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Research Article

Cytotoxic and cytostatic activity of five new imidazotetrazine derivatives on breast cancer cell cultures MDAMB231, BT474, and MCF-7

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Abstract

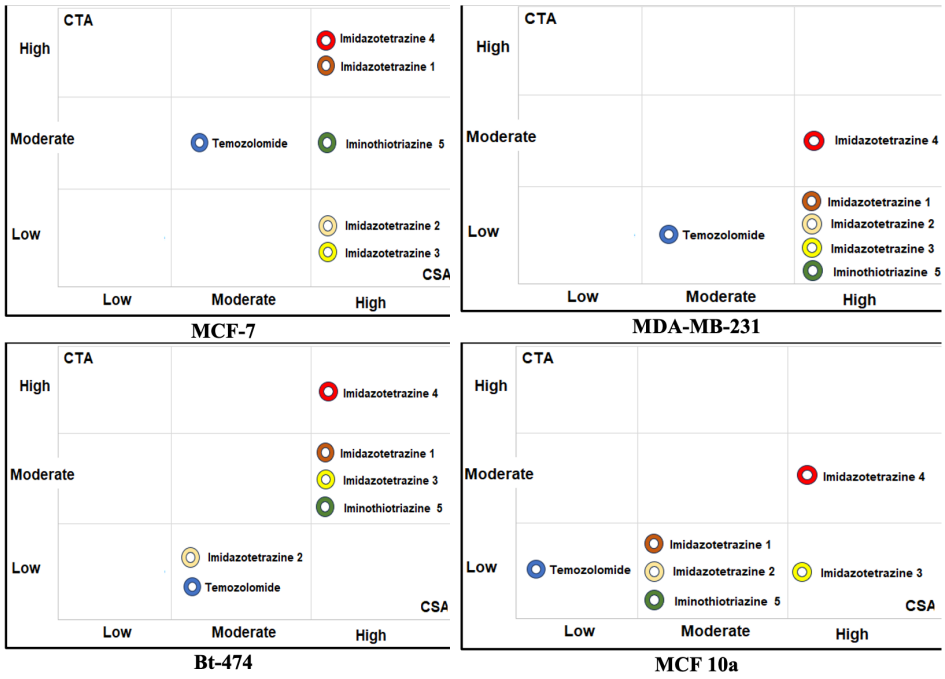
Introduction: The work presents the results of the study of new imidazotetrazine derivatives to establish the possibility of using them as anticancer agents, including for chemotherapy of metastatic breast cancer. The relevance of the work is due to the wide spread of oncological diseases and high cancer mortality, which dictates the need to constantly obtain cell lines and improve cultivation protocols for testing new antitumor drugs. The goal of this study is to check the potential of five new imidazotetrazine derivatives to become new antitumor drugs, in the scope of studying their cytotoxic and cytostatic activities on breast cancer cell cultures.

Materials and Methods: The culturing MCF-7, MDAMB231, BT474, and MCF-10a cells with determining cytotoxic and cytostatic activities of five new azolotetrazine derivatives are base methods used in this study.

Results: For the MCF-7 culture, MCS of comparison drug temozolomide was equal to 2.44 and IC_{50} was 6.81 mM/L; for other cultures CTA indicators were worse. Imidazotetrazine 2 and imidazotetrazine 3 demonstrated CTA indicators lower than those of temozolomide. IC_{50} was not achieved, and the MCS value varied between 1.34 and 1.74. These two derivatives were classified as the compounds with an extremely low CTA. Imidazotetrazine 1 and iminothiotriazine 5 showed cytotoxic activity higher than that of the comparison drug and we classified these compounds as the ones with a moderate CTA. Finally, we found imidazotetrazine 4 with IC_{50} of 0.85 mM/L and CTA of 7.34 as a compound with a potentially strong anticancer effect for further investigation. The cytostatic activity of four of the five azoloazine derivatives studied was in a narrow range corresponding to the survival rate from 0.21 to 0.32, depending on the compound and cell culture. Against this background, imidazotetrazine 4 demonstrated a higher CSA, determined by the survival rate from 0.17 to 0.20.

Conclusion: As a result of an *in vitro* study, we found that five new azolotriazine derivatives can be evaluated in the ascending order of these properties, as a combination of CTA+CSA in order imidazotetrazine 2, imidazotetrazine 3 < temozolomide < imidazotetrazine 1, iminothiotriazine 5 < imidazotetrazine 4, although the CSA of all the studied compounds turned out to be high. Thus, 3-Cyclohexyl-4-oxoimidazo[5,1-d]-[1,2,3,5]tetrazine-8-N-piperidinyl-carboxamide (imidazotetrazine 4) is an unconditional leader in the tested series of new azoloazine derivatives and we recommend it for further preclinical trials.

Graphical abstract



Cytotoxic and cytostatic activity of the azolotetrazine derivatives against the comparison drug Temozolomide in four cell cultures

Keywords

breast cancer, imidazotetrazine, cytotoxic activity, cytostatic activity, MCF-7 cell line, MDAMB231 cell line, BT474 cell line, MCF-10a cell line

Introduction

Malignant neoplasms are one of the main causes of mortality in the world. Breast cancer is the leading type of malignant tumors for women (Bray et al. 2018; Wilkinson and Gathani 2022). Breast cancer accounts for 23% of the total number of employees dealing with cancer, and 14% deaths from cancer, which makes it extremely relevant research in this area (Akram et al. 2017). In 2020, breast cancer was concentrated in everyone largely due to malicious people and the disease in 2.3 million people, and 685 thousand faced it (Rositch et al. 2020). Significant progress in the prevention and treatment of breast cancer has not yet revealed a long-term decrease in growth, morbidity, and mortality from any disease (Feiten et al. 2014; Akram et al. 2017; Azamjah et al. 2019). It is these moments that determine the urgent need to develop new chemotherapeutic agents that would cope with breast cancer (Hassan et al. 2010; Zhukova et al. 2021; Pakina et al. 2024).

Evaluation of cytotoxic and cytostatic activities when exposed to cell cultures of the compounds with a potential antitumor activity, already at the *in vitro* stage of the study, allows us to determine the most promising substances (Dai et al. 2017; Clegg et al. 2020). The greatest interest in this regard is the breast cancer cell line – MCF-7, which, admittedly, is a universal option for screening studies of new antitumor substances with a variety of mechanisms of action (Comşa et al. 2015). In turn, when developing potential antitumor agents at the screening stage, one of the key points is an adequate selection and use of several cell lines (Arnedos et al. 2015; Alexandrova et al. 2019).

Among the great number of anticancer drugs, we focused our attention on the study of imidazo[5,1-d][1,2,3,5]tetrazine derivatives, which have been used as antitumor agents for 40 years. The most well-known is temozolomide, which has proven its clinical efficacy in the treatment of lymphomas, brain tumors, metastatic melanoma, as well as an antiviral drug (Shirazi et al. 2011; Garza-Morales et al. 2018). To date, more than 30 imidazotetrazine derivatives have been synthesized, with prospects for use as antitumor agents (Moody and Wheelhouse 2014; Sadchikova 2016).

The **goal of this study** is to check the potential of five new azolotetrazine derivatives to become new antitumor drugs, in the scope of studying their cytotoxic (CTA) and cytostatic (CSA) activities on breast cancer cell cultures.

Materials and Methods

Tested substances

To assess the potential antitumor properties, we tested five azolotetrazine derivatives: ethyl ether of 3-n-propyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxylic acid as imidazotetrazine 1; ethyl ether of 3-cyclohexyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxylic acid as imidazotetrazine 2; 3-n-Propyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide as imidazotetrazine 3; 3-Cyclohexyl-4-oxoimidazo[5,1-d]-[1,2,3,5]tetrazine-8-N-piperidinyl-carboxamide as imidazotetrazine 4; and 5-(p-Toluyl)-8-ethoxycarbonyl-iminopyrazolo[5,1-d][1,2,3,5]thiotriazine as iminothiotriazine 5. The synthesis of these compounds was carried out at Ural State University (Sadchikova et al. 2013; Sadchikova 2016), which kindly provided them for testing by CTA and CSA. Temozolomide acted as a comparison drug.

Cell culture technique

For our study we selected three human breast cancer cell lines. MDAMB231 are basal-type triple negative breast cancer cells, and this line is an ideal model for the study of its chemotherapy. BT474 cells are a triple-positive breast cancer luminal type model with the most unfavorable clinical prognosis. MCF-7 is a transferable line of human breast cancer cells; luminal–type cells contain estrogen and progesterone receptors, but the HER2 receptors are absent. Currently, it is the most popular line for studying the cytotoxicity of antitumor compounds and the molecular mechanisms of cancer (Holliday and Speirs 2011). MCF-10a is a culture of untransformed cells of the luminal epithelium of the human breast. It is used as a biological control of the effect of the studied substances on healthy tissue.

After defrosting the cells, we washed them twice in Hanks' solution, using them for precipitation by centrifugation for 5 minutes at 500g. Culture vials with a capacity of 10 mL contained a standard complete medium of the composition Eagle MEM/DMEM + Glutamine + Antibiotics. The cells multiplied until the required amount was obtained at a temperature of 37°C in the presence of 5% CO_2 in the gas phase. If necessary, the nutrient medium was replaced, using as a criterion the results of determining the viability of cells in the trypan blue test and their morphology when visualized in an inverted Olympus microscope.

Cytotoxic and cytostatic activities

To determine CTA of the test substances, we used a methyl tetrazolium test (Stockert et al. 2018; Vajrabhaya and Korsuwannawong 2018). The first step was the seeding of each cell culture (MDAMB231, BT474, MCF-7 or MCF-10a) into a flask and growing it to a monolayer. To replant cultures on a 96-well plate, we removed the nutrient medium and replace the cells on a 0.25% trypsin-EDTA disintegrator solution with subsequent centrifugation for 5 minutes at 500g. The precipitate was resuspended in 2 mL of standard complete medium and a suspension was prepared at the rate of 10^6 cells per 1 mL. Then we placed 100 µL of suspension containing 10^4 cells in each well of the plate and added the test substances in the required final concentrations of 0.25; 1.0; 2.5; 5.0 and 10.0 µmol/L. In addition, the plate had: negative control as 1% DMSO and positive control as 10% DMSO. The plate with the introduced compounds was placed for 1 hour in a CO_2 incubator. Then to stain the cells, we added 100 µL of 2.5% tetrazolium dye into the wells and placed the plates in an incubator for 2 hours. After substitution of the liquid phase in wells for DMSO to dissolve formazane crystals, we conducted plate photometry and calculated the cell survival rate as the ratio of the sample optical density to the control optical density, and the maximum suppression of cell survival rate (MCS) as the ratio of the optical density in the control series to the minimum optical density in the experiment. The concentration of the substance that causes 50% cell death (IC_{50}) we calculated using a dose-dependent curve with the Origin software (OriginLabCorporation). The value of the indicator was expressed in µmol/L of the test substance.

The CSA determination had the similar protocol with the following differences from the previous one. For initial growth, 5000 cells per 100 µL of standard complete medium were seeded into 96-well plates. After 24 hours of incubation, the test substances were added in the same final concentrations and the plates were sent to the incubator for another 24 hours. Then the medium was changed to a new one and after 72 hours of incubation, cell viability was measured using a methyl tetrazolium test. Cell viability was determined as the ratio of optical density in wells with the addition of the tested compounds and that in control wells.

Statistical method

The data were analyzed with Statistica 12.0 (Dell, USA) package programs. After Shapiro-Wilk, test we excluded the normal distribution and presented all the quantitative data as median and quartiles (Me [Q1÷Q3] form). The intra-group comparative analysis was carried out according to the Kraskel-Wallis criterion (a nonparametric version of ANOVA), followed by multiple Bonferroni-Dann comparisons. The comparison between the groups was carried out according to the Mann-Whitney test. The differences were considered statistically significant when $P<0.05$.

Results

Temozolomide

The comparison drug reduced cell viability in all cultures used in a dose-dependent manner; the maximum suppression of viability MCS was seen at the concentration of 10.0 mM/L. It was 2.44 for the MCF-7 line, 1.63 for the MDA-MB-231 line, 1.82 for the Bt-474 line, and 1.45 for the untransformed MCF-10a cell line. The calculated IC_{50} turned out to be 6.81 mM/L when tested on MCF-7 cell culture, and in other cases it exceeded the maximum tested concentration of 10.0 mM/L. As a result, we regarded the CTA of temozolomide on MCF-7 as moderate, whereas in respect to other cell lines it was regarded as low.

Cell viability after administration of temozolomide in all tumor cultures ranged from 0.76–0.83 at a concentration of 0.25 mM/L to 0.46–0.55 at a concentration of 10.0 mM/L and remained at a higher level in the culture of non-tumor MCF-10a cells as Table 1 illustrates. According to the test results, temozolomide

showed moderate CSA against tumor cells and low activity against non-tumor cells.

Imidazotetrazine 1

This substance reduced the cell survival of the taken lines over the entire range of studied concentrations, as Table 2 illustrates. Though it was dose-dependent for the cancer cell lines, there was no concentration dependence for the line of untransformed MCF-10a cells. MCS differed between tumor cell lines, and it was 2.50 for the MCF-7 line, 1.59 for the MDA-MB-231 line, and 2.38 for the Bt-474 line at a concentration of 10 mM/L. In relation to the MCF-10a cell line, the value of the indicator was only 1.33. Similarly, the IC_{50} was 7.72 mM/L for the MCF-7 line and 5.09 mM/L for the Bt-474 line. In other cases, IC_{50} was not reached in the studied concentration range. The CTA of the compound on MCF-7 and Bt-474 cell lines was moderate, and it was low for MDA-MB-231 and MCF-10a cell lines.

Imidazotetrazine 1 suppressed the viability of tumor cells more than twice as much as the comparison drug. The effect was dose-dependent for the MCF-7 and Bt-474 lines, and there were no signs of concentration dependence for the cells of the MDA-MB-231 line. As a result, we concluded for CSA of imidazotetrazine 1 to be sufficiently high in relation to tumor cells. In MCF-10a line, the cell viability decreased in a dose-dependent manner from 0.76 at a concentration of 0.25 mM/L to 0.48 at a concentration of 10.0 mM/L, which indicated

Imidazotetrazine 2

Table 3 shows the results of testing imidazotetrazine 2. This compound reduced the survival rate of tumor cells to varying degrees, so that the MCS was 1.69 for the MCF-7 line, 1.28 for the MDA-MB-231 line and 1.92 for Bt-474 line. For the non-tumor cell line, the value of the indicator was 1.30 at a concentration of 10.0 mM/L. Accordingly, IC_{50} in the studied concentration range was not achieved in any of the cases. We attributed imidazotetrazine 2 to the compounds with an extremely low CTA against tumor and non-tumor epithelial cells of the human breast.

Cell viability due to testing imidazotetrazine 2 on tumor cell lines varied from 0.42 to 0.21 depending on the dose, which we generally regarded as a high CSA exceeding the same one of temozolomide by 2.19–4.16 times. The viability of MCF-10a cells turned out to be only 1.13 times lower than the index value for comparison drug, which indicated moderate CSA of imidazotetrazine 2 against non-tumor cells of the MCF-10a line.

Imidazotetrazine 3

Table 4 contains data on the effect of imidazotetrazine 3 on cell survival and viability in the four cell cultures. For MCF-7 cells, the effect was minimal; the MCS was 1.74 at a concentration of 5 mM/L; for MDA-MB-231 cells, the value of the indicator was 1.47, and for MCF-10a cells, it was 1.25. IC_{50} was not achieved in these cases. Only for Bt-474 cells, we saw a more pronounced effect.

Table 1. Quantitative indicators of cytotoxic and cytostatic activity of temozolomide in four cell cultures

Temozolomide concentration, mM/L	Cell line			
	-7 MCF	MDA-MB-231	Bt-474	MCF-10a
Cytotoxic activity (cell survival rate, %)				
Control	1.00 [0.96 ÷ 1.05]	1.00 [0.94 ÷ 1.05]	1.00 [0.95 ÷ 1.07]	1.00 [0.96 ÷ 1.07]
0.25	0.85 [0.77 ÷ 0.94]	0.82 [0.74 ÷ 0.90]	0.84 [0.76 ÷ 0.90]	0.95 [0.88 ÷ 1.01]
1.0	0.79 * [0.70 ÷ 0.86]	0.79 * [0.70 ÷ 0.85]	0.76 * [0.70 ÷ 0.83]	0.88 [0.81 ÷ 0.96]
2.5	0.63 *# [0.55 ÷ 0.69]	0.76 * [0.69 ÷ 0.84]	0.67 *# [0.60 ÷ 0.73]	0.84 [0.77 ÷ 0.92]
5.0	0.52 *# [0.47 ÷ 0.58]	0.69 * [0.62 ÷ 0.73]	0.61 *# [0.56 ÷ 0.66]	0.79 * [0.72 ÷ 0.86]
10.0	0.41 *# [0.36 ÷ 0.45]	0.61 * [0.55 ÷ 0.68]	0.55 * [0.48 ÷ 0.61]	0.69 * [0.62 ÷ 0.75]
Cytostatic activity (cell viability, %)				
Control	1.00 [0.95 ÷ 1.07]	1.00 [0.95 ÷ 1.06]	1.00 [0.93 ÷ 1.04]	1.00 [0.94 ÷ 1.05]
0.25	0.83 [0.75 ÷ 0.90]	0.82 [0.75 ÷ 0.90]	0.76 * [0.69 ÷ 0.84]	0.92 [0.83 ÷ 1.03]
1.0	0.75 * [0.68 ÷ 0.83]	0.77 * [0.71 ÷ 0.84]	0.60 *# [0.54 ÷ 0.67]	0.84 [0.75 ÷ 0.92]
2.5	0.66 * [0.59 ÷ 0.73]	0.72 * [0.66 ÷ 0.77]	0.54 *# [0.48 ÷ 0.63]	0.77 * [0.69 ÷ 0.85]
5.0	0.60 * [0.54 ÷ 0.67]	0.61 * [0.55 ÷ 0.68]	0.47 *# [0.42 ÷ 0.53]	0.72 * [0.66 ÷ 0.79]
10.0	0.55 * [0.48 ÷ 0.60]	0.54 * [0.48 ÷ 0.60]	0.46 *# [0.39 ÷ 0.51]	0.69 [0.62 ÷ 0.77]

Note: * – significant differences when compared to the value in the control samples; # –significant differences between the values in the cultures of tumor and non-tumor cells.

Table 2. Quantitative indicators of cytotoxic and cytostatic activity of imidazotetrazine 1 in four cell cultures

Imidazo-tetrazine 1 concentration, mM/L	Cell line			
	-7 MCF	MDA-MB-231	Bt-474	MCF-10a
Cytotoxic activity (cell survival rate, %)				
Control	1.00 [0.95 ÷ 1.06]	1.00 [0.94 ÷ 1.05]	1.00 [0.95 ÷ 1.07]	1.00 [0.96 ÷ 1.07]
0.25	0.76 *# [0.68 ÷ 0.82]	0.92 [0.83 ÷ 1.00]	0.53 *# [0.46 ÷ 0.60]	0.93 [0.86 ÷ 1.02]
1.0	0.68 * [0.61 ÷ 0.74]	0.81 [0.72 ÷ 0.91]	0.54 *# [0.47 ÷ 0.62]	0.75 * [0.67 ÷ 0.83]
2.5	0.56 *# [0.50 ÷ 0.61]	0.79 [0.70 ÷ 0.78]	0.49 *# [0.44 ÷ 0.54]	0.77 * [0.69 ÷ 0.86]
5.0	0.45 *# [0.41 ÷ 0.49]	0.71 * [0.65 ÷ 0.80]	0.47 *# [0.43 ÷ 0.56]	0.86 [0.78 ÷ 0.95]
10.0	0.40 *# [0.36 ÷ 0.43]	0.63 * [0.57 ÷ 0.69]	0.42 *# [0.37 ÷ 0.50]	0.78 * [0.70 ÷ 0.87]
Cytostatic activity (cell viability, %)				
Control	1.00 [0.95 ÷ 1.07]	1.00 [0.95 ÷ 1.06]	1.00 [0.93 ÷ 1.04]	1.00 [0.94 ÷ 1.05]
0.25	0.36 *# [0.32 ÷ 0.40]	0.25 *# [0.23 ÷ 0.29]	0.24 *# [0.21 ÷ 0.28]	0.76 * [0.69 ÷ 0.84]
1.0	0.30 *# [0.26 ÷ 0.32]	0.24 *# [0.21 ÷ 0.28]	0.22 *# [0.19 ÷ 0.24]	0.65 * [0.60 ÷ 0.72]
2.5	0.26 *# [0.24 ÷ 0.29]	0.24 *# [0.20 ÷ 0.27]	0.23 *# [0.20 ÷ 0.25]	0.61 * [0.54 ÷ 0.66]
5.0	0.26 *# [0.23 ÷ 0.29]	0.27 *# [0.24 ÷ 0.30]	0.25 *# [0.21 ÷ 0.28]	0.53 * [0.47 ÷ 0.57]

Note: * – significant differences when compared to the value in the control samples; # –significant differences between the values in the cultures of tumor and non-tumor cells.

Table 3. Quantitative indicators of cytotoxic and cytostatic activity of imidazotetrazine 2 in four cell cultures

Imidazo-tetrazine 2 concentration, mM/L	Cell line			
	-7 MCF	MDA-MB-231	Bt-474	MCF-10a
Cytotoxic activity (cell survival rate, %)				
Control	1.00 [0.96 ÷ 1.05]	1.00 [0.94 ÷ 1.05]	1.00 [0.95 ÷ 1.07]	1.00 [0.96 ÷ 1.07]
0.25	0.65 * [0.59 ÷ 0.72]	0.94 [0.84 ÷ 1.03]	0.82 [0.73 ÷ 0.94]	0.84 [0.75 ÷ 0.94]
1.0	0.77 * [0.68 ÷ 0.86]	0.85 [0.76 ÷ 0.95]	0.76 * [0.68 ÷ 0.83]	0.86 [0.77 ÷ 0.99]
2.5	0.60 * [0.53 ÷ 0.67]	0.80 [0.71 ÷ 0.89]	0.69 * [0.62 ÷ 0.76]	0.78 * [0.70 ÷ 0.85]
5.0	0.59 *# [0.53 ÷ 0.66]	0.77 * [0.69 ÷ 0.85]	0.57 *# [0.51 ÷ 0.64	0.83 [0.75 ÷ 0.92]
10.0	0.67 * [0.60 ÷ 0.73]	0.78 * [0.70 ÷ 0.87]	0.52 *# [0.47 ÷ 0.63	0.77 * [0.69 ÷ 0.86]
Cytostatic activity (cell viability, %)				
Control	1.00 [0.95 ÷ 1.07]	1.00 [0.95 ÷ 1.06]	1.00 [0.93 ÷ 1.04]	1.00 [0.94 ÷ 1.05]
0.25	0.42 *# [0.38 ÷ 0.47]	0.27 *# [0.24 ÷ 0.32]	0.24 *# [0.22 ÷ 0.28]	0.81 [0.69 ÷ 0.84]
1.0	0.31 *# [0.27 ÷ 0.34]	0.27 *# [0.24 ÷ 0.31]	0.23 *# [0.19 ÷ 0.26]	0.77 * [0.70 ÷ 0.88]
2.5	0.29 *# [0.26 ÷ 0.33]	0.28 *# [0.25 ÷ 0.33]	0.27 *# [0.22 ÷ 0.31]	0.64 * [0.58 ÷ 0.71]
5.0	0.26 *# [0.23 ÷ 0.30]	0.24 *# [0.21 ÷ 0.26]	0.21 *# [0.17 ÷ 0.25]	0.59 * [0.52 ÷ 0.64]
10.0	0.24 *# [0.22 ÷ 0.27]	0.24 *# [0.22 ÷ 0.26]	0.21 *# [0.18 ÷ 0.24]	0.55 * [0.51 ÷ 0.61]

Note: * – significant differences when compared to the value in the control samples; # –significant differences between the values in the cultures of tumor and non-tumor cells.

The MCS turned out to be 2.44; the IC_{50} was calculated as 5.66 mM/L. As a result, we recognized that imidazotetrazine 3 has a moderate CTA on Bt-474 cells and a low Cytotoxic activity on other cell lines.

The minimum cell viability in all cell cultures was manifested at concentrations of 5.0–10.0 mM/L without signs of dependence on this concentration. The CSA effect of the compound was 1.41–1.72 times higher in comparison to temozolomide, and we regarded it as high.

Imidazotetrazine 4

As Table 5 demonstrates, this compound significantly reduced cell survival of the used lines in a clear dose-dependent manner over the entire range of the studied concentrations. The MCS was 7.34 for the MCF-7 line, 2.17 for the MDA-MB-231 line, 3.13 for the Bt-474 line, and 2.78 for the MCF-10a line. The calculated IC_{50} turned out to be 0.85 mM/L, 7.02 mM/L, 2.75 mM/L, and 7.18 mM/L, respectively. It is worth noting that imidazotetrazine 4 is one of the most toxic of the entire tested sample; its CTA alters in a dose-dependent manner. The CTA of imidazotetrazine 4 was found to be high for all tumor cell lines and the highest of the five studies compounds.

The maximum suppression of cell viability in imidazotetrazine 4 study was achieved in all cases at a concentration of 10.0 mM/L; it was 2.70–3.24 times higher than the value of the reference drug for tumor cells, and it was 1.73 times higher than this indicator for the MCF-10a cell line. As a result, we conclude about the high CSA of the compound in relation to the applied cell lines.

Iminothiotriazine 5

Table 6 shows that iminothiotriazine 5 reduced cell viability in the studied concentration range; for all cell lines, excluding MCF-7, the dependence on concentration is obvious. The MCS in all cases was achieved at a concentration of 10 mM/L. It was 2.84 for the MCF-7 line, 1.82 for the MDA-MB-231 cell line, 2.38 for the Bt-474 line, and 1.33 for non-tumor cells of the MCF-10a line. Accordingly, we were able to calculate the IC_{50} for iminothiotriazine 5 as equal to 6.92 mM/L for the MCF-7 line and as equal to 6.02 mM/L for the Bt-474 line. As a result, we assumed that iminothiotriazine 5 was a compounds with moderate CTA.

According to the results of cell viability determination, we also discovered a high CSA of iminothiotriazine 5 against tumor cells, which exceeded the similar effect of the comparison drug by 1.92–3.84 times. We also recognized the CSA of the compound against non-tumor cells of the MCF-10a line as moderate.

Discussion

The modern paradigm of how to develop new drugs required their chemical structure to evidently imply a certain pharmacological, for example, antitumor activity; these compounds have clearly marked targets of action, demonstrate high specific activity *in vitro* and *in vivo*, have minimal toxic effects on healthy tissues, and do not cause rapid development of resistance (Shirazi et al. 2011; Clegg et al. 2020; Yan and Yue 2023).

Table 4. Quantitative indicators of cytotoxic and cytostatic activity of imidazotetrazine 3 in four cell cultures

Imidazo-tetrazine 3 concentration, mM/L	Cell line			
	-7 MCF	MDA-MB-231	Bt-474	MCF-10a
Cytotoxic activity (cell survival rate, %)				
Control	1.00 [0.95 ÷ 1.06]	1.00 [0.94 ÷ 1.05]	1.00 [0.95 ÷ 1.07]	1.00 [0.96 ÷ 1.07]
0.25	1.09 [0.96 ÷ 1.14]	0.77 [0.69 ÷ 0.88]	0.51 *# [0.44 ÷ 0.56]	0.86 [0.78 ÷ 0.95]
1.0	0.90 [0.80 ÷ 0.99]	0.75 [0.67 ÷ 0.87]	0.51 *# [0.45 ÷ 0.58]	0.81 [0.74 ÷ 0.90]
2.5	0.62 *# [0.55 ÷ 0.69]	0.86 [0.77 ÷ 0.95]	0.49 *# [0.44 ÷ 0.57]	0.97 [0.86 ÷ 1.05]
5.0	0.59 *# [0.54 ÷ 0.66]	0.75 [0.68 ÷ 0.86]	0.41 *# [0.36 ÷ 0.46]	0.93 [0.84 ÷ 1.03]
10.0	0.68 * [0.61 ÷ 0.75]	0.68 * [0.60 ÷ 0.75]	0.44 *# [0.39 ÷ 0.48]	0.80 [0.71 ÷ 0.89]
Cytostatic activity (cell viability, %)				
Control	1.00 [0.95 ÷ 1.07]	1.00 [0.95 ÷ 1.06]	1.00 [0.93 ÷ 1.04]	1.00 [0.94 ÷ 1.05]
0.25	0.35 *# [0.32 ÷ 0.39]	0.25 *# [0.22 ÷ 0.28]	0.23 *# [0.20 ÷ 0.27]	0.72 * [0.66 ÷ 0.80]
1.0	0.36 *# [0.33 ÷ 0.40]	0.25 *# [0.22 ÷ 0.20]	0.24 *# [0.21 ÷ 0.29]	0.60 * [0.54 ÷ 0.67]
2.5	0.34 *# [0.30 ÷ 0.38]	0.24 *# [0.20 ÷ 0.28]	0.23 *# [0.19 ÷ 0.26]	0.57 * [0.52 ÷ 0.63]
5.0	0.32 *# [0.29 ÷ 0.36]	0.24 *# [0.21 ÷ 0.29]	0.23 *# [0.10 ÷ 0.27]	0.50 * [0.44 ÷ 0.56]
10.0	0.36 *# [0.32 ÷ 0.41]	0.26 *# [0.22 ÷ 0.30]	0.25 *# [0.22 ÷ 0.30]	0.49 * [0.44 ÷ 0.54]

Note: * – significant differences when compared to the value in the control samples; # – significant difference between the values in the cultures of tumor and non-tumor cells.

Table 5. Quantitative indicators of cytotoxic and cytostatic activity of imidazotetrazine 4 in four cell cultures

Imidazo-tetrazine 4 concentration, mM/L	Cell line			
	-7 MCF	MDA-MB-231	Bt-474	MCF-10a
Cytotoxic activity (cell survival rate, %)				
Control	1.00 [0.95 ÷ 1.07]	1.00 [0.93 ÷ 1.05]	1.00 [0.95 ÷ 1.06]	1.00 [0.94 ÷ 1.07]
0.25	0.57 * [0.51 ÷ 0.64]	0.73 * [0.65 ÷ 0.81]	0.55 * [0.49 ÷ 0.61]	0.63 * [0.57 ÷ 0.70]
1.0	0.27 *# [0.24 ÷ 0.32]	0.67 * [0.60 ÷ 0.75]	0.44 * [0.39 ÷ 0.50]	0.58 * [0.52 ÷ 0.64]
2.5	0.28 *# [0.24 ÷ 0.33]	0.64 * [0.57 ÷ 0.72]	0.46 * [0.41 ÷ 0.52]	0.49 * [0.44 ÷ 0.55]
5.0	0.29 *# [0.26 ÷ 0.34]	0.48 * [0.43 ÷ 0.54]	0.37 * [0.33 ÷ 0.42]	0.52 * [0.47 ÷ 0.57]
10.0	0.14 *# [0.10 ÷ 0.17]	0.46 * [0.40 ÷ 0.51]	0.32 * [0.28 ÷ 0.36]	0.36 * [0.33 ÷ 0.40]
Cytostatic activity (cell viability, %)				
Control	1.00 [0.96 ÷ 1.05]	1.00 [0.94 ÷ 1.05]	1.00 [0.95 ÷ 1.08]	1.00 [0.96 ÷ 1.07]
0.25	0.25 *# [0.21 ÷ 0.30]	0.25 *# [0.21 ÷ 0.28]	0.24 *# [0.20 ÷ 0.27]	0.62 * [0.56 ÷ 0.68]
1.0	0.24 *# [0.21 ÷ 0.28]	0.24 *# [0.21 ÷ 0.27]	0.22 *# [0.19 ÷ 0.26]	0.55 * [0.50 ÷ 0.62]
2.5	0.21 *# [0.18 ÷ 0.25]	0.24 *# [0.21 ÷ 0.29]	0.22 *# [0.18 ÷ 0.26]	0.47 * [0.42 ÷ 0.53]
5.0	0.19 *# [0.16 ÷ 0.21]	0.21 *# [0.17 ÷ 0.24]	0.21 *# [0.17 ÷ 0.24]	0.44 * [0.39 ÷ 0.49]
10.0	0.17 *# [0.14 ÷ 0.20]	0.20 *# [0.16 ÷ 0.24]	0.18 *# [0.15 ÷ 0.22]	0.40 * [0.35 ÷ 0.44]

Note: * – significant differences when compared to the value in the control samples; # –significant differences between the values in the cultures of tumor and non-tumor cells.

Table 6. Quantitative indicators of cytotoxic and cytostatic activity of iminothiotriazine 5 in four cell cultures

Iminothio-triazine 5 concentration, mM/L	Cell line			
	-7 MCF	MDA-MB-231	Bt-474	MCF-10a
Cytotoxic activity (cell survival rate, %)				
Control	1.00 [0.95 ÷ 1.07]	1.00 [0.93 ÷ 1.05]	1.00 [0.95 ÷ 1.06]	1.00 [0.94 ÷ 1.07]
0.25	0.63 *# [0.55 ÷ 0.70]	0.71 *# [0.64 ÷ 0.79]	0.74 *# [0.66 ÷ 0.83]	1.05 [0.98 ÷ 1.11]
1.0	0.54 *# [0.46 ÷ 0.61]	0.62 *# [0.55 ÷ 0.69]	0.67 *# [0.60 ÷ 0.75]	1.01 [0.95 ÷ 1.09]
2.5	0.74 * [0.67 ÷ 0.82]	0.59 *# [0.52 ÷ 0.66]	0.59 *# [0.52 ÷ 0.66]	0.92 [0.82 ÷ 1.01]
5.0	0.72 * [0.65 ÷ 0.79]	0.58 *# [0.51 ÷ 0.65]	0.44 *# [0.39 ÷ 0.50]	0.85 [0.76 ÷ 0.95]
10.0	0.35 *# [0.31 ÷ 0.40]	0.55 *# [0.49 ÷ 0.62]	0.42 *# [0.37 ÷ 0.48]	0.75 * [0.66 ÷ 0.84]
Cytostatic activity (cell viability, %)				
Control	1.00 [0.96 ÷ 1.05]	1.00 [0.94 ÷ 1.05]	1.00 [0.95 ÷ 1.08]	1.00 [0.96 ÷ 1.07]
0.25	0.68 *# [0.60 ÷ 0.75]	0.60 *# [0.52 ÷ 0.68]	0.59 *# [0.52 ÷ 0.66]	0.89 [0.79 ÷ 0.96]
1.0	0.65 * [0.58 ÷ 0.72]	0.56 *# [0.49 ÷ 0.63]	0.55 *# [0.48 ÷ 0.62]	0.76 * [0.68 ÷ 0.83]
2.5	0.61 * [0.54 ÷ 0.68]	0.52 * [0.46 ÷ 0.59]	0.51 *# [0.43 ÷ 0.57]	0.68 * [0.61 ÷ 0.75]
5.0	0.57 * [0.51 ÷ 0.64]	0.49 * [0.43 ÷ 0.54]	0.48 * [0.42 ÷ 0.55]	0.57 * [0.50 ÷ 0.64]
10.0	0.31 *# [0.27 ÷ 0.35]	0.26 *# [0.23 ÷ 0.31]	0.24 *# [0.20 ÷ 0.29]	0.51 * [0.44 ÷ 0.57]

Note: * – significant differences when compared to the value in the control samples; # –significant differences between the values in the cultures of tumor and non-tumor cells.

In this study, we focused on the study of new azoloazine derivatives, similar in structure to the well-known antitumor drugs mitozolomide and temozolomide (Dwyer et al. 2013; Horishny et al. 2020). The class of compounds consisting of imidazotriazine and imidazotetrazine derivatives we have chosen is attractive in this regard both because of quite well-established representatives, andas well as the emergence of protocols for the synthesis of new compounds with potentially promising properties. So, we conducted the screening of cytotoxic and cytostatic activities of five new azoloazine derivatives synthesized at the Department of Organic Synthesis Technology of Ural Federal University named after the first President of Russia B.N. Yeltsin (Sadchikova et al. 2013; Sadchikova 2016).

The present study confirmed the comparison drug temozolomide to have very moderate cytotoxic and cytostatic activities. For the MCF-7 culture, temozolomide MCS was 2.44 and IC_{50} was 6.81 mM/L; for other cultures CTA indicators were worse. Similarly, the moderate CSA of the drug was confirmed. The pharmacodynamic properties of temozolomide are well-known and they are like those of other azolotetrazine derivatives. The drug is most active in the phases of the cell cycle, accompanied by the most intensive DNA synthesis. After intercalation between DNA base pairs, it stabilizes the topoisomerase II-DNA complex, which leads to irreversible rupture of the DNA strand. For temozolomide, cytotoxicity against breast cancer has been proven both *in vitro* and *in vivo,* and several other human tumor cells; and cell death became stronger with increasing drug concentration (Khodadadi et al. 2019). Therefore, temozolomide can be considered a classic comparison drug for preclinical trials, which are designed to find new more effective drugs for tumor chemotherapy.

Figure 1 illustrates this fact for five studied compounds to have a different CTA and CSA.

According to the results of testing, imidazotetrazine 2 and imidazotetrazine 3 demonstrated CTA indicators lower than that of temozolomide. IC_{50} was not achieved; and the MCS value varied between 1.34 and 1.74. As a result, these two derivatives were classified as compounds with extremely low CTA. Imidazotetrazine 1 and iminothiotriazine 5 showed cytotoxic activity higher than in the comparison drug. According to the test results, we classified these compounds as the ones with moderate CTA. Finally, we found imidazotetrazine 4 with IC_{50} of 0.85 mmol/L and CTA of 7.34 as a compound with a potentially strong anticancer effect for further investigation.

The cytostatic activity of four of the five azoloazine derivatives studied was in a narrow range corresponding to the survival rate from 0.21 to 0.32, depending on the compound and cell culture. Against this background, imidazotetrazine 4 demonstrated a higher CSA, determined by survival rate from 0.17 to 0.20. All these indicators corresponded to a high CSA, which is a characteristic feature of chemotherapeutic drugs with alkylating action (Hassan et al. 2010; Khodadadi et al. 2019; Zhang et al. 2021).

It is important that the cytotoxic and cytostatic effects of the studied drugs on non-tumor cells in all cases were lower than similar effects on breast cancer cell cultures. This fact indicates that the action of the new derivatives is based on the same mechanisms of antitumor action as the

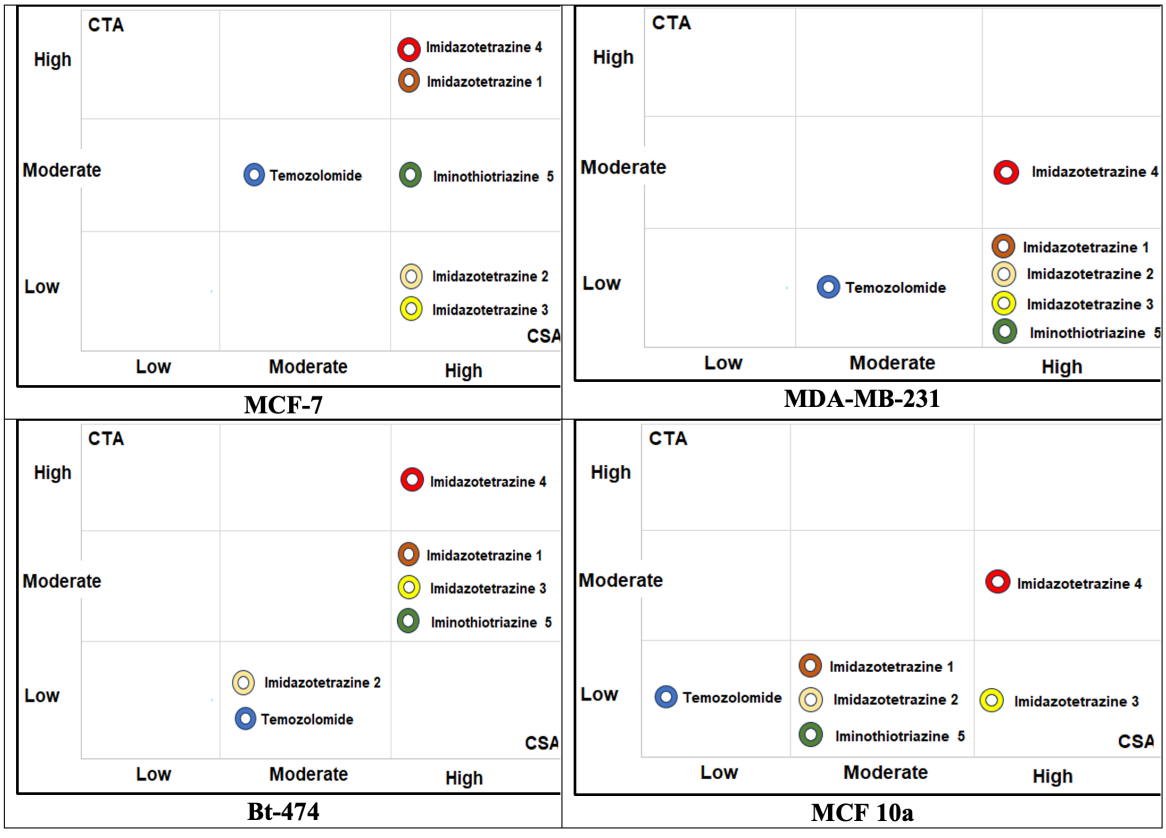


Figure 1. Cytotoxic and cytostatic activities of the studied derivatives against the comparison drug Temozolomide in four cell cultures. Along the abscissa axis, substances are arranged in accordance with the CTA integral assessment, along the axis they are arranged in CSA gradation.

character for other alkylating agents. They are associated with the blockade of DNA replication and repair, and therefore the drug actively attacks rapidly dividing cells, and to a much lesser extent affects untransformed epithelial cells.

A *in vitro* comparison of CTA and CSA of five azoloazine derivatives on cell cultures showed that, despite the similar structure and physico-chemical properties, these compounds exhibit unequal activity with respect to breast cancer cells and untransformed human breast epithelial cells. The known reasons that cause differences in the antitumor effects of homologous molecules are, first of all, differences in the nature of distribution in the body during parenteral administration, and, secondly, the ability to penetrate target cells. The experimental values of the cell survival rate and cell viability *in vitro* obtained by us are consistent with the results of studying other alkylating compounds (Sztanke et al. 2008; Shirazi et al. 2011; Arnedos et al. 2015; Wang et al. 2020) and confirm the important role of the second mechanism in the realization of the pharmacological effects of these compounds.

The development of medical biotechnology and advances in the targeted synthesis of organic compounds with predicted properties have become the basis for a new era in development and implementation of modern chemotherapeutic agents (Nakhjavani and Shirazi 2017; Mansinho et al. 2019; Yan and Yue 2023). Regarding the subject of this study, it should be emphasized that solving the problem of timely diagnosis, treatment and rehabilitation of women suffering from breast cancer is one of the priorities of world health. The selected class of compounds such as azoloazine derivatives is attractive from these positions due to quite well-established representatives, including mitozolamide and temozolomide, as well as the production of new compounds with potentially promising properties.

Conclusion

As a result of an *in vitro* study of cytotoxic and cytostatic activities of five new azolotriazine derivatives on three human breast cancer cell cultures and a culture of non-tumor MCF-10a cells, we found that these substances can be evaluated in ascending order of these properties, as a combination of CTA + CSA in order imidazotetrazine 2, imidazotetrazine 3 < temozolomide < imidazotetrazine 1, iminothiotriazine 5 < imidazotetrazine 4, although the CSA of all the studied compounds turned out to be high. Thus, 3-Cyclohexyl-4-oxoimidazo[5,1-d]-[1,2,3,5]tetrazine-8-N-piperidinyl-carboxamide (imidazotetrazine 4) is an unconditional leader in the tested series of new azoloazine derivatives and we recommend it for further preclinical trials.

Conflict of interests

The authors declare that they have no conflicts of interests.

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Data availability

All of the data that support the findings of this study are available in the main text.

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