

**Short Thesis For The Degree of Doctor of Philosophy
(PhD)**

**The Role of Monoamine Signaling and Rexinoid-Based
Combination Treatments in Breast Cancer Chemoprevention**

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Introduction

Epidemiology of Breast Cancer and Risk Assessment Opportunities

Breast cancer is one of the most frequently diagnosed malignant tumor types among women worldwide and remains a significant cause of mortality. According to GLOBOCAN 2022 estimates, more than 2.3 million new cases are diagnosed annually, and nearly 670,000 deaths are attributable to the disease. The increasing incidence can be attributed to the combined effects of multiple factors, including lifestyle changes, the rising prevalence of obesity, alterations in reproductive life stages, and aging. Survival rates show marked regional disparities, reflecting differences in access to healthcare services and the effectiveness of screening programs (Bray Bsc et al. 2024).

The risk of developing breast cancer is determined by the interplay of genetic, hormonal, environmental, and lifestyle-related factors. Mutations in the BRCA1 and BRCA2 genes confer the highest genetic risk, resulting in more than a tenfold increase in breast cancer risk and frequently being associated with the development of aggressive, hormone receptor-negative tumors (Saleem et al. 2020). Hormonal risk factors, such as early menarche, late menopause, and nulliparity primarily increase the risk of estrogen-dependent breast cancer (Clamp, Danson, and Clemons 2002). Lifestyle-related factors, including obesity, alcohol consumption, smoking, and physical inactivity also play a substantial role in disease incidence (Friedenreich, Ryder-Burbidge, and McNeil 2020; Luo et al. 2011; Romieu et al. 2015). In addition, increased connective tissue stiffness within the breast tissue can be identified as an independent risk factor.

One of the key elements of effective breast cancer prevention is accurate risk assessment, which enables the targeted application of screening strategies and chemoprevention (Cyr and Kennard 2023; Harkness, Astley, and Evans 2020). Currently available risk assessment methods integrate multiple data sources, including clinical and epidemiological parameters, genetic testing results, imaging modalities, and tissue biomarkers (Usher-Smith et al. 2023). The widely used Gail model estimates breast cancer risk based on age, reproductive factors, and family history, whereas the Tyrer–Cuzick model additionally incorporates genetic predisposition and mammographic density (Kurian et al. 2021; Stevanato et al. 2022). Genetic approaches are increasingly complemented by polygenic risk scores (Easton et al. 2015; Ho et al. 2021). In recent years, artificial intelligence-based (AI) methods have gained growing importance in breast cancer risk assessment (Khanna et al. 2022). These AI-based approaches enable the integrated analysis of heterogeneous datasets, thereby improving the accuracy of risk stratification. However, their clinical implementation is currently limited by data heterogeneity, the lack of standardized benchmark datasets, and constraints related to methodological validation (Logan, Kennedy, and Catchpoole 2023; Sourlos et al. 2024).

Molecular Classification of Breast Tumors

Breast cancer is not a uniform disease but a biologically and clinically heterogeneous condition composed of multiple distinct molecular subtypes. Molecular classification fundamentally determines tumor prognosis, biological behavior, and therapeutic responsiveness. The classification applied in clinical practice primarily distinguishes subtypes based on estrogen and

progesterone receptor status, as well as the expression of Human Epidermal Growth Factor Receptor 2 (HER2) (Tsang and Tse 2020).

Accordingly, hormone receptor-positive, HER2-positive, and triple-negative breast tumors can be distinguished. The incorporation of gene expression profiling enabled the introduction of the PAM50 test, which classifies tumors into five molecular subgroups: Luminal A, Luminal B, HER2-enriched, Basal-like, and Normal-like (Orrantia-Borunda et al. 2022). These groups not only determine prognosis but also reflect which signaling pathways are active within the tumor, thereby predicting differential therapeutic sensitivity. Hormone receptor-positive tumors typically exhibit slower progression and respond well to hormone receptor targeted therapies, whereas hormone receptor-negative tumors, particularly the triple-negative subtype, are associated with a more aggressive clinical course and less favorable prognosis (Kensler et al. 2018). In the case of triple-negative subtypes, however, targeted therapeutic and preventive options remain limited.

Based on these considerations, taking the molecular classification of breast tumors into account is essential for the development of novel hormone receptor-independent chemopreventive strategies, as well as for the identification of new preventive or therapeutic targets.

Classical Chemopreventive Approaches and Their Target Populations

Chemopreventive strategies currently applied in clinical practice for breast cancer are predominantly based on the inhibition of estrogen signaling. The mechanism of action of selective estrogen receptor modulators and aromatase inhibitors is directed at reducing estrogen-dependent proliferation. They have therefore proven effective primarily in the prevention of hormone receptor-positive breast tumor development. The target population of these classical approaches typically includes individuals with genetic or hormonal risk factors, including BRCA1 and BRCA2 mutation carriers, as well as women with a medical history indicative of increased estrogen exposure (Bao, Prowell, and Stearns 2006; Chlebowski 2014).

However, the efficacy of chemopreventive approaches targeting estrogen signaling is closely linked to the hormone receptor status of the premalignant breast cells, as the preventive effects of these agents are less pronounced for the development of hormone receptor-negative subtypes. The clinical application of hormone-dependent chemoprevention is significantly limited by the unfavorable side-effect profile of these agents. Thromboembolic complications, endometrial alterations, as well as metabolic and skeletal adverse effects are frequently cited as side effects that limit preventive efficacy (Cuzick et al. 2020).

Overall, while the effectiveness of classical chemopreventive approaches is well defined, it is restricted to a narrower target population. This limitation clearly highlights the need for hormone receptor status-independent chemopreventive strategies that can be applied to a broader population and provide preventive benefit with a more favorable side-effect profile.

Application of Retinoids and Reginoids in Chemoprevention

Retinoid signaling plays a central role in the regulation of cellular differentiation, proliferation, and apoptosis, and therefore the potential application of retinoids in breast cancer

chemoprevention was recognized early on (Simeone and Tari 2004). Preclinical and clinical studies have demonstrated that retinoids can inhibit the development of breast tumors, however, their clinical use is also limited by significant toxicity and unfavorable side-effect profile. Adverse effects associated with long-term administration, including dermatologic, mucosal, and metabolic alterations (hypertriglyceridaemia), substantially reduce the feasibility of their prolonged use for preventive purposes (Veronesi et al. 1999; Zanardi et al. 2006).

Retinoids represent a subgroup of retinoids that exert their effects selectively through retinoid X receptors. In preclinical models, bexarotene effectively inhibited the development of hormone receptor-negative breast tumors (Li et al. 2008). However, dose-dependent adverse effects have also been observed in the case of retinoid-based monotherapy (Uray, Dmitrovsky, and Brown 2016). The application of retinoids for chemopreventive purposes may be facilitated through combination strategies. This approach creates the opportunity to achieve effective chemoprevention at lower drug doses and with reduced toxicity, and it directly provides the rationale for investigating retinoid-based combination treatments.

The Role and Opportunities of Monoamine Signaling in Hormone-Independent Chemoprevention

Monoamine signaling pathways are not exclusively active in the central nervous system but are also functionally present in peripheral tissues, including the mammary gland (Field et al. 2025; Hernandez et al. 2009; Matsuda et al. 2004; Stull et al. 2007). Monoamine mediators exert their effects primarily through G protein-coupled receptors, which activate distinct intracellular signaling pathways.

Based on retrospective clinical studies, widely used drug classes that influence monoamine signaling, such as adrenergic receptor antagonists and 5-HT transporter (SERT) inhibitors, have been associated with the regulation of breast cancer development and progression (Díaz-Santana et al. 2025; Løfling et al. 2022; Ma et al. 2025; Niu et al. 2024; Strell et al. 2024; Wernli et al. 2009). Therefore, the targeted investigation of monoamine signaling may be particularly relevant for the prevention of hormone receptor-negative breast tumors.

Overall, monoamine signaling systems represent clinically well-characterized and pharmacologically accessible targets whose modulation may open new opportunities for hormone receptor-independent chemoprevention. These targets may be suitable for the development of monotherapeutic or combination-based strategies.

The Role of Serotonin Signaling and Homeostasis in the Function of Mammary Cells

Serotonin, also known as 5-hydroxytryptamine (5-HT), is classically recognized as a neurotransmitter, but it also functions as a local regulatory factor in several peripheral tissues. In the mammary gland, epithelial cells can produce, transport, and sense 5-HT. Therefore, serotonin should not be interpreted merely as a systemic signal, but also as an autocrine and paracrine regulator of tissue homeostasis. Previous studies have shown that mammary epithelial cell-derived 5-HT contributes to lactational homeostasis, tight junction regulation, feedback control of milk production, and the initiation of involution (Matsuda et al. 2004; Stull et al. 2007; Hernandez et al. 2009; Field et al. 2024).

The biosynthesis of 5-HT starts from tryptophan, and its rate-limiting step in peripheral, non-neuronal tissues is mainly catalyzed by tryptophan hydroxylase 1 (TPH1). The intracellular and extracellular distribution of serotonin is regulated by the serotonin transporter (SERT, SLC6A4), which mediates 5-HT reuptake from the extracellular space. Inhibition of SERT by selective serotonin reuptake inhibitors (SSRIs) may shift the balance between extracellular receptor activation and intracellular serotonin metabolism. Importantly, 5-HT uptake is not exclusively dependent on SERT. Other monoamine transporters, including dopamine transporter (DAT), norepinephrine transporter (NET), members of the organic cation transporter (OCT) family, and plasma membrane monoamine transporter (PMAT), may also contribute to serotonin transport. This creates a particularly complex regulatory framework in peripheral cells (Daws 2009; Duan and Wang 2010; Li et al. 2013).

The extracellular effects of serotonin are mediated by several receptor families. 5-HT₁ receptors are mainly G_i-coupled, 5-HT₂ receptors are G_q-coupled, whereas 5-HT₄, 5-HT₆, and 5-HT₇ receptors activate G_s-coupled signaling. These pathways are connected to cAMP/PKA, PLC/IP3/DAG, Ca²⁺, Erk, Akt, and RhoA/ROCK signaling, thereby potentially influencing cell proliferation, survival, morphology, and motility (Hoyer, Hannon, and Martin 2002; Nichols and Nichols 2008). Since the 5-HT system can be pharmacologically modulated at multiple levels, including TPH1 inhibition, SSRI or SNRI treatment, receptor agonism or antagonism, and monoamine oxidase inhibition, it may represent a useful model system for examining how peripheral monoamine signaling is linked to early cellular state changes relevant to breast cancer chemoprevention.

The Role and Potential of Extracellular Matrix Composition in Chemoprevention

The extracellular matrix (ECM) is a fundamental determinant of the structural and functional integrity of breast tissue and actively participates in the regulation of epithelial cell proliferation, differentiation, and migration (Paavolainen and Peuhu 2021). During tumorigenesis, the composition and mechanical properties of the ECM undergo significant alterations, which are not merely consequences but active contributors to tumor initiation and progression (Boyd et al. 2010; Levental et al. 2009). Increased stromal stiffness and reorganization of the collagen network are associated with poor prognosis in multiple breast cancer subtypes (Acerbi et al. 2015). ECM remodeling is detectable even in pre-tumorigenic conditions, including premalignant lesions and obesity-associated breast tissue (Ellies 2016; Kuziel, Moore, and Arendt 2023). In these states, increased collagen deposition, a higher number of cross-links, and elevated expression of matrix-organizing proteins create a tumor-promoting microenvironment. The altered matrix not only affects mechanosensory cellular responses but may also form a physical barrier to the diffusion of therapeutic agents and support therapeutic resistance through integrin–growth factor receptor activation (Brown and Wendt 2014; Jain 1987).

Based on these findings, the extracellular matrix has emerged as a novel and independent chemopreventive target. In preclinical models, inhibition of enzymes regulating matrix remodeling, such as lysyl oxidase (LOX), which plays a key role in collagen cross-linking, reduced stromal stiffness and decreased the invasive and metastatic potential of breast tumors (Cetin et al. 2024; Ferreira et al. 2021). Although inhibition of matrix metalloproteinases did not lead to a breakthrough in early clinical trials, these results highlight that dynamic regulation of the ECM may represent a relevant intervention point from a preventive perspective (Winer, Adams, and Mignatti 2018).

Overall, modulating the organization and composition of the extracellular matrix offers an opportunity to intervene at early events that may influence the direction of tumor development. This particularly justifies the identification and investigation of molecular regulatory factors capable of modulating the interaction between epithelial cells and the matrisome, thereby forming the basis for novel hormone receptor-independent chemopreventive strategies.

Objectives

General Objectives

The general objective of the present doctoral research was to explore the role of monoamine signaling in the regulation of normal mammary epithelial cell function, with particular emphasis on mechanisms influencing proliferation and on identifying patterns and relationships among G protein-coupled signaling pathways. In our investigations, we aimed to systematically characterize the cellular responses induced by the activation of cell surface monoamine receptors and to distinguish the biological consequences of intracellular and extracellular monoamine effects.

The second major objective of this work was to determine whether pharmacological modulation of monoamine signaling is suitable for enhancing the efficacy of rexinoid-based chemopreventive treatments and for reducing the applied drug doses.

As a third general objective, we aimed to identify target genes regulated by various rexinoid-based synergistic combinations and to perform a detailed functional characterization of a selected target gene, Zymogene Granule Protein 16B (ZG16B), in normal mammary epithelial cells and breast tumor cells. Within this framework, we investigated the role of ZG16B in the regulation of processes relevant to proliferation, cell motility, extracellular matrix remodeling, epithelial–mesenchymal transition (EMT), and tumor progression.

Scientific Questions and Specific Objectives

The central question of the present doctoral research was whether the combined pharmacological modulation of peripheral monoamine signaling pathways and rexinoid signaling may be suitable for revealing hormone receptor status-independent chemopreventive mechanisms. The basic assumption of this work was that monoaminergic signaling networks present in normal mammary epithelial cells can influence proliferation, motility, and microenvironmental remodeling. Accordingly, the aim of the research was not to describe the effect of a single compound, but to characterize signaling mechanisms and molecular targets that may provide the basis for safer, lower-dose preventive strategies applicable to a broader patient population.

The objectives were formulated along the following interconnected scientific questions and hypotheses:

1. Can serotonin signaling be pharmacologically modulated in normal mammary epithelial cells?

The initial hypothesis was that normal mammary epithelial cells possess the main components required for 5-HT synthesis, transport, and receptor-mediated signaling. Therefore, the aim was to determine whether these cells are capable of producing 5-HT, whether they express the basic

elements of serotonin signaling, and what biological responses are induced by pharmacological modulation of the 5-HT transporter in mammary epithelial cells.

2. What is the significance of the intra- and extracellular distribution of serotonin in the regulation of proliferation?

The second hypothesis was that the effect of 5-HT can be interpreted as a dual biological system. Extracellular 5-HT may initiate signaling processes through cell surface receptors, whereas intracellular uptake may be linked to monoamine degradation-associated mechanisms. Therefore, the aim was to compare SERT inhibition, external 5-HT stimulation, and receptor-selective pharmacological modulation in order to distinguish the role of receptor-mediated proliferation control from intracellular monoamine-related effects. In parallel, we aimed to identify signaling elements that may highlight the serotonin system as a potential chemopreventive target.

3. Is receptor isoform identity or the associated signaling pathway more decisive in the regulation of proliferation?

The third hypothesis was that the effect of monoamine receptors on proliferation is not determined exclusively by which receptor family is activated. It may be equally important which G protein-coupled signaling pathway channels the extracellular signal. Accordingly, the aim was to examine whether Gs-, Gi-, and Gq-coupled receptor pathways differentially influence the growth response of normal mammary epithelial cells. Beyond serotonin receptors, the aim was also to test this signaling principle through the pharmacological modulation of other monoamine receptor families, thereby identifying potential preventive targets more broadly.

4. Can targeted modulation of serotonin signaling potentiate the growth-inhibitory effect of the chemopreventive rexinoid bexarotene in normal mammary epithelial cells?

The fourth hypothesis was that monoaminergic pathways and the RXR agonist bexarotene may be functionally connected in normal mammary epithelial cells. Previous results obtained with the carvedilol and bexarotene combination, together with the monoaminergic stimulation-based antiproliferative mechanism identified in our work, raised the possibility that compounds other than carvedilol may also potentiate the effect of bexarotene. Therefore, the aim was to determine whether modulation of serotonin signaling can synergistically enhance the growth-inhibitory effect of bexarotene.

5. Which common molecular mechanisms and combination-specific target genes may underlie monoamine- and rexinoid-based synergies?

The fifth hypothesis was that the synergy between monoamine signaling modulators and rexinoids may be linked to combination-specifically regulated molecular targets. To investigate this, the aim was to identify gene expression and chromatin-level alterations that may be associated with the effects of multiple synergistic combinations. A key question was whether bexarotene-based combinations can modify the expression of target genes through epigenetic regulation, for example by influencing ARID1A and SWI/SNF complex activity. These target genes may participate in the regulation of processes that support tumor initiation or progression, including proliferation, cell motility, and microenvironmental reprogramming.

6. Which biological processes are regulated by the suppression of ZG16B through rexinoid-monoaminergic synergistic combinations?

The sixth hypothesis was that Zymogene Granule Protein 16B is one of the target genes of rexinoid-based combination treatments regulated through ARID1A and the SWI/SNF complex. Based on previous literature, ZG16B is a secreted regulator that may functionally contribute to tissue remodeling processes supporting tumor formation and progression. Accordingly, the aim was to examine whether altered ZG16B expression or extracellular ZG16B availability influences the proliferation, migration, adhesive behavior, and gene programs related to extracellular matrix organization and epithelial plasticity in normal mammary epithelial cells and breast tumor cells. This objective placed ZG16B at the center of our investigations as a possible functional mediator of combination treatments and as a novel preventive target.

Materials and Methods

Cell Lines and Cell Culture

hTert-immortalized human mammary epithelial cell line was used for the modelling of normal breast epithelium. These cells preserve the fundamental morphological and functional characteristics of non-transformed cells and are suitable for investigating early, proliferation-related alterations.

For tumor cell-based experiments, human breast tumor cell lines with different hormone receptor statuses (ER+: MCF7, T47D; Triple-negative: MDA-MB-231, MDA-MB-468, HCC1143) were applied, enabling subtype-specific evaluation of the observed effects. The cell lines were maintained under standard cell culture conditions with controlled temperature, CO₂ levels, and nutrient supply. Authentication of the cell lines was performed using deep-sequencing, and mycoplasma-free status was regularly verified using a Promokine PCR-based test. In all experiments, cells were used exclusively before passage 15.

Cloning of ZG16B into an Expression Vector

To produce the secreted ZG16B factor, a custom expression system was established based on a pEGFP-Empty mammalian expression vector. ZG16B cDNA was generated from RNA samples isolated from HME-hTert cells using RT-PCR and subsequently amplified by PCR. Restriction enzyme cleavage sites for EcoRI and SalI were introduced at both ends of the amplified cDNA, and following enzymatic digestion, the ZG16B sequence was ligated into the pEGFP-C3 vector using T4 DNA ligase.

HEK293 cells were used as the host system because they do not express ZG16B under normal conditions. Therefore, the protein detected in the supernatant of transfected HEK293 cells was unequivocally derived from the introduced vector. During vector construction, EGFP co-expression was ensured to allow monitoring of the transfection efficiency. The signal peptide directing ZG16B secretion was cleaved during translation together with EGFP, therefore the native structure of the secreted ZG16B was not affected by the fluorescent labeling.

Pharmacological and Biological Treatments

For pharmacological investigations, a compound library was established comprising selective modulators of monoamine G protein-coupled receptors. In addition, selective inhibitors of

downstream signaling elements (adenylate cyclase, protein kinase A), the rexinoid bexarotene, several selective serotonin reuptake inhibitors (SSRIs) (citalopram, sertraline, paroxetine), and the non-selective β -adrenergic blocker carvedilol were used as pharmacological treatments. Compounds were obtained from Tocris – Bio-Techne W&D Systems Kft. and MedChemExpress – Bio-Kasztel Ltd. The compounds were stored according to the manufacturers' recommendations and dissolved in the following solvents as recommended: DMSO–EtOH (1:1), PBS, 1 M NaOH, or 1 M HCl.

Treatments were applied within a concentration range of 0.1–10 μ M, and cellular responses were evaluated relative to the appropriate vehicle controls. Rexinoid-based combinations were assessed in dose–response experiments to quantify synergy.

For biological treatments, conditioned supernatants containing recombinant, secreted ZG16B were used after concentration by size-exclusion filtration (10–30 kDa molecular weight range). These supernatants were collected from HEK293 cells previously transfected with pEGFP-ZG16B. As a negative control, supernatants from HEK293 cells transfected with the pEGFP-Empty vector were used, which underwent the same processing procedure. HEK293 cells were selected as the host cell line because they do not endogenously express ZG16B.

2D Proliferation Assay

For the primary quantitative evaluation of antiproliferative effects, a proliferation assay performed in 2D cell culture was applied. The division rate of normal mammary epithelial cells represents a suitable and relevant readout for modeling early steps of tumorigenesis, as the manipulation of proliferative rate is one of the most general features of breast cancer initiation. Cells were seeded at low density into 96-well optical plates, and pharmacological or biological treatments were added for an incubation period of 3–5 days. The application of the proliferation assay enabled the functional comparison of modulators targeting different G protein-coupled receptors and allowed the estimation of which signaling pathways, when activated or inhibited, lead to an antiproliferative response in normal mammary epithelial cells. Thus, the 2D proliferation system served as a fundamental screening platform for our compound library.

Colony Formation Assay

To assess the long-term growth potential and transformation status of cells, a colony formation assay was performed. In these experiments, breast tumor cell lines were cultured in a low-melting-point agarose matrix, which forced the cells to survive and proliferate in a non-adherent environment, a key step of malignant transformation. Cells were subjected to pharmacological treatments prior to embedding into the matrix, and treatments were maintained throughout the entire culture period via the supernatant.

Colony formation was evaluated after several weeks of culture based on microscopic imaging. The number and size of colonies were determined from projected images generated from Z-stack acquisitions using automated image analysis. This approach enabled objective and quantitative comparison of the effects of different treatment conditions in terms of transformation potential. In the case of MCF7 cells, RNA was isolated from matrix-grown cells following the colony formation assay for further analysis.

Assessment of Cell Migration, Adhesion, and Morphology

Cell migration was evaluated using a wound-healing assay in normal mammary epithelial and breast tumor cell lines. After pretreatment, cells were seeded into Ibidi inserts, and following insert removal, closure of the cell-free area was monitored by microscopic imaging. The extent of migration was quantified by measuring the area covered by the cells over defined time intervals.

Cell adhesion properties were assessed using a trypsin-based adhesion assay. In this assay, detachment of cells adhered to the culture surface was monitored over time, and the number of remaining attached cells was compared under different treatment conditions.

Cell morphology was analyzed using microscopic image-based quantification (CellProfiler), during which cell shape and geometric parameters were assessed quantitatively. Morphological changes were characterized at the single-cell level by calculating the ratio of the longitudinal and shorter cell axes, as well as by measuring circularity.

Immunostaining, Immunohistochemistry, and Fluorescence Microscopy

To examine the expression and localization of intracellular and extracellular proteins, immunostaining and immunohistochemical methods were applied. Following fixation and permeabilization, cells were incubated with specific primary antibodies, and the proteins of interest were visualized using fluorescently labeled secondary antibodies. Changes in cell morphology and protein expression were evaluated based on fluorescence microscopy images. Fluorescence intensity values were quantified using image analysis-based methods (ImageJ, CellProfiler, CellProfiler Analyst).

For tumor tissues derived from *in vivo* experiments, indirect immunohistochemical staining was performed on paraffin-embedded sections. During staining, tissue distribution and relative expression levels of target proteins were evaluated relative to the area defined by hematoxylin nuclear counterstaining.

Western Blot Analysis

To investigate changes in cellular signaling, western blot analysis was performed. Cell lysates intended for analysis were diluted to a uniform final concentration, denatured, and proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Target proteins were detected using specific primary antibodies and fluorescently labeled secondary antibodies. The activation status of Erk 1/2, Src, and Akt proteins was analyzed using phosphorylation-specific antibodies. Time- and dose-dependent changes in phosphorylation patterns were monitored in a time-dependent manner (0; 10; 60 minutes). Western blot images were quantitatively evaluated by densitometry using ImageJ. Levels of phosphorylated proteins were normalized to the corresponding total protein levels.

Quantification of Secondary Messengers (IP3, cAMP)

To assess signaling responses associated with activation of G protein-coupled receptors, intracellular levels of the secondary messengers IP3 and cAMP were determined. Measurements were performed in normal mammary epithelial cells. Changes in IP3 levels were used to characterize the activity of the G_q -coupled signaling pathway, whereas quantification of cAMP enabled assessment of the functional state of G_s - and G_i -coupled receptors. cAMP levels were measured using a dedicated competitive ELISA kit, while IP3 levels were assessed indirectly using an HTRF technology-based kit designed to monitor its stable derivative, IP1. IP1 levels directly correlate with IP3 levels.

Transcriptomics, RNA Sequencing, and RT-qPCR

To comprehensively investigate gene expression changes induced by pharmacological and biological treatments, transcriptomic analysis was performed. RNA isolation was carried out using TRIzol (UD-Genomed Kft.) and the Macherey-Nagel RNA isolation kit according to the manufacturers' recommendations. Total RNA isolated from normal mammary epithelial cells served as the basis for high-throughput RNA sequencing (RNA-seq) analyses. Following RNA isolation, sample quality control, library preparation, and sequencing were performed with the assistance of UD-Genomed Kft. Bioinformatic processing of RNA-sequencing data using the Galaxy platform focused on identifying differentially expressed genes. Functional interpretation of gene expression data was performed using gene ontology analysis (ShinyGO) and gene set enrichment analysis (GSEA), enabling biological contextualization of transcriptional responses. RNA-sequencing results were validated by real-time quantitative PCR (RT-qPCR).

Chromatin Immunoprecipitation, Sequencing, and qPCR

To investigate the epigenetic background of transcriptional regulation, chromatin immunoprecipitation (ChIP) was performed. Cells were fixed with formaldehyde, and following mechanical fragmentation of chromatin, immunoprecipitation was carried out using specific antibodies to isolate DNA fragments associated with target proteins or histone modifications. DNA samples obtained from ChIP experiments were subjected to high-throughput sequencing to map genome-wide binding patterns, and targeted ChIP-qPCR analyses were also performed to examine predefined genomic regions. Enrichment in ChIP-qPCR-based evaluations was determined relative to input controls.

Real-Time Quantitative PCR

To quantitatively determine the expression levels of selected genes, real-time quantitative PCR (RT-qPCR) was applied. cDNA generated by reverse transcription from total RNA served as the template. PCR reactions were performed using a fluorescence-based detection system with TaqMan probes, enabling real-time monitoring of amplification. Gene expression levels were normalized to β -actin. RT-qPCR measurements confirmed key transcriptional changes identified by RNA sequencing.

Animal Experiments

To investigate the *in vivo* effects of pharmacological interventions, two breast tumor models with distinct biological backgrounds were applied. For the evaluation of chemopreventive effects, MMTV-ErbB2 transgenic mice were used, which, due to HER2 overexpression, serve as a widely applied preclinical model for studying breast tumor development and early progression. The primary experiments related to the MMTV-ErbB2 mouse model were not part of the present doctoral research, however, immunohistochemical processing of tissue samples derived from these experiments was included in this work. (Uray et al. 2015)

To analyze effects associated with tumor progression, SCID mice xenografted with human breast tumor cell lines were included in experiments investigating the effects of bexarotene + carvedilol. During *ex vivo* sample processing, the expression of ZG16B and fibronectin-1 (FN1) was examined using immunohistochemical methods. All procedures performed in experiments involving xenografted SCID mice were conducted in accordance with applicable ethical and animal welfare guidelines (16/2020/DEMÁB).

Processing of Data Derived from Genomic Databases

To compare ZG16B expression across tumor types, publicly available genomic databases were utilized. The analyses were based on The Cancer Genome Atlas (TCGA Pan-Cancer) database, which comprises a total of 12,839 samples from various human tumor types. For detailed investigation of breast tumors, the TCGA Breast Cancer (BRCA) dataset was applied, enabling comparison of ZG16B expression between normal breast tissues and breast tumor samples. Data processing was performed using the UCSC Xena browser interface.

Statistical Analysis and Figure Preparation

Statistical evaluation and graphical representation of experimental data were performed using GraphPad Prism software. In all cases, data represents results from at least three independent experiments. Results were presented as mean $\pm$ SEM, according to the experimental design. For comparison of two groups, Student's t-test was applied, whereas one- or two-way analysis of variance (ANOVA) with appropriate post-hoc tests was used for simultaneous comparison of multiple groups. Statistical significance was defined in all cases as at least $p < 0.05$.

Results

SERT Inhibition and 5-HT Stimulation Exert Antiproliferative Effects in HME-hTert Cells

The investigation of the effects of 5-HT signaling on mammary epithelial cell proliferation was initiated by pharmacological modulation of the serotonin transporter (SERT) in HME-hTert cells. In a comparison of clinically used SSRIs, sertraline and citalopram did not significantly affect cell number, whereas paroxetine induced a dose-dependent antiproliferative effect. The effect of paroxetine was attributed to its high affinity for SERT, which was further confirmed by a similar reduction in cell number following siRNA-mediated silencing of SERT (Hyttel 1993).

The growth-inhibitory effects of 5-HT and paroxetine were also confirmed in 3D spheroid models. Exogenously applied 5-HT alone reduced cell proliferation, and this effect was further enhanced by paroxetine pretreatment, indicating that the intracellular and extracellular effects of 5-HT can be functionally distinguished. Collectively, these results suggested that SERT inhibition and activation of cell surface 5-HT signaling play a decisive role in the regulation of proliferation in normal mammary epithelial cells.

Normal Mammary Epithelial Cells Express Proteins Required for 5-HT Production and Signaling

To interpret the antiproliferative effects induced by 5-HT, we first confirmed that normal mammary epithelial cells possess the molecular components necessary for 5-HT synthesis, transport, and sensing. Immunofluorescence analysis detected intracellular accumulation of 5-HT in HME-hTert cells, which was significantly reduced following paroxetine treatment, indicating functional SERT activity in this cell line.

In addition to SERT expression, we also confirmed the expression of the TPH1 enzyme, which plays a key role in 5-HT synthesis. Transcriptomic data revealed that the cells simultaneously expressed multiple 5-HT receptor isoforms, however, the transcript level of the 5-HT₇ receptor exceeded that of the other examined isoforms by orders of magnitude. These results confirmed the presence of key molecular components required for 5-HT signaling in the investigated normal mammary epithelial cells.

Paroxetine Induces Transcriptional Changes in Mammary Epithelial Cells

Transcriptomic and GSEA analyses of genes induced by paroxetine revealed increased expression of pathway components primarily associated with cell surface receptor activation and signaling processes. Based on expression data, paroxetine not only reduced intracellular serotonin levels through transporter inhibition but also increased the expression of several genes related to receptor-mediated signaling.

In parallel, paroxetine treatment led to decreased expression of numerous genes. Suppression was observed in genes associated with cell cycle regulatory checkpoints. In addition, genes involved in DNA repair and metabolic pathways also showed reduced expression. These findings provided a basis for further investigation of 5-HT effects mediated through cell surface signaling and those arising from intracellular accumulation.

Intracellular 5-HT Increases ROS Production and Induces DNA Damage in Mammary Epithelial Cells

During the investigation of intracellular effects of serotonin, we found that 5-HT entering mammary epithelial cells increased the production of reactive oxygen species (ROS). In parallel with elevated ROS levels, markers indicative of DNA damage were detected, suggesting that intracellular 5-HT induces oxidative stress in these cells. Inhibition of SERT with paroxetine reduced intracellular 5-HT levels and attenuated ROS production as well as the extent of DNA damage.

5-HT Receptors Expressed on Normal Mammary Epithelial Cells Are Functionally Active and Modulate Erk 1/2 Activity

Investigation of the cell surface mechanism of serotonin action demonstrated that 5-HT receptors expressed by normal mammary epithelial cells are functionally active. Following serotonin treatment, Erk 1/2 phosphorylation decreased. Erk 1/2 dephosphorylation was further enhanced in the presence of paroxetine, consistent with the observation that SERT inhibition amplifies cell surface 5-HT effects.

These data indicated that changes in Erk 1/2 activity primarily result from signaling mediated through cell surface 5-HT receptors rather than from direct intracellular effects of serotonin. Based on these findings, it became justified to further investigate in detail the extent to which individual 5-HT receptor isoforms contribute to the regulation of proliferation in normal mammary epithelial cells.

Screening of Selective 5-HT Receptor Modulators Identified Potentially Antiproliferative Compounds in HME-hTert Cells

To further clarify the functional role of cell surface serotonin receptors, a dose–response-based screening of selective 5-HT receptor modulators was performed in HME-hTert cells. During these experiments, agonists and antagonists targeting different 5-HT receptor isoforms influenced cell proliferation to varying extents. Agonists of G_s -coupled serotonin receptors, as well as antagonists of G_i - and G_q -coupled receptors, frequently induced a marked reduction in cell number.

Based on dose–response analyses and IC_{50} values, several compounds were identified that exerted significant antiproliferative effects at low concentration ranges ($< 4 \mu M$). These findings confirmed that pharmacological modulation of 5-HT receptors can influence the proliferation of normal mammary epithelial cells and provided a basis for comparative evaluation of the observed effects according to G protein coupling.

Finally, pharmacological investigation of downstream elements of G_s -coupled signaling (adenylate cyclase, protein kinase A - PKA) confirmed that activation of the 5-HT₇–adenylate cyclase–cAMP–PKA signaling axis is largely responsible for the antiproliferative effect of serotonin.

G Protein Coupling of Receptors Determines the Proliferative Outcome of Receptor Isoform Activation

Integrated analysis of the screening results obtained with selective 5-HT receptor modulators revealed a clear association between receptor G protein coupling and the effect on proliferation. Independent of which specific 5-HT receptor isoform was targeted, selective activation of G_s -coupled receptors, as well as inhibition of G_i - and G_q -coupled receptors, generally led to an antiproliferative response in HME-hTert cells. In contrast, agonists of G_i - and G_q -coupled receptors typically did not reduce cell number.

To further investigate the general mechanism, the compound library was expanded to include agonists and antagonists specific to other GPCR receptor families (dopamine, histamine, muscarinic acetylcholine receptors). The uniform pattern observed across different receptor families confirmed that the cellular response is primarily determined not by the receptor isoform itself, but by the G protein-mediated signaling pathway it activates. These results indicated that activation of the adenylate cyclase–cAMP axis plays a key role in mediating the antiproliferative effect.

Cell Surface Stimulation of Serotonin Receptors Synergistically Potentiates the Antiproliferative Effect of Bexarotene in Normal Mammary Epithelial Cells

In normal mammary epithelial cells, combined treatment with bexarotene and serotonergic agents resulted in a stronger antiproliferative effect than individual treatments. Dose–response-based combination studies showed that a synergistic interaction between bexarotene and paroxetine occurred within a defined concentration range. Similarly, the combination of bexarotene and 5-HT exhibited strong synergy even at low concentrations, which was further enhanced in the presence of paroxetine. These findings demonstrated that cell surface stimulation of 5-HT receptors synergistically enhances the antiproliferative effect of bexarotene in normal mammary epithelial cells.

Experiments performed in 3D HME-hTert spheroids demonstrated that the combination of bexarotene and paroxetine significantly reduced the proportion of cells positive for the proliferation marker Ki-67 and decreased spheroid size.

Bexarotene-Based Synergistic Combinations Regulate ZG16B expression via ARID1A

During the investigation of the molecular background of bexarotene-based synergistic combinations, we identified that ZG16B expression consistently decreased in response to these treatments in normal mammary epithelial cells. Mechanistic analysis of ZG16B suppression demonstrated that this effect was ARID1A-dependent, as the ZG16B-reducing effect of the combinations was abolished in the absence of ARID1A.

Chromatin immunoprecipitation analyses revealed that the combination of bexarotene and carvedilol promoted ARID1A enrichment at the enhancer region associated with ZG16B, while H3K27ac enrichment at this genomic locus was reduced. This alteration in chromatin state indicated condensation of the region and repression of ZG16B transcription. These findings confirmed that ZG16B expression can be pharmacologically modulated through a well-defined, ARID1A-mediated epigenetic mechanism by rexinoid-based synergistic combinations.

ZG16B Enhances Proliferation in Normal Mammary Cells and Hormone Receptor-Positive Breast Tumor Cells

To clarify the biological role of ZG16B suppressed by bexarotene-based synergistic combinations, functional analyses were performed in normal epithelial and breast tumor cell lines. Normal mammary epithelial cells treated with conditioned supernatant from ZG16B-expressing HEK293 cells exhibited increased proliferation. A similar proliferation-enhancing effect was

observed in hormone receptor-positive breast tumor cell lines, whereas proliferation remained unchanged in hormone receptor-negative cells.

Signaling analyses showed that ZG16B treatment increased Akt phosphorylation in all examined cell lines, while changes in Erk 1/2 and Src phosphorylation depended on the transformation status and hormone receptor profile of the given cell line.

ZG16B Increases Motility in Normal Mammary Epithelial and Breast Tumor Cells

Following the characterization of the proliferative effects of ZG16B, its impact on cell migration and motility was investigated in normal mammary epithelial and breast tumor cells. ZG16B treatment resulted in increased motility in both normal mammary epithelial cells and breast tumor cell lines. The observed response manifested as enhanced migratory capacity and reduced adhesion.

These findings were consistent with previous observations indicating that ZG16B functions as a migration- and invasion-promoting factor in other tumor types (Park et al. 2011; Youn et al. 2022).

The Migration-Stimulating Effect of ZG16B Is Mediated by Activation of the PI3K–Akt Signaling Pathway

To elucidate the molecular background of ZG16B-induced increased cell motility, activation of major mitogenic signaling pathways was examined in normal mammary epithelial and breast tumor cells. Signaling analyses consistently demonstrated increased Akt phosphorylation in all investigated cell lines following ZG16B treatment. This observation suggested that activation of the Akt pathway represents a central component of the signaling response induced by ZG16B.

Pharmacological inhibition of PI3K using LY294002 effectively reduced the ZG16B-induced migratory response and concurrently attenuated Akt activation. These results clearly demonstrated that the migration-promoting effect of ZG16B is primarily mediated through the PI3K–Akt signaling pathway.

Pathways Related to ECM Remodeling and Cell Motility Are Regulated in an Inverse Manner by ZG16B and the Combination of Bexarotene and Carvedilol

To elucidate the molecular background of the phenotypic responses induced by ZG16B, transcriptomic analysis was performed in normal mammary epithelial cells. ZG16B treatment primarily increased the expression of genes involved in extracellular matrix organization. The upregulated genes showed enrichment mainly in pathways related to ECM–receptor interaction, focal adhesion, and regulation of the actin cytoskeleton, which were consistent with the observed alterations in cell motility.

In contrast, combined treatment with bexarotene and carvedilol resulted in suppression of a substantial proportion of genes belonging to these same pathways. Comparison of the transcriptional profiles generated by the two treatments demonstrated that processes related to

ECM remodeling and cell motility are regulated in an inverse manner by ZG16B stimulation and the bexarotene + carvedilol combination. These findings suggested that suppression of ZG16B plays an important role in the migration-inhibitory effects of synergistic rexinoid-based combinations.

ZG16B Induces EMT in Normal Mammary Epithelial and Breast Tumor Cells

Based on the migration- and ECM-related changes induced by ZG16B, we examined whether the protein could induce epithelial–mesenchymal transition (EMT) in normal and breast tumor cells. ZG16B treatment resulted in gene expression and phenotypic changes characteristic of EMT in both normal mammary epithelial cells and breast tumor cell lines. Specifically, we detected reduced expression of the epithelial marker CDH1 and increased expression of mesenchymal markers (FN1, CDH2). In addition to alterations in EMT markers, morphological analysis of normal cells revealed changes consistent with a mesenchymal phenotype.

ZG16B and the Combination of Bexarotene and Carvedilol Inversely Regulate the Colony-Forming Capacity of Breast Tumor Cells

In the presence of ZG16B, breast tumor cells exhibited increased colony-forming capacity irrespective of hormone receptor expression status, indicating enhanced transformation potential. In contrast, combined treatment with bexarotene and carvedilol significantly reduced the colony-forming ability of breast tumor cells. These findings demonstrated that ZG16B and synergistic rexinoid-based combinations regulate the long-term proliferative and survival capacity of breast tumor cells in opposite directions.

The Combination of Bexarotene and Carvedilol Reduces ZG16B and FN1 Expression in Normal Mammary Tissue and Xenograft Breast Tumor Tissue

To validate the previous *in vitro* observations *in vivo*, tumor-free or tumor-bearing mammary tissues from two distinct animal models were examined by immunohistochemistry. In normal mammary tissue of MMTV-ErbB2 transgenic mice, combined treatment with bexarotene and carvedilol reduced ZG16B and FN1 expression. A similar decrease in expression was detected in breast tumor tissues derived from SCID mouse xenograft models established with the MDA-MB-231 cell line.

Immunohistochemical analyses indicated that the effect of the combined treatment was also evident in hormone receptor-negative breast tumors. These results confirmed that the bexarotene + carvedilol combination effectively reduces ZG16B and FN1 expression *in vivo* and supported the role of ZG16B regulation in inhibiting both breast tumor initiation and progression.

Discussion

The results of the present doctoral research demonstrate that monoamine GPCR signaling represents a previously underappreciated, yet biologically and translationally relevant chemopreventive target in breast cancer prevention.

An increasing body of clinical and preclinical evidence suggests that β -adrenergic receptor blockers may influence tumor progression and the risk of relapse (Albitre et al. 2023; Fjæstad et al. 2025; Madden, Szpunar, and Brown 2011). Adrenergic modulators hold translational value because they constitute a well-characterized and widely used class of drugs, which many patients already receive long term for non-oncological indications. Similarly, antidepressants and antihistamines are also broadly and chronically administered medications. Among oncology patients, the use of antidepressants is more frequent than in the general population due to the psychological burden associated with cancer. Consequently, the population-level overlap between breast cancer patients and users of 5-HT signaling modulators may be clinically substantial. Despite this, the role of signaling pathways associated with these receptor families in breast tumors has largely remained unexplored.

Selective serotonin reuptake inhibitors (SSRIs), widely used in clinical practice, prolong and potentiate serotonin signaling through inhibition of SERT. Although retrospective studies on SSRI use have yielded inconsistent results, some reports have described a reduced risk of breast cancer (Díaz-Santana et al. 2025; Wernli et al. 2009). The contradictory findings are likely attributable, at least in part, to the frequent lack of consideration of molecular subtype and receptor status heterogeneity in breast tumors in these analyses.

A fundamental determinant of homeostatic 5-HT signaling in breast tissue is the ability of normal mammary epithelial cells to synthesize serotonin endogenously. Our results confirmed that the key enzyme of 5-HT biosynthesis, tryptophan hydroxylase 1 (TPH1), is expressed in normal mammary epithelial cells. Previous literature has primarily described the physiological role of 5-HT in breast tissue in the regulation of secretory activation and involution. In our investigations, intracellular accumulation of 5-HT was detectable by immunostaining.

Literature data indicate that expression of the tryptophan hydroxylase enzyme (TPH1) is often higher in breast tumor cells compared with normal cells, presumably leading to increased 5-HT production (Gautam et al. 2016; Pai et al. 2009). Regulation of 5-HT signaling, however, is not determined solely by synthesis but also by the activity of the serotonin transporter (SERT), which mediates serotonin uptake from the extracellular space. Transporter function depends on the electrochemical sodium and potassium gradient maintained by the ATP-dependent sodium–potassium pump (Yang and Gouaux 2021). Transporter activity influences not only intracellular 5-HT concentration but also the temporal and spatial patterning of receptor-mediated signaling.

In proliferation assays, silencing of the SERT gene inhibited proliferation of normal mammary epithelial cells. This effect was confirmed pharmacologically, as paroxetine treatment induced a similar antiproliferative response. In contrast, other tested SSRIs, such as citalopram and sertraline, did not exhibit antiproliferative effects. This difference is most likely explained by the distinct binding affinities of these compounds for SERT, as paroxetine displays the strongest binding affinity among the tested inhibitors (Hyttel 1993). Accordingly, paroxetine was selected for subsequent experiments to functionally distinguish intracellular and extracellular effects of 5-HT. These findings also indicate that retrospective studies investigating the association between SSRI use and breast cancer risk may be biased not only by the lack of molecular classification of tumors but also by neglecting pharmacological differences among individual SSRIs.

Consistent with the antiproliferative effect, we demonstrated that paroxetine treatment reduced the amount of 5-HT entering the cells. This finding confirmed that SERT is not only expressed but also functionally active in normal mammary epithelial cells.

Transcriptomic analyses revealed that paroxetine treatment increased the expression of numerous genes associated with cell surface receptor-mediated signaling. Pathway analysis demonstrated enhanced expression of genes related to direct G protein-mediated signaling processes, calcium homeostasis, and synaptic signaling. These results suggest that upon transporter inhibition, cells upregulate components required for receptor-mediated signaling at the cell surface. The observed transcriptomic changes likely do not represent direct consequences of acute GPCR activation but rather reflect an adaptive response to sustained and prolonged extracellular 5-HT exposure, which may involve initial receptor activation followed by desensitization and induction of compensatory mechanisms.

Transcriptomic analysis also identified multiple pathways whose associated genes were significantly downregulated following paroxetine treatment. These primarily involved processes related to cell cycle regulation, maintenance of genomic integrity, and the endoplasmic reticulum stress response. In interpreting these changes, it is important to consider that normal mammary epithelial cells physiologically have access to 5-HT, which can exert effects through intracellular targets. Inhibition of SERT by paroxetine not only increases extracellular 5-HT levels and receptor-mediated stimulation but simultaneously reduces the amount of 5-HT entering the cells. Intracellular degradation of 5-HT generates reactive oxygen species, a phenomenon we also demonstrated experimentally. Literature data indicate that monoamine oxidase activity may act as a pro-tumorigenic factor in breast tumors by increasing oxidative stress and genomic instability (Ahmadimanesh et al. 2019; Jorgensen et al. 2022; Sáez-Freire et al. 2018). In our experiments, reduction of intracellular 5-HT levels was associated with decreased reactive oxygen species and a significant reduction in the proportion of γ H2AX-positive cells, indicative of reduced DNA damage. Taking these findings into account, the suppressed transcriptional programs observed following paroxetine treatment likely reflect partial attenuation of intracellular 5-HT-mediated effects.

Our results indicate that reduction of intracellular 5-HT levels upon paroxetine treatment leads to gradual attenuation of intracellular 5-HT-mediated effects, which may contribute primarily during the early phase of treatment to the observed transcriptional changes. However, during prolonged treatment, it remains uncertain over what time interval cell surface 5-HT receptor-mediated signaling remains functionally relevant for modulating cellular functions. In proliferation assays, we found that inhibition of the transporter significantly enhanced the antiproliferative effect of 5-HT, suggesting that the reduction in proliferation induced by paroxetine is primarily attributable to enhanced and prolonged stimulation of cell surface receptors.

To further substantiate the antiproliferative nature of cell surface 5-HT signaling, we analyzed the activity of several mitogenic signaling pathways essential in mammary epithelial cells. The ERK 1/2, Akt, and Src-associated signaling axes are central regulators of cell growth, survival, and adhesion. In our experiments, only rapid dephosphorylation of ERK 1/2 proteins was observed following 5-HT treatment, indicating inactivation of mitogenic signaling and consistent with the observed antiproliferative response. According to literature data, the effect of 5-HT on ERK 1/2 activity is highly cell type-dependent, as activation has been described in some systems,

whereas inhibition has been reported in others (Lione et al. 2000; Della Rocca et al. 1999; Sheehan et al. 2003). Based on our results, ERK 1/2 dephosphorylation in normal mammary epithelial cells is most likely a consequence of signaling mediated through cell surface 5-HT receptors, further supported by the observation that the effect was more pronounced in the presence of paroxetine. In parallel, activation of other G_s -coupled receptors, such as β_2 -adrenergic receptors, has also been shown to induce ERK 1/2 dephosphorylation, raising the possibility that a similar cAMP-mediated molecular mechanism may underlie the observed phenomenon in the case of 5-HT (Hamed et al. 2021).

Based on these findings, subsequent investigations focused on detailed analysis of the functional role of cell surface 5-HT receptors and their associated signaling. In line with previous literature observations, our transcriptomic data confirmed that normal mammary epithelial cells simultaneously express multiple 5-HT receptor isoforms (Pai et al. 2009). Among these, the 5-HT₇ receptor exhibited by far the highest transcript level in HME-hTert cells, although expression of several other isoforms was also detectable. Dominant expression of 5-HT₇ may increase the likelihood that serotonin preferentially binds this receptor at low ligand concentrations. However, the affinity and binding kinetics of different 5-HT receptors may vary substantially, and consequently, cellular responses may display receptor preference shifts in a concentration-dependent manner.

The signaling response is further complicated by crosstalk between individual G protein-coupled pathways, which can influence each other. Even moderate G_i -mediated activity may effectively suppress a dominant G_s -driven cAMP response. Conversely, a receptor expressed at lower levels may exert disproportionately strong biological effects through efficient downstream signal amplification, particularly in MAPK- or Ca^{2+} -dependent pathways. Signaling may also be modified by receptor homo- and heterodimerization, which can fundamentally alter canonical signaling patterns attributed to individual isoforms (Herrick-Davis 2013). In addition, different 5-HT receptors display distinct desensitization and internalization kinetics, meaning that during sustained ligand exposure, an initially dominant signaling pathway may gradually diminish. Overall, although our transcriptomic data clearly confirms dominant expression of the 5-HT₇ receptor in the investigated model system, expression level alone is insufficient to conclude that the antiproliferative effects of paroxetine and 5-HT are exclusively attributable to this isoform.

Further studies therefore aimed to validate the functional roles of 5-HT receptor isoforms expressed in normal mammary epithelial cells using endogenous 5-HT as well as selective agonists and antagonists. Signaling responses corresponding to receptor G protein coupling were monitored by measuring secondary messengers. Adenylate cyclase activity of G_s - and G_i -coupled receptors was assessed via cAMP quantification, whereas phospholipase C activity of G_q -coupled receptors was examined by IP1 measurement. As representative targets, we selected three receptors with distinct G protein coupling and confirmed expression in the model system: 5-HT_{1D}, 5-HT_{2B}, and 5-HT₇.

Following 5-HT treatment, both cAMP and IP1 levels increased, confirming functional 5-HT receptor signaling. In experiments using selective modulators, each receptor elicited the expected canonical secondary messenger response, while modulators did not affect receptors with different G protein coupling. This supports isoform specificity of the applied compounds and indicates simultaneous activation of multiple 5-HT receptors in the model system.

Subsequently, a targeted compound library was assembled to determine which of the three canonical signaling pathways mediates the antiproliferative response. Dose-response screening identified several compounds with significant proliferation-inhibitory effects. The overall antiproliferative pattern indicated activation of the G_s -coupled pathway and inhibition of G_q - and G_i -coupled pathways, highlighting the central role of adenylate cyclase- and cAMP-mediated signaling.

Although certain exceptions were observed, activation of G_s -coupled signaling emerged as particularly important for the antiproliferative response of normal mammary epithelial cells. This is consistent with literature data regarding 5-HT₆ and 5-HT₇ receptors, which have been reported to mediate antiproliferative effects in normal cells, whereas their role in tumor tissue may differ (Pai et al. 2009; Zhang et al. 2022).

In addition to activation of G_s -coupled signaling, inhibition of G_i -coupled receptors also played a substantial role in the antiproliferative response. Previous studies in bovine mammary epithelial cells demonstrated that pertussis toxin exerts antiproliferative effects and attenuates serum-derived growth factor-induced proliferation (Shamay et al. 1990). Pertussis toxin inactivates G_i -mediated inhibitory signaling via ADP-ribosylation of the G_i subunit, thereby releasing adenylate cyclase activity and enabling cAMP production mediated by G_s -coupled receptors. A similar mechanism may have been operative in our model system, as antagonism of G_i -coupled 5-HT receptors may have shifted endogenous 5-HT signaling toward activation of non-inhibited and dominantly expressed G_s -coupled receptors. Modulation of G_q -coupled receptors displayed a similar pattern, as antiproliferative effects were observed exclusively with antagonists of these isoforms. Overall, these results support the conclusion that inhibition of proliferation in normal mammary epithelial cells is primarily determined by activation of G_s -coupled signaling together with inhibition of G_i - and G_q -mediated pathways.

The role of the 5-HT₇ receptor in mediating the antiproliferative effect of 5-HT was validated through complementation experiments. Cells were pretreated with selective 5-HT receptor antagonists, as well as adenylate cyclase and PKA inhibitors, followed by stimulation with 5-HT, paroxetine, or their combination. The antiproliferative effect was abolished exclusively by inhibition at any point along the 5-HT₇-adenylate cyclase-PKA signaling axis. These findings indicate that proliferation reduction induced by 5-HT and paroxetine is primarily mediated by 5-HT₇ receptor-driven G_s -coupled signaling, consistent with the exceptionally high expression of this receptor in the model system.

Because G_s -mediated signaling can also be activated through other GPCRs, we hypothesized that the mechanism linked to 5-HT₇ may reflect a more general regulatory principle. To examine this possibility, analyses were extended to other GPCR families, including muscarinic acetylcholine, dopamine, and histamine receptors. The pattern of antiproliferative effects remained consistent across these systems. Agonists of G_s -coupled receptors and antagonists of G_i - and G_q -coupled receptors most effectively reduced proliferation. Collectively, these findings reveal a unified regulatory pattern in which activation of G_s -coupled signaling and inhibition of G_i - and G_q -mediated pathways lead to antiproliferative responses in normal mammary epithelial cells.

Considering that multiple endogenous GPCR ligands may be continuously present in the investigated cell model, several receptors may operate in a partially activated basal state,

potentially attenuating the effects of agonists. In contrast, antagonists may more effectively shift the balance of GPCR signaling by reducing basal signaling tone and relieving G_i-mediated inhibition, thereby producing more readily detectable antiproliferative cellular responses.

Investigation of combinations between rexinoids and monoamine receptor modulators was based on previous observations by our research group demonstrating that combined treatment with the non-selective beta-blocker carvedilol and bexarotene exerts synergistic antiproliferative effects in normal mammary epithelial cells *in vitro* and *in vivo* (Jdeed, Lengyel, and Uray 2022; Uray et al. 2015). In the present doctoral work, we therefore examined whether modulation of 5-HT signaling could similarly potentiate the antiproliferative effect of bexarotene.

Dose–response studies demonstrated that the combination of bexarotene and paroxetine resulted in synergistic inhibition of proliferation in normal mammary epithelial cells. Following confirmation of synergy, the antiproliferative effect of the combination was also validated in a 3D spheroid model derived from HME-hTert cells. Combination-treated spheroids exhibited significantly reduced size and a lower proportion of Ki-67-positive cells compared with controls. Since our previous findings indicated that the antiproliferative effect of paroxetine is predominantly mediated via cell surface 5-HT receptors, we also examined the direct combination of bexarotene and 5-HT. This pairing demonstrated even more pronounced synergy than that observed with paroxetine, which was further enhanced by simultaneous SERT inhibition with paroxetine.

The translational relevance of these findings is considerable, as the compounds used in our studies possess well-established clinical applicability and safety profiles. Paroxetine, as a widely prescribed antidepressant, represents a realistic starting point for the development of a potentially long-term, low-dose preventive strategy. In contrast, selective pharmacological modulation of 5-HT₇ or downstream signaling components represents an important direction for future research, including detailed evaluation of toxicity profiles.

Although synergy between bexarotene and monoamine signaling modulators was demonstrated across multiple combinations, the precise molecular mechanism underlying this synergy remained unknown. To address this question, we sought tumor-biologically relevant molecular markers that were consistently regulated across several independent rexinoid-based combinations. Based on these criteria, ZG16B emerged as a key candidate, as its expression in normal mammary epithelial cells was significantly reduced by both the bexarotene–paroxetine and the bexarotene–carvedilol combinations. Selection of this gene was further supported by TCGA data demonstrating elevated ZG16B expression in breast tumors compared with normal breast tissue, with breast cancer representing one of the tumor types exhibiting the highest ZG16B expression levels.

Based on prior results from our research group, it became evident that the synergistic antiproliferative effect of bexarotene and carvedilol is accompanied by epigenetic transcriptional reprogramming mediated by the ARID1A- and BRG1-containing SWI/SNF chromatin-remodeling complex (Jdeed, Erdős, et al. 2022; Jdeed, Lengyel, et al. 2022). In light of this, we investigated the role of this mechanism in the regulation of ZG16B expression. ChIP-sequencing identified a distal enhancer region associated with the ZG16B gene that showed ARID1A enrichment upon combined bexarotene–carvedilol treatment. In parallel, histone H3 acetylation decreased, indicating a more condensed chromatin state and reduced transcriptional activity.

The functional role of ARID1A in ZG16B suppression was confirmed by siRNA-mediated silencing. In the absence of ARID1A, the ZG16B-reducing effect of the bexarotene and carvedilol combination was reversible, whereas in its presence, ZG16B suppression proved to be a phenomenon specific to the combined treatment. We also validated these findings in normal mammary tissue from the MMTV-ErbB2 transgenic mouse model using immunohistochemistry, where the combination reduced ZG16B protein levels *in vivo* as well.

Collectively, these data indicate that a clinically available combination of compounds with known toxicity profiles can target an ARID1A-dependent epigenetic regulatory node that, through inhibition of ZG16B expression, can modulate tumor progression-associated processes already at an early stage of breast tumor development.

ZG16B, previously described as Pancreatic Adenocarcinoma Upregulated Factor (PAUF), is a secreted lectin-like protein that likely binds glycosaminoglycans through its carbohydrate-binding properties. Its functional role has been primarily characterized in pancreatic ductal adenocarcinoma, where elevated expression brought it into focus (Kim et al. 2012). Multiple studies have demonstrated that ZG16B can reprogram innate immune cells, thereby promoting the formation of a tumor progression-supporting, inflammatory, and immunosuppressive microenvironment (Kim et al. 2024; Song et al. 2016). Mechanistically, it induces ERK activation and tumor-promoting cytokine production through TLR signaling, while inhibiting NF- κ B-mediated responses (Park et al. 2011). In *in vivo* models, ZG16B increases the accumulation of myeloid-derived suppressor cells and enhances tumor cell migration, invasion, and metastatic potential, primarily via CXCR4- and FAK-associated pathways (Song et al. 2016).

ZG16B has also been implicated in other tumor types. For example, in colorectal tumors, it has been linked to cell cycle regulation and the Wnt/ β -catenin pathway (Escudero-Paniagua et al. 2020). Clinically, ZG16B may also be relevant as a prognostic biomarker, as it is frequently upregulated in several cancers, including breast cancer. However, paradoxically, in breast tumors, high ZG16B expression was associated with better survival (Lu et al. 2020). From a therapeutic perspective, inhibition of ZG16B in pancreatic cancer was shown to increase sensitivity to gemcitabine, suggesting a role in drug resistance (Cho et al. 2017). In addition, tumor-secreted circulating ZG16B may contribute to the development of cachexia and can therefore be considered a mediator of systemic tumor-associated complications (Yoo et al. 2021).

To date, the tumor-biological significance of ZG16B has been studied primarily in the context of late-stage tumor progression processes. In contrast, much less is known about its role in early events of tumor initiation and about how it affects cellular function in a normal tissue context. This is particularly true for breast tissue, where functional characterization of ZG16B in non-transformed and breast tumor cells has so far been lacking.

The most frequently described functional effect of ZG16B is enhancement of tumor cell migration and invasive capacity, as well as support of metastatic dissemination (Park et al. 2011; Youn et al. 2022). Accordingly, we examined the extent to which ZG16B influences cell migration in mammary epithelial cells and various breast tumor cell lines, and through which signaling mechanisms. Based on our results, unlike the proliferative responses, ZG16B treatment induced a pronounced increase in motility in all investigated breast-derived cell lines, independent of hormone receptor status. In addition, ZG16B was able to partially reverse the migration-inhibitory

effect induced by the bexarotene and carvedilol combination, suggesting that ZG16B may functionally contribute to the migration-inhibitory effect of the chemopreventive combination.

According to literature data, the migration- and invasion-promoting effects of ZG16B are mainly linked to TLR-associated ERK 1/2–MAPK signaling, CXCR4-mediated chemotaxis, and activation of FAK–Src and PI3K–Akt pathways (Lee et al. 2011; Park et al. 2011). In this context, we analyzed the phosphorylation status of ERK 1/2, Src, and Akt signaling components following ZG16B treatment. The responses differed across cell lines. Akt activation was observed in all examined cell lines, whereas ERK 1/2 activation occurred only in normal epithelial cells and ER-positive T47D cells. In normal cells, Src activation was also detected, inferred from a decrease in inhibitory phosphorylation (p-Tyr527).

Among the three signaling components examined, only Akt activation showed complete concordance with the migration results. To investigate the relationship between these phenomena, we assessed the effect of ZG16B on migration under PI3K inhibition. In normal epithelial cells and triple-negative breast tumor cells, in the presence of a PI3K inhibitor, ZG16B was unable to increase either migration or Akt phosphorylation. These findings clearly indicate that in mammary cells, the migration-promoting effect of ZG16B is primarily mediated through activation of the PI3K–Akt signaling pathway.

To more comprehensively characterize ZG16B-induced responses, RNA sequencing was performed on treated HME-hTert cells. The results largely confirmed our previous observations. Among differentially expressed genes, we identified factors involved in focal adhesion formation, actin cytoskeleton regulation, and PI3K–Akt pathway function, consistent with the PI3K–Akt axis-mediated migration-promoting effect of ZG16B in normal mammary epithelial cells. In addition, expression of multiple genes involved in processing extracellular matrix (ECM)-mediated signals was also increased. These transcriptomic changes closely paralleled previous findings in pancreatic tumor cells, where ZG16B was associated with activation of gene expression programs supporting tumor progression, invasion, and metastasis (Park et al. 2011).

Because in our earlier experiments ZG16B was able to partially compensate for the antiproliferative and migration-inhibitory effects induced by the bexarotene and carvedilol combination, we compared the transcriptional profiles generated by the two treatments. This comparison identified a substantial number of genes that were induced by ZG16B but suppressed by the bexarotene and carvedilol combination. Among these genes, several key factors involved in ECM remodeling, cell–ECM interactions, and regulation of migration stood out, including FN1, PLEC, and multiple laminin and collagen matrix components. These changes suggest that ZG16B does not regulate individual matrix components in isolation but rather initiates a complex, coordinated matrisome remodeling program that structurally and functionally supports increased cell migration.

Within the breast tumor microenvironment, matrisome reorganization fundamentally shapes cellular behavior. During tumor progression, ECM composition and structure undergo substantial changes, including increased deposition of collagen, fibronectin, and proteoglycans, thickening of fibers, and increased cross-linking (Insua-Rodríguez and Oskarsson 2016; Popova and Jücker 2022). These changes lead to progressive increases in tissue stiffness, more ordered matrix topography, and formation of invasion-supporting migratory tracks. The biomechanical

consequences of an altered matrix, such as increased tissue stress and interstitial pressure, can induce perfusion disturbances and hypoxia and can also inhibit tissue penetration of therapeutic agents (Schwartz 2010).

Cells actively sense this altered mechanical environment and translate it into mechanosensitive signaling responses. Increased stiffness promotes integrin-mediated adhesion, formation of focal adhesions, and cytoskeletal contractility, which can activate pathways supporting proliferation, survival, and the development of an invasive phenotype (Schwartz 2010). From the perspective of our investigations, it is particularly notable that ZG16B treatment initiated increased deposition of extracellular matrix components even in normal mammary epithelial cells, suggesting that ZG16B can reprogram the stromal and mechanical microenvironment already in a non-malignant state. This early matrix remodeling may, through changes in tissue stiffness and tensional state, trigger mechanosensitive programs that could later promote tumor progression and invasive behavior.

Structural and biomechanical alterations of the extracellular matrix are often accompanied by a shift toward epithelial–mesenchymal transition (EMT) (Brandão-Costa et al. 2020). Although EMT is classically linked to TGF- β signaling, it can also occur in normal epithelial cells in a physiological, reversible form, for example during wound healing (Yao et al. 2024). Because ZG16B treatment induced not only matrisome remodeling but also increased expression of mesenchymal markers, including FN1, we investigated EMT-associated changes. Our results showed that, in the presence of ZG16B, transcriptional and morphological changes indicative of EMT were detectable in both normal mammary epithelial cells and breast tumor cells. Consistent with this, in tumor-free mammary tissues from MMTV-ErbB2 transgenic mice, the combination of bexarotene and carvedilol reduced FN1 expression alongside decreased ZG16B levels.

Previous studies suggest that EMT and broad matrisome reorganization may promote the development of resistance to conventional chemotherapeutic agents. Given that ZG16B can influence both EMT processes and extracellular matrix remodeling, it may serve as a potential biomarker for predicting an invasive phenotype and therapeutic resistance. This concept is further supported by observations in pancreatic tumor cells, where suppression of ZG16B increased sensitivity to gemcitabine, indicating that ZG16B-mediated programs may also contribute to drug resistance (Cho et al. 2017).

EMT-associated cellular plasticity and ECM remodeling may enable tumor cells to gain an adaptive advantage in environments where physiological adhesion conditions are not available. To investigate this, we applied a soft agar colony formation assay as a measure of transformation potential. Our results showed that ZG16B increased colony-forming capacity in both estrogen receptor-positive and hormone receptor-negative breast tumor cells, whereas the ZG16B-suppressing combination of bexarotene and carvedilol significantly reduced colony formation. In colonies derived from MCF7 cells, combined treatment reduced transformation and decreased ZG16B expression. Consistent with these findings, immunohistochemical analyses of xenograft tumor tissues generated in SCID mice by implantation of MDA-MB-231 cells confirmed that ZG16B expression can be effectively reduced in hormone receptor-negative breast tumors by combined administration of bexarotene and carvedilol. Collectively, these results suggest that regulation of ZG16B may have potential therapeutic relevance not only for inhibiting breast tumor initiation but also for attenuating progression of established tumors.

In summary, we demonstrated that in normal mammary epithelial cells, the antiproliferative effect of 5-HT is mediated primarily via cell surface receptors, predominantly through activation of the 5-HT₇–adenylate cyclase–PKA axis, while reducing intracellular 5-HT attenuates oxidative stress and DNA damage. Across multiple GPCR families, a general pattern emerged in which activation of G_s-coupled signaling and inhibition of G_i- and G_q-coupled pathways consistently led to reduced proliferation. Interpretation of these findings is influenced by methodological and conceptual limitations. Complete separation of intracellular and extracellular 5-HT effects was technically constrained, and we have not yet been able to examine mechanisms of receptor dimerization and time-dependent shifts in receptor dominance. Pharmacological effects were primarily analyzed in normal mammary epithelial cells, which is relevant from a chemoprevention perspective, but further studies are required to delineate tumor subtype-specific differences.

A particularly important clinical implication is that modulation of serotonin signaling is feasible using SSRIs that are already in clinical use, and their combination with the rexinoid bexarotene produced synergistic antiproliferative effects at low doses. In exploring the molecular basis of these combination strategies, we identified ZG16B as a central, epigenetically regulated effector whose ARID1A-dependent suppression inhibits not only proliferation but also the invasive phenotype associated with EMT, matrisome remodeling, and cell migration in normal mammary epithelial and breast tumor cells. Based on our results, ZG16B can be interpreted as a potential prognostic biomarker and therapeutic target, as it may contribute to determining invasive behavior and risk of tumor progression.

Overall, our findings open new research directions in the investigation of serotonin signaling, GPCR-based chemoprevention, and ZG16B-linked tumor biological processes. In the future, particularly promising avenues include the development of molecular subtype-tailored preventive strategies based on GPCR expression profiles, extension of investigations to next-generation cell culture and animal models, and validation of ZG16B in clinical samples as a prognostic biomarker and therapeutic target.

Summary

The present doctoral research demonstrates that monoamine G protein-coupled receptor (GPCR) signaling constitutes a previously underexplored yet biologically and translationally relevant target in breast cancer chemoprevention. While monoaminergic modulators, including β -adrenergic blockers, antidepressants, and antihistamines, are widely used in clinical practice, their role in breast tumor biology has remained insufficiently characterized despite substantial population-level exposure. Our findings provide mechanistic insight into how modulation of these pathways can influence early tumor-associated cellular processes.

A central observation of this work is that serotonin signaling exerts antiproliferative effect in normal mammary epithelial cells. These effects are primarily mediated through cell surface receptors rather than intracellular mechanisms. Inhibition of the serotonin transporter using paroxetine reduced intracellular 5-HT levels, leading to decreased oxidative stress and reduced DNA damage, while simultaneously enhancing extracellular receptor-mediated signaling. Functional and pharmacological analyses demonstrated that the antiproliferative response is largely driven by activation of the 5-HT₇ receptor and its downstream adenylyl cyclase–cAMP–

PKA signaling axis. In parallel, inhibition of G_i- and G_q-coupled pathways further reinforced this effect.

Importantly, these findings extend beyond serotonin signaling. Across multiple GPCR families, including adrenergic, histaminergic, dopaminergic, and muscarinic receptors, a consistent pattern emerged: activation of G_s-coupled signaling, together with inhibition of G_i- and G_q-mediated pathways, resulted in reduced cellular proliferation. This suggests the existence of a generalizable signaling principle in normal mammary epithelial cells, where the balance of GPCR downstream pathways, rather than individual receptor identity alone, determines proliferative outcomes.

Building on this, we investigated the potential of combining monoamine signaling modulators with rexinoid-based chemoprevention. The rexinoid bexarotene, previously shown to exert preventive effects, demonstrated significant synergy when combined with paroxetine or with serotonin itself. These combinations produced synergistic antiproliferative effects in both 2D and 3D mammary epithelial models, supporting the feasibility of low-dose, combinatorial preventive strategies using clinically established compounds.

To identify molecular mediators underlying this synergy, transcriptomic and epigenetic analyses were performed. These studies revealed ZG16B as a central, consistently regulated target of rexinoid-based combinations. ZG16B expression was significantly reduced upon bexarotene + carvedilol treatment, and this effect was shown to depend on ARID1A-containing SWI/SNF chromatin remodeling complexes. Functional validation demonstrated that suppression of ZG16B contributes to the antiproliferative and anti-migratory effects of the combinations.

Further characterization established ZG16B as a key regulator of tumor-associated phenotypes. Exogenous ZG16B promoted cell migration, induced epithelial–mesenchymal transition-associated transcriptional and morphological changes, and enhanced extracellular matrix (ECM) remodeling in both normal mammary epithelial and breast cancer cells. Its pro-migratory effects were primarily mediated through activation of the PI3K–Akt signaling pathway. In addition, ZG16B increased tumorigenic transformation potential, as evidenced by enhanced colony formation in soft agar assays. Conversely, pharmacological combinations that suppressed ZG16B reduced these tumor-promoting properties *in vitro* and *in vivo*.

These findings position ZG16B as a functionally relevant mediator linking epigenetic regulation to extracellular matrix remodeling, cellular plasticity, and invasive behavior. Given its elevated expression in breast tumors and its role in promoting tumor-associated processes, ZG16B represents a promising candidate biomarker and potential therapeutic target in early-stage tumorigenesis.

In summary, this work establishes a novel chemopreventive paradigm in which modulation of monoamine GPCR signaling, particularly through enhancement of G_s-coupled pathways and inhibition of G_i or G_q signaling, suppresses proliferation in normal mammary epithelial cells. The demonstrated synergy between monoamine modulators and rexinoids highlights the translational potential of repurposing clinically available drugs for preventive applications. Furthermore, the identification of ZG16B as an epigenetically regulated effector provides mechanistic insight into how these combinations influence tumor biology associated cellular programs, including migration, EMT, and matrix remodeling.

Future directions include the development of subtype-specific preventive strategies based on GPCR expression profiles, validation of ZG16B in clinical cohorts, and further exploration of GPCR-driven signaling networks in advanced experimental models. Collectively, these results contribute to a deeper understanding of early tumor biology and open new avenues for targeted breast cancer chemoprevention.



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List of publications related to the dissertation

1. **Lengyel, M.**, Árkosy, P., Uray, I. P.: G protein subtype preference dictates paroxetine-enhanced serotonin receptor response in non-malignant breast epithelial cells.
Biochem. Pharmacol. 244, 1-16, 2026.
DOI: <http://dx.doi.org/10.1016/j.bcp.2025.117584>
IF: 5.6 (2024)
2. **Lengyel, M.**, Molnár, Á., Nagy, T., Jdeed, S., Garai, I., Horváth, Z., Uray, I. P.: Zymogen granule protein 16B (ZG16B) is a druggable epigenetic target to modulate the mammary extracellular matrix.
Cancer Sci. 116 (1), 81-94, 2025.
DOI: <http://dx.doi.org/10.1111/cas.16382>
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List of other publications

3. Vaiculeviciute, R., Alaburda, A., Uzielienė, I., Kistamás, K., **Lengyel, M.**, Dinnyés, A., Bergaud, C., Bernotiene, E.: Biomimetic electrospun scaffolds for engineered heart tissue: from design parameters to drug testing platforms.
Front. Bioeng. Biotechnol. 14, 1-26, 2026.
DOI: <http://dx.doi.org/10.3389/fbioe.2026.1711698>
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4. Jdeed, S., **Lengyel, M.**, Uray, I. P.: Redistribution of the SWI/SNF Complex Dictates Coordinated Transcriptional Control over Epithelial-Mesenchymal Transition of Normal Breast Cells through TGF- β Signaling.
Cells. 11 (17), 1-15, 2022.
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