Distinct GD3 expression in breast cancer subpopulations after novel thieno[2,3-b]pyridine derivative treatment

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DISTINCT GD3 EXPRESSION IN BREAST CANCER SUBPOPULATIONS AFTER NOVEL THIENO[2,3-B]PYRIDINE DERIVATIVE TREATMENT

DIPLOMA THESIS

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Secondly, I want to thank my father for encouraging me to start studying medicine in Split and guiding me through difficult times.

LIST OF ABBREVIATIONS

- AKT: protein kinase B
- AKT1: AKT Serine/Threonine Kinase 1
- AR: androgen receptor
- ATCC: American Type Culture Collection
- BCSC: breast cancer stem cell
- BL1: basal-like 1
- BL2: basal-like 2
- BRCA: breast cancer gene
- CSC: cancer stem cell
- CDH1: cadherin 1
- CK5: cytokeratin 5
- DCIS: ductal carcinoma in situ
- DMEM: Dulbecco's modified Eagle medium
- DNA: deoxyribonucleic acid
- EGFR: epidermal growth factor receptor
- ER: estrogen receptor
- FITC: fluorescein isothiocyanate
- GD: disialogangliosides
- GM: monosialoganglioside
- GMI: geometric mean fluorescence intensity
- GQ: tetrasialoganglioside
- GT: trisialoganglioside
- HER2: human epidermal growth factor receptor 2
- IM: immunomodulatory
- KMT2C: Lysine N-methyltransferase 2C
- LAR: luminal androgen receptor
- M: mesenchymal
- MSL: mesenchymal stem-like
- MUC16: mucin 16
- NCDB: National Cancer Data Base
- NF1: neurofibromin 1
- mTOR: mammalian target of rapamycin
- PARP: poly (ADP-ribose) polymerase

- pCR: pathological complete response
- PBS: phosphate-buffer saline
- PE: phycoerythrin
- PI3/K: phosphoinositide 3-kinase
- PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
- PKC: protein kinase C
- PR: progesterone receptor
- SD: standard deviation
- TP53: tumor protein 53
- TNBC: triple-negative breast cancer
- VEGF: vascular endothelial growth factor

1. INTRODUCTION

1.1 Cancer

As the second leading cause of death in the United States in 2020 cancer is considered to be a major health problem (1). Cancer is a group of diseases that share features regarding growth dysregulation (2). Due to Darwinian selection, cells with a growth or survival advantage are able to outcompete other cells. These advantages help a single cell to serve as a progeny during the tumor initiation phase. Hence, all cancers are considered to be clonal. Even after the phase of initiation, Darwinian selection continues. This phase is known as progression, during which daughter cells develop more advantageous characteristics for their own survival and spread (3). The hallmarks of cancer are general features shared by different types of cancer. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Fundamental for these hallmarks are genome instability and inflammation (4). The usual cause of cancer related death is due to metastatic spread, accounting for approximately 90% of cancer deaths (5). For metastatic spread to occur, the cancer cell has to detach from the primary tumor and invade a distant organ (6).

1.2 Triple Negative Breast Cancer (TNBC)

1.2.1 Definition

Breast cancer is defined as triple-negative when it is lacking the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (7). It is important to mention that TNBC and basal-like breast cancer cannot be used as synonyms. This is due to the fact, that up to 20% of basal-like breast cancers express ER or overexpress HER2 and therefore do not match the criteria to be determined as triple-negative (8).

1.2.2 Epidemiology

In 2020 breast cancer was the most common cancer to affect women. In fact, the incidence of breast cancer, which is rare in men, was so high that it turned out to be the most common new cancer even when looking at both sexes together (9). As shown in Figure 1A, 11.7% of all newly diagnosed cancers in both sexes in 2020 were breast cancer, with breast cancer making up 6.9% of all cancer related deaths. The impact of breast cancer gets even

further stressed when looking at its incidence and mortality specifically in females. As can be seen in Figure 1B, 24.5% of all new cancers in women in 2020 were breast cancers, which means that the incidence of breast cancer was approximately 2.25 million. Not only is breast cancer the most frequent one in women, it is also the cancer type responsible for most cancer related deaths. Out of 4.4 million cancer deaths, 15.5% were caused by breast cancer (Figure 1B).

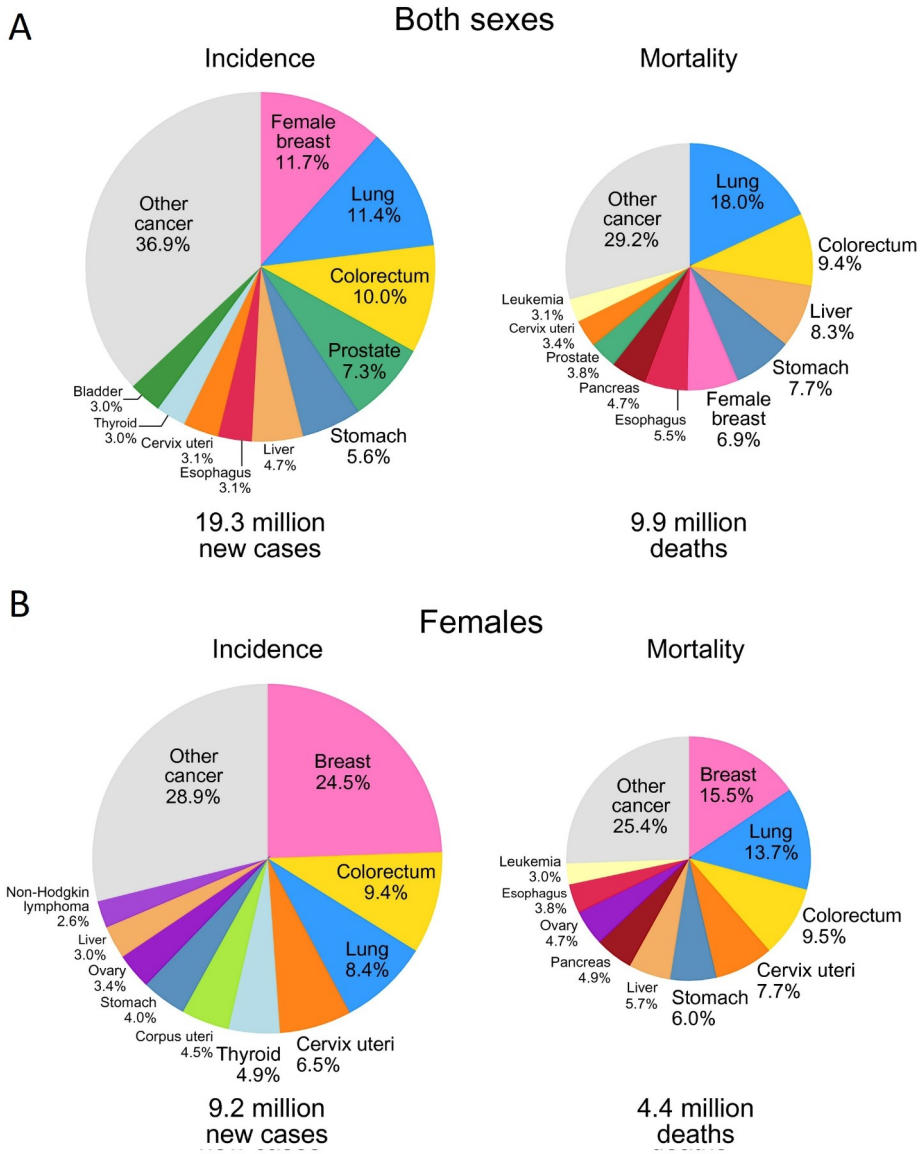


Figure 1 Pie chart showing the incidence and mortality of different cancer types for (A) Both sexes and (B) females. The category "other" encompasses nonmelanoma skin cancers with the exception of basal cell carcinoma for incidence.

Source: GLOBOCAN 2020 (9).

When looking at the incidence and mortality with an age-standardized rate per 100,000 significant differences across different areas of the world can be noticed. The highest incidence worldwide occurs in Belgium (9). This finding goes along with the overall high incidence in Western Europe of 90.7 per 100,000. Also, the other regions of Europe excluding Eastern Europe show a high incidence with 86.4 per 100,000 in Northern Europe and 79.6 per 100,000 in Southern Europe, respectively. Eastern Europe has an incidence of 57.1 per 100,000 which is significantly less than other parts of Europe, yet higher than all African and Asian areas, as well as South America and Central America. The incidences of Australia/ New Zealand with 95.5 per 100,000 and Northern America with 89.4 per 100,000 are also very high (Figure 2). The highest mortality worldwide was found in Barbados (9).

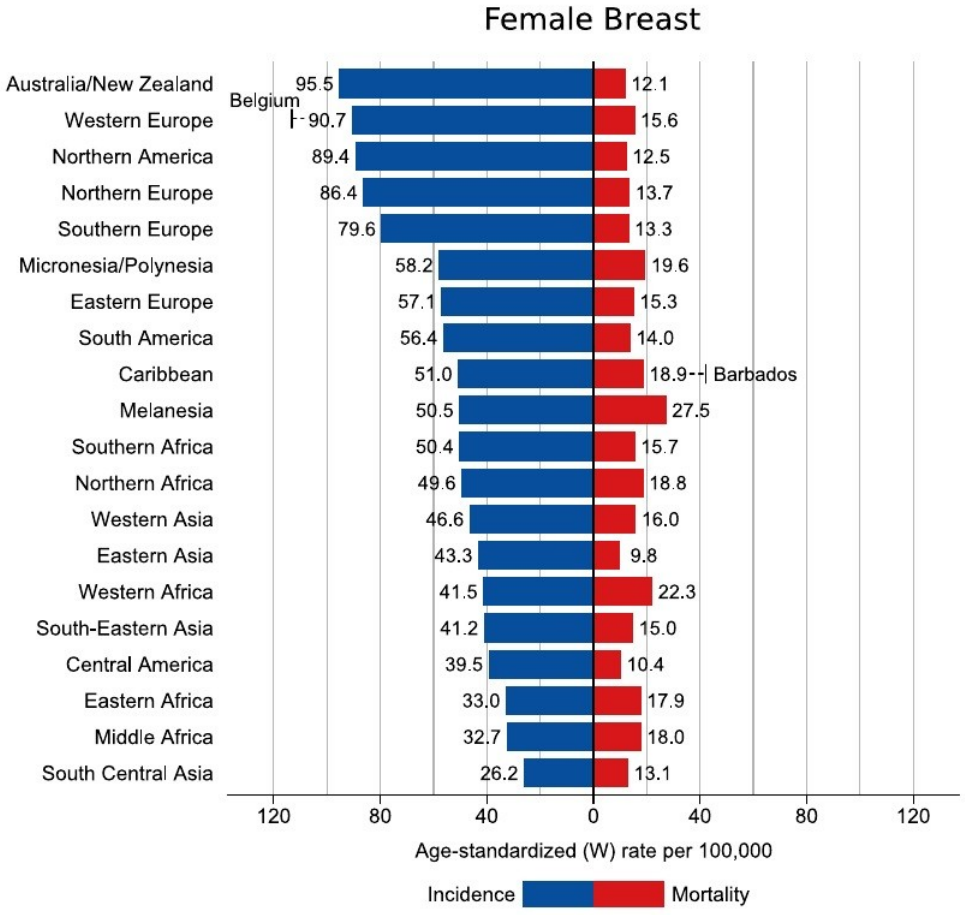


Figure 2. Bar chart showing incidence and mortality of breast cancer in women in different areas of the world. The countries with the highest incidence and mortality are added to the bar chart in respect to their area.

Source: GLOBOCAN2020 (9).

When looking at all invasive breast cancer cases diagnosed between 2010 and 2011 from the National Cancer Data Base (NCDB), it can be observed that the incidence of TNBC is significantly higher in women. Out of 295,801 cases in women 38,628 were triple-negative which is equal to approximately 13%. In comparison, only 185 out of 3,136 (6%) cases in men were triple-negative (10). Ethnicity seems to be an important factor when comparing the individual risk for TNBC. The lowest risk was observed in the Non-Hispanic white and Non-Hispanic Asian population with 11.4% and 11.2% of all breast cancer cases being TNBC. In contrary to this, the percentage of TNBC cases in Non-Hispanic black people was more than twice as much with 23.7% (Table 1). As shown in Table 1, there is an increased risk for TNBC in younger patients in comparison to older patients. In the group of patients younger than 30 years of age 23.3% of breast cancers were triple negative. In different to this, only 10% of breast cancers were triple-negative in the age group above 70. Additionally, there is a gradual decline in TNBC risk with an increase of age.

Table 1. Incidence of triple-negative tumors by sex, race/ethnicity, age, and geographic region. SOURCE: Pasilova et al (10).

	Total cancer number	Nontriple-negative number (%)			Triple-negative number (%)
		HR+ Her2−	HR+ Her2+	HR− Her2+	
Sex					
Female	295,801	214,052 (72.4%)	29,794 (10.1%)	13,327 (4.5%)	38,628 (13.1%)
Male	3,136	2587 (82.5%)	315 (10.0%)	49 (1.6%)	185 (5.9%)
Race/ethnicity					
Non-Hispanic white	235,082	175,760 (74.8%)	22,870 (9.7%)	9669 (4.1%).1%)	26,783 (11.4%)
Non-Hispanic black	33,970	20,255 (59.6%)	3744 (11.0%)	1904 (5.6%)	8,067 (23.7%)
Non-Hisp Asian/P.I.	9,294	6519 (70.1%)	1091 (11.7%)	639 (6.9%)	1,045 (11.2%)
Hispanic	15,536	10,476 (67.4%)	1847 (11.9%)	907 (5.8%)	2,306 (14.8%)
Other/unknown	5,055	3629 (71.8%)	557 (11.0%)	257 (5.1%)	612 (12.1%)
Age					
≤30	2,059	1014 (49.2%)	411 (20.0%)	155 (7.5%)	479 (23.3%)
31–40	15,094	8439 (55.9%)	2494 (16.5%)	978 (6.5%)	3,183 (21.1%)
41–50	51,793	34,894 (67.4%)	6422 (12.4%)	2627 (5.1%)	7,850 (15.2%)
51–60	72,543	49,920 (68.8%)	8051 (11.1%)	4149 (5.7%)	10,423 (14.4%)
61–70	77,870	59,173 (76.0%)	6724 (8.6%)	3061 (3.9%)	8,912 (11.4%)
>70	79,578	63,199 (79.4%)	6007 (7.5%)	2406 (3.0%)	7,966 (10%)
Geographic region					
East South Central	17,319	11,844 (68.4%)	1850 (10.7%)	888 (5.1%)	2,737 (15.8%)
West South Central	23,951	16,634 (69.5%)	2693 (11.2%)	1150 (4.8%)	3,474 (14.5%)
South Atlantic	65,180	46,520 (71.4%)	6502 (10.0%)	3035 (4.7%)	9,123 (14.0%)
East North Central	52,707	38,085 (72.3%)	5162 (9.8%)	2301 (4.4%)	7,159 (13.6%)
Middle Atlantic	46,259	33,908 (73.3%)	4699 (10.2%)	1982 (4.3%)	5,670 (12.3%)
West North Central	21,923	16,223 (74.0%)	2121 (9.7%)	949 (4.3%)	2,630 (12.0%)
Mountain	14,382	10,682 (74.3%)	1377 (9.6%)	619 (4.3%)	1,704 (11.8%)
Pacific	38,647	28,794 (74.5%)	3842 (9.9%)	1699 (4.4%)	4,312 (11.2%)
New England	18,569	13,949 (75.1%)	1863 (10.0%)	753 (4.1%)	2,004 (10.8%)

1.2.3 Pathology

The term triple-negative breast cancer might be misleading as it implies that it is a type of cancer with clearly defined histopathological features. However, it has been shown that there is a variety among TNBCs regarding those features. In a sample of 97 patients with TNBC, 92 of those cancers showed histopathological features of invasive ductal carcinoma. Additionally, cancers with features of apocrine, medullary, spindle cell, or small cell carcinoma were also observed (11). Common morphological features of ductal invasive carcinoma are lymphoid aggregation, high mitotic indices, central necrosis, nuclear pleomorphism, and expression of epidermal growth factor receptor (EGFR) and cytokeratin (CK5) (12).

Based on histological features, TNBCs can be divided into the common subtype, which is the invasive ductal carcinoma, as well as the special subtypes. The special subtypes encompass 47 different subtypes and include metaplastic carcinoma matrix producing type, squamous type, mixed type, and spindle cell type, as well as invasive lobular, adenoid cystic, glycogen-rich clear, and mucinous carcinoma (13). Characteristic pictures of the subtypes can be seen in Figure 3.

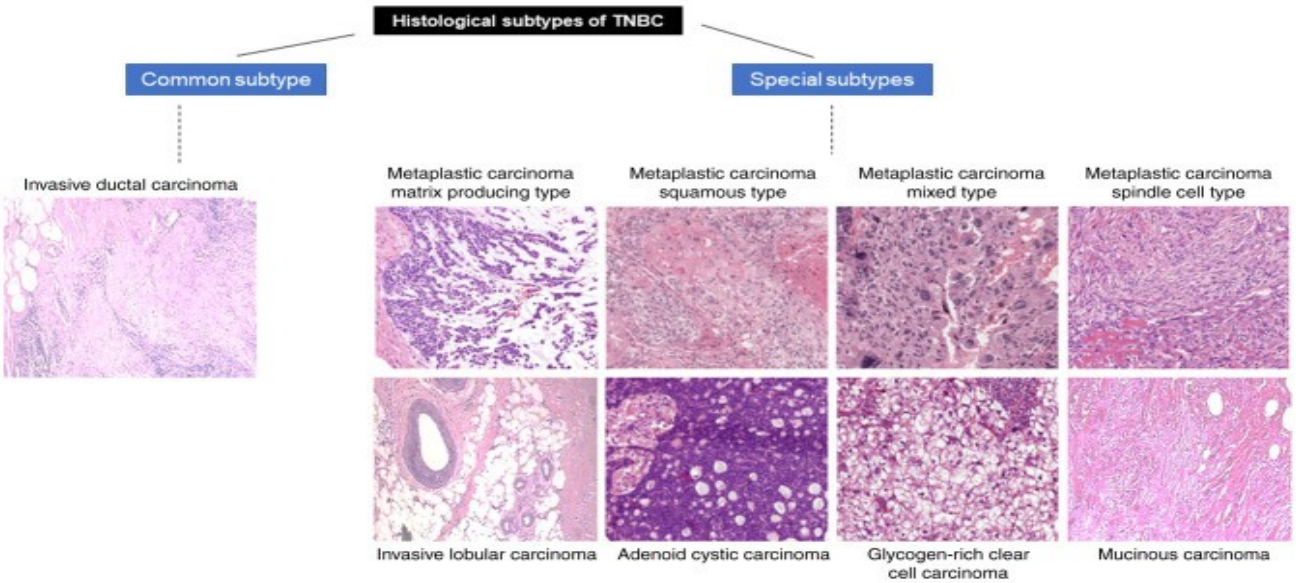


Figure 3. Histological subtypes of TNBC are shown with their characteristic appearance under the microscope.
SOURCE: Manjunath et al (13).

TNBC can also be classified and divided into molecular subtypes based on gene expression. Six different subtypes were identified this way: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) (14). Those subtypes have a heterogenous mutational profile. Overall, the LAR subtype has the highest mutational burden, whereas the MSL subtype shows a lower mutational burden. The most common mutated gene in exonic regions in all subtypes is the tumor protein 53 (TP53) followed by the mucin 16 (MUC16) and the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene. The most characteristic mutational profile can be seen in the LAR subtype which exhibits mutations in the PIK3CA, Lysine N-methyltransferase 2C (KMT2C), cadherin 1 (CDH1), neurofibromin 1 (NF1) and AKT Serine/Threonine Kinase 1 (AKT1) gene (15).

In the TNBC cell lines BT-549 and Hs 578T different subpopulations according to their expression of the cell surface markers CD44 and CD24 can be found. Those subpopulations are breast cancer stem cell (BCSC) mesenchymal-like cells ($CD44^+/CD24^-$), epithelial cells ($CD44^-/CD24^+$), hybrid cells showing both mesenchymal and epithelial characteristics ($CD44^+/CD24^+$) and $CD44^-/CD24^-$ cells (16). When comparing different histological breast cancer subtypes and their expression of CD44 and CD24, significant differences can be observed. Pure ductal carcinoma in situ (DCIS) has the highest percent of $CD44^+/ CD24^-$, followed by $CD44^+/ CD24^+$. The highest percent of $CD44^-/ CD24^+$ can be seen in DCIS with microinvasion. Invasive ductal carcinoma (IDC) is characterized by a high percent of $CD44^-/ CD24^-$ (17). It has been shown that lack of expression of CD44 and CD24 in luminal breast cancer is associated with metastatic properties (18). Furthermore, cells with expression of mixed epithelial and mesenchymal gene expression are highly aggressive. Plasticity and self-renewal are associated with the $CD24^+/CD44^-$ (epithelial) and the $CD24^-/CD44^+$ (mesenchymal) phenotypes respectively (19).

1.2.4 Therapeutic Options

Treatment modalities for TNBC range from surgery, radiation therapy, and chemotherapy, to molecular-directed targeted therapy. Surgical options include mastectomy and breast-conserving surgery (BCS) with subsequent radiation therapy. BCS is the treatment of choice for low grade tumors, whereas mastectomy is used for more advanced cancers as well as tumors with positive margins after previous BCS (20). The local recurrence rate of

TNBC is reduced by postoperative radiotherapy. It has been shown that patients with TNBC without lymph node metastases who underwent BCS with subsequent radiotherapy had a similar outcome to those who underwent mastectomy. Additionally, postoperative radiotherapy improves the outcome and leads to a lower local recurrence rate in patients T1/ T2 TNBC with 4 or more positive axillary lymph nodes (21). Despite TNBC being chemotherapy sensitive in early stages, no optimal therapeutic has been established so far. The pathological complete response (pCR) in patients treated solely with anthracyclines ranges from 14 to 47%. The pCR rate of platinum monotherapy ranges from 23 to 90% has been shown to be even higher in patients with breast cancer gene (BRCA) mutations. However, carboplatin has a significant toxicity. The monoclonal antibody pembrolizumab, which acts as an inhibitor of programmed cell death 1 (PD1), improves the pCR when combined with anthracycline and taxane chemotherapy (22). For metastatic disease, a taxane or anthracycline combination can be used as a drug regimen (23).

Another approach is the molecular-directed targeted therapy. Different molecular pathways that play a role in the pathogenesis of TNBC have been explored including vascular endothelial growth factor (VEGF), poly (ADP-ribose) polymerase (PARP), epidermal growth factor receptor (EGFR), mammalian target of rapamycin (mTOR), androgen receptor (AR), as well as anti-PD-1 agents (20).

VEGF is an important mediator of angiogenesis and contributes to growth of cancers, making it a logical therapy target (24). Bevacizumab is a VEGF-A-targeting monoclonal antibody and the first approved angiogenesis inhibitor (25). In a non-randomized phase II study bevacizumab in combination with either docetaxel or paclitaxel, as a first-line treatment for HER2-negative metastatic breast cancer, has led to a progression-free survival of 11.3 months and an overall survival of 35.1 months (26). In a randomized phase III trial, bevacizumab plus chemotherapy has been compared with chemotherapy alone in patients with HER2-negative locally recurrent or metastatic breast cancer after treatment with bevacizumab plus chemotherapy. Progression-free survival was 6.3 months for patients who received bevacizumab and 4.2 months in patients who received chemotherapy alone (27).

PARP-1 and PARP-2 play an important role in the repair of single-strand deoxyribonucleic acid (DNA) breaks (28). Veliparib acts as a PARP inhibitor (29). Adding veliparib to a combination of carboplatin and paclitaxel has been proven to improve progression-free survival in BRCA-mutated advanced breast cancer (30).

Expression of EGFR predominates in TNBC making it a potential target for therapeutic approaches (31). The chimeric mouse-human IgG1 antibody cetuximab targets EGFR signaling and has been used in different cancers (32). Unfortunately, cetuximab did not show promising results in randomized controlled trials which might be due to its partial agonistic action on EGFR (33, 34).

TNBC frequently shows alterations in the phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT)/ mTOR pathway, leading to the exploration of potential therapeutic drugs which act on this pathway (35). Everolimus is a derivative of rapamycin that acts on mTOR and has been shown to reduce growth of breast cancer cells *in vitro* (36, 37). However, addition of everolimus to a combination of paclitaxel and cisplatin in stage II/III TNBC leads to an increase in adverse reactions without improving pCR (38).

AR expression is a characteristic feature of the LAR subtype, making it a possible target for anti-AR drugs (39). The AR inhibitor enzalutamide, which has been shown to be effective in the treatment of prostate cancer, can inhibit 5-alpha-dihydrotestosterone (DHT)-mediated proliferation of breast cancer lines *in vitro* (40, 41). In a phase II clinical study, enzalutamide has been proven to not cause severe side effects in the treatment of AR expressing TNBC (42).

1.3 Gangliosides

1.3.1 Definition

Gangliosides are defined as sialic acid-containing glycosphingolipids and were discovered by Ernst Klenk who isolated them postmortem from brain tissue of patients with amaurotic idiocy (43). The number of sialic acid residues determines the nomenclature of gangliosides and differentiates between monosialogangliosides (GM), disialogangliosides (GD), trisialogangliosides (GT) and tetrasialoganglioside (GQ), according to Svennerholm (44, 45).

1.3.2 Function

Gangliosides are present in all tissues and fluids but can be mostly found in the nervous system (46). The significance of gangliosides has been demonstrated in knock-out mice which were lacking the genes encoding for GM2 and GM3 resulting in a fatal

neurodegenerative outcome (47). Furthermore, loss of the gene encoding for GM3 resulted in hearing loss in knock-out mice due to degeneration of the organ of Corti (48).

It appears that the gangliosides GT1b, GD3, and GM3 inhibit the activity of protein kinase C (PKC) in cow mammary glands and thereby interfere with PKC mediated signal transduction (49). PKC plays an important role in cell growth, survival, migration, as well as adhesion (50).

1.3.3 Biosynthesis

The synthesis of ceramide in the endoplasmatic reticulum marks the beginning of the synthesis of gangliosides (51, 52). In the next step UDP-glucose ceramide glycosyltransferase transfers a glucose residue onto ceramide generating glucosylceramide. Following this, glucosylceramide gets converted into lactosylceramide which is the precursor of GM3 (53, 54). After the generation of GM3 ganglioside synthesis occurs via two different pathways named “a” and “b” pathway (Figure 4).

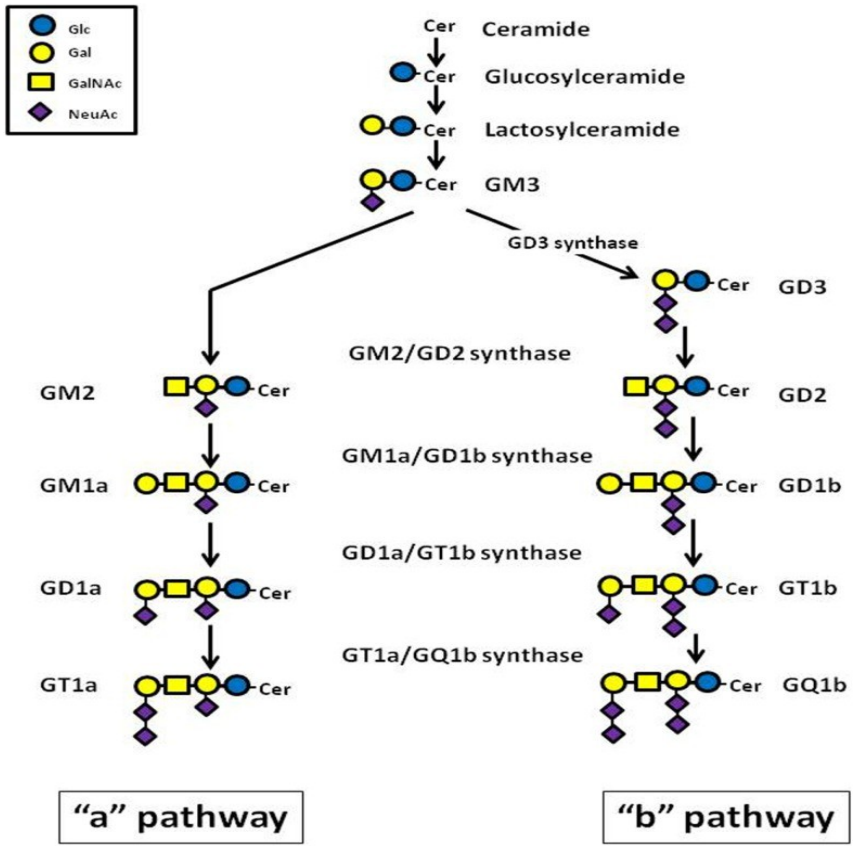


Figure 4. Schematic illustration of ganglioside biosynthesis and the “a” and “b” pathway
SOURCE: Berois et al (54).

1.3.4 Ganglioside GD3

GD3 is a very important ganglioside in embryonic or undifferentiated cells (55). Studies in knockout mice showed that GD3 appears to be significant for the self-renewal of neural stem cells in the mouse brain (56). Furthermore, alterations of GD3 expression have been demonstrated in Creutzfeldt-Jakob disease suggesting a role in the pathogenesis of this disease (57).

In glioblastoma cell cultures it has been demonstrated that acetylated GD3 aids in tumor survival (58). In addition to this, GD3 can regulate cell proliferation and differentiation via VEGF stimulation (44).

It has been demonstrated, that GD3 levels are 1.7 times increased in breast cancer tissue (59). The importance of GD3 in this type of cancer is further emphasized by the ability of GD3 to activate EGFR signaling in breast cancer cell lines (60).

2. OBJECTIVES AND HYPOTHESES

2.1 Objectives

The aim of this study was to determine the effect of the newly synthesized thieno[2,3-*b*]pyridine anticancer compound (compound 1) on breast cancer stem cells, by focusing on its impact on GD3 expression in the human cell line MDA-MB-231 and its $CD44^+/CD24^+$, as well as $CD44^-/CD24^-$ subpopulations.

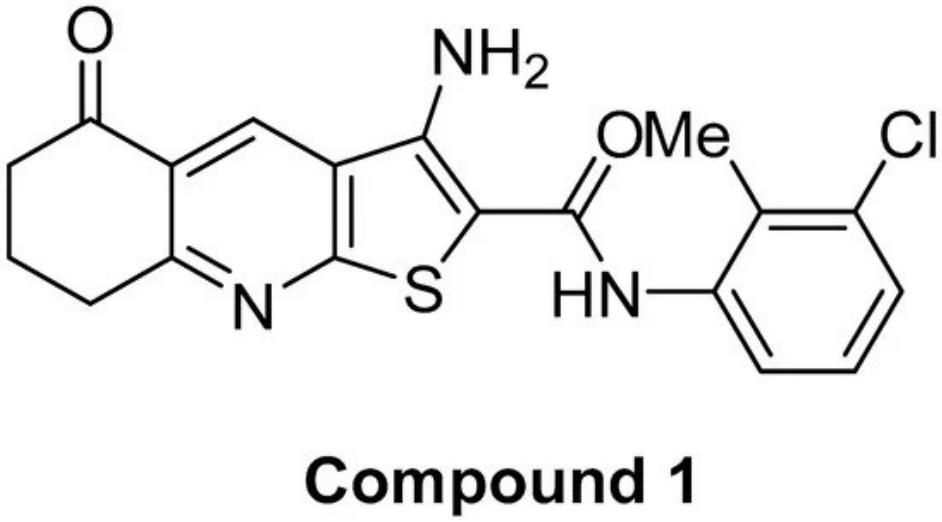
2.2 Hypotheses

1. Treatment with compound 1 leads to a reduction of the GD3 expression in $CD44^+/CD24^-$ CSCs
2. Treatment with compound 1 leads to a reduction of the GD3 expression in $CD44^-$ epithelial cells
3. Treatment with compound 1 leads to a reduction of the GD3 expression in $CD44^+/CD24^+$ hybrid cells

3. MATERIALS AND METHODS

3.1 Chemistry and cell line

Compound 1 was prepared in a 3-step fashion from 1,3-cyclohexanedione as reported in Marijan et al (61). The chemical structure of 3-Amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3- b]quinoline-2-carboxamide (compound 1) can be seen in Figure 5. Cells were obtained from the American Type Culture Collection (ATCC, LGC Standards). Cell line MDA-MB-231 was grown in a humidified incubator at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Steinheim, Germany) containing 10% fetal bovine serum (EuroClone, Milan, Italy) and 1% antibiotics (EuroClone) (61). Characteristic features of the cell line MDA-MB-231 are shown in Table 2 (62).



Compound 1

Figure 5. Structure of the anticancer thieno[2,3-*b*]pyridine derivative
SOURCE: Marijan et al (61)

Table 2. Characteristics of MDA-MB-231 cell line

SOURCE: https://www.lgcstandards-atcc.org (62)

Characteristics	
Organism	Homo sapiens
Cell Type	epithelial cell
Tissue	Breast; Mammary gland
Age	51 years
Gender	Female
Morphology	Epithelial
Growth properties	Adherent
Disease	Adenocarcinoma
Product format	Frozen
Storage conditions	Vapor phase of liquid nitrogen
Intended use	Laboratory research only
Biosafety level	1
Growth conditions	37°C, 100% air
Reagents for cryopreservation	Complete growth medium supplemented with 5% (v/v) DMSO (ATCC 4-X)
Receptor Expression	Epidermal growth factor (EGF), expressed; transforming growth factor alpha (TGF alpha), expressed

3.2 Flow cytometric analysis

An identical number of cells were plated onto six-well plates and treated with 2 M compound 1 for 48 h, as well as the untreated controls, trypsinized and washed with a phosphate-buffered saline (PBS). After an incubation time of 20 minutes with PBS-diluted primary antibodies against GD3 (mouse IgG3) (produced by laboratory of Dr. J. Muthing)(63) at room temperature, cells were incubated with diluted anti-CD44-fluorescein isothiocyanate (FITC) (1:13; BD Biosciences), anti-CD24-phycoerythrin (PE), and secondary antibody to anti-GD3s conjugated with eFluor 660 fluorochrome (1:10; eBioscience) for 20 min in a lightproof area. Following this, cells were resuspended in PBS, and then analyzed via flow

cytometry. The acquisition of data of triple-stained samples was done by a BD Accuri C6 cytometer (BD Biosciences) and analyzed with the FlowLogic Software.

3.3 Geometric Mean Fluorescence Intensity (GMI)

The geometric mean fluorescence intensity (GMI) of GD3s (GD3s expression/ cell), was determined with FlowLogic from a number of cells and the log of fluorescence in a FL4 flow cytometer channel. A standard optical filter of 675/25 nm has been used. The emission wavelength of immunofluorescence of eFluor 660 fluorochrome that is conjugated to a secondary anti-GD3 antibody is 660 nm.

GMI is calculated through the multiplication of cell numbers with the matching fluorescence and adding all results. The following total sum is divided with the total cell number, yielding the GMI (61).

3.4 Statistical analysis

The statistical analysis was done with the student t-test using statistical software Graph-Pad Prism 7.0 (San Diego, Ca, USA) and the significance was set at $P < 0.05$.

4. RESULTS

4.1 GD3 expression in $CD44^+/CD24^-$ CSC

The treatment with compound 1 resulted in an increased percentage of $GD3^+$ cells in $CD44^+/CD24^-$ CSCs. The percentage of $GD3^+$ cells was 8.9 in the untreated control and 32.5 after treatment (P <0.01) (Figure 6).

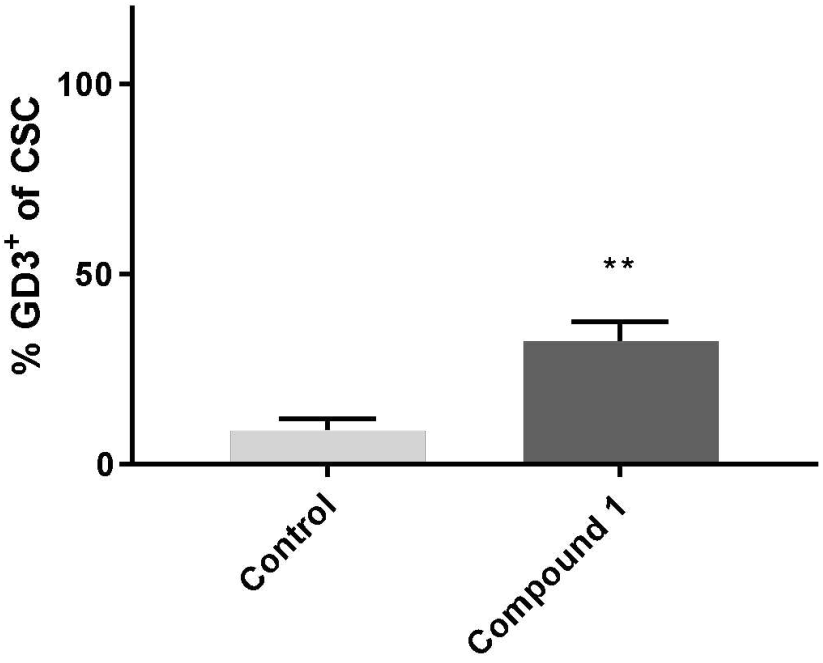


Figure 6. Percentage of $GD3^+$ CSCs after compound 1 treatment.

Notes: Percentage of GD3+ of CSCs after treatment with compound 1 for 48 h in MDA-MB-231. Data represent are expressed as a mean from experiment performed in triplicate ― SD. Columns, mean of cells; bars, SD; $^{**}P < 0.01$

Abbreviations: CSCs cancer stem cells, SD standard deviation.

Geometric mean fluorescence intensity (GMI) of GD3 (GD3 expression/cell) in CSCs after treatment with compound 1 was 8,673.75 and therefore decreased in comparison to untreated cells with a GMI of 10,682.85 (Figure 7).

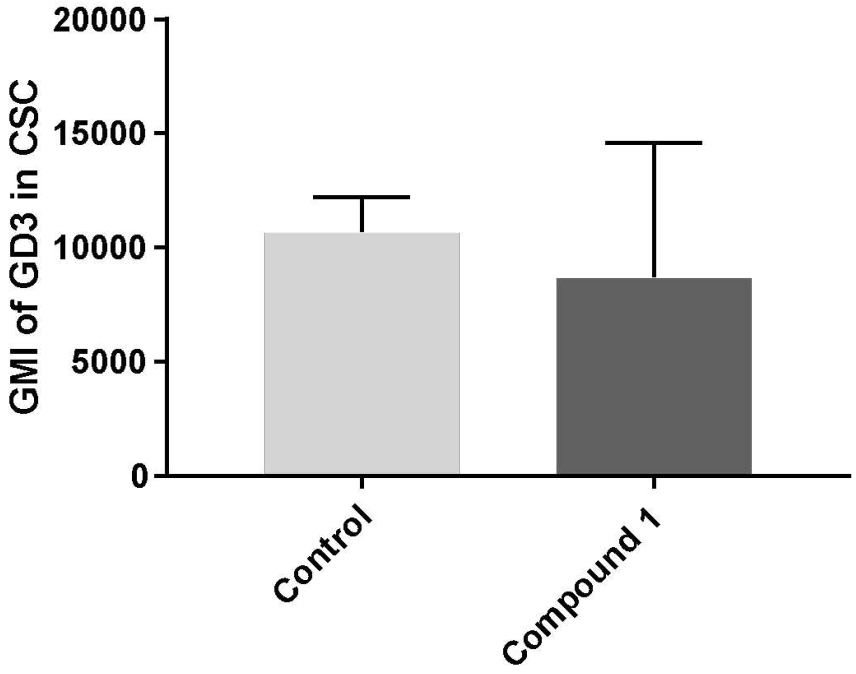


Figure 7. GMI of GD3 in CSCs after compound 1 treatment.

Notes: Expression of GD3 in CSCs after treatment with compound 1 for 48 h in MDA-MB-231. Data represent are expressed as a mean from experiment performed in triplicate ― SD. Columns, mean of cells; bars, SD

Abbreviations: CSCs cancer stem cells, SD standard deviation

4.2 GD3 expression in $CD44^-$ epithelial cells

The treatment with compound 1 resulted in an increased percentage of $GD3^+$ cells in $CD44^-$ epithelial cells. The percentage of $GD3^+$ cells was 22.2 in the untreated control and 40.6 after treatment (P <0.05) (Figure 8).

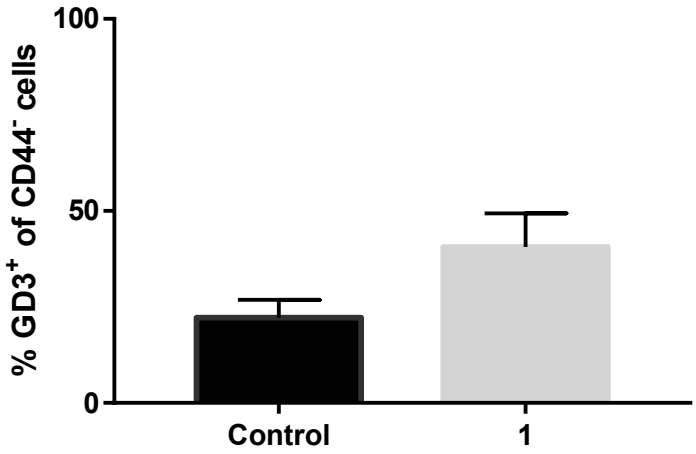


Figure 8. $CD44^-$ cells after compound 1 treatment.

Notes: Percentage of $GD3^+$ of $CD44^-$ epithelial cells after treatment with compound 1 for 48 h in MDA-MB-231. Data represent are expressed as a mean from experiment performed in triplicate ― SD. Columns, mean of cells; bars, SD;

Abbreviations: SD standard deviation.

Geometric mean fluorescence intensity (GMI) of GD3 (GD3 expression/cell) in $CD44^-$ epithelial cells after treatment with compound 1 was 11,986 and therefore decreased in comparison to untreated cells with a GMI of 5,390 ($P < 0.01$) (Figure 9).

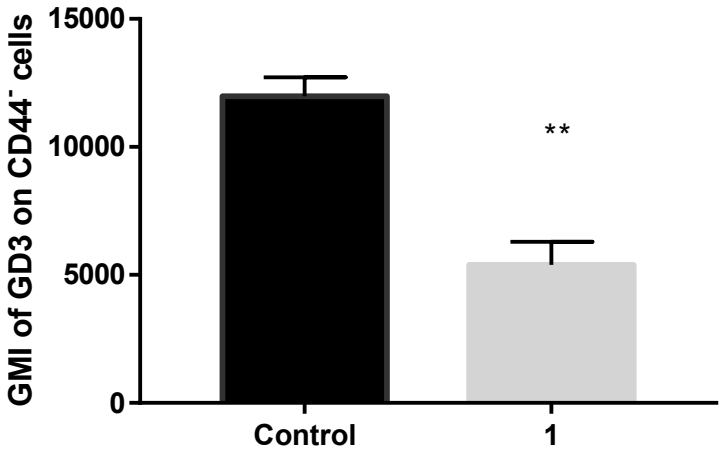


Figure 9. GMI of GD3 in $CD44^-$ epithelial cells after compound 1 treatment.

Notes: Expression of GD3 in $CD44^-$ epithelial cells after treatment with compound 1 for 48 h in MDA-MB-231. Data represent are expressed as a mean from experiment performed in triplicate ― SD. Columns, mean of cells; bars, SD. $^{**}P < 0.01$

Abbreviations: SD standard deviation

4.3 GD3 expression in $CD44^+/CD24^+$ hybrid cells

The treatment with compound 1 resulted in an increased percentage of $GD3^+$ cells in CD $44^+/CD24^+$ hybrid cells. The percentage of $GD3^+$ cells was 76.3 in the untreated control and 82.9 after treatment ($P > 0.5$) (Figure 10).

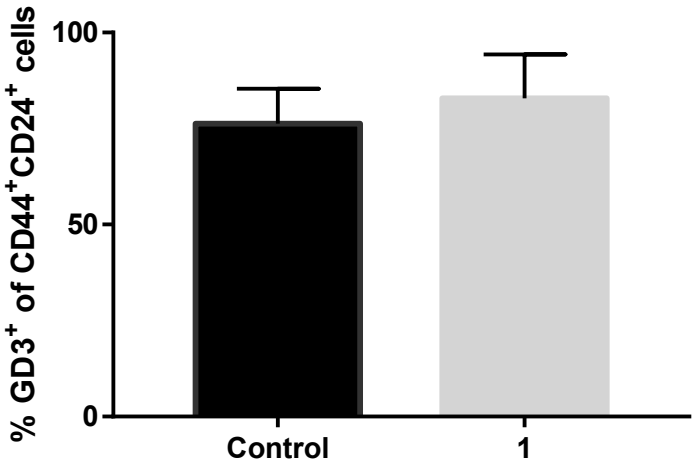


Figure 10. $CD44^+$/ $CD24^+$ cells after compound 1 treatment.

Notes: Percentage of $GD3^+$ of $CD44^+$/$CD24^+$ hybrid cells after treatment with compound 1 for 48 h in MDA-MB-231. Data represent are expressed as a mean from experiment performed in triplicate ― SD. Columns, mean of cells; bars, SD;

Abbreviations: SD standard deviation.

Geometric mean fluorescence intensity (GMI) of GD3 (GD3 expression/cell) in $CD44^+$/$CD24^+$ hybrid cells after treatment with compound 1 was 43,559 and therefore increased in comparison to untreated cells with a GMI of 42,049 ($P > 0.05$).

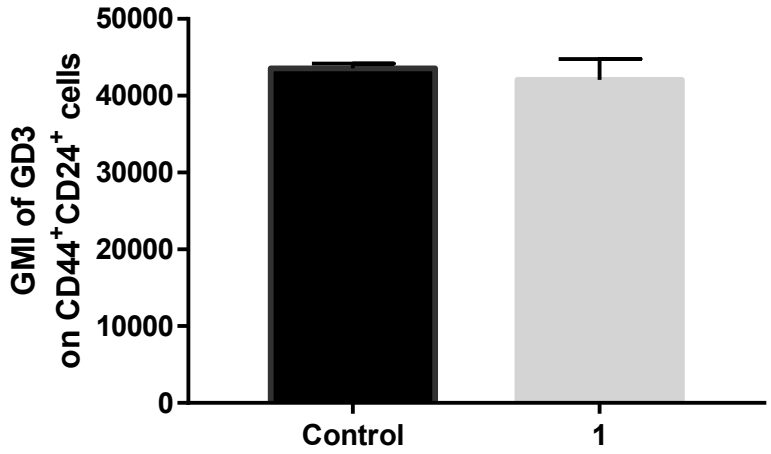


Figure 11. GMI of GD3 in $CD44^+$/ $CD24^+$ hybrid cells after compound 1 treatment.

Notes: Expression of GD3 in $CD44^+$/$CD24^+$ hybrid cells after treatment with compound 1 for 48 h in MDA-MB-231. Data represent are expressed as a mean from experiment performed in triplicate ― SD. Columns, mean of cells; bars, SD.

Abbreviations: SD standard deviation

5. DISCUSSION

When taking into consideration that up to this day an optimal treatment for TNBC has not been established, exploring new therapeutic options is a logical consequence (22). It has been demonstrated that 3-amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide (compound 1) is not only able to reduce CSCs in the MDA-MB-231 cell line, but also has an impact on morphology and cell migration (61). Its dose- and time-dependent cytotoxicity has been proven in previous studies (64). Therefore, in order to unravel the different effects that compound 1 has on the MDA-MB-231 cell line, we wanted to determine its effect on GD3 expression in different breast cancer subpopulations.

We observed that treatment with compound 1 resulted in an increased percentage of GD3 in CSCs (Figure 6). Interestingly, the GMI of GD3 was decreased in the cells, that were treated with compound in comparison to the untreated control group (Figure 7). A similar observation was made in $CD44^-$ epithelial cells, which showed a reduction of GMI of GD3 upon treatment with compound 1 (Figure 8, Figure 9). This shows, that reduction of GD3 expression has occurred in cells which were treated with compound 1. As mentioned before, GD3 expression is advantageous for cancer cells. It plays a role in different signaling pathways including those which involve VEGF, as well as EGFR (44, 60). Furthermore, GD3 has been linked to aid in tumor survival (58). We can conclude that treatment with compound 1 has an impact on GD3 linked signaling pathways, as well as survivability in CSCs and $CD44^-$ epithelial cells via the reduction of GD3 expression in these subpopulations.

In different to other subpopulations, hybrid cells ($CD44^+/CD24^+$) did not show a significant reduction in GMI of GD3 (Figure 11). However, it has been shown previously that compound 1 is able to reduce the expression of CD15s in the $CD44^+/CD24^+$ subpopulation (61). It appears that the effect of compound 1 in this subpopulation is more significant in CD15s expression than in GD3 expression.

It has to be mentioned that limiting factors for this study have to be taken into consideration. As an *in vitro* study, no conclusions regarding the safety of the drug in patients can be drawn. However, the experiments were performed in triplicates, eliminating further major limitations of this study. Therefore, one could conduct a new study and explore GD3 expression after treatment with compound 1 in other breast cancer cell lines and compare those results with this study.

Overall, this study shows that GD3 expression in breast cancer subpopulations after novel thieno[2,3-*b*]pyridine derivative treatment, varies upon different subpopulations. A reduction of GD3 expression has been confirmed in CSCs, as well as $CD44^-$ epithelial cells. In order to determine a therapeutic dose tolerable for patients, as well as the effect of compound 1 on an organism, further *in vivo* studies have to be conducted.

6. CONCLUSIONS

- Treatment with compound 1 leads to a reduction of the expression of GD3 in $CD44^{+}/CD24^{-}$ CSCs
- Treatment with compound 1 leads to reduction of the expression of GD3 in $CD44^{-}$ epithelial cells
- Treatment with compound 1 does not have a significant impact on GD3 expression in $CD44^{+}/CD24^{+}$ hybrid cells
- When taking into consideration the role of GD3 in survivability of cancer cells and the impact of compound 1 on its expression in different breast cancer subpopulations, further studies to explore its utility *in vivo* should be conducted.

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8. SUMMARY

Objectives: The goal of this study was to determine GD3 expression after treatment with the novel thieno[2,3-*b*]pyridine derivative, by focusing on $CD44^+/CD24^-$ CSCs, $CD44^-$ epithelial cells, as well as $CD44^+/ CD24^+$ hybrid cells.

Methods: The MDA-MB-231 cell line was treated with 3-amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-b]quinoline-2-carboxamide (compound 1). Cells were plated onto six-well plates and treated with 2 M compound 1 for 48 h. Following this, cells were stained with anti-GD3, anti-CD44 and anti-CD24 antibodies, and then analyzed via flow cytometry. The acquisition of data of triple-stained samples was done by a BD Accuri C6 cytometer (BD Biosciences) and analyzed with the FlowLogic Software.

Results: Treatment of MDA-MB-231 cells with compound 1 has led to a reduction of the expression of GD3 in $CD44^+/CD24^-$ CSCs, as well as $CD44^-$ epithelial cells. GD3 expression in $CD44^+/CD24^+$ hybrid cells was not significantly reduced.

Conclusion: Due to its ability to reduce GD3 expression in different breast cancer subpopulations, compound 1 has a potential to be used in the treatment of triple-negative breast cancer.

9. CROATIAN SUMMARY

Ciljevi: Cilj ove studije bio je utvrditi ekspresiju GD3 nakon tretmana novim derivatom tieno [2,3-b] piridina, posebice na $CD44^+/CD24^-$ matičnim stanicama raka (CSC), $CD44^-$ epitelnim stanicama, kao i $CD44^+/CD24^+$ hibridnim stanicama.

Metode: Stanična linija MDA-MB-231 tretirana je s 3-amino-N-(3-kloro-2-metilfenil)-5-okso-5,6,7,8-tetrahidrotienom[2,3-b]kinolin-2-karboksamid (spoj 1). Stanice su nasađene na ploče sa šest jažica i tretirane s 2 M spojem 1 tijekom 48 sati. Nakon toga, stanice su obojane s protu-GD3, protu-CD44 i protu-CD24 protutijelima, a zatim analizirane protočnom citometrijom. Prikupljanje podataka trostruko obojenih stanica rađeno je citometrom BD Accuri C6 (BD Biosciences) i analizirano softverom FlowLogic.

Rezultati: Tretman MDA-MB-231 stanica spojem 1 doveo je do smanjenja ekspresije GD3 na $CD44^+/CD24^-$ CSC, kao i $CD44^-$ epitelnim stanicama. Ekspresija GD3 na hibridnim stanicama $CD44^+/CD24^+$ nije bila značajno smanjena.

Zaključak: Zbog svoje sposobnosti smanjenja ekspresije GD3 u različitim subpopulacijama raka dojke, spoj 1 ima potencijal za upotrebu u liječenju trostruko-negativnog raka dojke.

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