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**UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE**

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**THE CYTOTOXIC EFFECT AND APOPTOSIS RATE
OF BREAST CANCER STEM CELLS TREATED WITH THE
NOVEL THIENO[2,3-B] PYRIDINE ANTICANCER COMPOUND**

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I'll see you again.

LIST OF ABBREVIATIONS

- ASCO: American Society of Clinical Oncology
- ATCC: American Type Culture Collection
- BARD1: heterodimerizes with BRCA1
- BC: breast cancer
- BCC: breast cancer cell
- BCS: breast-conserving surgery
- BCSC: breast cancer stem cell
- BRCA1 / 2: mutated tumor suppressor genes
- CSC: cancer stem cell
- DMEM: Dulbecco's Modified Eagle's Medium
- DMSO: Dimethyl sulfoxide
- EGF: epidermal growth factor
- ER: estrogen receptor
- FBS: fetal bovine serum
- FISH: fluorescence in situ hybridization
- HER2: human epidermal growth factor receptor 2
- H&E: hematoxylin and eosin
- IBC: invasive breast cancer
- IHC: immunohistochemistry
- MDA-MB-231: triple-negative breast cancer stem cell line
- MRI: magnetic resonance imaging
- MTT: Method of Transcriptional and Translational assay,
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
- NST: neoadjuvant systemic treatment
- OC: oral contraceptive (pill)
- p53: TSG p53, TP53/tumor protein 53
- PALB2: partner and localizer of BRCA2
- PBS: phosphate-buffered saline
- PET: positron emission tomography
- PLC: phospholipase C
- PLC C- γ 2: phospholipase C- γ 2
- PR: progesterone receptor

- PV: pathologic variant
- RAD5D / C: DNA helicase
- RT: radiation therapy
- SC: stem cell
- SD: standard deviation
- TGF alpha: transforming growth factor alpha
- TNBC: triple-negative breast cancer
- TSG: tumor suppressor gene
- US: ultrasound
- WHO: World Health Organization
- WNT7B: oncogene

1. INTRODUCTION

1.1 Cancer

Cancer is with increasing significance a major global health problem, and it represents a group of diseases which are most importantly characterized by abnormal cell growth with the potential to invade and or metastasize to other sides in the human body. This distinguishes cancers from benign tumors, which cannot spread across the side of its origin. However, several research studies suggest that cancers are monoclonal in their origin, implying that the malignant transformation started in one single cell. The cellular process of developing cancer cells is a complex and long-lasting one, which requires several stages to turn healthy tissue into cancer. This process is called tumor progression (1).

According to the newest model of "The Hallmarks of Cancer" by Douglas Hanahan and Robert Weinberg, all known cancers have ten traits in common which enable them to modulate themselves from a normal to a cancerous cell, presented in the following list:

1. Self-sufficient in growth signals;
2. Insensitive to inhibitory anti-growth signals;
3. Resistant to apoptosis;
4. Limitless replication potential;
5. Stimulate angiogenesis;
6. Invade surrounding tissues and metastasize;
7. Disturbed metabolic pathways;
8. Evade the immune system;
9. Genomic instability;
10. Inflammation (2).

The differences in etiology, pathology, and clinical presentation of cancers lead to various therapeutic angles, but unfortunately, it mostly results in the lack of a sufficient and standardized treatment regime (1,2).

1.2 Triple-negative breast cancer

1.2.1 Definition of TNBC

The term triple-negative breast cancer was first introduced in 2005, and is generally abbreviated as TNBC. Overall it refers to a genomically heterogenic group of breast cancers which make up 12-17% of all breast cancers and are defined by their unifying lack of expression of estrogen receptor (ER) and progesterone receptor (PR), and the absence of human epidermal growth factor receptor 2 amplification (HER2) (3,4). Furthermore, it has to be emphasized that TNBC is not a synonym for basal-like cancer. Basal-like breast cancers are often used as a surrogate term for triple-negative breast cancers even though only 80% of basal-like breast cancers are truly triple-negative in their receptor expression. 10% are ER+/HER2- and 2% are even HER2+ (5).

1.2.2 Epidemiology and etiology

Breast cancer is a prevalent and significant disease in females with several potentially severe adverse effects on a woman's life. The importance of this cancer stressed by the fact that in 2018, breast cancer was the most common type of cancer in females in 154 countries worldwide, according to the World Health Organization (WHO), accounting for 2.08 million new cases in women (Table 1). Furthermore, breast cancer is not only the most commonly diagnosed female cancer type in the world, constituting for 24.2% out of 8.6 million cases of all newly diagnosed female cancer patients, but also together with lung cancer, it is the most frequently diagnosed cancer in general, as shown in Figure 1 A and B. The incidence of breast cancer cases in 2018, for males and females together, is 11.6% out of 18.1 million new cancer cases, same as for lung cancer, but 99.12% of the new breast cancer cases are in females, highlighting the immense female predominance of this cancer (Figure 1B, Table 1). Concerning cancer mortality, breast cancer is one of the leading causes of cancer-related deaths. In 2018, breast cancer accounted for 0.6336 million deaths. 99.43% of these were women, making it the 5th most lethal cancer in the world (Table 1). Lung cancer is in the general population the leading cause of cancer-related deaths in humans being responsible for 18.4% out of 9.6 million deaths, but breast cancer constitutes to the highest female cancer mortality with 15% out of 4.2 million deaths and is, therefore, the principal cause of cancer-related deaths in women in at least 103 countries according to the WHO (Figure 1, Table 1) (6).

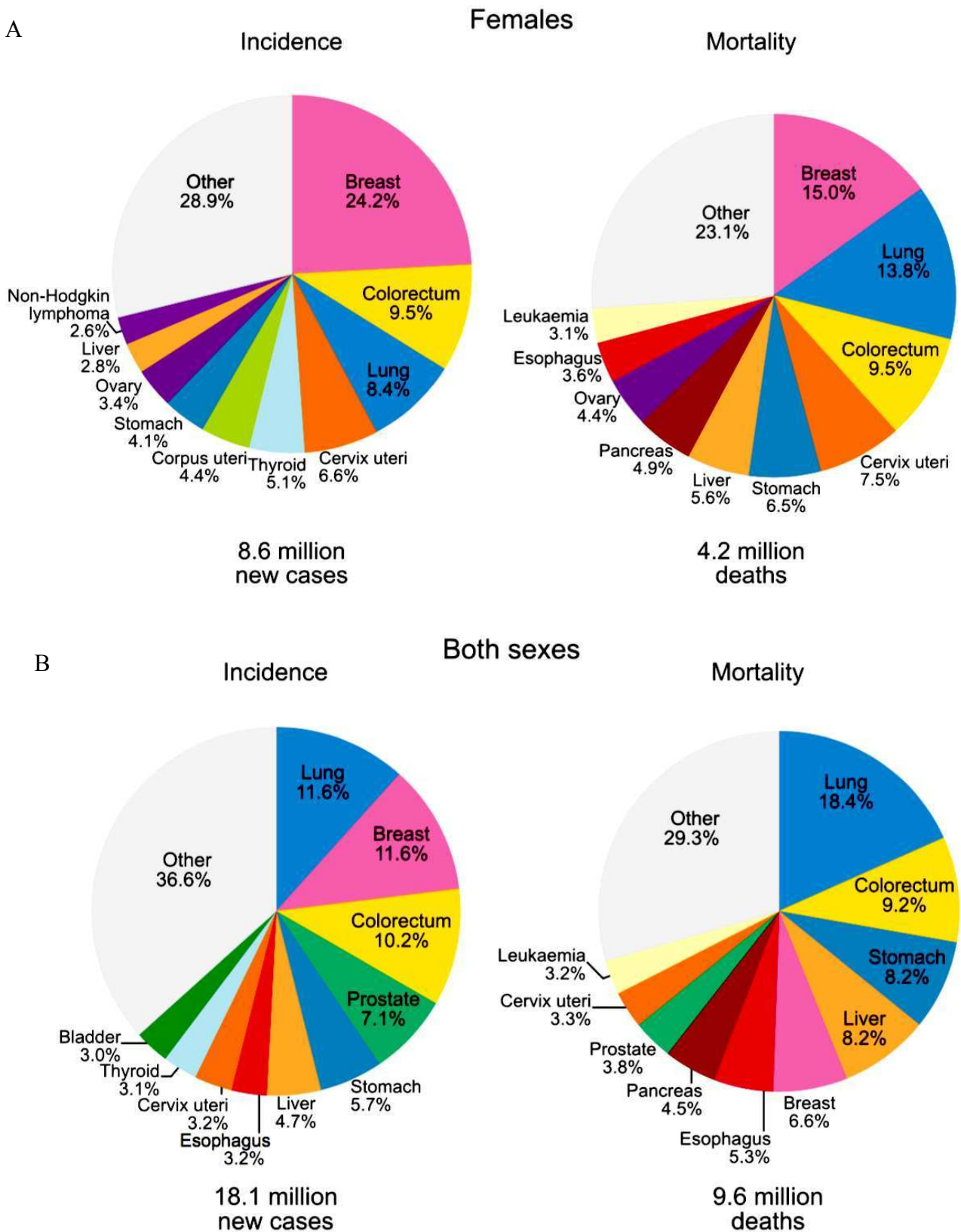


Figure 1. Pie charts depicting for the ten most common cancers the distribution of new cases and deaths in 2018 for (A) females and (B) both sexes. The area of the pie chart represents the proportion of the total number of new cases or deaths; the category "others" includes nonmelanoma skin cancers.

SOURCE: Globocan 2018, World Health Organization (6).

Table 1. Cancer and breast cancer incidence and mortality in 2018.

2018	Incidence of new cancer cases	Incidence of new breast cancer cases	Mortality of cancer	Mortality of breast cancer
Females	8.6 million	2.0812 million	4.2 million	0.63 million
Both sexes	18.1 million	2.0996 million	9.6 million	0.6336 million
Percentage of Females	47.5%	99.12 %	43.75 %	99.43%

The table above shows for both sexes and females the amount of people newly diagnosed with cancer and breast cancer and the mortality of cancer and breast cancer in 2018, as well as the percentage.

SOURCE: Globocan 2018, World Health Organization.

The incidence of TNBC among breast cancer depends on several factors like, for instance, sex, race, and age of the patient. Not only is breast cancer itself more common in females than in males but also the TNBC type occurs mainly in females with an incidence of 13% and 6% respectively. Taking into account the race of a patient it was noticed that for example, in the African-American population, the incidence is the highest one yet determined accounting for 35% of all breast cancers diagnoses of this group. To be more precise it is stated that it is most prominent among non-Hispanic black and Hispanic patients (23.7% and 14.8%) and in the Caucasian population it only makes up around 15% (7). The lowest occurrences so far measured are in Filipino patients (8.9%), albeit Filipino women are known to be at a heightened risk for HER2+ cancers (7,8). For all ethnic groups, it is deemed to be that younger patients are more prone to have TNBC, with notable distinctions between Caucasian and African-American patients. As seen in Figure 2, for patients younger than 30-years of age, the two racial groups start with a roughly similar overall incidence of TNBC. In Caucasian patients, the TNBC percentage is highest for the age group around 30 years, and then it continuously declines, while in African-American patients, there is no significant decrease until the patients are older than 60-years, but even a slight increase during their thirties. Nonetheless, the graph shown in Figure 2 points out that African-American women of all age groups are at any time during their life more predisposed to have TNBC compared to Caucasian patients (7).

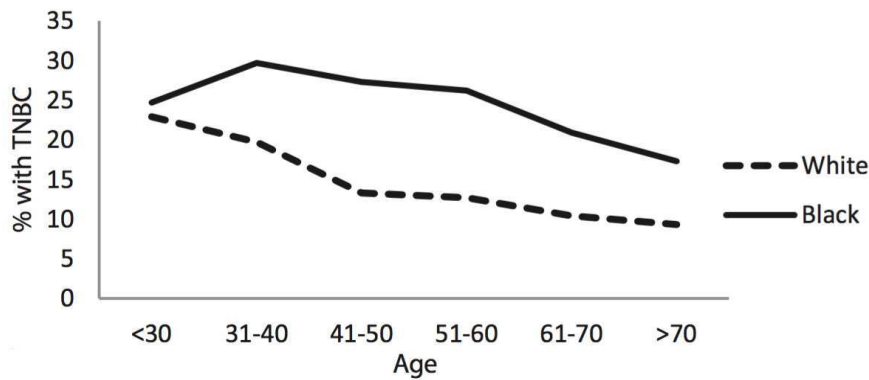


Figure 2. Percentage of TNBC based on patient age and race.

TNBC: triple-negative breast cancer.

SOURCE: Plasilova et al (7).

TNBC is of course also associated with genetic, pathologic variants (PV), which underline how important it is to consider the race of a patient as the incidence of the different PVs varies immensely depending on whether a patient is of Caucasian ancestry or African-American. For example, an increased incidence in patients with germline BRCA1 mutations is commonly seen in Caucasians with TNBC, the most striking PVs with the highest incidence are BRCA1 and PALB2 PVs which have a lifetime risk for TNBC of 18% and 10% respectively, followed by BARD1 with 7%, 6% BRCA2, and 5% RAD51D, as shown in Figure 3A and B. Even though the same pathologic variants are found in African-American people, it has to be emphasized that the impact is far more drastic on their TNBC risk estimates for overall breast cancer, with 81% BRCA1, 62% BRCA2, 41% PALB2, and 39% BARD1 (Figure 3C and D) (9). Furthermore, around 70% of breast cancers diagnosed in people with an inherited BRCA mutation, particularly BRCA1, are classified as triple-negative (10). To summarize, one has to add that African-Americans have a large and diverse mutation profile with more genetic variants on numerous genes per patient, while Caucasians have PVs on fewer genes and also the deleterious PVs per patient are significantly less prevalent in Caucasians (11).

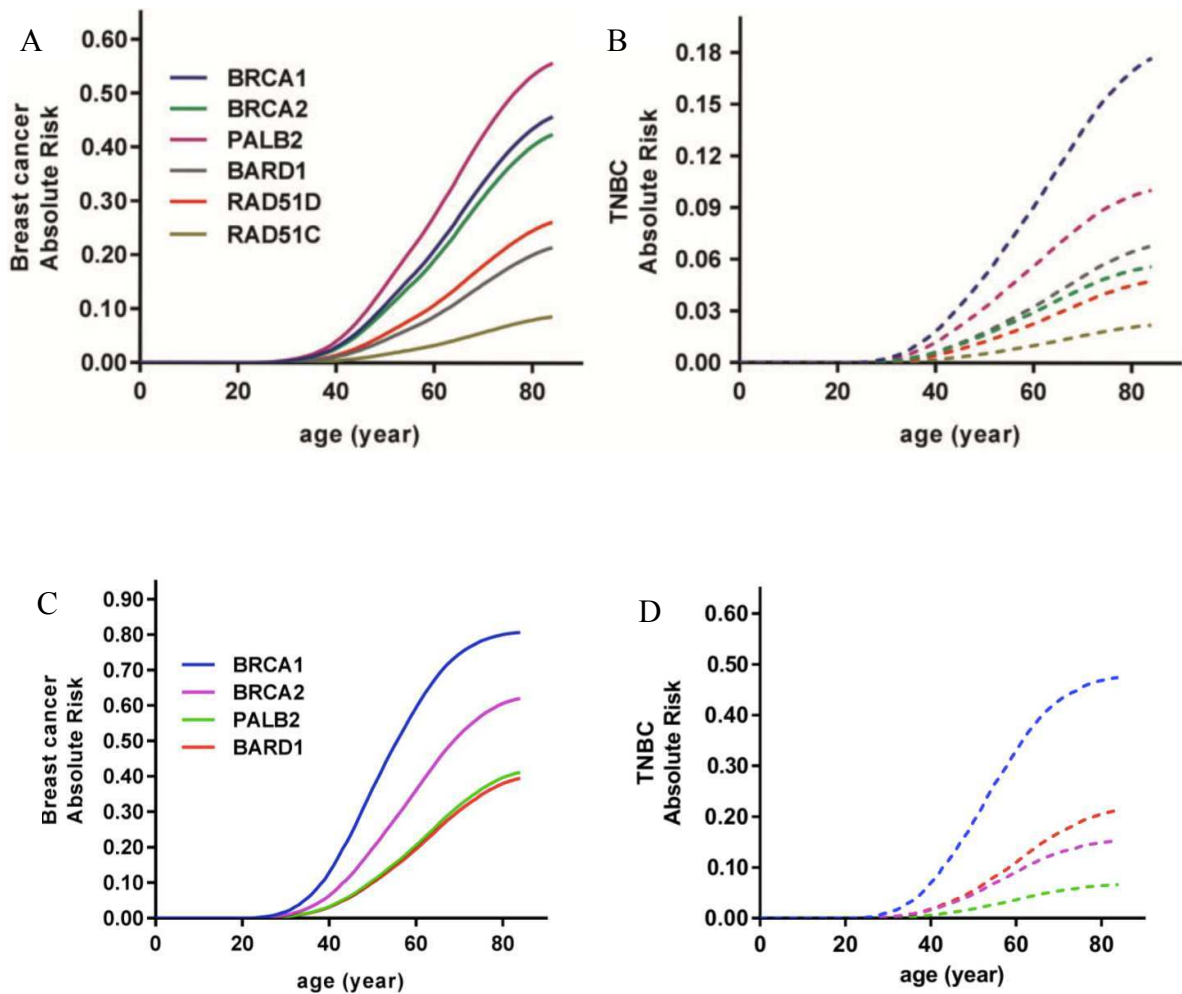


Figure 3. Absolute risk of specific pathologic variants for Caucasian (A and B) and African-American (C and D) accessed to age 85 the overall breast cancer and triple-negative breast cancer risk. (A) Age-related risk curves for Caucasian overall breast cancer for six genes as color lines. (B) Age-related risk curves for Caucasian TNBC for six genes as colored dashed lines. (C) Age-related risk curves for African-American overall breast cancer for four genes as color lines. (D) Age-related risk curves for African-American TNBC for four genes as colored dashed lines.

TNBC: triple-negative breast cancer.

SOURCES: <http://www.seer.cancer.gov> and Shimelis et al (9).

Another apparent risk factor which is still under investigation is the oral contraceptive pill (OC). A study from 2009 compares the impact of taking OC on the risk of getting TNBC in women who took birth control pills for less than one year or never. The article states that taking OCs for at least one year causes a 2.5 times increase in risk for TNBC in females, and if the women are around the age 40 the increase is even 4.2 times. Furthermore, they also concluded that the prolonged duration of OC administration leads to an increased TNBC risk (12).

However, in another study from 2011, which also covers the association of the patients' reproductive history and their oral contraceptive usage in relation to the risk of getting TNBC, the increased risk of OCs for getting TNBC was not found. Albeit, it finds an association between nulliparity and the risk of breast cancer, showing a decreased incidence in TNBC patients but an increase in ER-positive ones (13). Another study from the same year strengthens the relevance of number of pregnancies in breast cancer patients, as it also states that the frequency of nulliparity is lowest in TNBC patients with 13% compared to other types of breast cancer, but it also says that multiparity, more precisely ≥ 3 kids is more common in TNBC (14,15). Aside from that, another known and relevant factor that decreases the risk of developing TNBC is breastfeeding for a prolonged duration or breastfeeding several children (15).

Besides, metabolic syndrome, characterized by obesity and insulin resistance, and a higher waist/hip ratio is also more prevalent in TNBC than it is in other breast cancers (15,16,17). Last but not least, another study found that alcoholism has a decreasing effect on the risk of getting TNBC and also concluded that smoking is not associated with TNBC (18).

1.2.3 Pathophysiology

The formation of breast cancer development is, like in most other cancers, set off by a combination of multiple external environmental factors and their interaction in a genetically susceptible host (1). The CD44⁺/CD24⁻ phenotype, which is known to be numerously common in cancer stem cells (CSC), represents the tumorigenic cells with stem cell-like properties, meaning the ability to extensively self-renew and give rise to phenotypically different cells with a decreased proliferative and developmental potential. The cell surface glycoprotein CD44 is crucial for the quick cancer progression as it plays a critical role in the adhesion, migration, and invasion of breast cancer cells (BCC), as well as in proliferation and metastasis (19,20). These CSC qualities do not only lead to the production of tumorigenic cells but also to the production of several phenotypically distinct nontumorigenic cells that make up the overall tumor mass. Due to the fact, that the only unifying characteristic of TNBC is the lack of the expression of ER, PR, and HER2, and due to the stem cell (SC) qualities of this cancer group, the creation of phenotypically heterogenic tumors is expected (4,21). The latest definition presented by the American Society of Clinical Oncology (ASCO) in their guidelines about the three main biomarkers ER, PR, HER2 states, that ER/PR is considered positive if $\geq 1\%$ immunohistochemistry (IHC) and HER2 is considered positive if there

is protein expression of 3+, and/or HER2/neu gene amplification is ≥ 2.0 by fluorescence in situ hybridization (FISH). This means that the contemporary proper pathologic definition of TNBC actually is defined as 0% by IHC for ER and PR, and for HER2 negativity it states IHC expression of 0-1+ or lack of HER2/neu gene amplification (FISH < 2.0) (22).

The most known genes responsible for breast cancer development are BRCA mutations and the germline mutations of the tumor protein 53 (p53) tumor suppressor gene (TSG). Even though BRCA mutations are only responsible for 5-10% of breast cancer cases in women, 70% of these are triple-negative, chiefly BRCA1 variants (10,23).

Besides, to the age of 80-years, these harmful mutations lead to an estimated cumulative risk of getting breast cancer of 72% and 69%, for BRCA1 and BRCA2 respectively (24). On top of this, the BRCA mutations form TSG complexes, where the BRCA1 modification combines with BARD1 and causes the heterodimer BRCA1-BARD1, which interacts with RAD51 and the TSG complex BRCA2-PALB2. BRCA2 works together with PALB2, forming the TSG complex BRCA2-PALB2, PALB2 stands for partner and localizer of BRCA2, and both combined mutations have increased risks for breast cancer development and make targeted therapy even more difficult (25). Lastly, TP53 causes Li-Fraumeni syndrome and is, therefore, responsible for 4% of breast cancers among females under the age of 30-years (26).

1.2.4 Clinical features and prognostics

Table 2 states the most important clinical and diagnostic factors concerning TNBC, but for further details, one can say that clinically, TNBC mostly presents as a palpable lump in the breast of younger females and is therefore usually not detected by the standard breast cancer screenings, which usually start around the age of 50. For women who attend regular screening programs, TNBC is known to typically present as an interval cancer, meaning between two mammographies. Withal, at the time of detection it commonly first manifests in a more unfavorable histopathologic condition, as it is often attended with lymphovascular invasion when it is diagnosed and the tumor is generally more massive in size, tends to present with clinically metastatic disease, and overall mostly in a higher cancer stage. Strictly speaking at the time of diagnosis, a striking majority of 79.8% are grade III and the mean tumor size is 2.78 cm (Table 3). Only as little as 2.4% are grade I at the time of their first presentation. (7,27). Until in 2016, the newer study by Plasilova et al. was

published, it was believed that there is no correlation between increasing tumor size and lymph node involvement in TNBC, unlike in other breast cancer types, but as shown in Figure 4, positive lymph nodes are generally less common in TNBC, even though in both BC groups the overall percentage of lymph node positivity is the same (7,28,29). Furthermore, it usually presents more aggressively and preferentially spreads hematogenously, leading to frequent visceral relapses in the brain and lungs (16).

Table 2. Key points about TNBC characteristics.

Main characteristic features of TNBC phenotype:
Younger women (<50 years)
More frequent in African-American and black ethnicities
Presents as interval cancer
Less likely to have lymph node involvement
Steep increase in risk of recurrence after diagnosis
Peak risk of recurrence at 1-3 years
Distal recurrence more common than local ones; no distant relapse after 8 years
Higher mortality rate first 5 years; all deaths occur within 10 years of diagnosis
Short survival after distant recurrence
More aggressive with increase incidence of visceral metastasis (brain, lung)

SOURCES: Plasilova et al (7); Kumar et al (27); Dent et al (28).

Speaking of visceral relapses, the pattern of recurrences shows a significant difference in TNBC compared to other BCs. First of all, distant metastases are more common than locoregional ones and also considerably more common in TNBC (33.9%) than in different subtypes (20.4%). After surgery, the relapse rate as shown in Figure 5, shows a striking increase in the first three years with the mean time to distant metastasis being 2.6 years compared to five years in other BCs, and interestingly the rate for distant metastases decreases quickly until it is rather uncommon eight years following the diagnosis. Furthermore, distal recurrences are rarely preceded by local ones, only in 25% of women treated with breast-conserving surgery, which are only slightly more common in TNBC than in other types of breast cancers, but occur notably sooner in TNBC after an estimated 2.8 years versus 4.2 years respectively (27,28).

Table 3. Specific characteristic differences for TNBC compared to other types of breast cancers.

Characteristic:	TNBC n (%)	P*
T stage:		
T ₁	46.4%	<0.001
T ₂	38.1%	
T ₃	8.4%	
T ₄	7.1%	
N stage:		
N ₀	72%	<0.001
N ₁	20.1%	
N ₂	4.7%	
N ₃	3.3%	
M stage:		
M ₀	94.1%	<0.001
M ₁	5.9%	
Mean tumor size (cm±SE):	2.78±0.012	<0.001
Tumor grade		
I	2.4%	<0.0001
II	17.8%	
III	79.8%	

*P values were calculated using the x² test.

SE=standard error; LVI, lymphovascular invasion.

Full table shown in supplements (Table 2).

SOURCE: Plasilova et al (7).

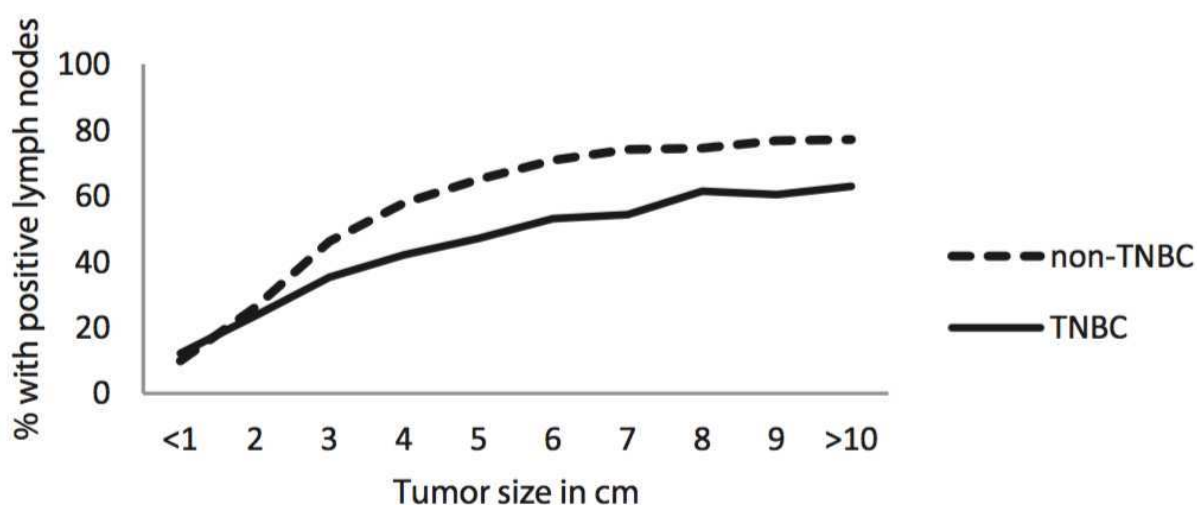


Figure 4. Comparison of TNBC and non-TNBC regarding their tumor size and lymph node positivity in percentage (%).

Abbreviations: TNBC, triple-negative breast cancer.

SOURCE: Plasilova et al (7).

The overall outcome in TNBC is significantly worse than in the other subtypes, as 42.2% of the patients with TNBC died compared to 28%. TNBC has an especially higher mortality rate in the first five years following diagnosis with 70% of all TNBC-related deaths occurring during this time, but all deaths occur within ten years of diagnosis, while for the other subtypes the mortality rate continues for around 18 years (28). But as TNBC represents a heterogeneous group, not every single type has a poor prognosis, for example, adenoid cystic and secretory carcinoma, present with a rather indolent course and therefore have a better prognosis even without chemotherapy being used as the treatment option (30). Albeit as shown in Figure 6, the overall probability of survival in TNBC is continuously lower than in other BCs. TNBC is an exceptionally rapidly progressing cancer, with a survival time of only nine months from distant recurrence on, compared to 20 months in other breast cancers (28).

Last but not least, patients who are older than 70 years at the time of diagnosis have a superior prognosis compared to younger women with TNBC, and black ethnicities have generally as a whole an inferior prognosis (11,31).

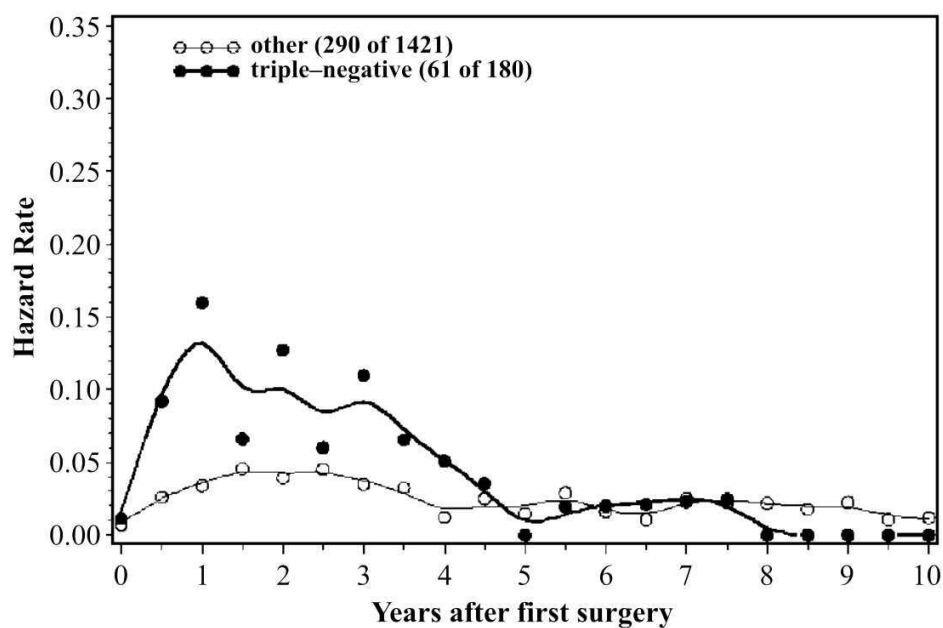


Figure 5. Distant recurrences after surgery in TNBC and other types of breast cancers. SOURCE: Dent et al (28).

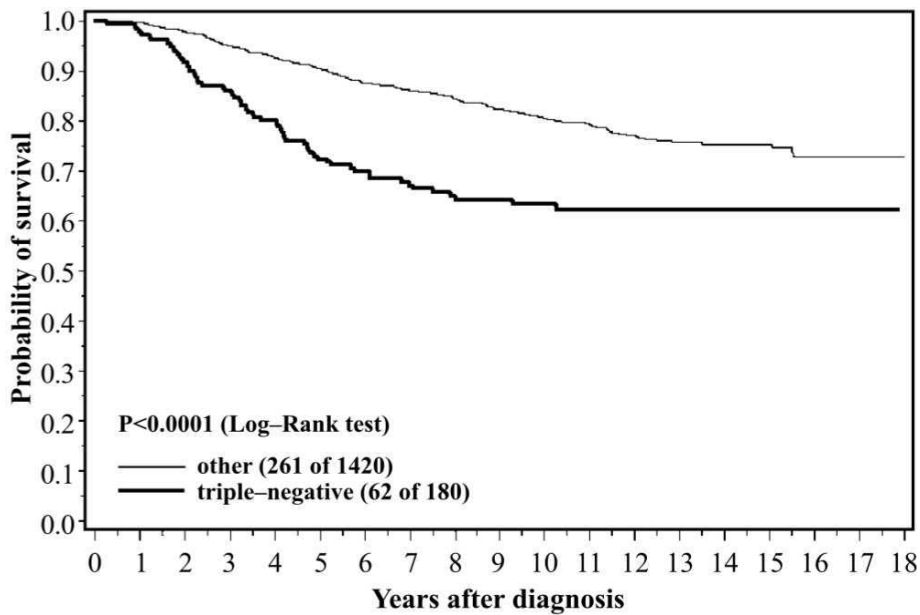


Figure 6. Comparison of survival rates in TNBC and other breast cancers (28).
SOURCE: Dent et al (28).

1.2.5 Diagnostics

As TNBC is a very aggressive subtype and usually presents in advanced stages, it is crucial to know its main imaging features, especially as they often lack the typical suspicious features for malignancies, like an irregular shape, speculated margins and calcifications. On mammography, TNBC usually appears as an interval cancer, as in spite of its large size at the time of diagnosis, it is often occult on initial mammography scans (18%). Distinct characteristics of mammography for TNBC are in contrast to the ones for other breast cancer types, and thus it may be useful in the diagnosis and differentiation of TNBC, as TNBC usually presents as a well-circumscribed mass in 37.2% of the cases and without calcification in around 49-100% of the cases (27,32,33,34). More precisely speaking, calcifications are absent in 83.3% of visible masses, and there is total lack of calcifications in 74.1% of scans, which are significantly higher percentages compared to non-TNBCs. The shape is mostly round to oval (58.1%) and sometimes lobular (30.2%) (34).

Ultrasound (US) has a very high sensitivity for triple-negative breast cancers (92-100%), but caution as TNBC usually presents with prevalent signs of benign neoplasms, cysts, and abscesses, meaning as a distinct mass that has with well-circumscribed margins (21-27%) and shows posterior acoustic enhancement (24-41%), which can also be indicative for a tumor necrosis associated fluid component (27,32,34).

Magnetic resonance imaging (MRI) is the most sensitive method to detect TNBC, with a visualization rate of 100%. The lesions appear with smooth margins, but also with rim enhancement, which is a highly predictive characteristic of malignancy. Furthermore, features are persistent enhancement patterns and, on T2-weighted images, a high intratumoral signal intensity (35). A study from 2007 with a rather small sample 29 patients states that at the time of diagnosis, in 21% of the cases multifocality is visible, as well as in 34% prominent skin enhancement indicating dermal lymphatic invasion in tumors smaller than five centimeters. The mean tumor of this study is size 4.1 ± 2.7 cm and 79% of the cases presented at least at grade II (36,37). Dynamic MRI shows vascular differences between TNBC and the other subgroups, observing a significantly higher outflow rate and smaller leakage space in TNBCs (38). To sum up, the TNBC visualization rates of the three main diagnostic methods, mammography, US, and MRI are very high, 91%, 93% and 100% respectively (34).

Another imaging procedure, rather than a primary diagnostic method, that can be performed is [¹⁸F]2-fluoro-2-deoxy-D-glucose positron emission tomography (¹⁸F-FDG-PET), it is useful for detecting metastasis and to follow-up the response to chemotherapy (32,39).

1.2.6 Classification of breast cancer

According to the WHO classification from 2012, there are for the time being 21 morphologically distinct subtypes of invasive breast cancer (IBC), which make it a very heterogeneous group with distinct pathological and molecular characteristics. The whole list of all acknowledged breast cancers is added in the supplement section, Figure 2. The most common IBC type is the invasive carcinoma of no special type, also known as invasive ductal carcinoma, which makes up between 40 to 75% in published series (40). Invasive ductal carcinoma comprises even a more significant percentage among TNBCs with a 90% majority of TNBCs being unifocal, invasive ductal carcinomas. All the other histological types of TNBC are classified as either medullary, secretory, adenoid cystic, apocrine, metaplastic, invasive lobular carcinoma, or microglandular adenosis carcinomas (27).

Up to this point, the morphological diagnosis and the contemporary staging systems are rather inadequate and disorganized; consequently, they are currently evolving into a multi-classification system which integrates the morphological diagnosis of cancer, immunohistochemical assessment, DNA microarray analysis, and CIRCOS Plot to improve the therapy response rate and

overall outcomes (41). However, for the time being, the guidelines for breast cancer therapy focus on a combination of pathological classification, the histological grade of cancer, ER, PR, and HER2 status (42). But, due to the advancement of molecular procedures, such as DNA microarray analysis which focuses on gene expression profiling, the concept of heterogeneity of breast cancer has become accepted and the importance in diagnosis shifted to "Molecular Classification" of breast cancers — leading to the development of targeted therapeutics and individualized treatment (43).

Figure 7 shows eleven distinct histopathological pictures of eight different types of breast cancers stained with hematoxylin and eosin (H&E). The different classes show slight differences, but the cancer cells are highly coherent. Histopathological slides show significant variations making differentiation of breast cancer types hard (44).

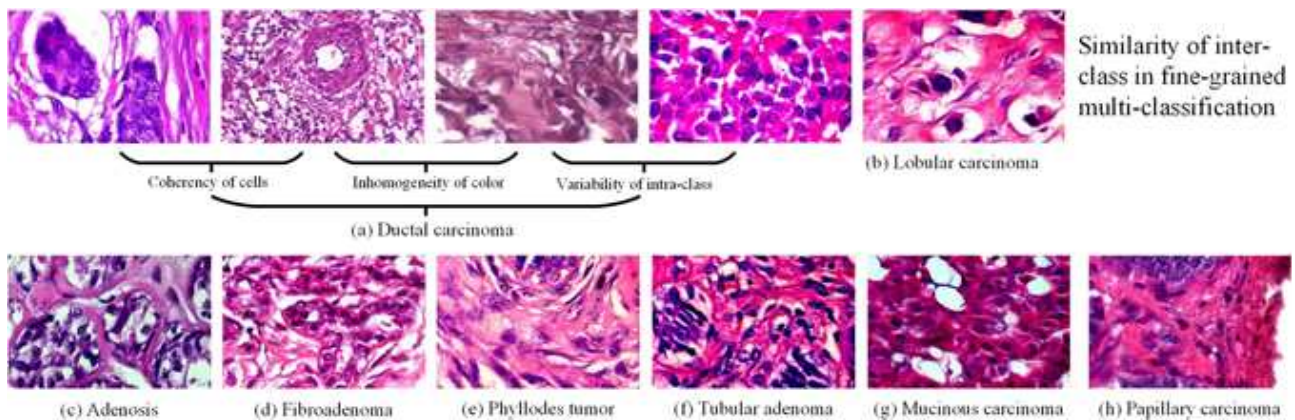


Figure 7. Histopathological images of the eight most common breast cancer types at a magnification factor of 400.
SOURCE: Han et al (44).

The importance of immunohistochemistry is presented in Figure 8, which shows the differences in H&E staining and immunohistochemical patterns for various breast cancer types, with a focus on TNBC divided by its basal-like subtype and non-classified TNBCs. Most commonly ER, PR, and HER2 are used in IHC, which divides IBC into TNBC, luminal, and HER2 breast cancer subtypes. With further IMC surrogates, more subdivisions can be made, like for example Ki-67, cytokeratin 5, and EGFR separating the basal-like breast cancer subtype from TNBC, and luminal breast cancers in A and B subtypes (42).

Figure 8. Histological images with hematoxylin-eosin stain (H&E) and immunohistochemical patterns for seven molecular breast cancer subtypes.

Stains	Luminal BC			HER2 Positive BC			TNBC	
	Luminal A Subtype	Luminal B Subtype (Ki67≥14%)	Luminal B Subtype (PR<20%)	Luminal HER2 PR (≥1%)	Luminal HER2 PR (<1%)	HER2 Enriched	Basal-like subtype	Non-classified subtype
H&E								
ER								
PR								
HER2								
KI-67								
CK5								
EGFR								

Abbreviations: BC, breast cancer; BLBC, basal-like subtype; CK5, high-molecular weight cytokeratin expressed in normal myoepithelial cells; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; NCBC, nonclassified subtype; PR, progesterone receptor; TNBC, triple-negative breast cancer.

SOURCE: Tang et al (42).

Subclassifying the TN phenotype is somewhat tricky, considering that the only unifying feature being the lack of three biomarkers. Figure 9 emphasizes this heterogeneity by showing various histopathological TNBC subtypes and their degree of low- to high-grade cancers. Although progression to high-grade cancers is typical in TNBC it is with varying rates depending on the histological type, i.e., prevalent in acidic cell carcinoma and rare in salivary gland-like breast tumors, resulting in some subgroups having a low histologic grade and rather indolent behavior, even though in general TNBC is known for its aggressive presentation (45). For example, adenoid cystic and secretory carcinomas of the breast, are TNBCs with an indolent course and especially a better prognosis even without chemotherapy (29).

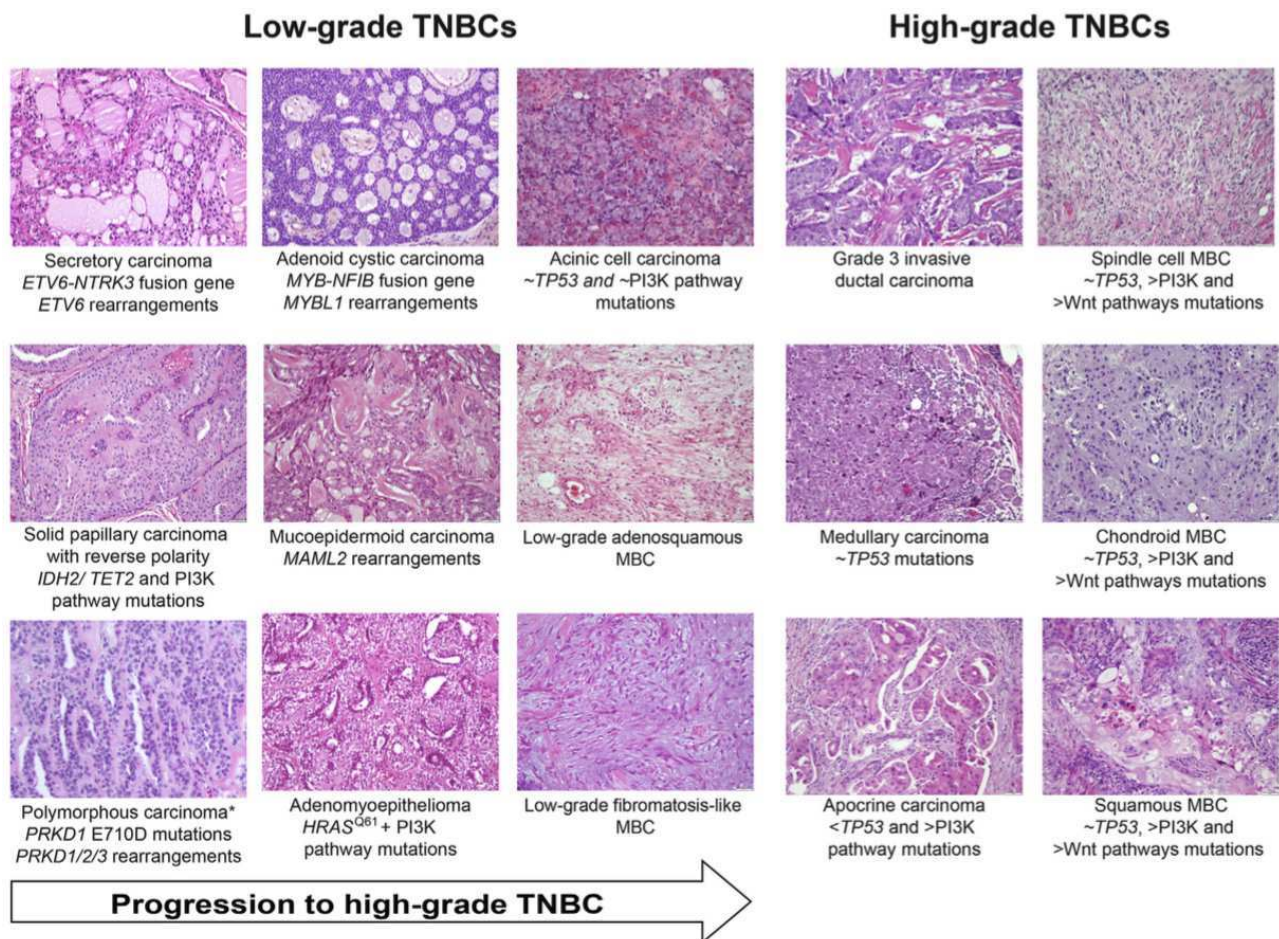


Figure 9. Spectrum of low to high-grade TNBCs. Various histopathological subtypes of TNBC are connected to their known specific genetic alterations. Progression from low to high-grade TNBC is common but occurs at different rates. *Evidence for polymorphous carcinoma of the breast mutation *PRKD1* E710D or *PRKD1/2/3* rearrangements still needs to be documented. Magnification factor of 200.

Abbreviations: PI3K, phosphatidylinositol 3-kinase.; TNBC, triple-negative breast cancer. SOURCE: Geyer et al (45).

1.2.7 Therapeutic options and advances

Due to the fact that there are no proper standard treatment regimens or even options available for TNBC, there are various therapeutic approaches, ranging from local therapy, meaning surgery and radiation to systemic approaches involving chemotherapy, targeted therapies, immunotherapeutic approaches, to the option of breast cancer vaccines, but most potentially more potent therapeutics are still under investigation so far in clinical or like our compound in preclinical trials (27).

Local therapy for TNBC is applied in a comparable way as to other IBCs and involves removal of the cancerous lump by either breast-conserving surgery (BCS) or mastectomy supported by radiotherapy (RT), as there are no TNBC-specific local therapy recommendations. The breast-conserving procedure remains the standard therapy for small T1 and T2 BCs, and mastectomy is for more extensive, multifocal cancers, or in patients with positive margins after BCS (8,27). A study from 2017 highlights the point that there is insufficient data, to imply that mastectomy is better than BCS, but also points out that systemic recurrence remains higher in TNBC patients emphasizing the importance of more efficient systemic therapeutic options (46). There are no distinguished guidelines for adjuvant RT in TNBC, and the better outcome in BCS is determined by the fact that three-year relapse-free survival is 79.6% in patients treated with RT compared to 57.9% in patients without RT, even though the patients with RT had a higher cancer stage (27).

Since appropriate targets in TNBC for systemic approaches are missing, chemotherapy remains the primary treatment for TNBC, with no difference in the outcome whether or not the therapy is started pre- or postoperative in early-stage TNBCs, but several other systemic treatment options are momentarily under investigation (27,47). Even though the response to chemotherapy in TNBC is superior compared to other BC types, the five-year survival is by less than 30% of women with metastatic disease (48). So far anthracycline-taxane-based chemotherapy remains the standard neoadjuvant systemic treatment (NST), but the addition of platinum-based agents in NST is considered, in defiance of the added toxicity several papers say that it shows better clinical response to systemic therapy and could possibly improve local therapy, however some state that it does not improve survival (47,48).

Many promising therapies are under investigations to allow personalized treatment strategies in this heterogeneous disease, like immunotherapeutic approaches and the oncolytic vaccinia virus, which does not only show promising results in preventing metastasis but also in treating it (27). Another approach is targeted therapies concentrating on, for example, PARP inhibitors, anti-androgen therapy, PI3K inhibitors, MEK inhibitors, and inhibitors of cancer stem cells. This study focuses on a novel therapeutic compound to counter the BCSC population, which is known to be more resistant to chemotherapy than cancers without stem cells (48).

2. OBJECTIVES AND HYPOTHESES

2.1 Objectives

The aim of this study is to determine the effects of treating breast cancer stem cells with the newly synthesized thieno[2,3-b]pyridine anticancer agent, by focusing on its cytotoxic and apoptotic effects on the investigated human cell line MDA-MB-231.

2.2 Hypotheses

1. Treating the MDA-MB-231 cell line with compound 1 will have a cytotoxic effect on the breast cancer stem cells.
2. Compound 1 will lead to an increased apoptotic rate in the MDA-MB-231 breast cancer stem cell line.
3. The percentage of the breast cancer stem cell subpopulation will be decreased following the treatment with compound 1.
4. The effects of compound 1 will be in a dose- and time-dependent manner.

3. MATERIALS AND METHODS

3.1 Chemistry and cell line

3-Amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-b]quinoline-2-carboxamide (compound 1) (Figure 10) was dissolved in dimethyl sulfoxide (DMSO). Cells were purchased from the American Type Culture Collection (ATCC, LGC Standards), the cell micrograph is shown in Figure 11. The MDA-MB-231 breast cancer stem cells were grown in a Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Steinheim, Germany) with 10% fetal bovine serum (FBS, EuroClone) supplemented with 1% antibiotics (Penicillin-Streptomycin, EuroClone). The culture conditions were in a humidified incubator at 37°C with an atmosphere of 5% CO₂. The known characteristics of the cell line are presented in the table below (Table 4).

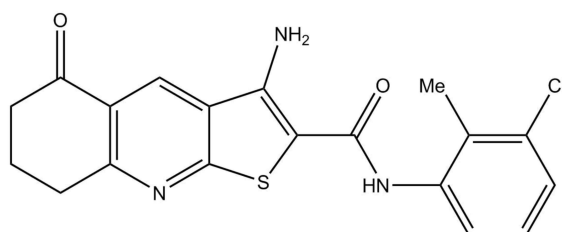


Figure 10. The structure of the newly synthesized anticancer agent (compound 1).

Note: Compound 1, 3-Amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-b]quinoline-2-carboxamide.

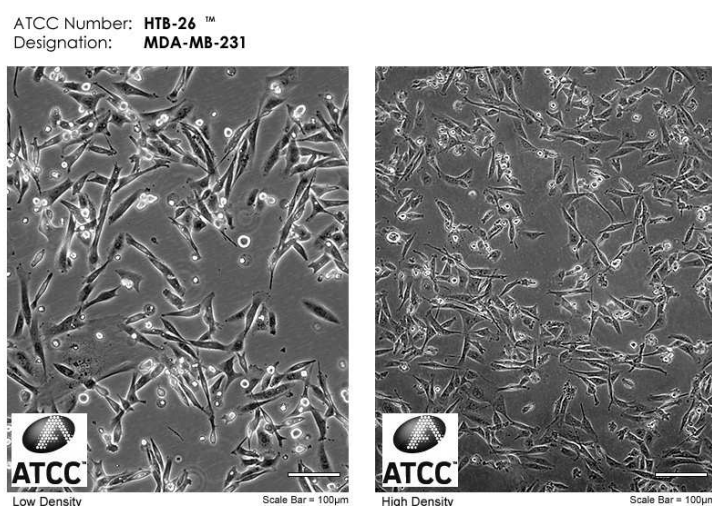


Figure 11. Cell micrograph

SOURCE: <https://www.lgcstandards-atcc.org> (49).

Table 4. Characteristics of MDA-MB-231 cell lines

Characteristics:	
Organism:	<i>Homo sapiens</i> , human
Tissue:	mammary gland/breast; derived from metastatic site: pleural effusion
Disease:	adenocarcinoma
Cell Type:	epithelial
Age:	51 years adult
Gender:	female
Ethnicity:	Caucasian
Morphology:	epithelial
Culture Properties:	adherent
Biosafety Level:	1 Biosafety classification is based on U.S. Public Health Service Guidelines
Product Format:	frozen
Applications:	these cells are a suitable transfection host
Storage Conditions:	liquid nitrogen vapor phase
Receptor Expression:	epidermal growth factor (EGF), expressed transforming growth factor alpha (TGF alpha), expressed
Oncogene:	the cells express the WNT7B oncogene

SOURCE: <https://www.lgcstandards-atcc.org> (49).

3.2 Colorimetric MTT assay

A tetrazolium-based colorimetric MTT assay was performed, to analyze the cell viability and in doing so determining the cytotoxic effect of the novel anticancer compound on triple-negative breast cancer stem cells. The cell proliferation was measured by first diluting the cells in a solution of trypan blue and Neubauer chambers counted by MOTIC AE30, an inverted binocular microscope. The cell number was calculated according to the formula: number of counted cells $\times 10 \times 10^4/\text{mL}$. A homogenous number of cells were then seeded in 96-well plates at a density of 104 cells/100 μL and incubated during the night to allow attachment (Figure 12). The cells were treated in a complete medium and the experiments performed in triplicate, for 4, 24, 48 and 72 h with specific solutions of compound 1 at a concentration of 50 nM, 250 nM, 500 nM, 1 μM and 5 μM . Thereupon, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed, and the treated cells were incubated with 0.5 g MTT/L at 37°C for 1 hour, the medium was removed and in the end dimethylsulphoxide (10% DMSO) was added and incubated for another 10 minutes at 37°C with shaking. Absorbance was measured photometrically at a signal wavelength of 570 nm and depends on the degree of formazan formation (Figure 13), which indicates cell viability and their metabolic activity. The collected data was calculated in comparison to the untreated control (100%) from three independent measurements.



Figure 12. MDA-MB-231 in 96-well plates and treated with compound 1.
SOURCE: Biochemistry Laboratory, Medical Faculty of Split.

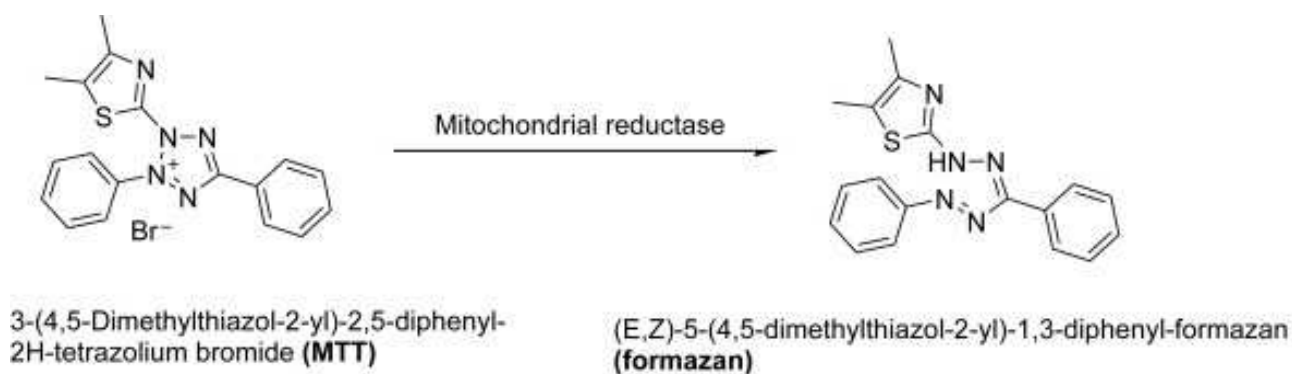


Figure 13. Enzymatic formazan formation in MTT.
SOURCE: Kuete et al (50).

3.3 Flow cytometric analysis

A homogenous number of cells were plated onto six-well plates at a density of 104 cells/100 μ L and treated with 2 μ M compound 1 for 48 h and afterward analyzed for apoptosis. The combined staining of propidium iodide and Annexin-V-FITC makes it possible to properly distinguish between early (Annexin-V+/PI-) and late (Annexin-V+/PI-) apoptotic cells, necrotic cells, and live cells. Following the treatment with compound 1 the cells were trypsinized, then washed with phosphate-buffered saline (PBS) and at last resuspended in 100 μ L of the binding buffer containing 5 μ L of propidium iodide and/or 5 μ L Annexin-V-FITC (Annexin-V-FITC Apoptosis Detection Kit I, BD Biosciences, San Jose, CA, USA). Then, the cells were incubated in the dark at room temperature for 15 minutes and after that flow cytometric analysis was carried out (BD Accuri C6, BD Biosciences). Using the FlowLogic Software (Inivai), the degree of apoptosis (Annexin-V positive cells) was analyzed and presented as mean \pm SD.

Besides, to detect the percentage of CD44+/CD24- cells, the cells administered with compound 1 and the controls were trypsinized and washed with PBS. Furthermore, the cells were prepared with an Fc receptor-blocking reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to avoid the occurrence of nonspecific binding from happening. Following another incubation at room temperature for 15 minutes with diluted anti-CD15s (BD Biosciences), the cells were additionally diluted with anti-CD44-FITC (BD Biosciences), anti-CD24-PE (eBioscience, Inc., San Diego, CA, USA) and secondary antibody conjugated with eFluor 660 fluorochrome (eBioscience, Inc.) and anew incubated for 15 minutes in the dark. Eventually, the cells were resuspended in PBS and then analyzed by flow cytometry (BD Accuri C6; BD Biosciences).

3.4 Statistical analysis

The results were statistically analyzed using the statistical software Statistica issued for Windows version 7.0 (Stat Soft, Tulsa, OK, USA), where $P < 0.05$ indicates a statistically significant difference.

The colorimetric MTT assay results were statistically analyzed by a one-way ANOVA followed by post-hoc Tukey test (MTT results after 4, 48, and 72 hours) or post-hoc Dunn test (MTT results after 24 hours). Data represent the percentage of live cells and mean \pm SD.

From the flow cytometric analysis the results are presented as mean \pm SD of Annexin-V⁺/PI⁻ cells and CD44⁺/CD24⁻ cells. Percentage of early apoptosis, as well as the percentage of CD44⁺/CD24⁻ cells is determined using a two-tailed paired t test.

4. RESULTS

4.1 Compound 1: dose- and time-dependent cytotoxicity

In this study, we analyzed cell viability by MTT assay after 4, 24, 48, and 72 hours treated with compound 1 is shown in Figure 10. Cytotoxic effect of compound 1 is already observable after 4 hours of treatment with a concentration of 0.5 μM . Even ten times lower concentrations (50 nM) are effective after a prolonged period of 48 hours. Maximal cytotoxicity was obtained after 72 hours treatment with 5 μM compound 1, for merely 47% of cells.

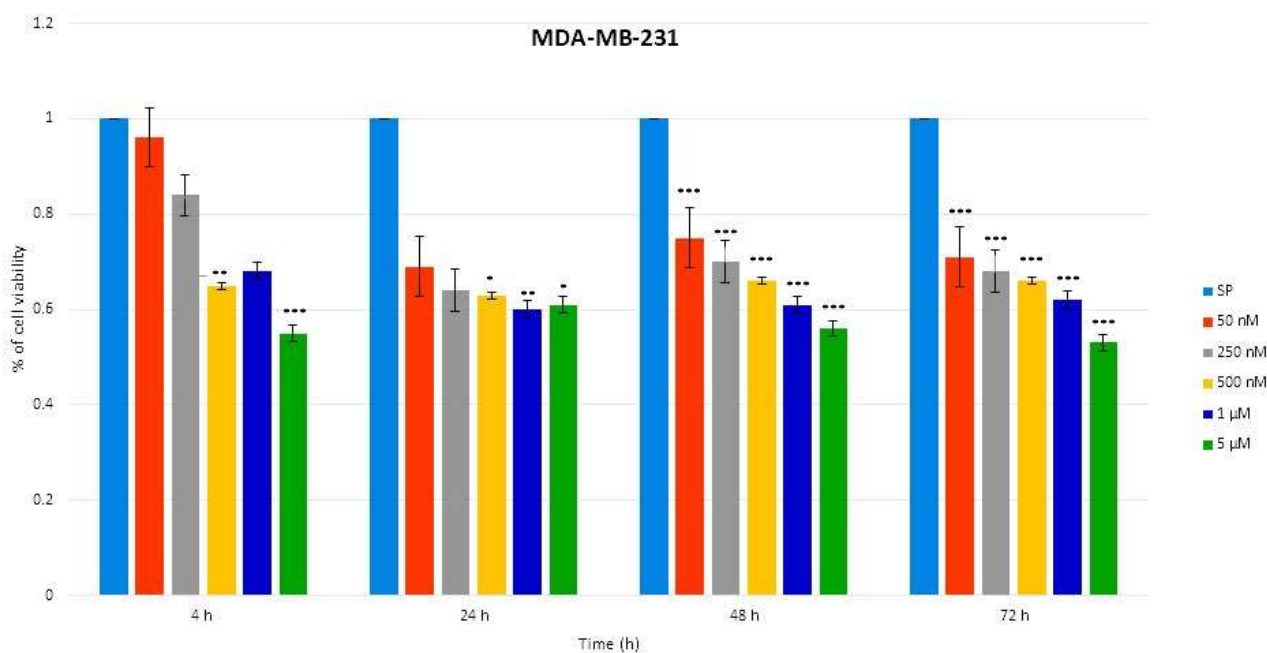


Figure 14. Cell viability after drug treatment.

Notes: Compound 1 produces a dose- and time-dependent metabolic defect in MDA-MB-231 cell line. Cells were treated with a dose-dependent curve of 1 as shown in the Figure for 4, 24, 48 and 72 h and cell metabolism evaluated by the MTT assay. Data represent the mean from experiment performed in triplicate \pm SD. Columns, mean of viable cells; bars, SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: SD, standard deviation; SP, control.

4.2 Compound I: cell death of breast cancer stem cells

To determine the cause of the MTT findings, we subsequently determined the type of cell death induced by 48-h treatment with 2 μ M compound 1. Compound 1 shows a prominent increase in early apoptosis in MDA-MB-231 cells compared to non-treated cells, as the percentage of early apoptosis in treated cells is around 9% and in untreated cells less than 2%. Therefore we can conclude that cell death mainly occurs due to treatment-induced apoptosis (Figure 15).

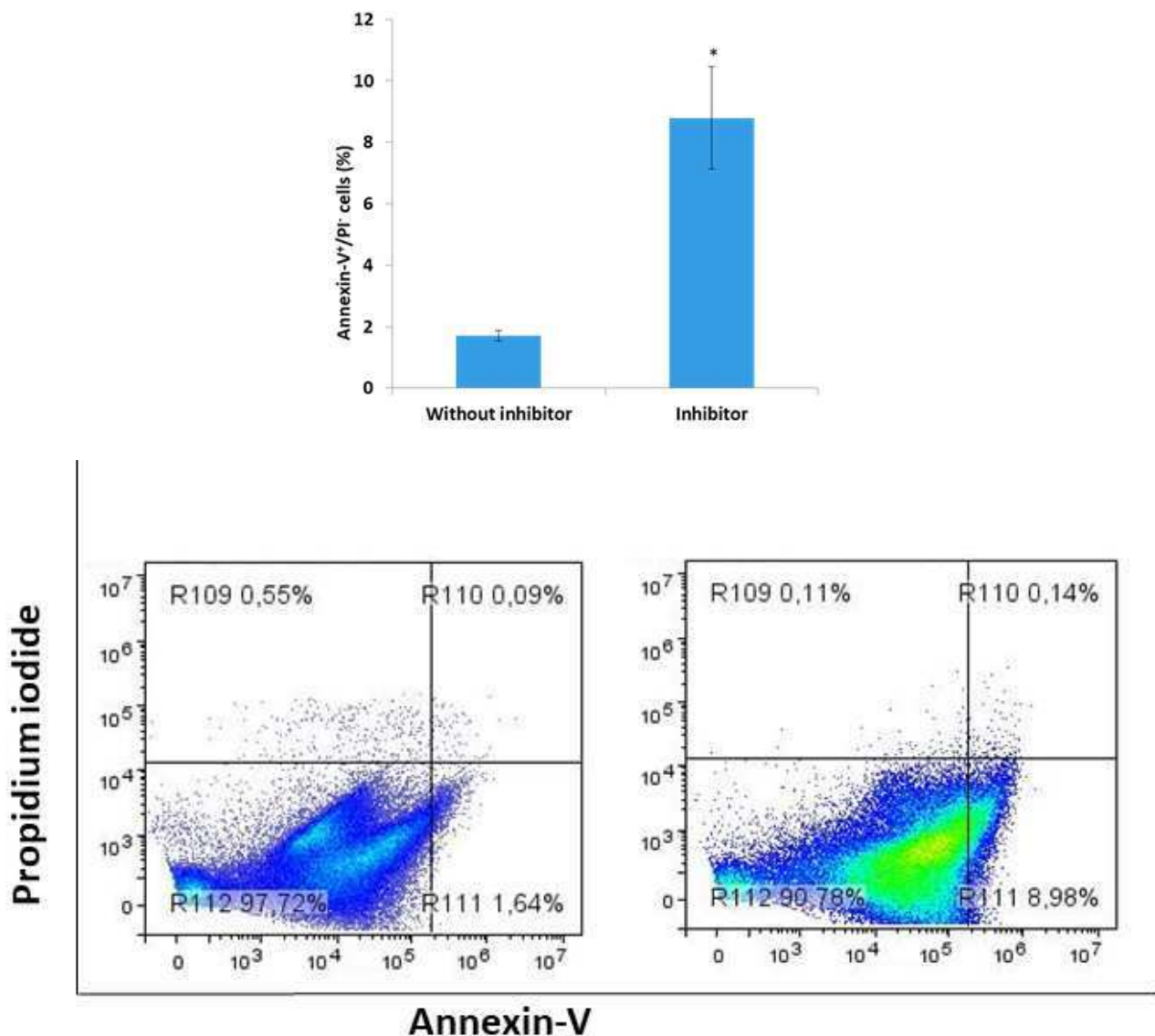


Figure 15. Apoptosis after drug treatment.

Notes: Apoptosis after treatment with 2 μ M compound 1 (inhibitor) for 48 h in MDA-MB-231. Data represent the mean \pm SD of Annexin-V⁺/PI⁻ cells. Percentage of early apoptosis is given in bar charts for treatment compared to the control. Columns, mean of cells; bars, SD; * $P < 0.05$.

Abbreviations: SD, standard deviation.

4.3 Compound I: decrease in number of BCSC

In the TNBC cell line MDA-MB-231, the marker combination CD44⁺/CD24⁻ is known to be numerous common in CSC as mentioned before. The treatment with compound 1 shows a statistically significant decrease of the CD44⁺/CD24⁻ subpopulation, with 89,86% in untreated control and 55.54% after treatment with 2 μ M compound 1 for 48 hours ($t=8.324$ $df=5$ $P < 0.001$).

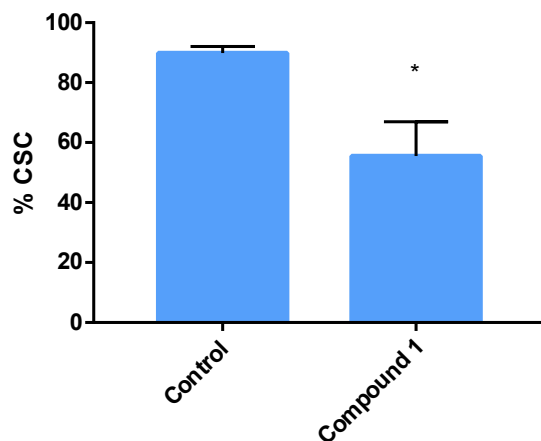


Figure 16. CSCs (CD44⁺/CD24⁻) after drug treatment.

Notes: Percentage of CSCs after treatment with 2 μ M compound 1 for 48 h in MDA-MB-231. Data represent the mean \pm SD. Percentage of CSC is given in bar charts for treatment compared to the control. Columns, mean of cells; bars, SD; * $P < 0.001$.

Abbreviations: CSCs, cancer stem cells; SD, standard deviation.

5. DISCUSSION

Acknowledging that targeting CSC is an auspicious strategy to defeat tumor recurrence and immunity to chemotherapy, the focus of this study was to determine, whether the newly synthesized putative phospholipase C (PLC) inhibitor, 3-amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-b]quinoline-2-carboxamide (compound 1; Figure 10), has cytotoxic properties and is able to induce cell death and therefore a potentially new therapeutic approach to treat TNBC (51). As it is ascertained that thieno[2,3-b]pyridine works against the phospholipase C- γ 2 (PLC- γ 2) isoform, a novel therapeutic target for molecular anticancer treatments which is an efficient anticancer agent against various cancer cell lines, we wanted to determine its potency against TNBC (52).

Performing a colorimetric MTT assay to analyze the cell viability of TNBC cells treated with different concentrations for various time intervals, we found that the newly synthesized anticancer compound shows a dose- and time-dependent cytotoxicity for breast MDA-MB-231 cancer cells. The novel compound under investigation is already cytotoxic at the lowest concentration tested after a prolonged period of 48 hours of treatment compared to the ten times higher concentration which is already cytotoxic after only four hours, the maximal cytotoxicity was achieved after treatment with maximal concentration at the longest time interval, but only for 47% of cells (Figure 14). This shows how increasing the dose and time raises the cytotoxic effect of compound 1 on MDA-MB-231. The performance of this experiment was done in triplicate to exclude any errors., however, the MTT assay used in our investigation, does not take into account whether the viable cells measured undergo active cell division or not. This means, we cannot exclude cytostatic qualities of the novel compound, that it for example, induces G2/M phase cell cycle arrest, and measured discrepancy is probably due to different analytical methods.

To determine the type of cell death, flow cytometric analysis was performed and we tested the compound at a concentration of 2 μ M after 48 hours. The results show a significant increase in early apoptosis in MDA-MB-231 cancer cells in comparison to non-treated cells, meaning that the majority of cells died by treatment-induced apoptosis (Figure 15). The same test was used to find out whether or not the percentage of BCSC is also declining, showing a prominent decrease in the CD44⁺/CD24⁻ subpopulation, which represent the tumorigenic stem cell-like properties. The high level of treatment-induced apoptosis and the significantly lowered BCSC subpopulation is so relevant because SCs are responsible for extensive self-renewal, proliferation, quick cancer progression, as well as metastasis disease in cancer patients, and up to this point no satisfying

therapy exists for TNBC, so it seems promising for treating TNBC, especially the metastatic form (19,20). However, in the context of this diploma thesis, we did not take into account the glycosphingolipid expression on the surface of CSCs which are responsible for cancer relapse and therapy resistance in TNBC, so we cannot say which receptors are associated and working together with the new compound and mediating the treatment-induced apoptosis and therefore also lowering the percentage of BCSCs (53).

In a similar study, another compound from the thieno[2,3-b]pyridine class was also used on the MDA-MB-231 cell line, showing comparable results. For the MTT assay the same time intervals were used but coupled with higher concentrations, 0.5 μM , 1 μM , 5 μM , 10 μM , and 25 μM of the thieno[2,3-b]pyridine compound. Anyways, it was also noticed that the compound produces a dose- and time-dependent cytotoxicity with regular growth retardation for MDA-MB-231 cells. Furthermore, it was also investigated whether the MTT results are caused by cell death or cell cycle arrest. Determination of the type of cell death was done by flow cytometric analysis with the same standards that were used in this study and it shows that compound-induced cell death occurs also mostly by early apoptosis, but it destroys a higher percentage than the compound tested in this study. Moreover, the decrease in BCSC was also observed but was not as prominent as in this study. However, they were also able to associate increased levels of GM3 expression on MDA-MB-231 cells to a higher apoptotic rate and a lower percentage of BCSC subpopulation.

Limiting factors to the study must be considered when interpreting and applying the conclusion. However, no major limitations to this study are established as every experiment was performed in triplicate. Albeit, one could test this novel compound on another TNBC cell line to confirm its therapeutic effects and compare the results to this study, allowing better comparison of the cytotoxic strength of this compound on TNBC stem cells.

This study conclusively confirms that the novel thieno[2,3-b]pyridine compound has a cytotoxic effect on the MDA-MB-231 TNBC stem cell line in not only a time- but also a dose-dependent manner. The most significant results obtained by this research concluded that the treatment-induced cell death is mainly due to an increased apoptotic rate. Furthermore, compound 1 considerably decreases the CSC subpopulation percentage found in TNBC.

Taking into account that several studies working with compounds of the thieno[2,3-b]pyridine class described efficient anticancer qualities against several types of cancers, these compounds targeting PLC- γ 2 should be paid more attention to, especially for TNBC which has no sufficient therapeutic regime yet (52,53). However, so far only in vitro trials on cancer cell lines were done and for the future the compounds have to be tested for whether or not they are only cytotoxic for cancerous cells or also for healthy tissue, to exclude any unwanted side effects.

6. CONCLUSIONS

- According to the results we obtained in this study, we were able to demonstrate that the treatment of human MDA-MB-231 TNBC with compound 1 works and has cytotoxic effects on the investigated cell line.
- The results show that compound 1 leads to a higher level of apoptosis and a significantly lowered percentage of the CSC subpopulation.
- Furthermore, to be more precise the results concluded that compound 1 affects TNBC in a dose- and time-dependent cytotoxic manner.
- This study conclusively shows that if one considers the fact that TNBC's characteristic property is an increased percentage of BCSCs and knowing its connection to being at a higher risk for metastatic disease and mortality, the thieno[2,3-b]pyridine class of compound 1 definitely deserves further attention as a potentially new therapeutic approach for treating TNBC.

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8. SUMMARY

Objectives: The purpose of this study is to determine the effects of treating breast cancer stem cells with the newly synthesized thieno[2,3-*b*]pyridine anticancer agent, by focusing on its cytotoxic and apoptotic effects on the investigated human cell line MDA-MB-231.

Methods: The MDA-MB-231 triple-negative breast cancer cell line was treated with a newly developed thienopyridine anticancer compound (3-amino-N-(3-chloro-2-methylphenyl)-5-oxo-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide, **1**) to determine its cytotoxic effect on triple negative breast cancer, the type of cell death it causes, and the cancer stem cell percentage after treatment. The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was performed, to analyze the cellular metabolic activity and determine the cytotoxic effect. Flow cytometric analysis was used in combination with Annexin-V-FITC and propidium iodide staining to assess the type of cell death after 48h of treatment with compound **1** (2 μ M). Furthermore, flow cytometry also provided the percentage of CD44⁺/CD24⁻ cancer stem cells after treatment.

Results: Compound **1** was cytotoxic for breast cancer cells in a dose- and time-dependent manner; cell death occurs mainly by apoptosis. The percent of cancer stem cells decreased four times.

Conclusion: Due to its cytotoxic effect on the percentage of triple negative breast cancer stem cells compound **1** can be a potential treatment for triple-negative breast cancer.

9. CROATIAN SUMMARY

Citotoksični učinak i apoptoza matičnih stanica karcinoma dojke tretiranih novosintetiziranim protutumorskim spojem tieno [2,3-b] piridinom

Ciljevi: Cilj ovog istraživanja je utvrditi učinke tretmana matičnih stanica karcinoma dojke novosintetiziranim protutumorskim spojem tieno [2,3-b] piridinom, s naglaskom na njegove citotoksične i apoptotske učinke na ispitivanu staničnu liniju MDA-MB-231.

Materijali i metode: Trostruko negativna stanična linija karcinoma dojke MDA-MB-231 tretirana je novosintetiziranim tieno-piridinskim spojem (3-amino-N-(3-kloro-2-metilfenil)-5-okso-5,6,7,8-tetrahidrotieno[2,3-b]kinolin-2-karboksamid, 1) kako bi se utvrdio citotoksični učinak, tip stanične smrti i postotak matičnih stanica karcinoma nakon tretmana. Napravljen je MTT (3-(4,5-dimetiltiazolil-2)-2,5-difeniltetrazolij bromid) test kako bi se analizirala stanična metabolička aktivnost i utvrdio citotoksični učinak. Protočna citometrija u kombinaciji s bojanjem aneksin-V-FITC-om i propidij jodidom, korištena je kako bi se utvrdio tip stanične smrti nakon 48h tretmana spojem 1 (2 μ M). Nadalje, protočnom citometrijom je također utvrđen postotak CD44+/CD24-matičnih stanica karcinoma nakon tretmana.

Rezultati: Spoj 1 bio je citotoksičan za stanice karcinoma dojke te citotoksičnost korelira s koncentracijom spoja i vremenom inkubacije. Stanična smrt je nastupila prvenstveno zbog apoptoze. Postotak matičnih stanica karcinoma smanjio se četiri puta.

Zaključci: Zbog citotoksičnog učinka na postotak matičnih stanica trostruko negativnog karcinoma dojke, spoj 1 može biti potencijalna terapija za trostruko negativni karcinom dojke.

10. CURRICULUM VITAE

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Education:

2005 — 2013: Luise-von-Duesberg Gymnasium, Kempen, Germany
2010 — 2011: International American School of Cancún, Mexico
WS 2012/13: International Management, Akademie für Unternehmensmanagement, Mohnheim, Germany
2013 — 2019: University of Split, School of Medicine, Medical Studies in English

Clinical traineeships:

August 2018: Krankenhaus Spittal/Drau GmbH: Internal medicine
September 2018: Krankenhaus Spittal/Drau GmbH: Anesthesiology and intensive care
January 2019: Krankenhaus Spittal/Drau GmbH: General surgery and trauma surgery

Other activities:

2010 — 2013: Privatpraxis G. Willmen, Kempen, Germany:
Assistance at a general practitioners office
2011 — 2013: Tutoring of pupils: Maths, English, German, Latin, and Spanish
SS 2015: University of Split, School of Medicine, Medical Studies in English:
Anatomy tutor

Skills:

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Qualification in Latin
Basics in Chinese and Croatian
Sport: Skiing and horse riding

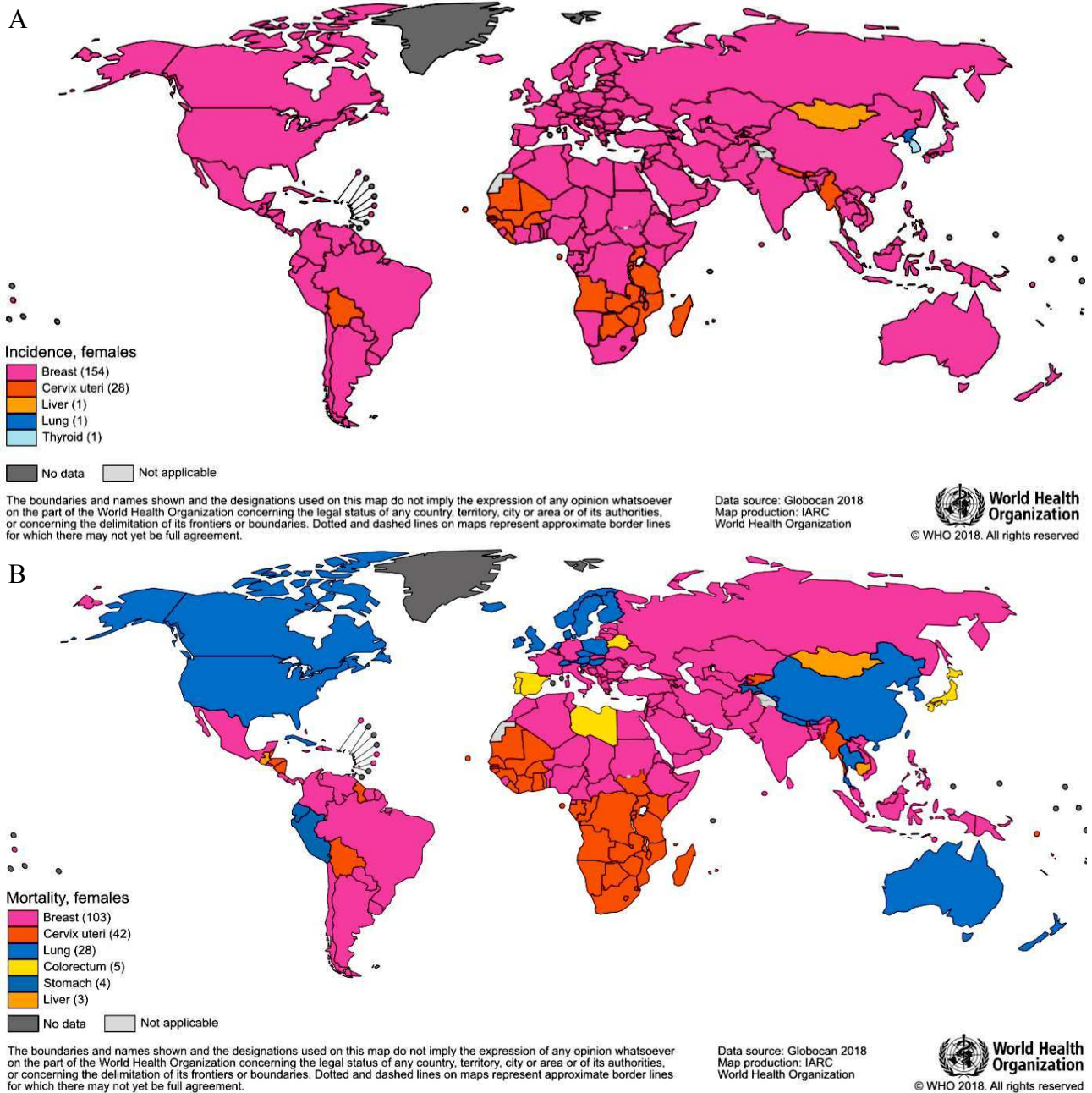


Figure 1. 1.2.2 Epidemiology and etiology. World maps illustrating the most common type of cancer (A) incidence and (B) mortality by country in 2018 among females.

SOURCE: Globocan 2018, World Health Organization (6).

Table 1. Frequency of PVs in African-American and Caucasian American.

Gene Symbol	% Variants in AA	% Variants in CA	Gene Symbol	% Variants in AA	% Variants in CA	Gene Symbol	% Variants in AA	% Variants in CA
MUC4	78	90	CSPG4	22	0	MUM1	0	22
MUC6	61	79	MUC8	22	0	DND1	0	18
TP53	61	77	BOD1	18	0	CYP2D6	0	17
TTN	61	55	FLG	16	0	FOXD4	0	17
OR4C5	57	43	FLOT2	16	0	ACSL1	0	16
FRG1B	55	70	OR2T27	16	0	GMIP	0	16
KCNJ12	55	58	TCEAL6	16	0	OR11H7	0	16
OTOP1	53	46	TMTC1	16	0	TUBB4B	0	16
KMT2C	51	42	CEACAM1	14	0	C1orf94	0	14
PABPC1	47	51	DNAAF3	14	0	DDX46	0	14
AQP7	45	48	EPHB6	14	0	OR5AC1	0	14
FRG1	45	46	LNP1	14	0	RETNLB	0	14

Notes: 1.2.2 Epidemiology and etiology. Blue highlights the highest percentage of PVs found in both groups, red stands for mutations exclusively found in African-Americans, and green stands for PVs found only in Caucasian-Americans.

Abbreviations: PV, pathologic variant; AA, African-American; CA, Caucasian-American.

SOURCE: Chang et al (11).

Table 2. Specific characteristic differences for TNBC compared to other types of breast cancers.

	Nontriple-negative number (%)			Triple-negative number (%)	P ^a
	HR+ Her2-	HR+ Her2+	HR- Her2+		
T stage					
cT1	129,150 (68.0%)	13,787 (52.9%)	4,860 (42.9%)	15,923 (46.4%)	<0.001
cT2	46,109 (24.3%)	8,677 (33.3%)	4,024 (35.5%)	13,090 (38.1%)	
cT3	8,384 (4.4%)	1,846 (7.1%)	1,091 (9.6%)	2,893 (8.4%)	
cT4	6,356 (3.3%)	1,774 (6.8%)	1,358 (12.0%)	2,432 (7.1%)	
N stage					
cN0	164,877 (84.6%)	19,425 (71.9%)	7,644 (63.4%)	25,202 (72.0%)	<0.001
cN1	22,921 (11.8%)	5,726 (21.2%)	3,150 (26.1%)	7,021 (20.1%)	
cN2	4,535 (2.3%)	1,173 (4.3%)	731 (6.1%)	1,630 (4.7%)	
cN3	2,501 (1.3%)	704 (2.6%)	530 (4.4%)	1,139 (3.3%)	
M stage					
cM0	192,356 (96.0%)	25,643 (92.6%)	11,227 (90.8%)	33,682 (94.1%)	<0.001
cM1	8,052 (4.0%)	2,047 (7.4%)	1,131 (9.2%)	2,104 (5.9%)	
Grade					
1	61,993 (30.1%)	2,397 (8.5%)	210 (1.7%)	888 (2.4%)	<0.001
2	102,929 (50.0%)	12,129 (42.9%)	2,996 (24.2%)	6,562 (17.8%)	
3	40,741 (19.8%)	13,733 (48.6%)	9,187 (74.1%)	29,353 (79.8%)	
LVI					
Yes	33,864 (19.0%)	6,847 (29.1%)	3,240 (32.0%)	7,643 (25.0%)	<0.001
No	144,162 (81.0%)	16,649 (70.9%)	6,897 (68.0%)	22,881 (75.0%)	
Mean tumor size (cm±SE)	2.04±0.004	2.48±0.012	2.78±0.021	2.78±0.012	<0.001
Positive nodes					
>0	57,327 (30.3%)	9,820 (38.2%)	4,502 (40.0%)	10,768 (32.0%)	0.218
0	131,798 (69.7%)	15,874 (61.8%)	6,749 (60.0%)	22,852 (68.0%)	
OR unadjusted (95% CI)	Reference	1.42 (1.38–1.46)	1.53 (1.48–1.59)	1.08 (1.06–1.11)	<0.001
OR adjusted for tumor size and grade (95% CI)	Reference	1.06 (1.02–1.09)	0.95 (0.91–1.00)	0.59 (0.57–0.61)	<0.001
Surgery					
Lumpectomy	117,468 (58.2%)	12,951 (47.7%)	4,713 (39.5%)	17,809 (50.0%)	<0.001
Mastectomy	84,405 (41.8%)	14,182 (52.3%)	7,224 (60.5%)	17,776 (50.0%)	
OR unadjusted (95% CI)	Reference	0.66 (0.64–0.67)	0.47 (0.45–0.49)	0.72 (0.70–0.74)	<0.001
OR adjusted for tumor size, nodal status and grade (95% CI)	Reference	0.78 (0.76–0.81)	0.62 (0.60–0.65)	0.96 (0.94–0.99)	0.009
Chemotherapy					
Yes	67,007 (34.9%)	20,622 (75.2%)	10,407 (84.5%)	28,460 (81.4%)	<0.001
No	125,018 (65.1%)	6,799 (24.8%)	1,916 (15.5%)	6,490 (18.6%)	
OR unadjusted (95% CI)	Reference	5.7 (5.5–5.8)	10.1 (9.6–10.6)	8.2 (7.9–8.4)	<0.001
OR adjusted for tumor size, nodal status and grade (95% CI)	Reference	5.7 (5.5–5.9)	7.6 (7.1–8.2)	5.6 (5.4–5.8)	<0.001

Notes: 1.2.4 Clinical features and prognostics. TNBC compared to nonTNBC except the rows with odds ratios, where the P value represents TNBC compared to the reference, HR+ Her2.

Abbreviations: TNBC, triple-negative breast cancer; CI, confidence interval; HR, hormone receptor; Her2, human epidermal growth factor receptor; OR, odds ratio; SE, standard error.

SOURCE: Plasilova et al (7).

Table 3. Differences between TNBC and non-TNBC on mammography.

Characteristic:	TNBC N=54 (%)	Non-TNBC N=5372 (%)	P*
Mass:			
Yes	34 (63.0)	132 (35.5)	<0.0001
No	20 (37.0)	240 (64.5)	
Mass + calcifications:			
Yes	9 (16.7)	158 (42.5)	<0.0001
No	<u>45 (83.3)</u>	214 (57.5)	
Total mass:			
Yes	43 (79.6)	290 (78.0)	0.7810
No	11 (20.4)	82 (22.0)	
Total calcification:			
Yes	14 (25.9)	204 (54.8)	<0.0001
No	<u>40 (74.1)</u>	168 (45.2)	
Mass shape:	N=43	N=290	
Round/oval	25 (<u>58.1</u>)	64 (22.1)	<0.0001
Lobular	13 (30.2)	91 (31.4)	
Irregular	5 (11.6)	135 (46.6)	
Mass margins:	N=43	N=290	
Circumscribed	16 (<u>37.2</u>)	10 (3.4)	<0.0001
Obscured	7 (16.3)	18 (6.2)	
Microlobulated	11 (<u>25.6</u>)	87 (30.0)	
Indistinct	5 (11.6)	80 (27.6)	
Spiculated	4 (9.4)	95 (32.8)	

*P values were calculated using χ^2 test or the Student's t-distribution.

Notes: 1.2.5 Diagnostics.

SOURCE: Gao et al (33).

Figure 2. WHO Classification of tumours of the breast.

WHO classification of tumours of the breast		Malignant lymphoma	
EPITHELIAL TUMOURS		Diffuse large B-cell lymphoma	9680/3
Microinvasive carcinoma		Burkitt lymphoma	9687/3
Invasive breast carcinoma		T-cell lymphoma	
Invasive carcinoma of no special type (NST)	8500/3	Anaplastic large cell lymphoma, ALK-negative	9702/3
Pleomorphic carcinoma	8022/3	Extranodal marginal-zone B-cell lymphoma of MALT type	9689/3
Carcinoma with osteoclast-like stromal giant cells	8035/3	Follicular lymphoma	9690/3
Carcinoma with choriocarcinomatous features			
Carcinoma with melanotic features	8520/3		
Invasive lobular carcinoma			
Classic lobular carcinoma			
Solid lobular carcinoma			
Alveolar lobular carcinoma			
Pleomorphic lobular carcinoma			
Tubulolobular carcinoma			
Mixed lobular carcinoma			
Tubular carcinoma	8211/3		
Cribiform carcinoma	8201/3		
Mucinous carcinoma	8480/3		
Carcinoma with medullary features			
Medullary carcinoma	8510/3		
Atypical medullary carcinoma	8513/3		
Invasive carcinoma NST with medullary features	8500/3		
Carcinoma with apocrine differentiation			
Carcinoma with signet-ring-cell differentiation			
Invasive micropapillary carcinoma	8507/3*		
Metaplastic carcinoma of no special type	8575/3		
Low-grade adenosquamous carcinoma	8570/3		
Fibromatosis-like metaplastic carcinoma	8572/3		
Squamous cell carcinoma	8070/3		
Spindle cell carcinoma	8032/3		
Metaplastic carcinoma with mesenchymal differentiation			
Chondroid differentiation	8571/3		
Osseous differentiation	8571/3		
Other types of mesenchymal differentiation			
Mixed metaplastic carcinoma	8575/3		
Myoepithelial carcinoma	8982/3		
Rare types			
Carcinoma with neuroendocrine features			
Neuroendocrine tumour, well-differentiated	8246/3		
Neuroendocrine carcinoma, poorly differentiated (small cell carcinoma)	8041/3		
Carcinoma with neuroendocrine differentiation	8574/3		
Secretory carcinoma	8502/3		
Invasive papillary carcinoma	8503/3		
Acinic cell carcinoma	8550/3		
Mucopidermoid carcinoma	8430/3		
Polymporphous carcinoma	8525/3		
Oncocytic carcinoma	8290/3		
Lipid-rich carcinoma	8314/3		
Glycogen-rich clear cell carcinoma	8315/3		
Sebaceous carcinoma	8410/3		
Salivary gland/skin adnexal type tumours			
Cylindroma	8200/0		
Clear cell hidradenoma	8402/0*		
Epithelial–myoepithelial tumours			
Pleomorphic adenoma	8940/0		
Adenomyoepithelioma	8963/0		
Adenomyoepithelioma with carcinoma	8963/3*		
Adenoid cystic carcinoma	8200/3		
Precursor lesions			
Ductal carcinoma in situ	8500/2		
Lobular neoplasia			
Lobular carcinoma in situ			
Classic lobular carcinoma in situ	8520/2		
Pleomorphic lobular carcinoma in situ	8519/2*		
Atypical lobular hyperplasia			
Intraductal proliferative lesions			
Usual ductal hyperplasia			
Columnar cell lesions including flat epithelial atypia			
Atypical ductal hyperplasia			
Papillary lesions			
Intraductal papilloma	8503/0		
Intraductal papilloma with atypical hyperplasia	8503/0		
Intraductal papilloma with ductal carcinoma in situ	8503/2*		
Intraductal papilloma with lobular carcinoma in situ	8520/2		
Intraductal papillary carcinoma	8503/2		
Encapsulated papillary carcinoma	8504/2		
Encapsulated papillary carcinoma with invasion			
Solid papillary carcinoma	8504/3		
In situ	8509/2		
Invasive	8509/3		
Benign epithelial proliferations			
Sclerosing adenosis			
Acrocinne adenosis			
Microglandular adenosis			
Malignant lymphoma			
Diffuse large B-cell lymphoma			
Burkitt lymphoma			
T-cell lymphoma			
Anaplastic large cell lymphoma, ALK-negative			
Extranodal marginal-zone B-cell lymphoma of MALT type			
Follicular lymphoma			
Metastatic tumours			
Gynaecomastia			
Invasive carcinoma			
In situ carcinoma			
Clinical patterns			
Inflammatory carcinoma			
Bilateral breast carcinoma			
Fibroepithelial tumours			
Fibroadenoma	9010/0		
Phyllodes tumour	9020/1		
Benign	9020/0		
Borderline	9020/1		
Malignant	9020/3		
Periductal stromal tumour, low grade			
Hamartoma			
Tumours of the nipple			
Nipple adenoma	8506/0		
Syringomatous tumour	8407/0		
Paget disease of the nipple	8540/3		

* The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) (4638). Behaviour is coded 0 for benign tumours, 1 for unspecified, borderline or uncertain behaviour, 2 for carcinoma in situ and grade III intracapsular neoplasia, and 3 for malignant tumours. The classification is modified from the previous WHO histological classification of tumours [1413] taking into account changes in our understanding of these lesions. In the case of neuroendocrine neoplasms, the classification has been simplified to be of more practical utility in morphological classification. *These new codes were approved by the IASO-WHO Committee for ICD-O.

Notes: 1.2.6 Classification of breast cancer.
SOURCE: Makhani et al (40)

Table 4. MTT results.

	SP	50 nM	250 nM	500 nM	1 μM	5 μM
After 4 hours	0,132	0,135	0,133	0,106	0,109	0,102
	0,144	0,128	0,12	0,08	0,115	0,083
	0,126	0,135	0,088	0,078	0,113	0,077
	0,132	0,12	0,101	0,102	0,124	0,052
	0,154	0,108	0,128	0,12	0,123	0,049
Average	0,1376	0,1252	0,114	0,0972	0,1168	0,0726
Mean	1	0,909883	0,828488	0,706395	0,848837	0,527616
SD	0,081835	0,083084	0,137794	0,130247	0,047210	0,161412
After 24 hours	0,152	0,079	0,116	0,083	0,107	0,108
	0,139	0,089	0,126	0,124	0,074	0,128
	0,131	0,145	0,126	0,109	0,119	0,112
	0,131	0,148	0,142	0,118	0,107	0,111
	0,122	0,138	0,137	0,111	0,118	0,095
Average	0,135	0,1198	0,1294	0,109	0,105	0,1108
Mean	1	0,887407	0,958518	0,807407	0,777777	0,820740
SD	0,083312	0,244971	0,075831	0,116298	0,135273	0,087237
After 48 hours	0,267	0,209	0,198	0,19	0,17	0,142
	0,287	0,212	0,195	0,165	0,158	0,139
	0,274	0,187	0,198	0,175	0,177	0,149
	0,287	0,2013	0,209	0,172	0,184	0,141
	0,29	0,226	0,208	0,178	0,169	0,12
Average	0,281	0,1198	0,2016	0,176	0,1716	0,1382
Mean	1	0,736868	0,717437	0,626334	0,610676	0,491814
SD	0,035498	0,051029	0,022870	0,032713	0,034558	0,038608
After 72 hours	0,232	0,187	0,139	0,149	0,144	0,114
	0,225	0,161	0,138	0,133	0,141	0,12
	0,221	0,157	0,158	0,15	0,13	0,115
	0,216	0,167	0,156	0,162	0,135	0,132
	0,237	0,169	0,153	0,152	0,146	0,131
	0,242	0,15	0,154	0,146	0,158	0,122
Average	0,228833	0,165166	0,149666	0,148666	0,142333	0,122333
Mean	1	0,721777	0,654042	0,649672	0,621995	0,534595
SD	0,043297	0,055580	0,038561	0,041149	0,042338	0,033699

Notes: 4.1 Compound I: dose- and time-dependent cytotoxicity.