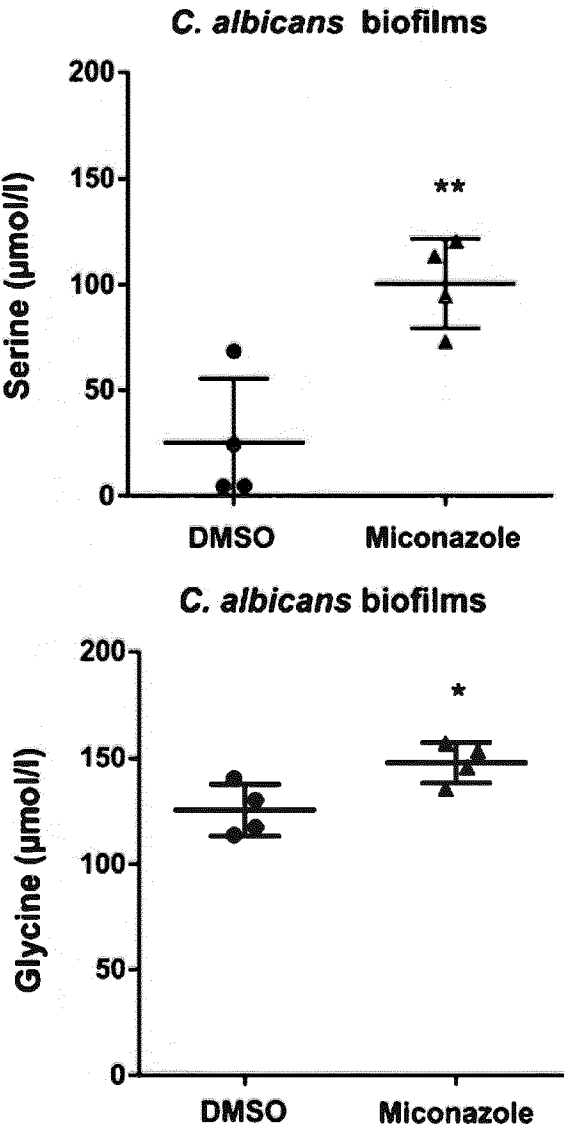


Figure 1

B.



C.

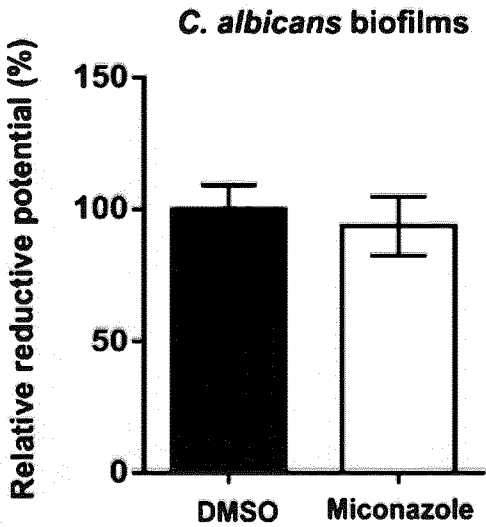
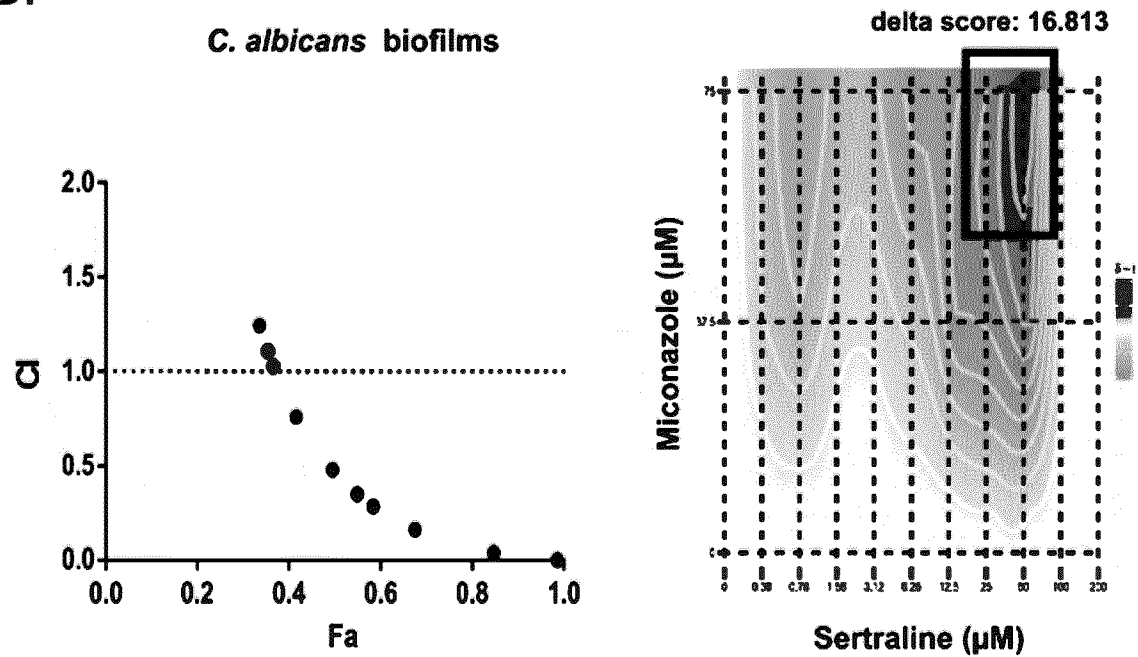


Figure 1 (continued)

D.



E.

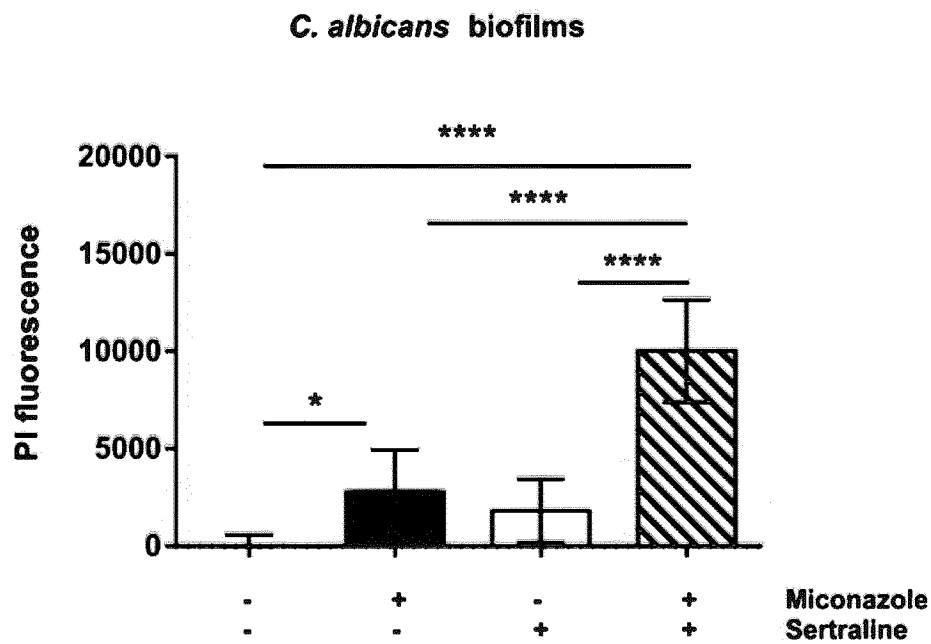


Figure 1 (continued)

4/23

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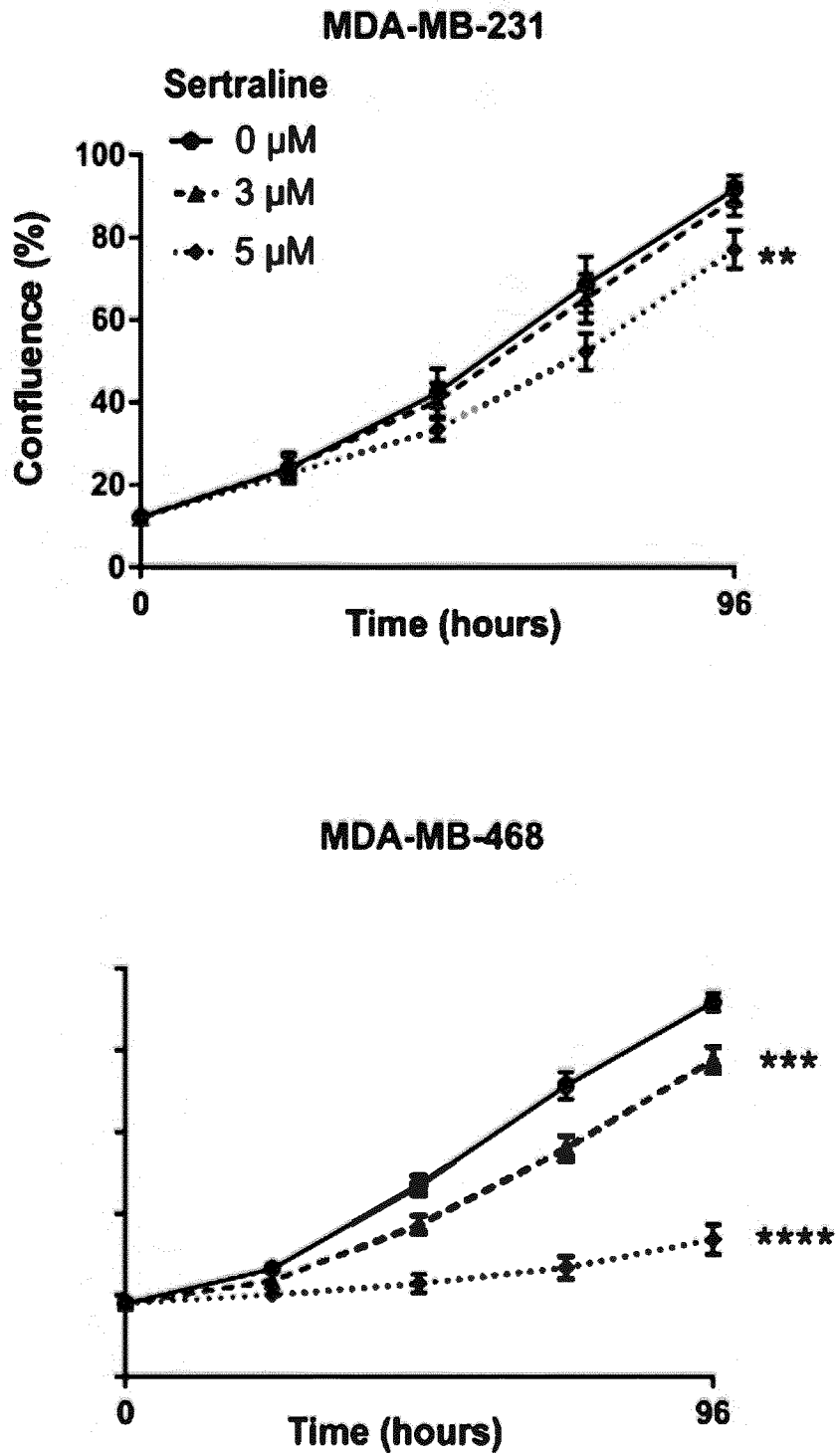


Figure 2

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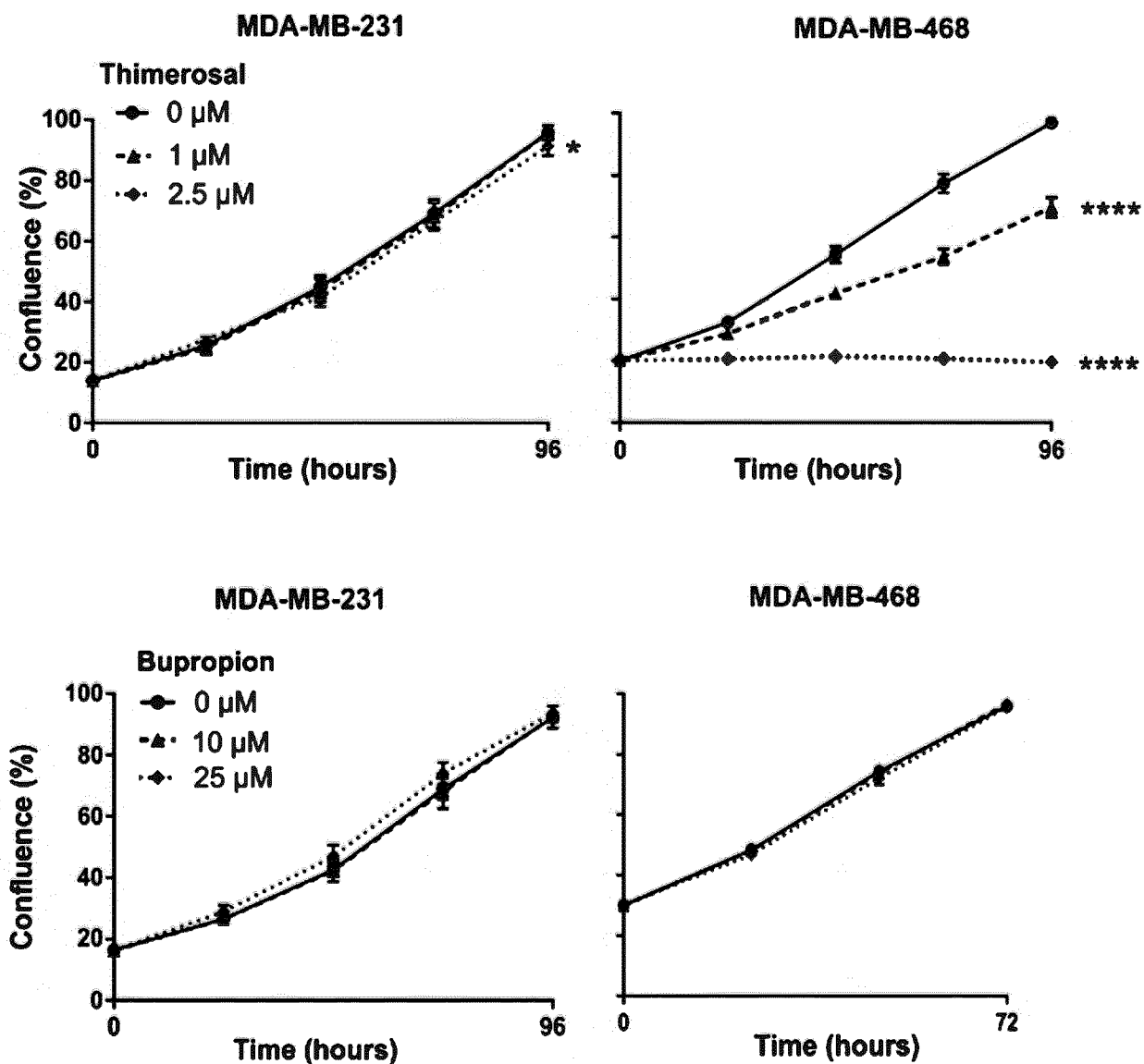


Figure 2(continued)

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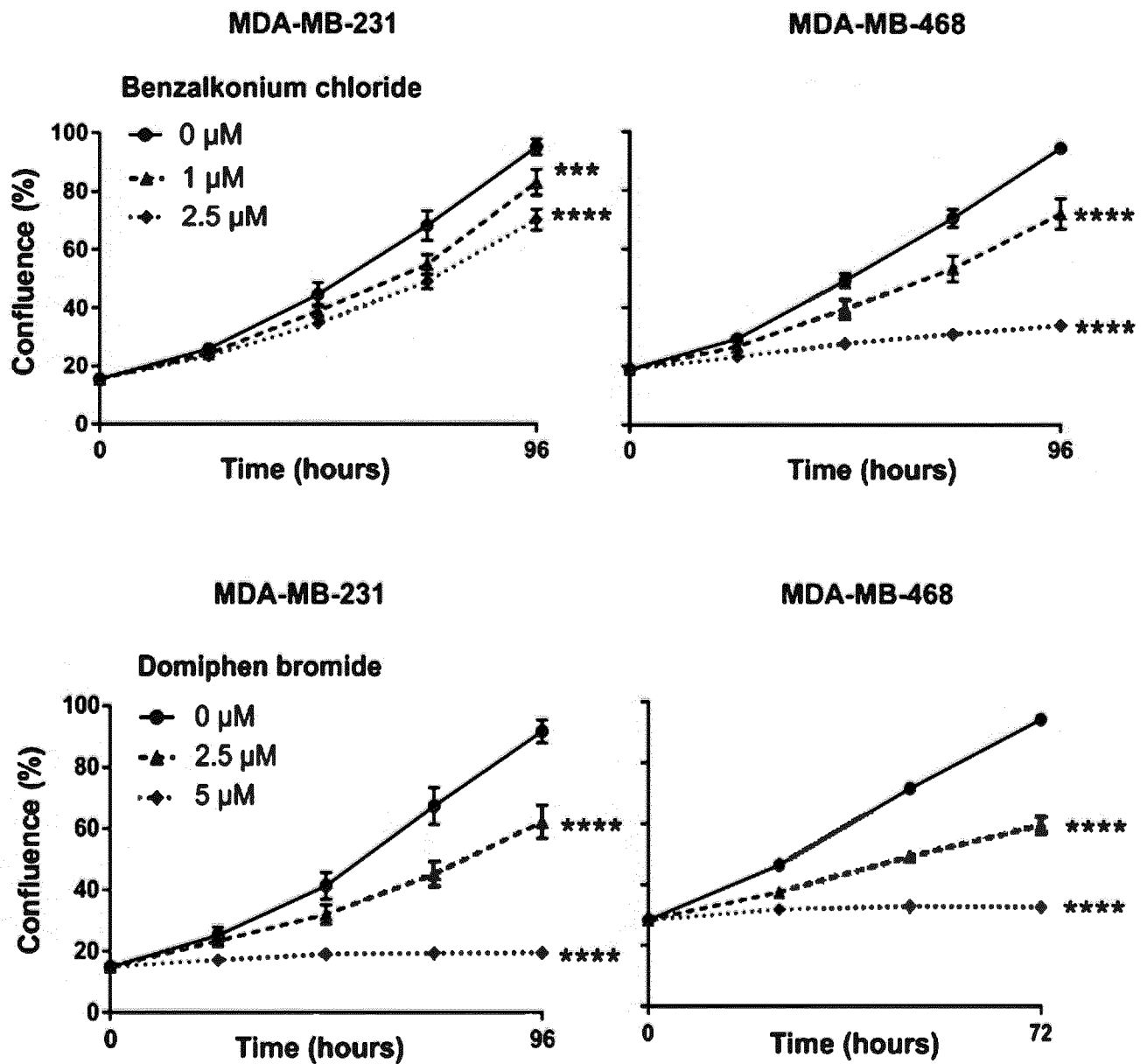


Figure 2(continued)

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A

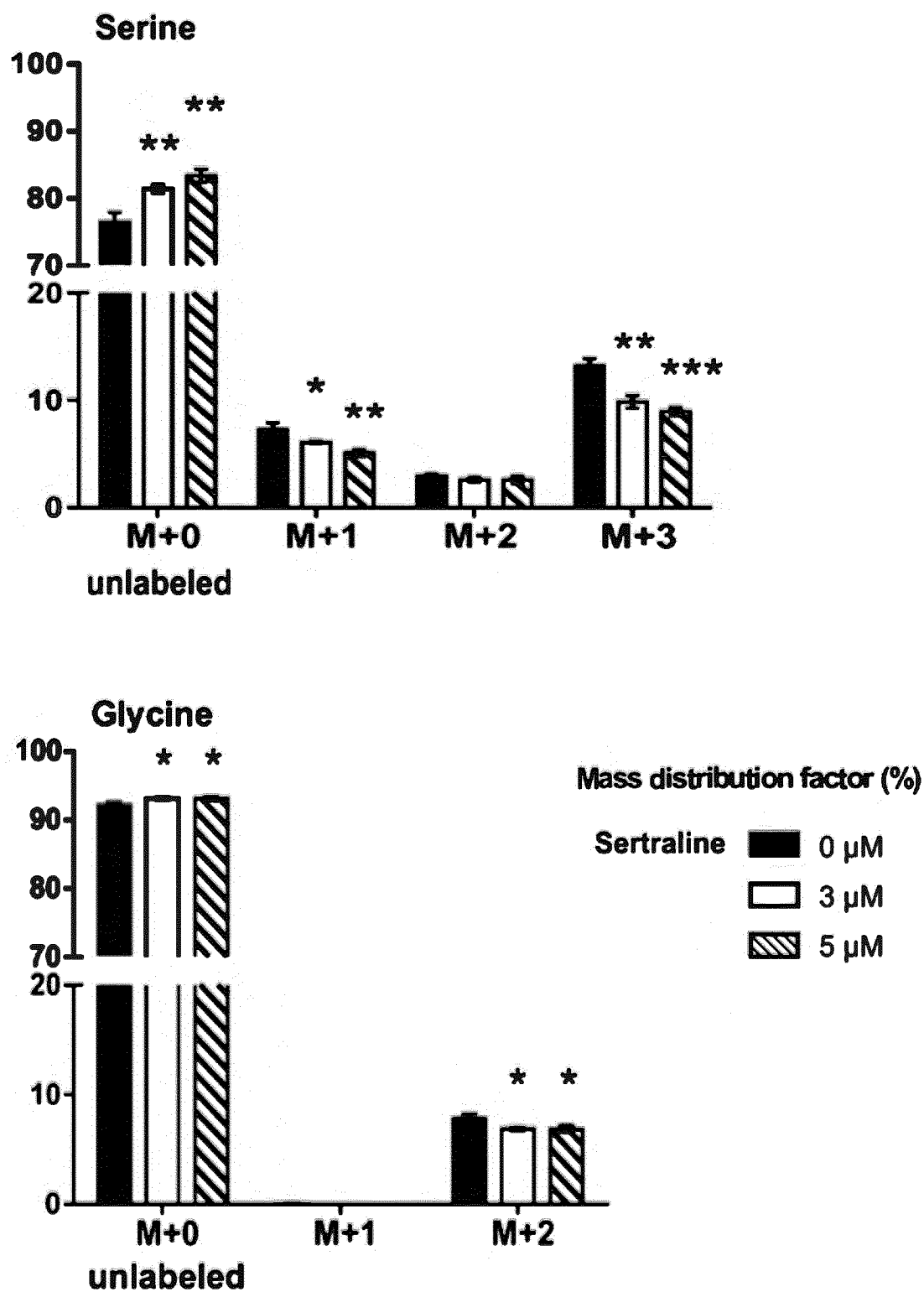
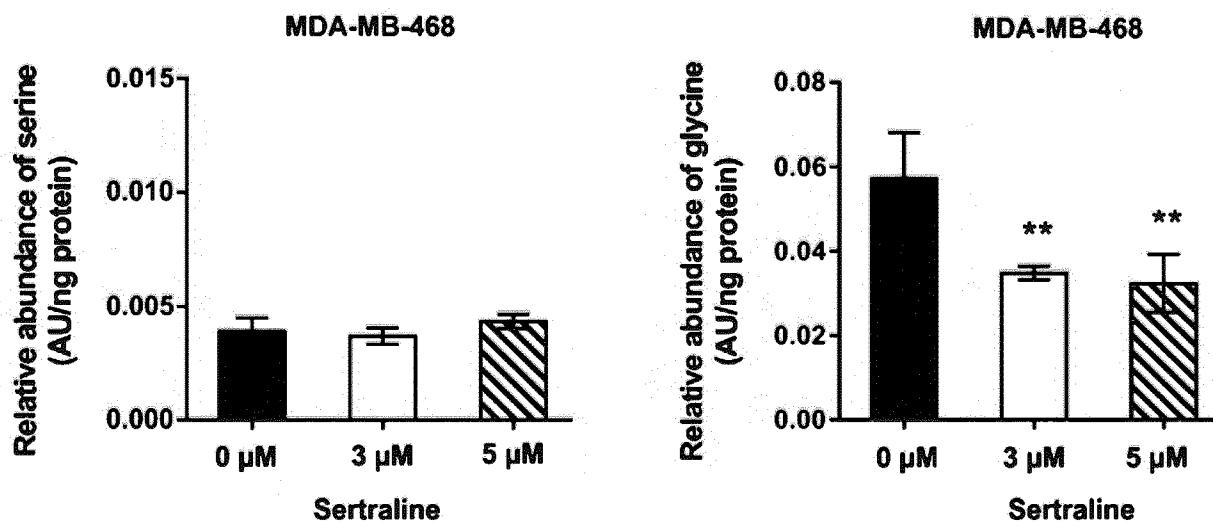


Figure 3

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B.



C.

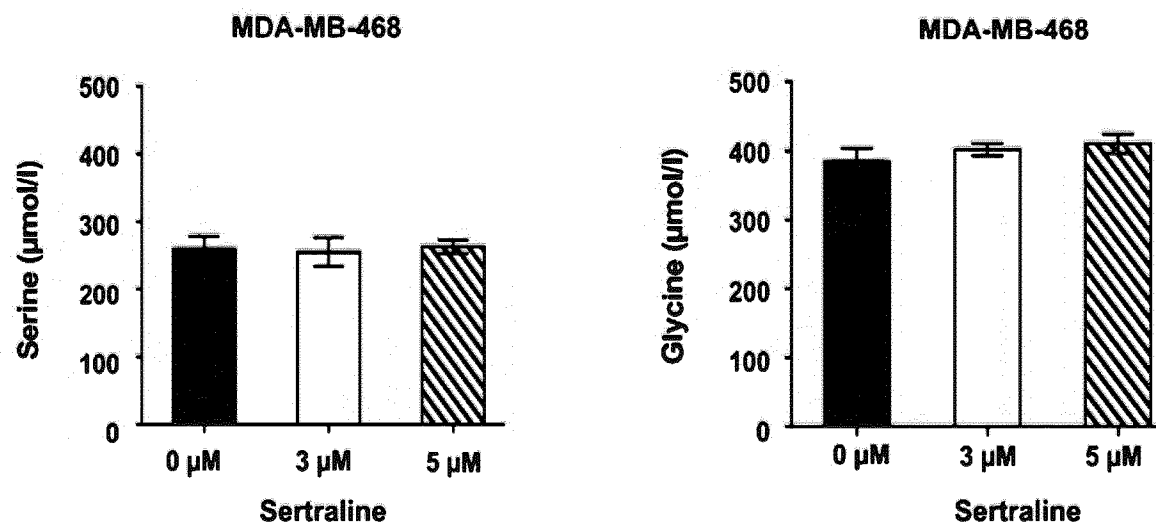


Figure 3 (continued)

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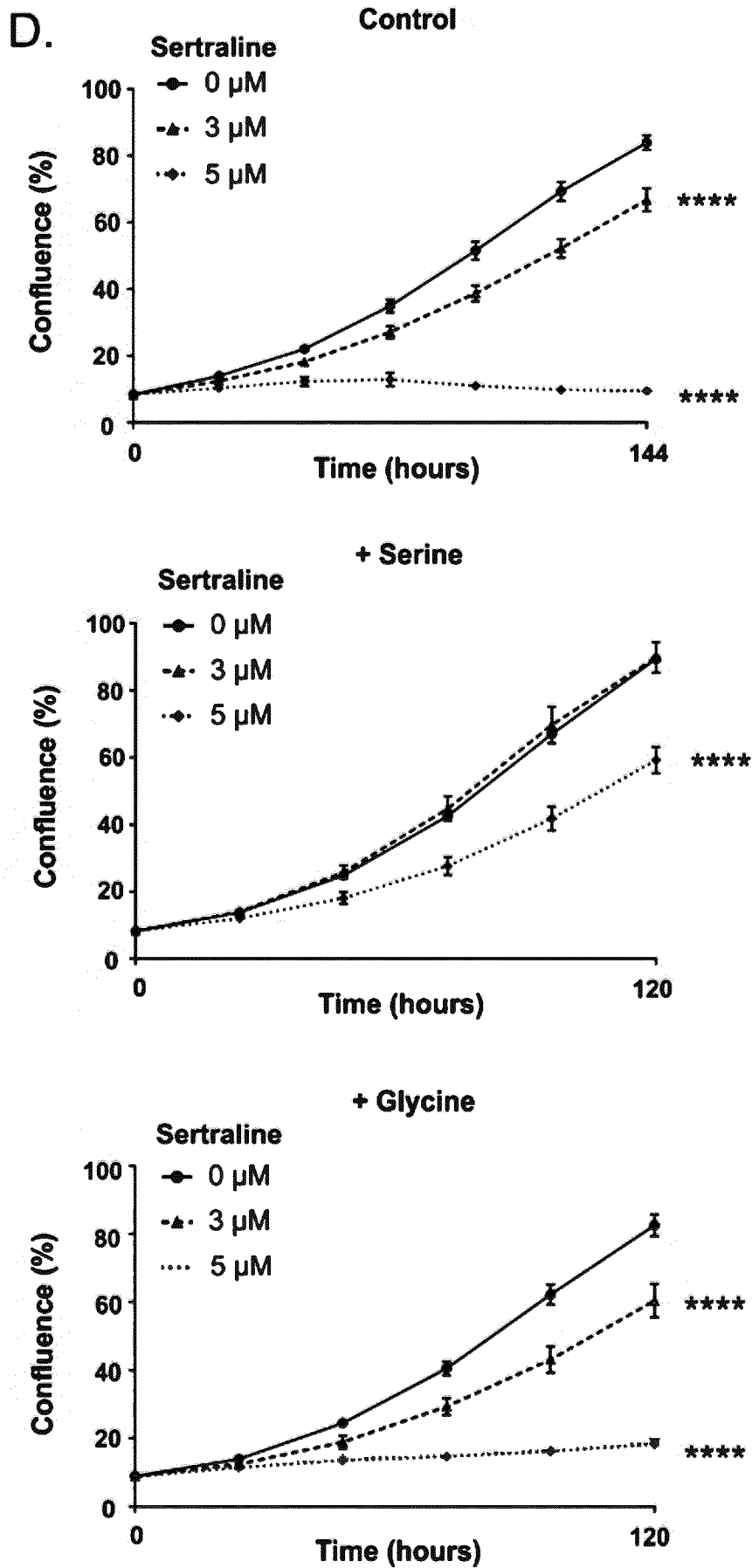
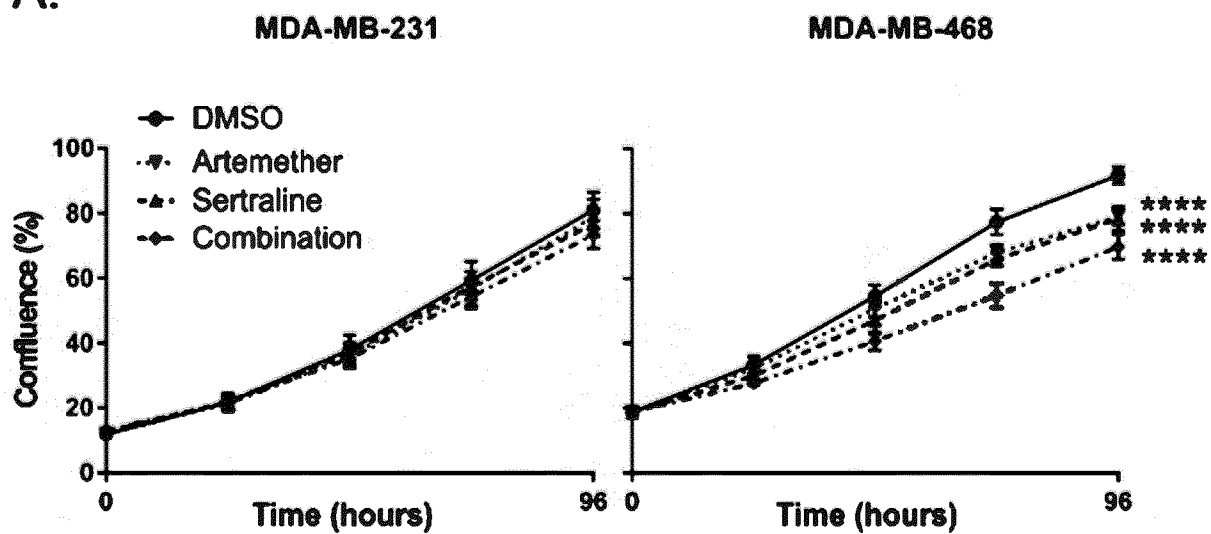


Figure 3 (continued)

10/23

A.



B.

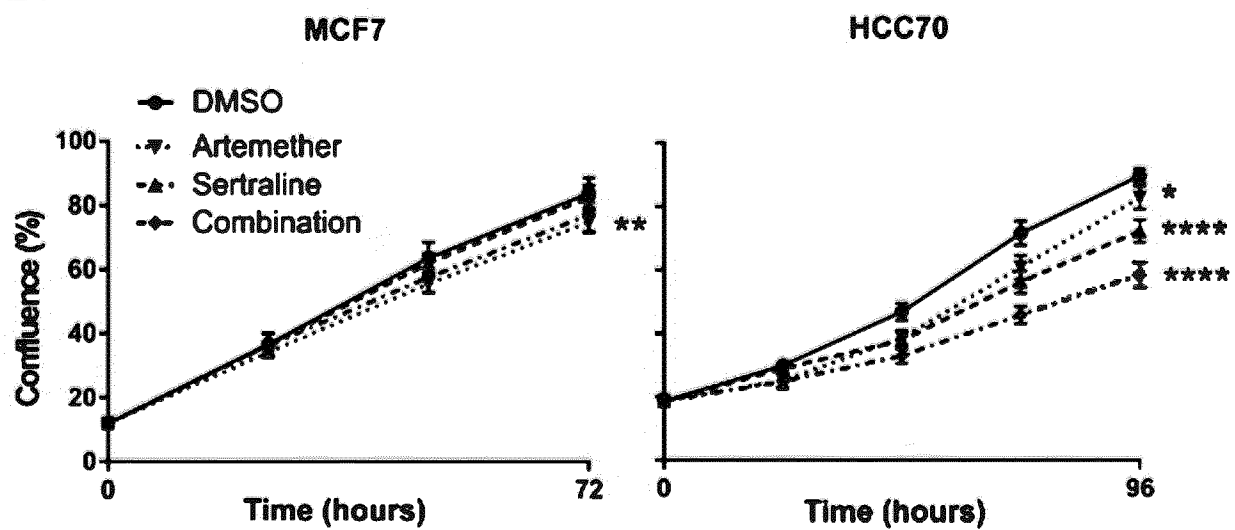


Figure 4

C

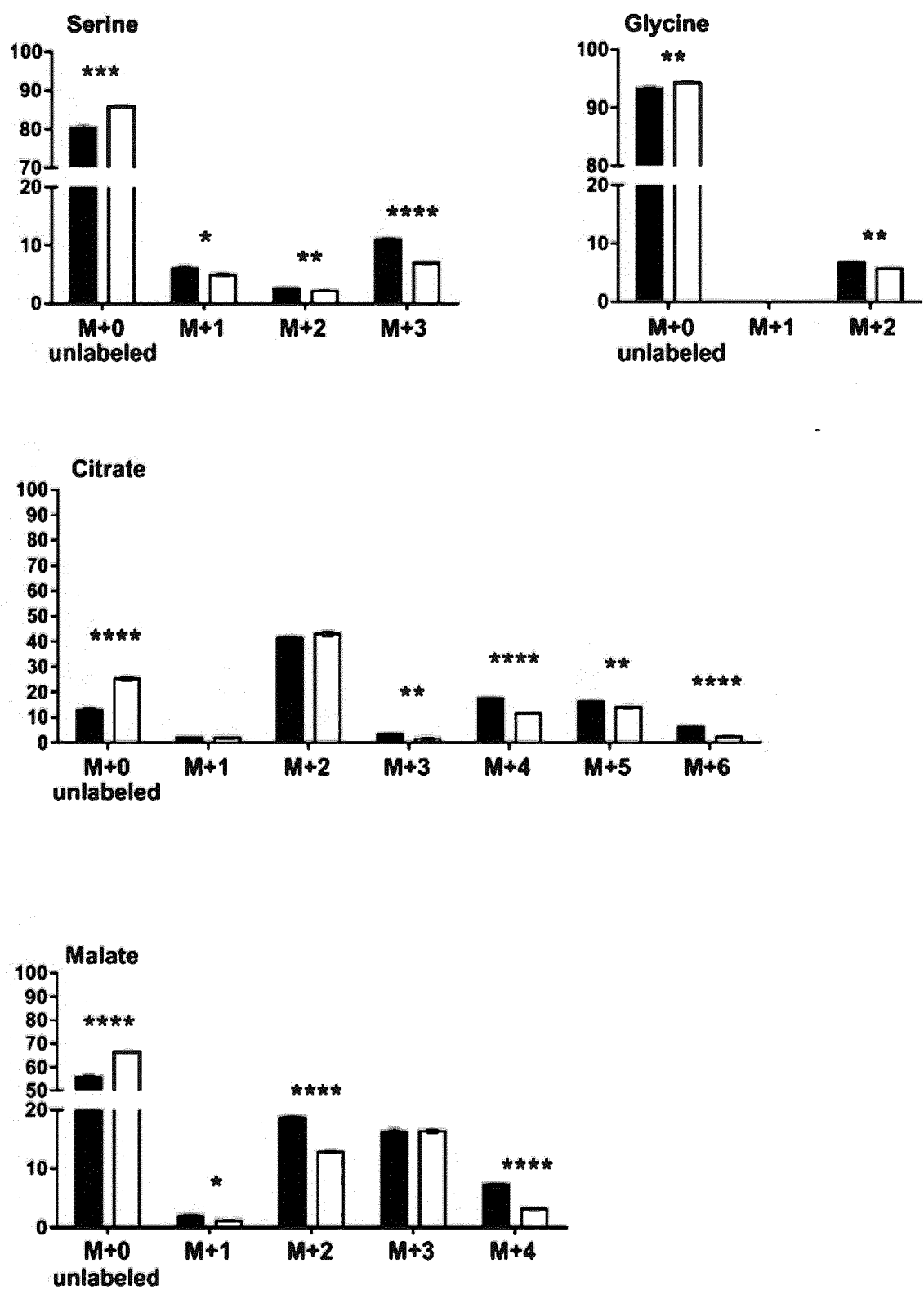


Figure 4 (continued)

12/23

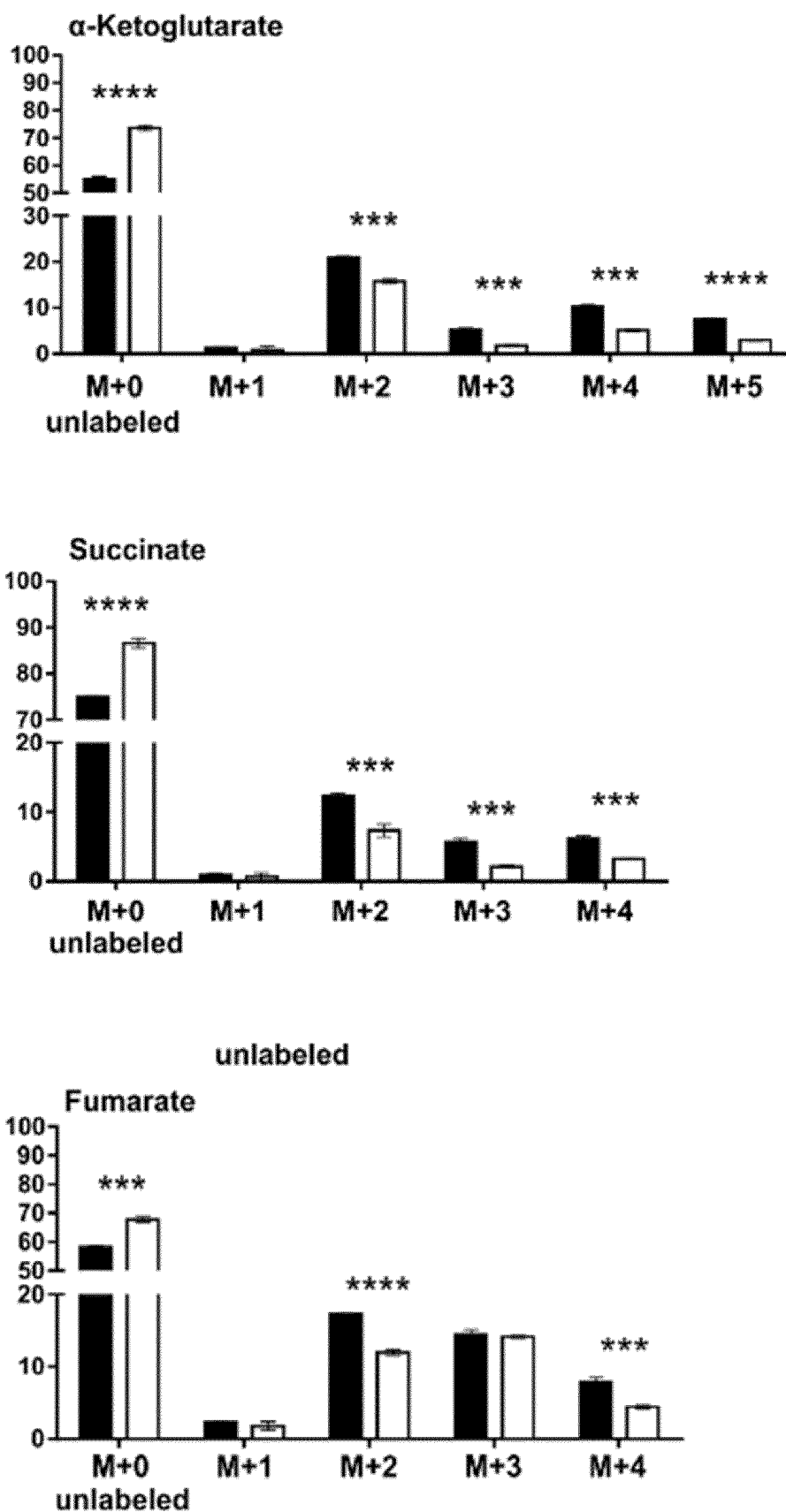
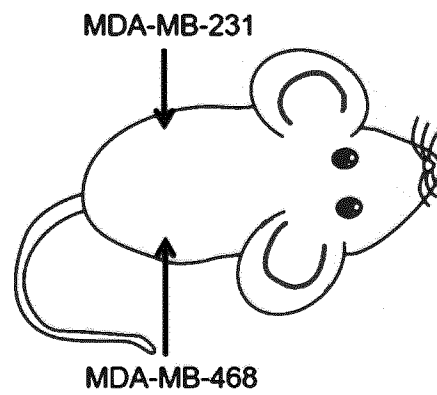


Figure 4 (continued)

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D.



E.

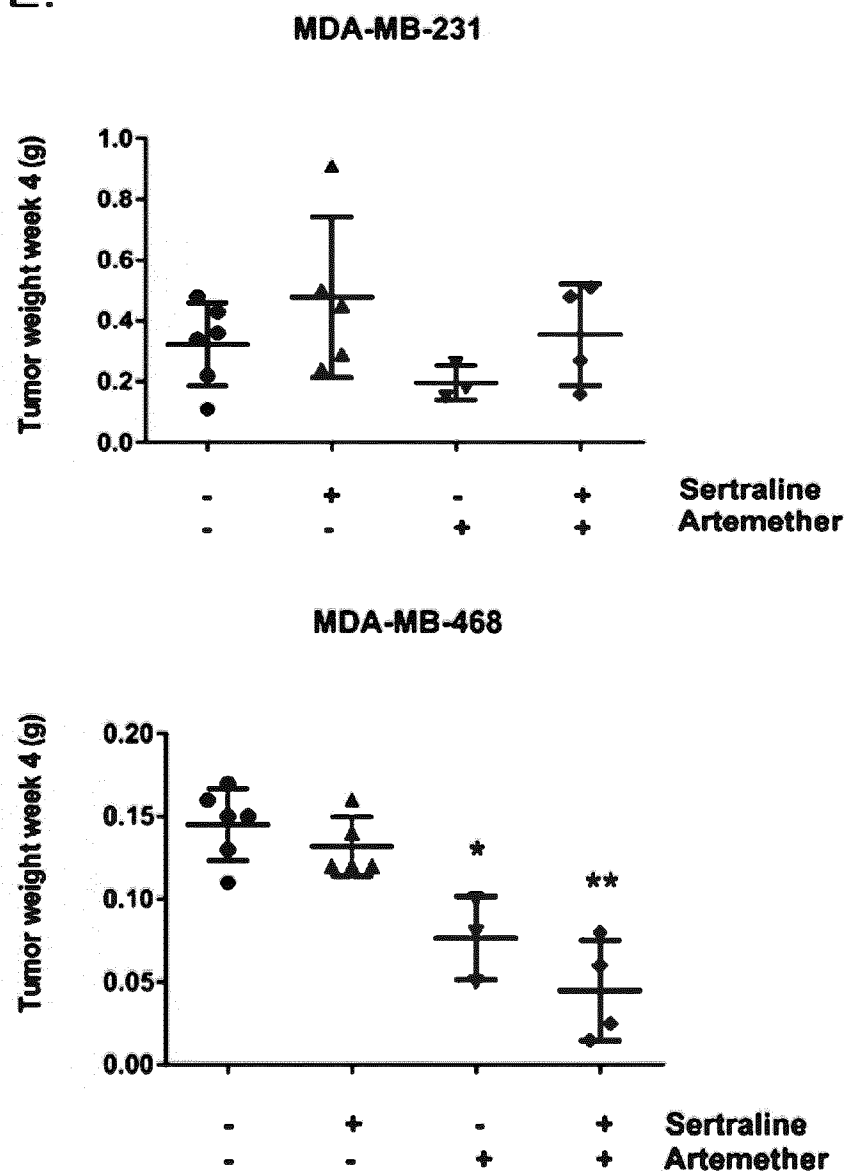
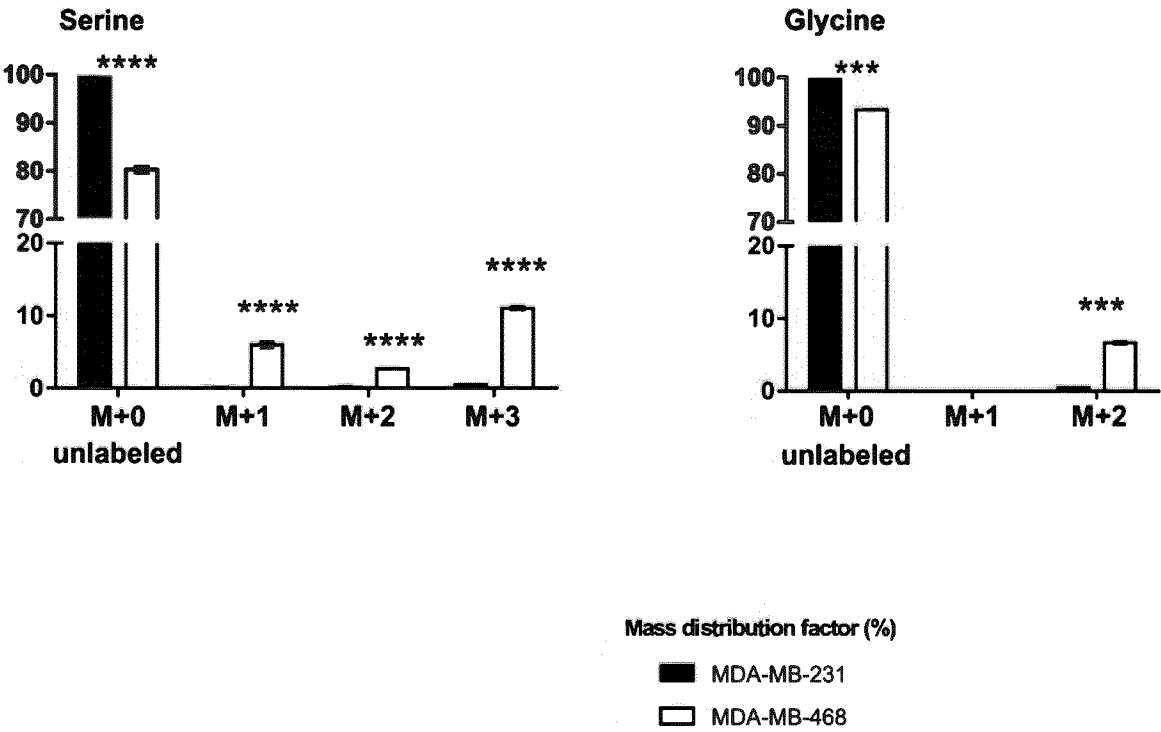


Figure 4 (continued)

A



B.

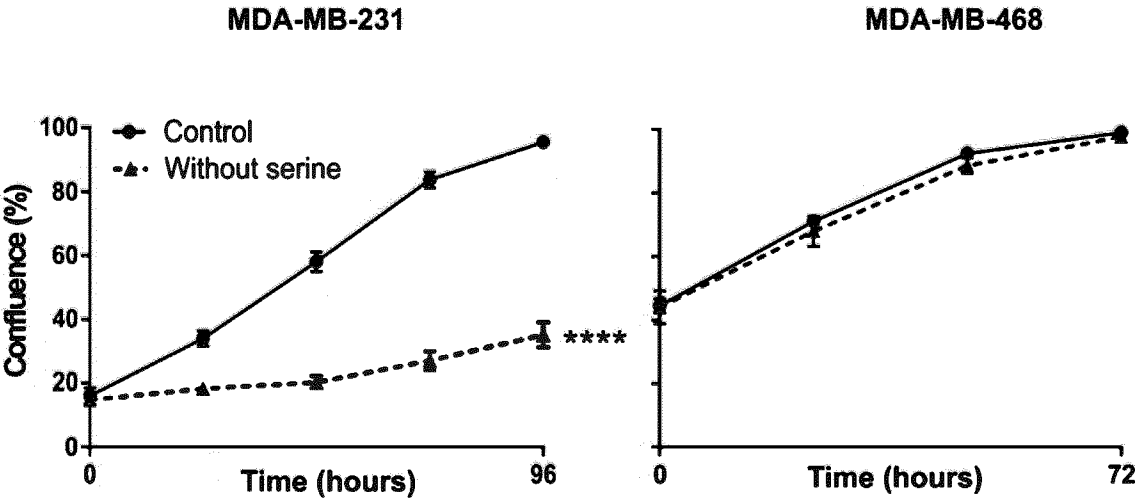


Figure 5

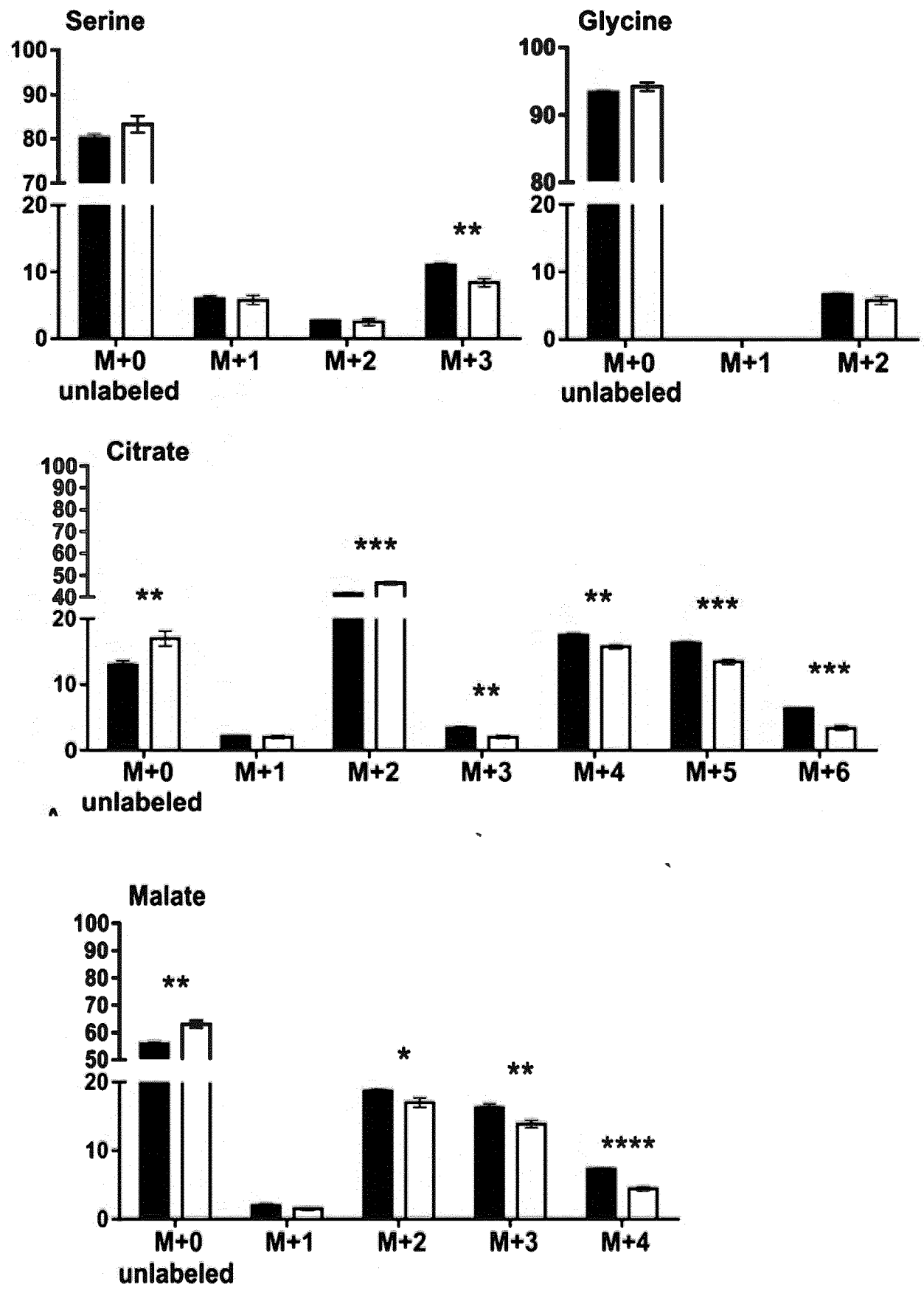


Figure 6

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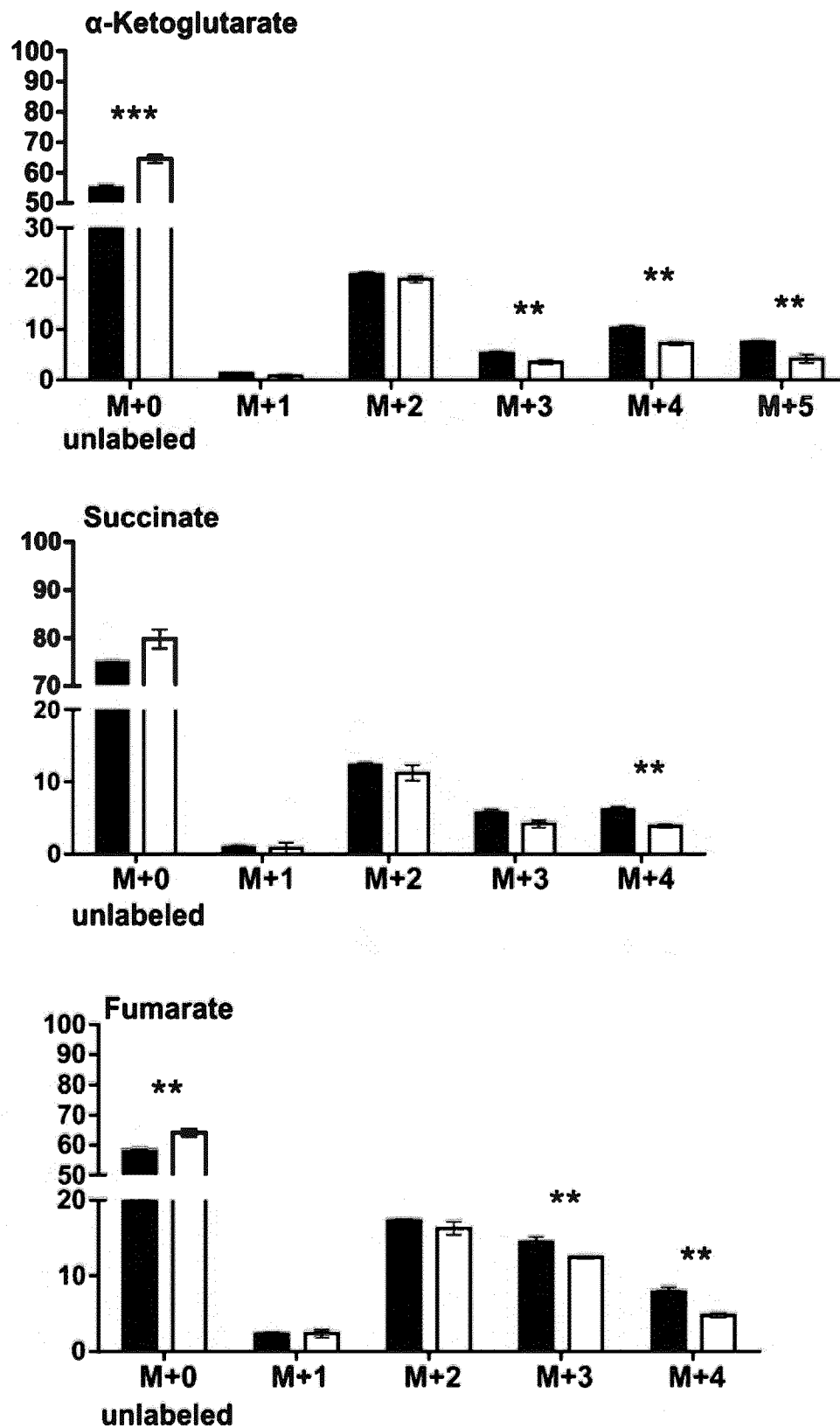


Figure 6 (continued)

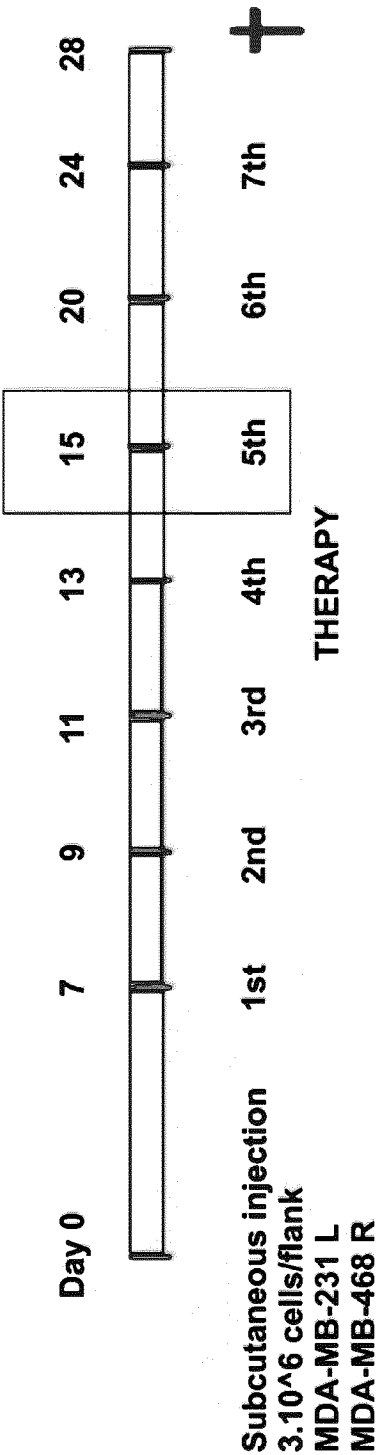


Figure 7

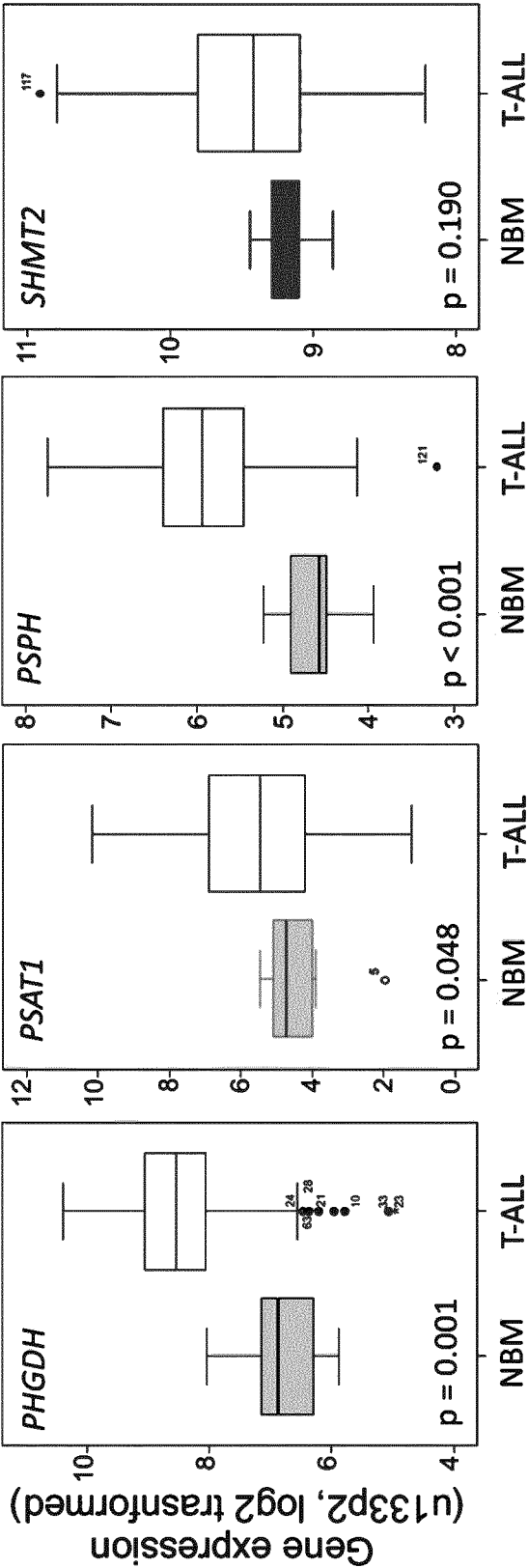


Figure 8

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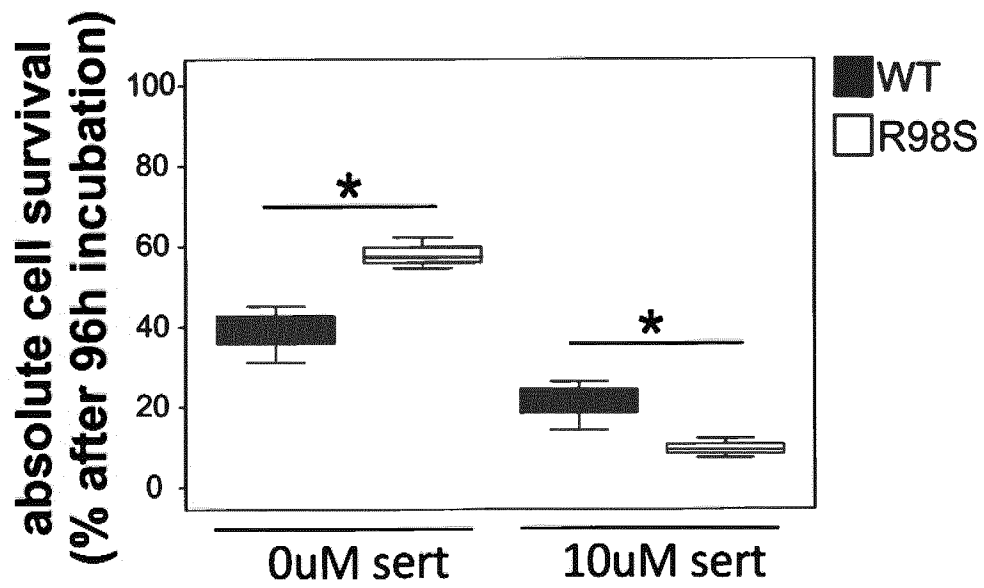


Figure 9

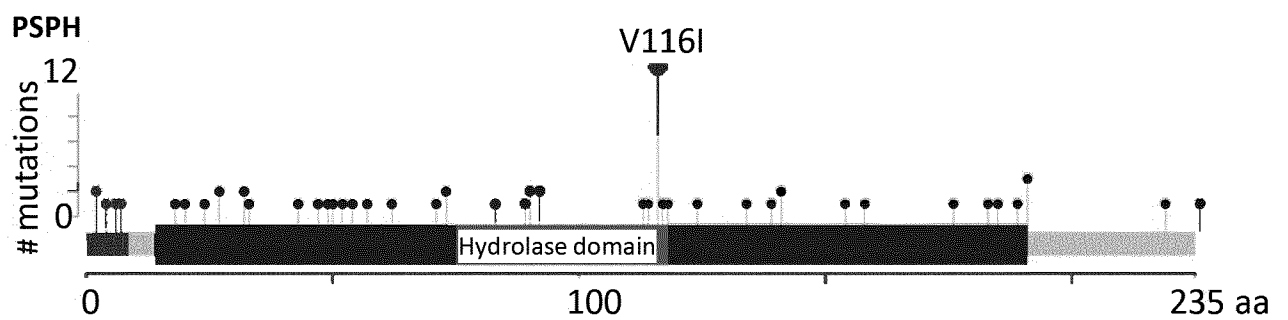


Figure 10

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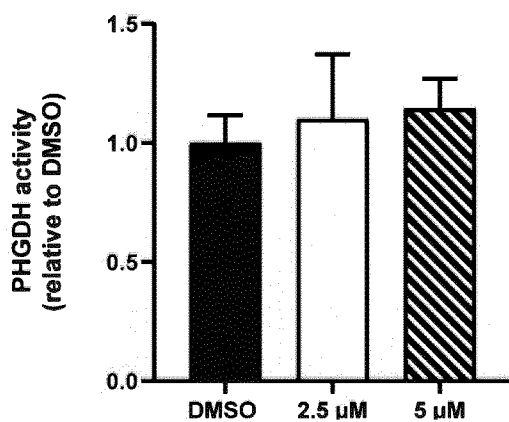
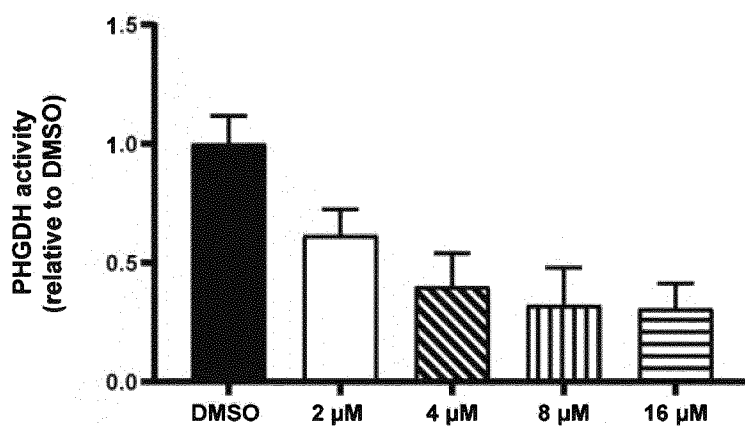
A**Sertraline****B****NCT-503**

Figure 11

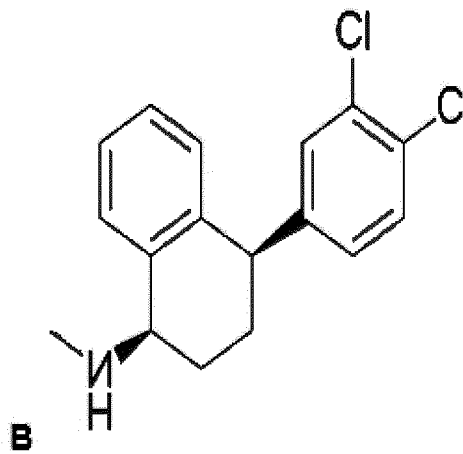
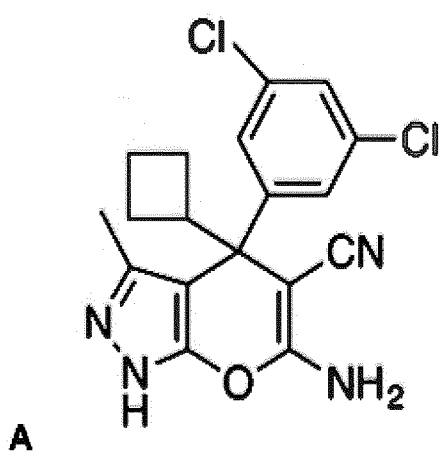


Figure 12

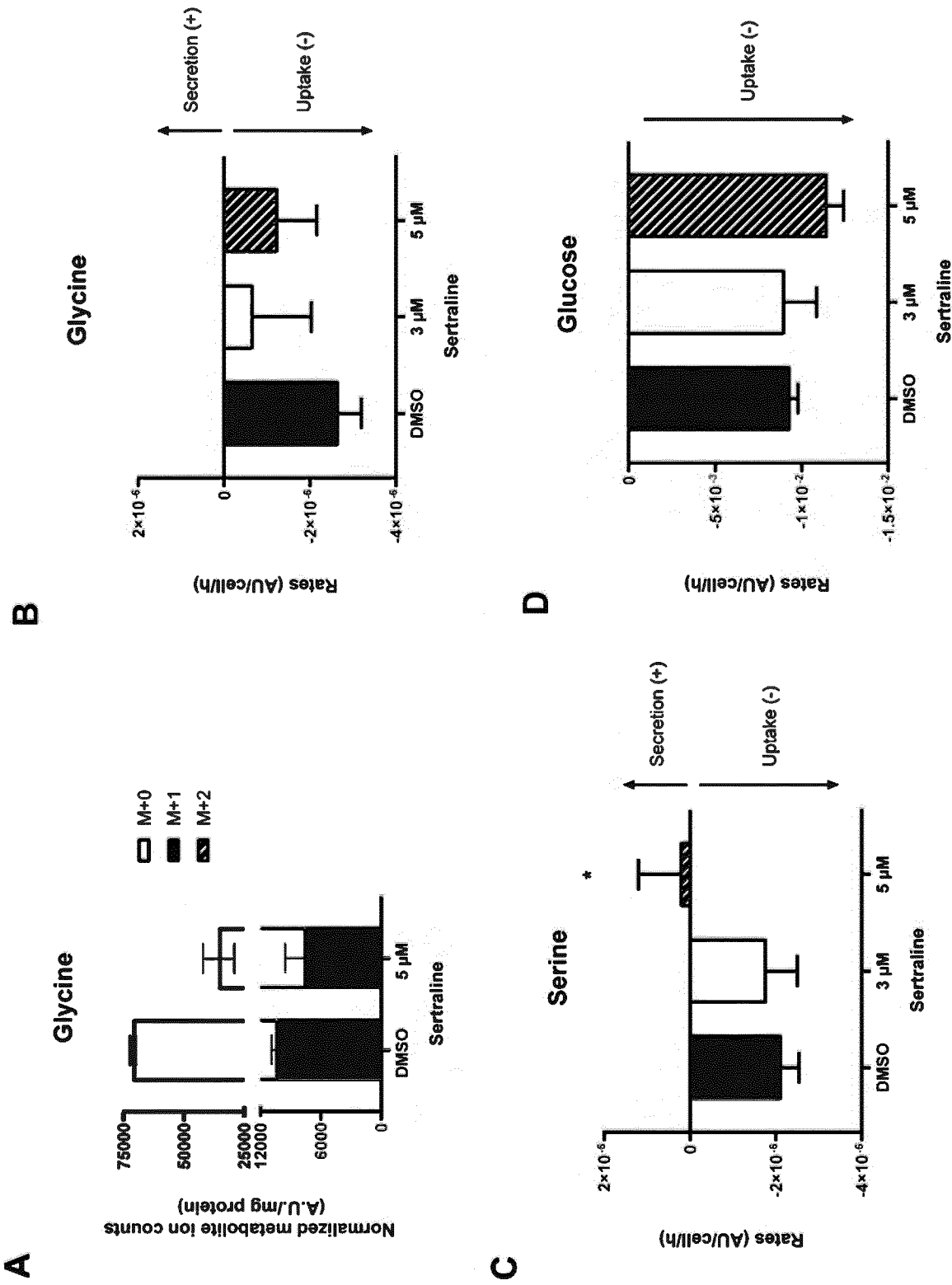


Figure 13

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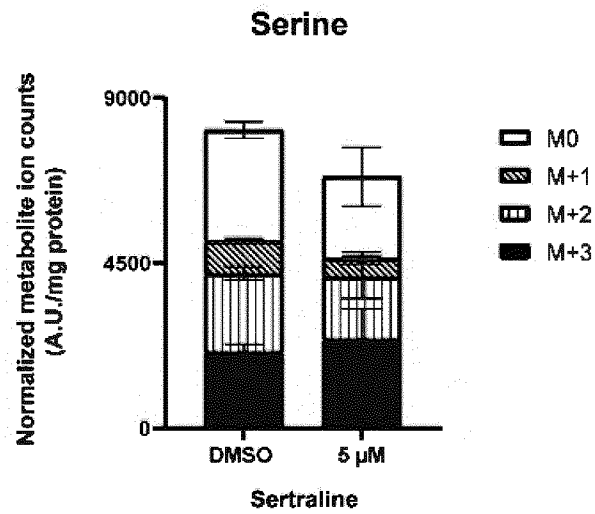
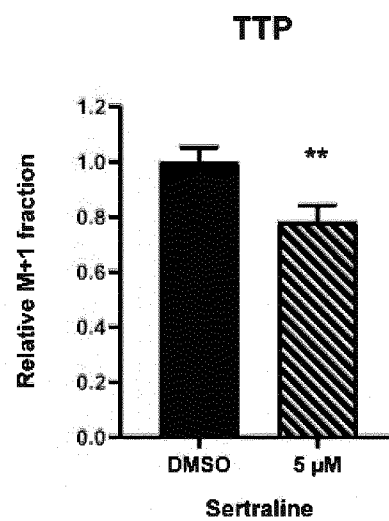
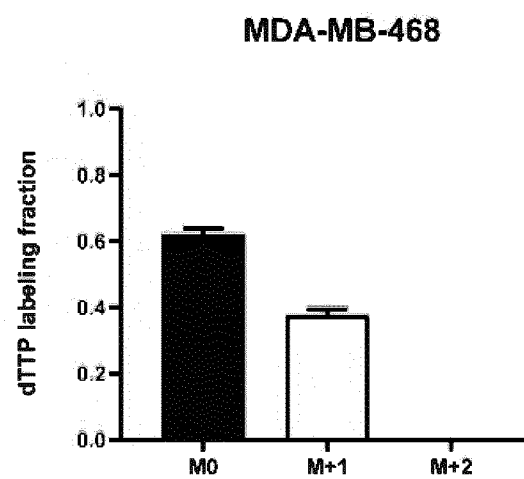
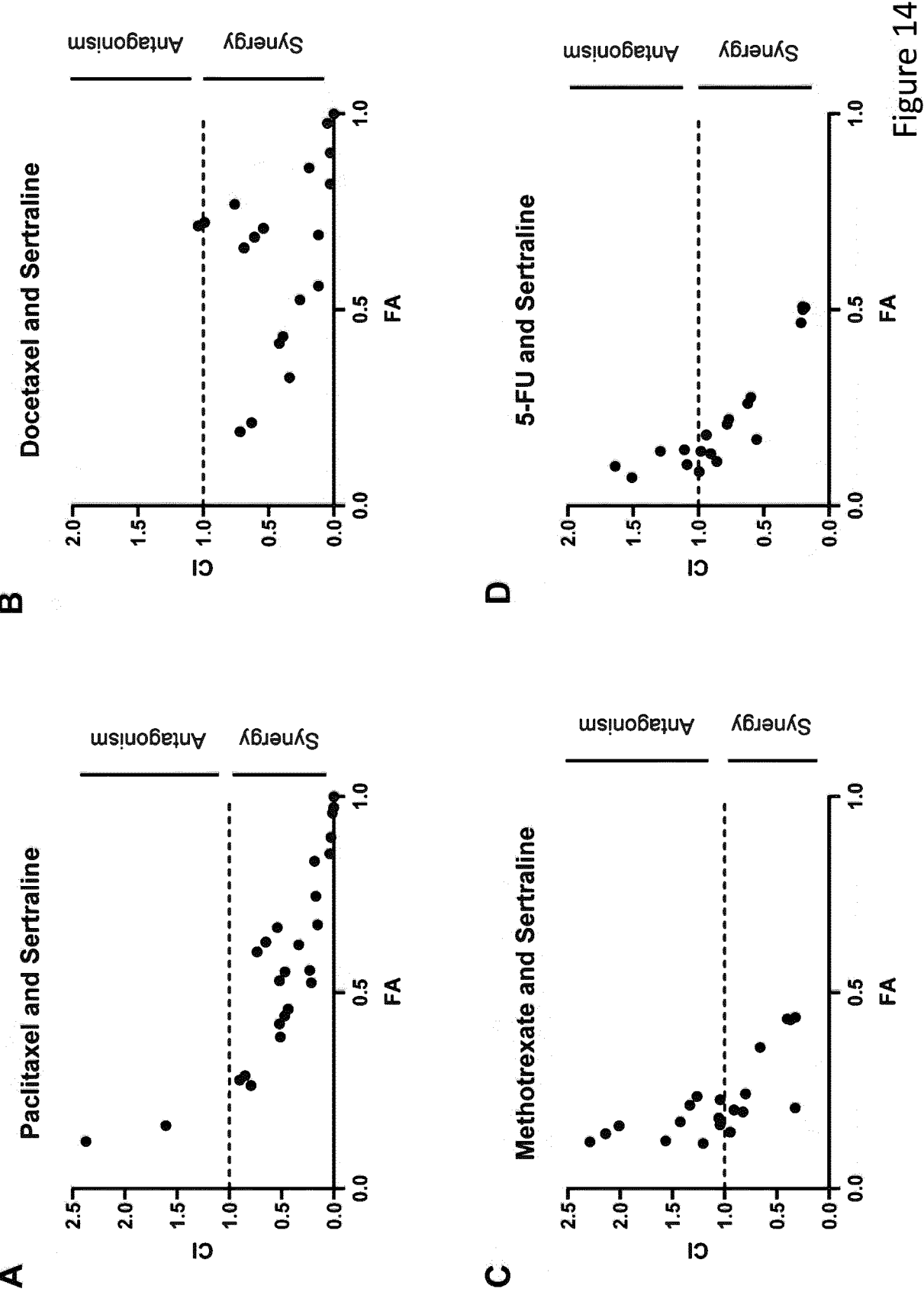
E**G****F**

Figure 13 (continued)



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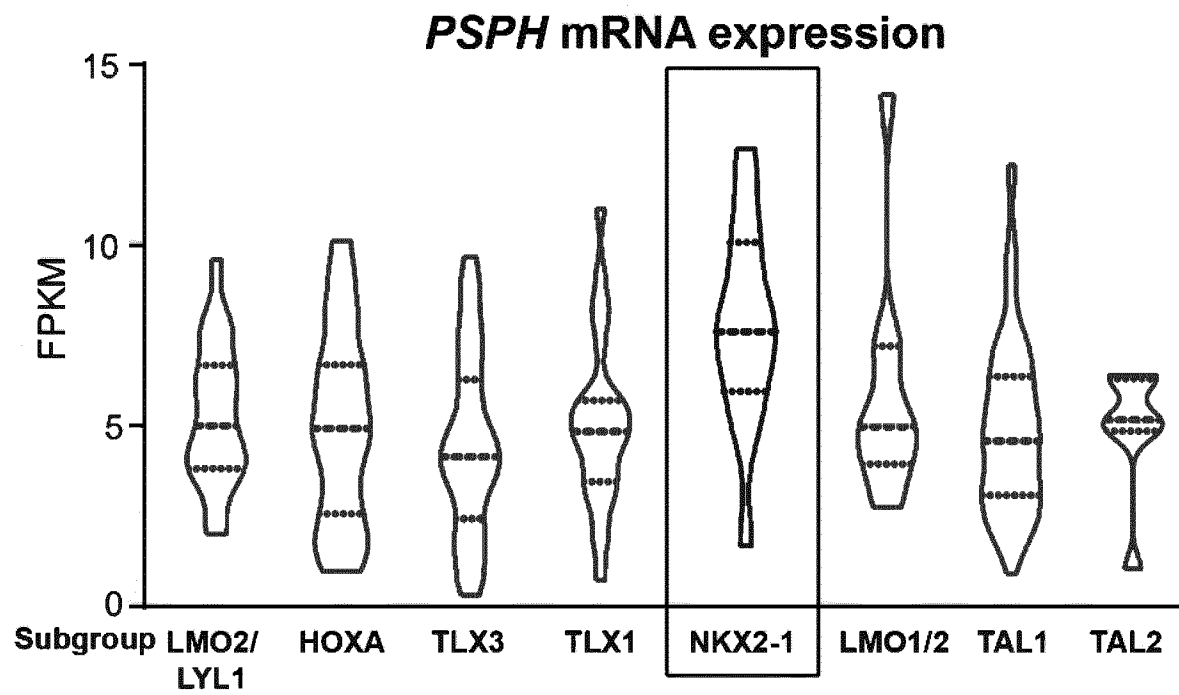


Figure 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/072826

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/135 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Y LI ET AL: "Identification of translationally controlled tumor protein in promotion of DNA homologous recombination repair in cancer cells by affinity proteomics", ONCOGENE, vol. 36, no. 50, 28 August 2017 (2017-08-28), pages 6839-6849, XP055625948, London ISSN: 0950-9232, DOI: 10.1038/onc.2017.289 abstract, Fig. 5; p. 6846, last of col. 1 ----- -/-	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

8 November 2019

Date of mailing of the international search report

26/11/2019

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Dahse, Thomas

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/072826

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WILLIAM D. GWYNNE ET AL: "Serotonergic system antagonists target breast tumor initiating cells and synergize with chemotherapy to shrink human breast tumor xenografts", ONCOTARGET, vol. 8, no. 19, 9 May 2017 (2017-05-09), XP055579817, DOI: 10.18632/oncotarget.16646 abstract, table 3; Fig. 5 -----	1-20
A	ROBIN M. HALLETT ET AL: "Serotonin transporter antagonists target tumor-initiating cells in a transgenic mouse model of breast cancer", ONCOTARGET, vol. 7, no. 33, 16 August 2016 (2016-08-16), XP055579828, DOI: 10.18632/oncotarget.10614 title, abstract -----	1-20
A	WO 2007/006799 A1 (CERENIS [FR]; REVAH FREDERIC [FR] ET AL.) 18 January 2007 (2007-01-18) Fig. 1B, Fig. 1C -----	1-20
A	WO 2010/135468 A1 (VIVIA BIOTECH S L [ES]; BALLESTEROS JUAN [ES] ET AL.) 25 November 2010 (2010-11-25) claim 55; [0180]; [0061]; [0106]; [0111], [0153], [0156], fig. 8, 9, 11, 28. -----	1-20
A	KATHERINE R. MATTAINI ET AL: "The importance of serine metabolism in cancer", THE JOURNAL OF CELL BIOLOGY : JCB, vol. 214, no. 3, 25 July 2016 (2016-07-25) , pages 249-257, XP055639289, US ISSN: 0021-9525, DOI: 10.1083/jcb.201604085 p. 251-252, in particular p. 252, penultimate of col. 1 -----	1-20
A	FRICK LUCIANA ROMINA ET AL: "Antidepressants: Influence on cancer and immunity?", LIFE SCIENCES, PERGAMON PRESS, OXFORD, GB, vol. 92, no. 10, 28 January 2013 (2013-01-28), pages 525-532, XP028988192, ISSN: 0024-3205, DOI: 10.1016/J.LFS.2013.01.020 p. 526: penultimate ; p. 527, sections 2.4. and 2.5. -----	1-20
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/072826

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	IRIT GIL-AD ET AL: "Evaluation of the potential anti-cancer activity of the antidepressant sertraline in human colon cancer cell lines and in colorectal cancer-xenografted mice", INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 33, no. 2, 1 August 2008 (2008-08-01) , pages 277-286, XP055274835, GR ISSN: 1019-6439, DOI: 10.3892/ijo_00000007 title, abstract -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/072826

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007006799 A1	18-01-2007	FR 2888507 A1	19-01-2007
		WO 2007006799 A1	18-01-2007
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		US 2017205395 A1	20-07-2017
		WO 2010135468 A1	25-11-2010