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Antitumor Activity of New Methylpyrazolotriazine and Pyrazolobenzotriazine Derivatives Tested on MCF-7, MDA-MB-231, and BT-474 Breast Cancer Cell Lines

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Introduction. Breast cancer (BC) remains a leading cause of cancer incidence and mortality among women worldwide. The high heterogeneity of tumor cells and their morphological and functional variability often complicate the selection of effective treatment regimens. A primary challenge in BC therapy is the development of drug resistance, underscoring the need for novel therapeutic agents.

Aim. To evaluate the potential of two new methylpyrazolotriazine derivatives (MPTA 1, MPTA 2) and two new pyrazolobenzotriazine derivatives (PBTA 1, PBTA 2) as anticancer agents, including their application in BC chemotherapy.

Materials and Methods. The study assessed the cytotoxic (CTA) and cytostatic (CSA) activity of these derivatives at concentrations ranging from 0.25 to 10.0 μM. The effects were tested on BC cell lines MCF-7, MDA-MB-231, and BT-474, and on non-tumorigenic human breast epithelial cells MCF-10A. CTA was determined using the methyl tetrazolium (MTT) assay**,** with the half-maximal cytotoxic concentration ($IC_{50}T$) calculated. CSA was evaluated after longer cultivation time (72 hours), with the $IC_{50}S$ defined as the concentration that inhibited cell growth by 50 %. Statistical analysis employed nonparametric tests (Kruskal–Wallis and Mann–Whitney) with a significance level of $p < 0.05$.

Results. The highest CTA against MCF-7 cells was observed for derivative MPTA 1 ($IC_{50}T$ 8.15 μM), against MDA-MB-231 cells for PBTA1 ($IC_{50}T$ 3.79 μM), and against BT-474 cells for MPTA 1 ($IC_{50}T$ 4.48 μM) and MPTA 2 ($IC_{50}T$ 6.15 μM). All tested compounds exhibited similar CSA against MCF-7 cells, averaging 1.20–1.45 times higher than the reference drug temozolomide (minimum cell viability 55 %). In MDA-MB-231 and BT-474 cultures, MPTA 1 and MPTA 2 exhibited superior CSA compared to PBTA 1, PBTA 2, and temozolomide, with a 1.16–1.50-fold increase. All the investigated derivatives of methylpyrazolotriazine and pyrazolobenzotriazine and the reference drug temozolomide showed low CSA against non-cancerous epithelial MCF-10A cells, with $IC_{50}S$ values exceeding the tested concentration range (> 10.0 μM). Ranking the new methylpyrazolotriazine and pyrazolobenzotriazine derivatives by the combined potency of CTA and CSA yields the following order: MPTA 2, PBTA 1 < temozolomide < PBTA 2 < MPTA 1.

Conclusion. Derivative MPTA 1 (3-(3'-Phenyl-4'-methoxycarbonyl-isoxazolyl)-7-methyl-pyrazolo[5,1-c]triazine) demonstrated the highest CTA and CSA among the tested derivatives and is recommended for further preclinical studies.

Keywords: pyrazolotriazines; cytotoxic activity; cytostatic activity; breast cancer; MCF-7; MDA-MB-231; BT-474; MCF-10A; *in vitro*

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Introduction

Among malignant neoplasms, breast cancer (BC) occupies a leading position among oncological diseases in terms of morbidity and mortality in women worldwide. According to the International Agency for Research on Cancer, the annual global incidence of BC is approaching one million new cases, with approximately 670,000 women dying from the disease each year [1, 2].

The genetic and morphological diversity of BC frequently impedes the selection of effective treatment regimens [3]; this challenge, compounded by the constantly increasing chemoresistance of tumor cells, dictates the constant need to develop new drugs to combat the disease [4, 5].

Alkylating agents are the basis of complex chemotherapy for metastatic BC [5]. Among these antitumor drugs, we studied the effects of new azoloazine derivatives, which are successfully used in clinical practice, with temozolomide being the most well-known [7]. Unfortunately, as for most classes of chemotherapeutic agents, many tumors are currently resistant to temozolomide, making the search for new pyrazolotetrazine and pyrazolotriazine derivatives an urgent priority [8].

The aim of the study was to evaluate the potential of two new methylpyrazolotriazine (MPTA) and two new pyrazolobenzotriazine (PBTA) derivatives as novel anticancer agents by assessing their cytotoxic (CTA) and cytostatic (CSA) activity in BC cell cultures.

Materials and Methods

Four pyrazolotriazine derivatives were studied: 3-(3'-Phenyl-4'-methoxycarbonyl-isoxazolyl)-7-methylpyrazolo[5,1-c][1,2,4]triazine (methylpyrazolotriazine 1, MPTA 1); 3-(4'-Methoxycarbonyl-thiadiazolyl)-7-methylpyrazolo[5,1-c][1,2,4]triazine (methylpyrazolotriazine 2, MPTA 2); 6,8-Dimethoxy-2-(p-tolyl)pyrazolo[5,1-c][1,2,4]benzo[e]triazine (pyrazobenzotriazine 1, PBTA 2); 6,8-Dimethoxy-2-(p-chlorophenyl)pyrazolo[5,1-c][1,2,4]benzo[e]triazine (pyrazolobenzotriazine 2, PBTA 2) synthesized at Ural Federal University named after the first President of Russia B.N. Yeltsin. As the structural formulas of the compounds indicate, MPTA 1 and MPTA 2 are based on a methylpyrazolotriazine core, while PBTA 1 and PBTA 2 are derivatives of pyrazolobenzotriazine (fig. 1).

The purity of the tested compounds, as determined by spectral analysis conducted during synthesis, ranged from 96.2 to 98.1 %.

Temozolomide produced by JSC "Research Institute of Chemical Diversity" (Russia, batch 04630030160045, valid until 20.10.26) was used as the reference drug.

The study utilized three human BC cell lines obtained from the Cell Line Bank of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg): MCF-7, a luminal BC cell line positive for estrogen and progesterone receptors and negative for HER2; MDA-MB-231, a triple-negative basal BC cell line commonly used as a test system for evaluating potential chemotherapeutic agents; and BT-474, a cell model of triple positive luminal BC associated with a less favorable clinical prognosis. To assess the effect of the tested compounds on non-transformed cells, the MCF-10a line of human mammary luminal epithelium was used. Upon thawing, cells were washed twice in Hanks' solution and pelleted by centrifugation at 500 g for 5 minutes. The cells were cultured in 10 ml flasks in a CO_2 incubator (MCO-19M, Sanyo, Japan) using Eagle MEM/DMEM medium supplemented with 1 % glutamine, 1 % streptomycin/penicillin solution, and 10 % fetal bovine serum (all components from PanEco, Russia) at 37 °C in a 5 % CO_2 atmosphere until a monolayer formed. Subsequently, the culture medium was removed, the cells were detached using a 0.25 % trypsin-EDTA solution, pelleted by centrifugation (500 g, 5 min), resuspended in fresh medium, and seeded into 96-well plates at a density of 1 × 10^4 cells per 100 µL per well for subsequent testing.

To determine CTA, a modified methyltetrazolium assay was employed [9, 10], with optimization of seeding density, adaptation time, and incubation period to account for the specific characteristics of BC cell. The final concentrations of the test compounds in the wells were 0.25, 1.0, 2.5, 5.0, and 10.0 µM. These concentrations were selected based on prior empirical testing of other azoloazine derivatives [11]. Negative control wells contained a 1 % dimethyl sulfoxide (DMSO) solution, and positive control wells contained a 10 % DMSO solution. After adding the compounds, the plates were incubated for 1 hour at 37 °C. The medium was then replaced with a 1 % methyltetrazolium solution, followed by a further 2-hour incubation to allow formazan crystal formation. The resulting crystals were dissolved in 96 % DMSO. The optical density of the final solutions in the wells was measured on a MARK flatbed photometer (BioRad, USA) at 530 nm, with immediate subtraction of the background absorbance at 620 nm. The percentage of viable cells was calculated as (sample optical density / negative control optical density) × 100 %. The concentration of the substance causing 50 % cell death ($IC_{50}T$, µM) was calculated using OriginLab software (USA).

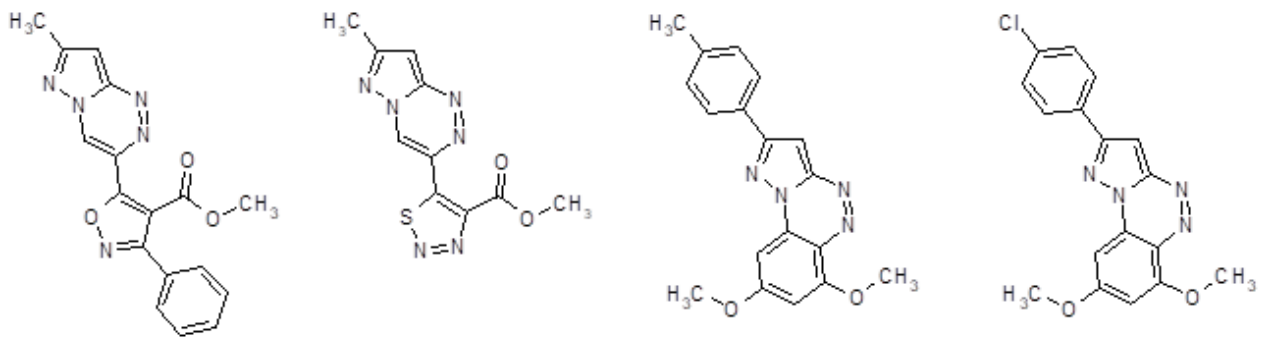

Fig. 1. Structural formulas of the tested methylpyrazolotriazine and pyrazolobenzotriazine derivatives

According to modern concepts, the cytostatic mechanism, which involves inhibiting the proliferation of tumor cells, is considered clinically more effective, since it does not trigger secondary tumor growth driven by colonization of vacated niches or dissemination of cells throughout the body. Therefore, the drug should have not only CTA, but also cytostatic potential [12]. A similar protocol was used to determine CSA, but for initial cultivation, initial cell seeding was performed in 96-well plates at a density of 5,000 cells per 100 μL of culture medium, and the empirically optimized testing period was extended to 72 hours. CSA was calculated as the ratio of optical density in wells containing the test compounds to that in negative control wells. The concentration causing 50 % inhibition of cell proliferation (IC_{50}S, μM) was then determined. This application of the methyltetrazole test is well-established in the phenotypic screening of potential antitumor agents [13].

The Statistica 12.0 software package (Dell, USA) was used for the analysis. After confirming a non-normal distribution via the Shapiro–Wilk test, data were presented as median and interquartile range (Me [Q1÷Q3]). The intragroup comparative analysis was performed using the Kruskal–Wallis test, and the comparison between groups was performed using the Mann–Whitney U test. Differences were considered statistically significant at $p < 0.05$.

Results

Cytotoxic activity of the studied compounds

A comparison of CTA and CSA of four new pyrazolotriazine derivatives on cell cultures of three different molecular genetic types of BC showed that, despite their similar structural and physicochemical similarities, these compounds exhibit distinct antitumor activity profiles.

In MCF-7 tumor cells, MPTA 1 demonstrated a moderate cytotoxic effect at concentrations of 5.0 and 10.0 **μM**, comparable to the reference drug temozolomide. MPTA 2 and PBTA 4 displayed lower cytotoxicity, while PBTA1 was practically inactive (tab. 1).

In MDA-MB-231 cells, the reduction in the percentage of viable cells following exposure to the tested compounds exceeded that of the reference drug in all cases. While the effects were generally similar, the highest CTA was observed for PBTA1 at a concentration of 10.0 μM.

In BT-474 tumor cell cultures, treatment with MPTA1 and MPTA2 resulted in a 2.5-fold decrease in viable cells, which was on average 1.39 times greater than the effect of temozolomide. Compounds PBTA 1 and PBTA 2 showed lower CTA compared to MPTA1 and, with activity comparable to the reference drug. All the described effects were achieved at pyrazolotriazine concentrations of 10.0 μM.

Regarding non-tumorigenic MCF-10a cells, MPTA1 caused a moderate reduction in viability across the entire concentration range, with the minimum percentage of viable cells observed at 5.0 and 10.0 μM. MPTA 2, PBTA 1, PBTA 2, and the reference drug had lower CTA, with minimum MCF-10a cell viability ranging from 67 % to 76 %. The maximal effects for these agents were achieved at concentrations of 5.0 μM (MPTA 2, PBTA 1) or 10.0 μM (MPTA 1, PBTA 2, temozolomide).

The calculation of the concentration causing 50 % cell death (IC_{50}T) for the MCF-7 cell line showed that IC_{50}T values below 10 μM were achieved only for MPTA1 and temozolomide. In other cases, the calculated IC_{50}T was not achieved because it exceeded the maximum tested concentration, indicating lower CTA (fig. 2).

For MDA-MB-231 cells, PBTA 1 demonstrated the strongest effect (lowest IC_{50}T), followed by MPTA 2 and PBTA 2. MPTA 1 and temozolomide showed the lowest activity (highest IC_{50}T) in this cell line.

For BT-474 triple negative BC cells, MPTA 1 exhibited the lowest IC_{50}T, MPTA 2 had slightly lower activity. The remaining compounds, including temozolomide, showed IC_{50}T values above 10.0 μM, indicating relatively lower CTA against this cell line.

Table 1. Percentage of viable cells (Me [Q1÷Q3]) following administration of the maximum tested concentration (10.0 μM) of the studied compounds

Compound	Cell line			
	MCF-7	MDA-MB-231	BT-474	MCF-10a
Methylpyrazolotriazine 1 (MPTA 1)	47 [42 ÷ 53] #	52 [45 ÷ 57]$	39 [35 ÷ 42] *#	59 [52 ÷ 65]
Methylpyrazolotriazine 2 (MPTA 2)	61 [55 ÷ 67] *	44 [39 ÷ 48] *#	40 [35 ÷ 44] *#	67 [60 ÷ 75]
Pyrazolobenzotriazine 1 (PBTA 1)	88 [79 ÷ 97] *$	40 [35 ÷ 42] *#	56 [50 ÷ 63] #$	76 [70 ÷ 85]$
Pyrazolobenzotriazine 2 (PBTA 2)	70 [63 ÷ 79] *$	45 [40 ÷ 51] *#	50 [43 ÷ 56] #$	72 [65 ÷ 80]
Temozolomide	41 [36 ÷ 45] #	61 [55 ÷ 68]	55 [48 ÷ 61] #	69 [62 ÷ 75]

In this and the following table, the * sign indicates statistically significant difference compared to temozolomide treatment within the same cell line, the # sign indicates statistically significant difference between tumor and non-tumor cell lines for the same compound, and the $ sign indicates statistically significant difference between the most active derivative and the other derivatives within the same cell line.

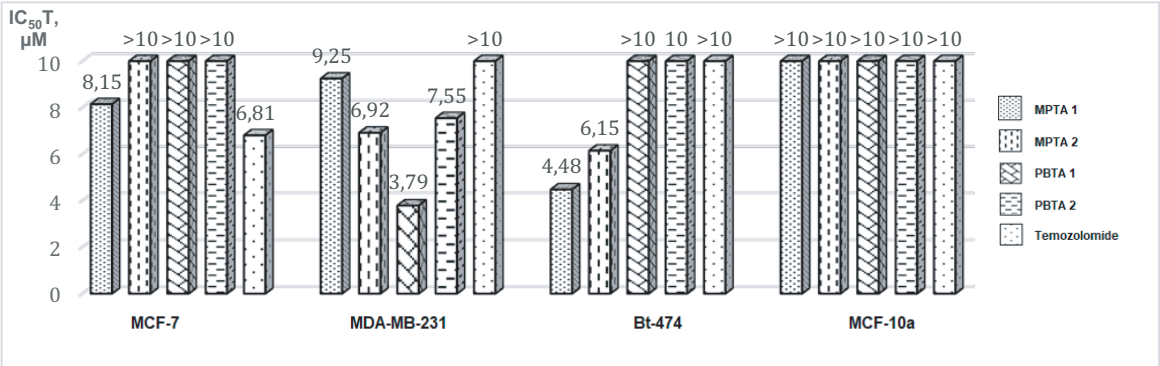

Fig. 2. The concentration causing 50 % cell death (IC50T, μM) after the introduction of the tested compounds into the culture medium

Table 2. Cell viability (Median [Q1÷Q3], % relative to negative control) following administration of the maximum tested dose (10.0 μM) of the studied compounds into the culture medium

Compound	Cell line			
	MCF-7	MDA-MB-231	BT474	MCF-10a
Methylpyrazolotriazine 1 (MPTA 1)	43 [38 ÷ 47] *#	42 [37 ÷ 48] *#	40 [34 ÷ 45] *#	71 [63 ÷ 78]
Methylpyrazolotriazine 2 (MPTA 2)	38 [34 ÷ 43] *#	36 [33 ÷ 41] *#	35 [33 ÷ 39] *#	67 [60 ÷ 75]
Pyrazolobenzotriazine 1 (PBTA 1)	42 [38 ÷ 47] *#	55 [49 ÷ 62] *#$	46 [41 ÷ 52] *#	75 [67 ÷ 84]
Pyrazolobenzotriazine 2 (PBTA 2)	46 [41 ÷ 52] *#	44 [40 ÷ 49] *#	41 [35 ÷ 47] *#	69 [61 ÷ 77]
Temozolomide	55 [48 ÷ 60] #	54 [48 ÷ 60] *	46 [39 ÷ 51] *#	69 [62 ÷ 77]

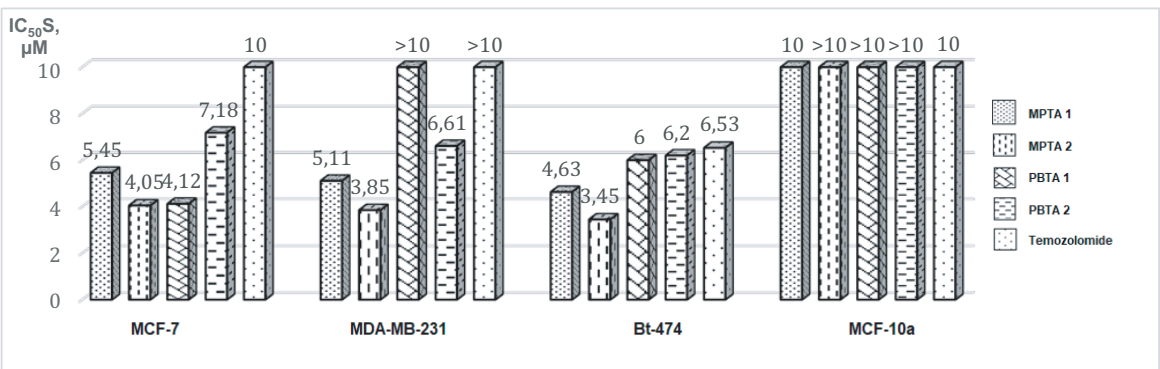

Fig. 3. Concentration causing 50 % inhibition of cell growth ($IC_{50}S$, μM)

All tested compounds showed lower cytotoxicity against non-tumorigenic MCF-10a epithelial cells, with $IC_{50}T$ values exceeding the measurement range (> 10.0 μM).

Cytostatic activity of the studied compounds

The percentage of viable cells following temozolomide administration in tumor cell cultures ranged from 46 to 55 % at a concentration of 10.0 μM, with a slightly higher viability observed in the MCF-10a culture (tab. 2).

In the MCF-7 cell culture, the minimum cell viability was achieved with the addition of the tested compounds at a concentration of 10 μM and was 1.20–1.45 times lower than with the reference drug; practically no differences in CSA were observed between the studied azolotriazines.

Against MDA-MB-231 cells, the compounds exhibited similar CSA, with maximum effects observed at 10.0 μM. The cytostatic effect of MPTA 1, MPTA 2, and PBTA 2 exceeded that of temozolomide by 1.23–1.50 times, while PBTA 1 showed activity comparable to the reference drug.

BT-474 cells demonstrated approximately the same sensitivity to the tested methylpyrazolotriazine and pyrazolobenzotriazine derivatives. After treatment with MPTA 1, MPTA 2, and PBTA 2, CSA was 1.12–1.31 times higher than that of temozolomide. PBTA 1 showed an effect similar to the reference drug.

When tested on non-tumorigenic MCF-10a cells, the CSA of the compounds was either comparable to (MPTA 2, PBTA 2) or slightly lower than (MPTA 1, PBTA 1) that of temozolomide.

The concentration causing 50 % inhibition of cell growth ($IC_{50}S$) for the reference drug, temozolomide, was below 10.0 μM only in the BT-474 cell line; in other cases, it was equal to or greater than this value (fig. 3).

Calculation of IC_{50}S values in MCF-7 cultures showed that for MPTA 1, MPTA 2, and PBTA 1, it ranged from 4.05 to 5.45 μM, slightly lower for PBTA 2, but remained below the value for temozolomide in all cases.

Against MDA-MB-231 tumor cells, only MPTA 1 and MPTA 2 demonstrated sufficiently high CSA. PBTA 2 showed lower activity, and for the other two compounds, the calculated IC_{50}S was not reached, as it exceeded the maximum tested concentration.

In BT-474 cells, the lowest IC_{50}S was observed for MPTA 2, followed by slightly higher values for MPTA 1 and PBTA 1. The remaining compounds, including temozolomide, exhibited IC_{50}S values above 6.0 μM, indicating even lower CSA.

All studied methylpyrazolotriazine and pyrazolobenzotriazine derivatives demonstrated relatively low CSA against non-tumorigenic MCF-10a epithelial cells, with IC_{50}S values exceeding the measurement range (> 10.0 μM). This observed low activity against untransformed human breast epithelial cells is important, as it suggests a potentially lower impact on surrounding healthy tissue and a reduced likelihood of adverse reactions.

As indicated by the results, all tested compounds demonstrated higher CSA than temozolomide against MCF-7 cells. In cultures of MDA-MB-231 and BT-474 cells, only MPTA1 and MPTA2 exhibited similarly activity.

Discussion

It is important to note that for nearly all combinations of "cell line – tested pyrazolotriazine," the CSA values were higher than CTA values. These findings are consistent with similar differences observed for new imidazotetrazine derivatives we previously studied [11], as well as experimental data obtained *in vitro* when studying other alkylating compounds [14, 15]. This confirms the significant role of the cytostatic mechanism in the overall pharmacological effect of this class of compounds. The fact that the CSA values for several derivatives were significantly higher than for temozolomide further validates the pursuit of new, more effective pyrazolotriazines as potential replacements.

The ratio of CTA and CSA is crucial for the ultimate efficacy of an antitumor agent. The cytotoxic mechanism eliminates tumor cells, reducing primary tumor volume, metastatic foci, and the number of circulating tumor cells. However, the vacated space can subsequently be colonized by cells resistant to this mechanism, leading to tumor recurrence. Polychemotherapy, or ideally, a single drug, should combine both modes of antitumor action: a strong CTA coupled with a no less pronounced CSA on cell population [16, 17]. This is exactly the dual activity we observed when testing the new methylpyrazolotriazine and pyrazolobenzotriazine derivatives.

The known reasons for differences in the antitumor effects of homologous molecules are, firstly, their distribution in the body and within target cells, and secondly, their ability to penetrate cells and exert the primary pharmacological effect [18]. he latter can be assessed via *in vitro* experiments.

Among the studied compounds, MPTA 1 and MPTA 2 differ in their core structure: MPTA 1 contains a phenyl group and an isoxazolyl ring as part of the main substituent, whereas MPTA 2 features a thiadiazolyl ring and lacks the phenyl group. PBTA 1 and PBTA 2 were based on 6,8-Dimethoxy-[5,1-c][1,2,4]benzo[e]triazine core and differ in the presence of a methylbenzene versus a chlorobenzene substituent, respectively (fig. 1).

Apparently, differences in the core pyrazolotriazine structure are responsible for the overall higher activity of MPTA 1 and MPTA 2 compared to PBTA 1 and PBTA 2. Their higher activity against different cell lines (MCF-7 and MDA-MB-231 for PTA1 and BT-474 for PTA2) may be explained by the distinct structure of their respective substituents. Some of the observed differences in cell line sensitivity to pyrazolotriazine derivatives seem to be related to the different expression of three key membrane markers: estrogen, progesterone, and HER2 receptors. Elucidating the precise mechanisms underlying these effects, of course, require separate, detailed research at the molecular genetic level.

Prospects for further research are seen, firstly, in the further preclinical study of the leader compound, as well as in the expansion of studies of cell lines of other histogenesis and localization.

According to the results of *in vitro* cytotoxicity and cytostatic properties testing on three human BC cell cultures and MCF-10a non-tumor cell culture of four new benzoloazolotriazine derivatives against the comparison drug temozolomide, it was shown that all of these substances possess CTA and CSA and can be arranged in the following order of increasing activity: methylpyrazolotriazine 2, pyrazolobenzotriazine 1 < temozolomide < pyrazolobenzotriazine 2 < methylpyrazolotriazine 1. The last derivative, (3-(3'-Phenyl-4'-methoxycarbonyl-isoxazolyl)-7-methylpyrazolo[5,1-c][1,2,4]triazine), is therefore the leader among the tested new compounds and is recommended for further preclinical trials. As a separate point, it is necessary to note the high sensitivity of Bt-474 triple negative BC cells to methylpyrazolotriazine 2 (3-(4'-Methoxycarbonyl-thiadiazolyl)-7-methylpyrazolo[5,1-c][1,2,4] triazine).

Conclusion

1. Compound MPTA 1 demonstrated cytotoxic activity against MCF-7 cells ($IC_{50}T$ = 8.15 μM), PBTA 1 was most potent against the MDA-MB-231 line ($IC_{50}T$ = 3.79 μM), and both MPTA 1 and MPTA 2 exhibited cytotoxicity against BT-474 cells ($IC_{50}T$ = 4.48 μM and 6.15 μM, respectively).

2. Compounds MPTA 1, MPTA 2 and PBTA 3 showed cytostatic action against against MCF-7 cells ($IC_{50}S$ = 4.12–5.45 μM), compounds MPTA 1 and MPTA 2 were effective against the MDA-MB-231 cell line ($IC_{50}S$ = 3.85 μM and 5.11 μM, respectively). All studied methylpyrazolotriazine and pyrazolobenzotriazine derivatives displayed cytostatic activity against BT-474 cells ($IC_{50}S$ = 3.45–6.20 μM).

3. Based on the combined profile of CTA and CSA, the most promising compound for further study is MPTA 1 (3-(3'-Phenyl-4'-methoxycarbonyl-isoxazolyl)-7-methylpyrazolo[5,1-c][1,2,4] triazine).

Conflict of interest
The authors declare no conflict of interest.

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Compliance with patient rights and principles of bioethics
The study was conducted in accordance with the ethical standards of the Declaration of Helsinki (2013 revision). The study protocol was reviewed and approved by the Ethics Committee of Volgograd State Medical University (Protocol No. 2021/049, dated May 27, 2021).

Authors' contributions
All authors confirm that their contributions comply with the International Committee of Medical Journal Editors (ICMJE) authorship criteria.All authors have read and approved the final version of the manuscript prior to its submission and and agree to be accountable for all aspects of the work, implying proper investigation and resolution of issues related to the accuracy or integrity of any part of the work.

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