

In vitro cytotoxic effect of 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine on human fibroblasts, peripheral blood mononuclear cells and breast cancer cells

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2-(Morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine having QSAR-predicted anti-tumor activity was tested for the cytotoxicity using MTT and LDH cell viability tests. The experiments were conducted using human fibroblasts, peripheral blood mononuclear cells and breast cancer cells and allowed to identify effective cytotoxic concentration and therapeutic range of this compound. The data obtained suggest the feasibility of the further studies of the test compound as a potential anti-cancer agent.

Keywords: anticancer agent, cytotoxicity, cell viability test, QSAR

NO donors find a variety of applications in current medicine. This is supported by diverse functions of nitrogen (II) oxide in living organisms [1, 2]. One of the most significant applications of nitrogen (II) oxide is the anti-tumor therapy [1, 2, 3, 4]. This work presents the data from a study of cytotoxic activity of 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine which was previously synthesized in Samara State Technical University, Russia [5].

The interest to 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine as a potential anti-tumor agent is supported by the fact that a number of 1,3,5-triazine amino derivatives having a morpholine moiety have already been tested and used as anti-tumor therapies [6]. The cytotoxic action of 1,3,5-triazine derivatives is associated with the formation of active metabolites covalently linking DNA molecules and thereby prevention cell division [7], as well as with the generation of nitrogen oxide damaging the macromolecules [8, 9, 10, 11, 12, 13]. Hexamethylmelamine (Altretamine, Figure 1A) which is used in ovarian cancer chemotherapy regimen is an example of such compounds. Another 1,3,5-triazine derivative, trimethyloltrimethylmelamine (trimelamol, Figure 1B), was used in clinical trials up to

Phase II but was ultimately refused due to its high toxicity, poor stability and solubility, despite good efficacy for a number of malignancies.

Therefore, there is a need in such compounds having good solubility and stability as well as low toxicity for normal cells. At the same time, partial replacement of amines for groups comprising polynitromethyl moieties could improve NO donor activity.

We have synthesized 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine (Figure 1C) which meets these requirements and potentially has an anti-tumor activity as estimated by QSAR/QSPR. 2-(Morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine, like a number of polynitromethyl triazine derivatives, can be an effective NO donor. Note that triazine derivatives with trinitroethoxy moieties have not previously been tested as the potential anti-tumor agents.

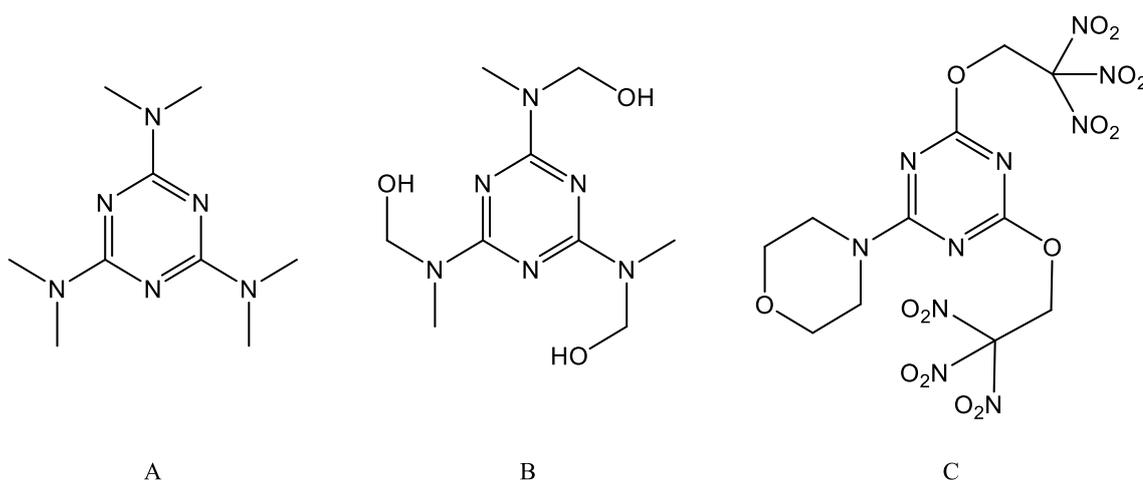


Figure 1. Structure formula of triazine compounds having an anti-tumor activity. A, altretamine; B, trimelamol; C, 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine.

The aim of this work is to study an in vitro cytotoxic effect of 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine on human fibroblasts, peripheral blood mononuclear cells and breast tumor cell cultures.

Experimental Part

Materials and Methods

The study was conducted in the Institute for Experimental Medicine and Biotechnologies, Samara State Medical University, Russia. 2-(Morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine synthesized in Samara State Technical University, Russia [5] was used in the experiments.

This compound was tested for physicochemical properties, potential biological effects and therapeutic targets using PASS Online (<http://www.way2drug.com/passonline/index.php>, [14]) web resource. A significant effect was assumed to meet activity level (Pa) ≥ 0.7 for separate enzymes and metabolic pathways and ≥ 0.6 for cytotoxicity towards tumor cell lines.

The compound was dissolved in 50 μL of dimethyl sulfoxide (Panreac Quimica SAU, Spain) and used *ex tempore* in appropriate concentrations. Doxorubicin (LENS Farm LLC, Russia) commonly used in standard cancer chemotherapy regimen was a reference drug. All experiments were performed at least in triplicate.

Human primary dermal fibroblast culture, PHA activated peripheral blood mononuclear cells (PBMC) and BT474 HEP2-positive breast cancer cell line were used to assess the compound cytotoxic activity. Such an approach would ensure comparison of the test compound effect on the malignant target cells, connective tissue cells presenting in most organs and body parts, and blood cells inherently contacting any substance being administered by parenteral mode.

Fibroblasts were grown using a primary explant technique [15] with complete cell culture medium (199 medium supplemented with 10% fetal calf serum and 40 $\mu\text{g}/\text{mL}$ gentamycine, PanEco Biolot, Russia) in 96-well plates inoculated with 2×10^4 cells/ cm^2 in MCO-17AI CO_2 incubator (Sanyo, Japan) at 37°C , 5% CO_2 and constant humidity. Before test, the culture was identified and characterized using morphological and genetical methods. This examination confirmed that the cells were unipotential fibroblastic lineage cells. PCR showed no culture contamination, including by mycoplasmas and cytomegalovirus. Upon obtaining a confluent monolayer, the

appropriate wells were added with the test compound in various concentrations and incubated for 5 days at 37°C, 5% CO₂ and constant humidity.

Mononuclear blood cells (lymphocytes) were isolated from the heparinized venous blood of adult healthy volunteers by gradient centrifugation with 1.077 g/cm³ Ficoll solution (PanEco, Russia). Cells were counted and assessed for viability in a counting chamber using 0.1% Tripan Blue, considering >90% viability satisfactory. Lymphocytes were incubated with 5 µg/mL phytohemagglutinin P (PHA, Sigma-Aldrich, USA) and the test compound for 5 days in the complete RPMI-1640 medium supplemented with L-glutamine, streptomycin, 20 mg/mL HEPES, 10% fetal calf serum (PanEco, Russia) at 37°C, 5% CO₂. Lymphocyte count was 800000 cells/400 µL. After 5 days, the plates were removed from an incubator and used for cytotoxicity testing. The wells with cells and no test compounds were used as control samples, and the wells without both cells and test compound were blanks.

Cytotoxicity tests used in our study included MTT and LDH tests [16, 17].

MTT test is based on a tetrazolium dye readily penetrating cell membranes and being converted to a colored formazan by mitochondrial enzymes in live cells. These enzymes are inactive in dead cells and cannot conduct such a conversion, therefore the color intensity is directly proportional to the number of live (i.e., survived after the exposure to an agent) cells in the well.

The test was performed as follows.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Merck, USA) was dissolved in the growth media for a concentration of 3 mg/mL. From each well, 100 µL of growth media was drawn and replaced for 100 µL of tetrazolium dye solution. The plate was covered, carefully stirred by circular motion, placed to a thermostat at 37°C and incubated for 3 hours.

After incubation, the medium was carefully removed from each well while holding the plate at an angle of 45 degrees. Then each well was added with 250 µL of dimethyl sulfoxide and pipetted until complete dissolution of the formazan.

Color intensity was measured at 540 nm on Tecan Infinite M200 plate reader (Tecan Instruments, Austria). Cell viability was calculated using a formula $s = \frac{A_{exp}}{A_c - A_b} \times 100\%$, wherein A_{exp} , A_c and A_b are absorbance values in test, control and blank wells, respectively.

LDH activity test is based on the measurement of activity of lactate dehydrogenase (LDH), an enzyme which is usually located within cells and only enters the medium upon the cell destruction. Thus, LDH activity in the medium is directly proportional to the number of destroyed (dead) cells.

This test was performed as follows.

At the first step, the medium from each well was transferred to a separate 1.5 mL tube. 0.1 mL of the medium was placed to a 3 mL quartz cell containing 2.9 mL of a buffered substrate including 0.1 M phosphate buffer (pH 7.8), 1% (w/w) sodium bicarbonate, 0.01 M sodium pyruvate and 1 mg/mL reduced nicotinamide adenosine dinucleotide (NADH₂, LDH cofactor) in the ratio of 25:1:1:3 by volume.

The absorbance was measured on SF-56 spectrophotometer (LOMO, Russia) in a kinetic mode against the buffered substrate without NADH₂ at 340 nm under room temperature for 5 min to record the initial (E1) and final (E2) absorbance values. The enzyme activity was determined as follows:

$$A1 = \Delta E \times 1000 \times 3 \times B/5 \times 6.22, \text{ wherein}$$

ΔE is the difference of E1 and E2;

6.22 is the extinction of 1 μM NADH₂ at 340 nm for optical length of 1 cm;

1000 is a factor to convert μM to nM;

3 is the cell volume in mL;

B is an adjustment for dilution (if any);

5 is recording time in min.

This step allowed to estimate LDH activity in a solution due to dead cell lysis.

Then the survived cells remaining in the well were lysed by adding 250 μ L of distilled water followed by 3 freeze-thaw cycles, and the LDH activity of these cells (A2) was measured in the resulting solution by a similar manner.

This allowed to estimate the number of survived cells. After this, a percentage of survived cells from the total number of both survived and dead cells was calculated using a formula:

$$A = \frac{A2}{(A1 + A2)} \times 100\%$$

The data were statistically processed in Microsoft Office Excel 2016. The results considered significant at $p < 0.05$.

Results and Discussion

Estimated solubility of the test compound in aqueous media is $\sim 10^{-4}$ M (ALOGPS 2.1, <http://www.vcclab.org/lab/alogs/>). The predicted bioactivity and cytotoxicity values are listed in Table 1.

Table 1

Predicted bioactivities and cytotoxicity of 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine

Pa	Pi	Activity	Cell line	Tumor
0.948	0.001	Increase in lipid catabolism	-	-
0.836	0.002	Interleukin agonist	-	-
0.799	0.001	Interleukin 12 agonist	-	-
0.778	0.004	Restenosis therapy	-	-
0.747	0.005	Inflammatory bowel disease treatment	-	-
0.691	0.030	Antiischaemic effect	-	-
0.701	0.076	Phobic disorders treatment	-	-
0.624	0.018	Antianginal effect	-	-

0.611	0.082	Membrane permeability inhibitor	-	-
0.578	0.013	Cytotoxicity	Kasumi 1	Childhood acute myeloid leukemia
0.578	0.017	Cytotoxicity	PC-3	Prostate carcinoma
0.554	0.002	Cytotoxicity	MDA-MB-361	Breast adenocarcinoma

Notes: P_a , probability of the activity; P_i , probability of lack of the activity

Table 1 shows that 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine potentially has antiinflammatory, antianginal, antiischaemic activities and can cause cytotoxic effect on childhood acute myeloid leukemia, prostate carcinoma and breast cancer cells. To confirm the information on the predicted cytotoxic activity of the test compound, *in vitro* studies were performed yielding the following results.

Fig. 2 shows MTT test results which illustrate that 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine below 100 nM did not affect fibroblasts, PHA-activated mononuclear cells and cancer cells. With higher concentrations, the test compound produced much more significant effect on BT474 breast cancer cells vs. fibroblasts and PBMC. The test compound significantly affected the tumor cells at concentrations higher than 600 nM ($p < 0.05$) whereas such cut-offs were approximately 1.5 μ M for fibroblasts and 2.0 μ M for PBMC. At the same time, LD_{50} for BT474 cells was $1.4 \pm 0.3 \mu$ M, and at higher concentrations of the test compound (2.5 - 5.0 μ M), BT474 viability was twice lower vs. normal cells.

Taken together, these results suggest that 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine could be considered as an anti-tumor drug candidate for HER2-positive breast cancer treatment and recommended for further pre-clinical studies.

Upon that, this compound preliminary therapeutic concentration range is 0.6 - 2.0 μM (as indicated with a green area in the Figure) as these concentrations significantly reduce cancer cells viability with no changes in the normal cells viability vs. control values.

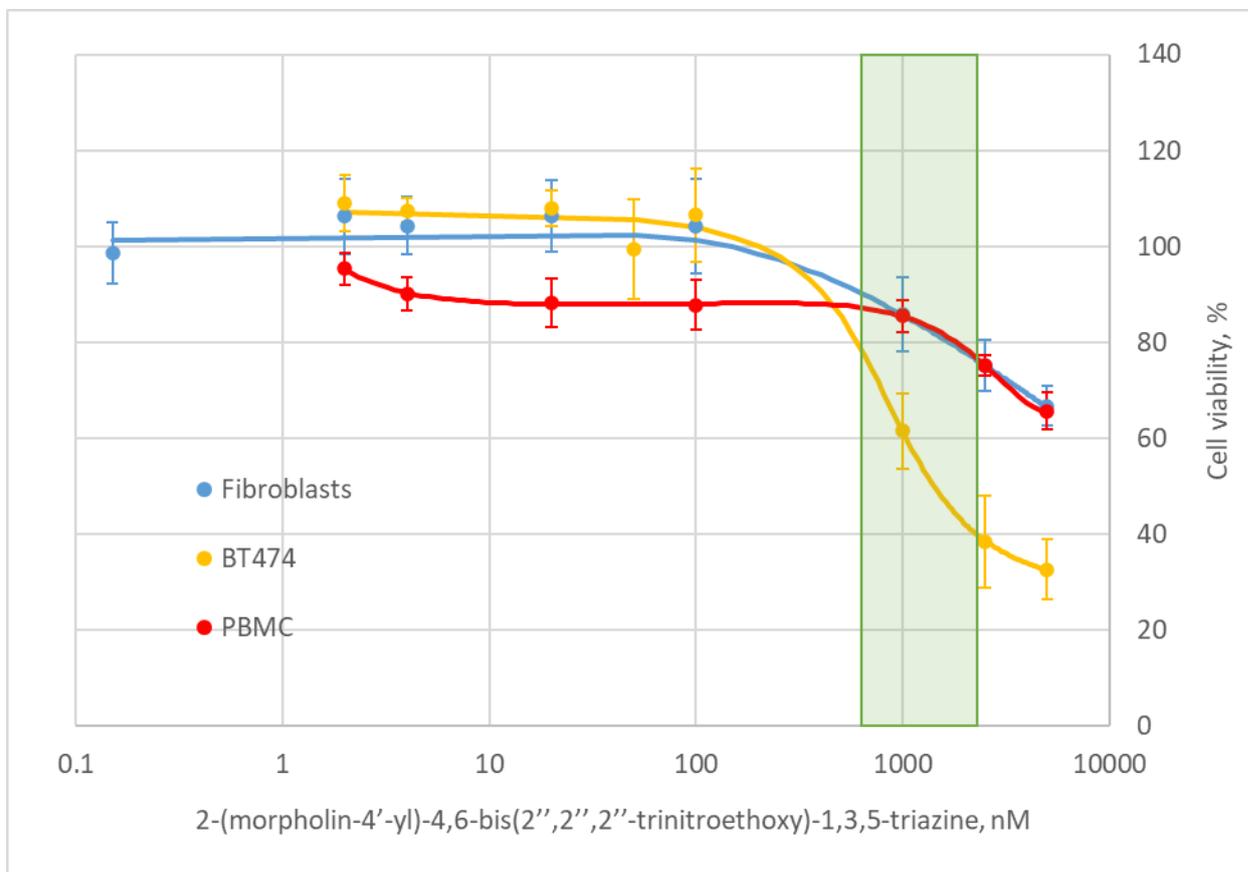


Figure 2. MTT test results. Cell viability against test compound concentration.

Because 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine had a similar concentration-dependent effect on fibroblasts and PBMC viability (correlation coefficient is 0,93, $p < 0,01$), LDH test was only performed for fibroblasts and BT474 cells.

The test results were essentially the same as for MTT test (Figure 3), except for the cut-off concentration value for fibroblasts which was of 3.6 μM , with LD50 for BT474 cells of $2.5 \pm 0.4 \mu\text{M}$.

Correlation coefficients for MTT vs. LDH tests were 0.78 and 0.96 ($p < 0.05$) for fibroblasts and BT474 cells, respectively. This means that both LDH and MTT

tests yield a similar estimation of the test agent cytotoxicity for cancer cells, with only an insignificant difference in the sensitivity for fibroblasts.

However, for the safety reasons we recommend to use the therapeutic range of 0.6 - 2.0 μM as determined by MTT test.

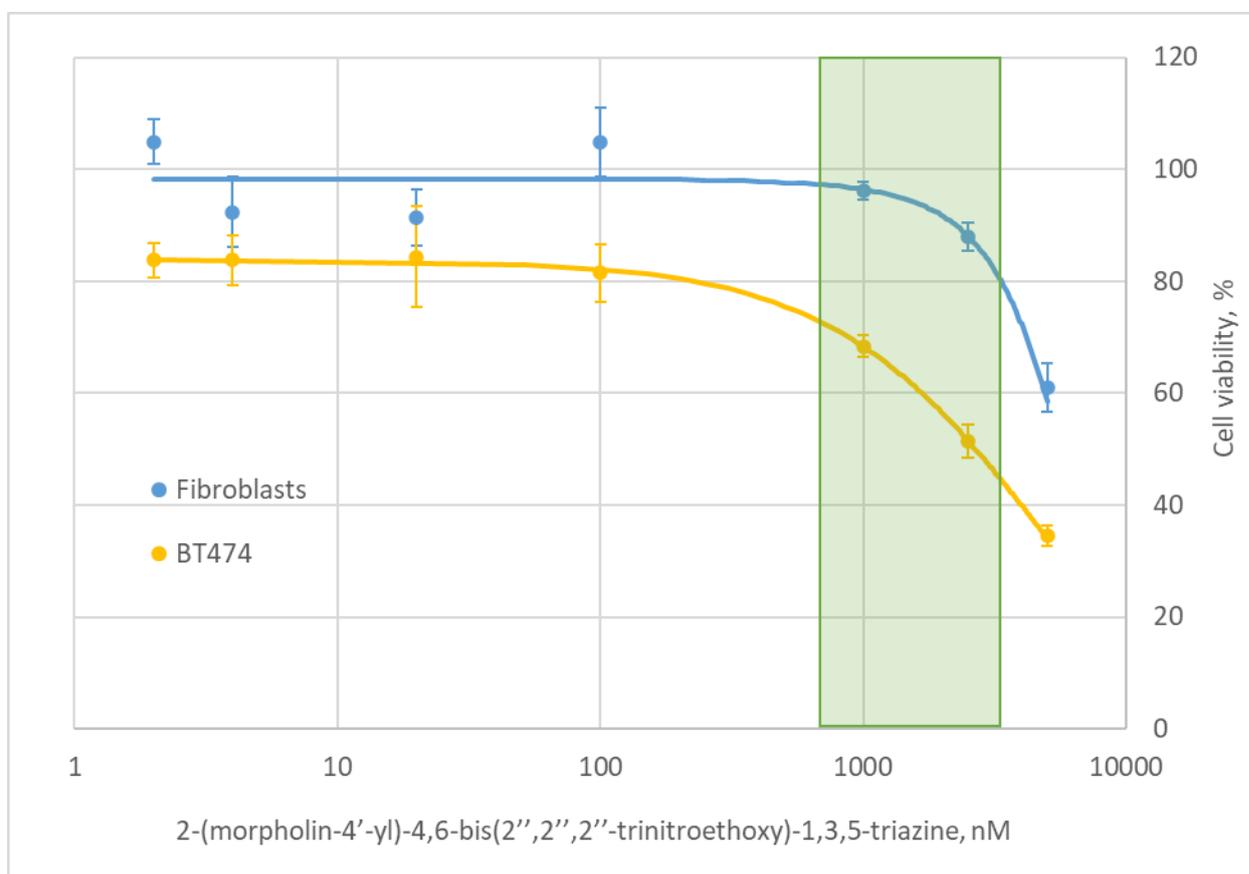


Figure 3. LDH test results. Cell viability against test compound concentration.

We also used MTT test to compare cytotoxicities of the test compound and a widely used anti-tumor drug, doxorubicin. This study has shown that doxorubicin in the concentration range less than 5 nM had a mild, statistically insignificant stimulatory effect on BT474 cells proliferation which disappeared with higher concentrations (5-30 nM), where the number of survived cells was actually the same as in the control (Figure 4). Significant cytotoxicity was only revealed using LDH test for doxorubicin concentrations of 30 - 50 nM, but MTT test did not suggest any effect of these concentrations on the tumor cells. At the same time, there are reports that, with 7-day exposure, LD_{50} of doxorubicin for BT474 cell line was about 10 nM, and

concentrations of about 100 nM caused almost 100% cell death [18]. This discrepancy seems to be associated with shorter incubation time (5 days) in our experiments. Note that other malignant cell lines are characterized by other effective doxorubicin concentrations vs. the above ones, e.g., LNCaP and HepG2 cells (prostate and liver cancer, respectively) are only susceptible to doxorubicin in micromolar range [19, 20], and doxorubicin therapeutic index (a ratio of 75% inhibitory to 10% lethal dose) in mice is about 0.3 [21].

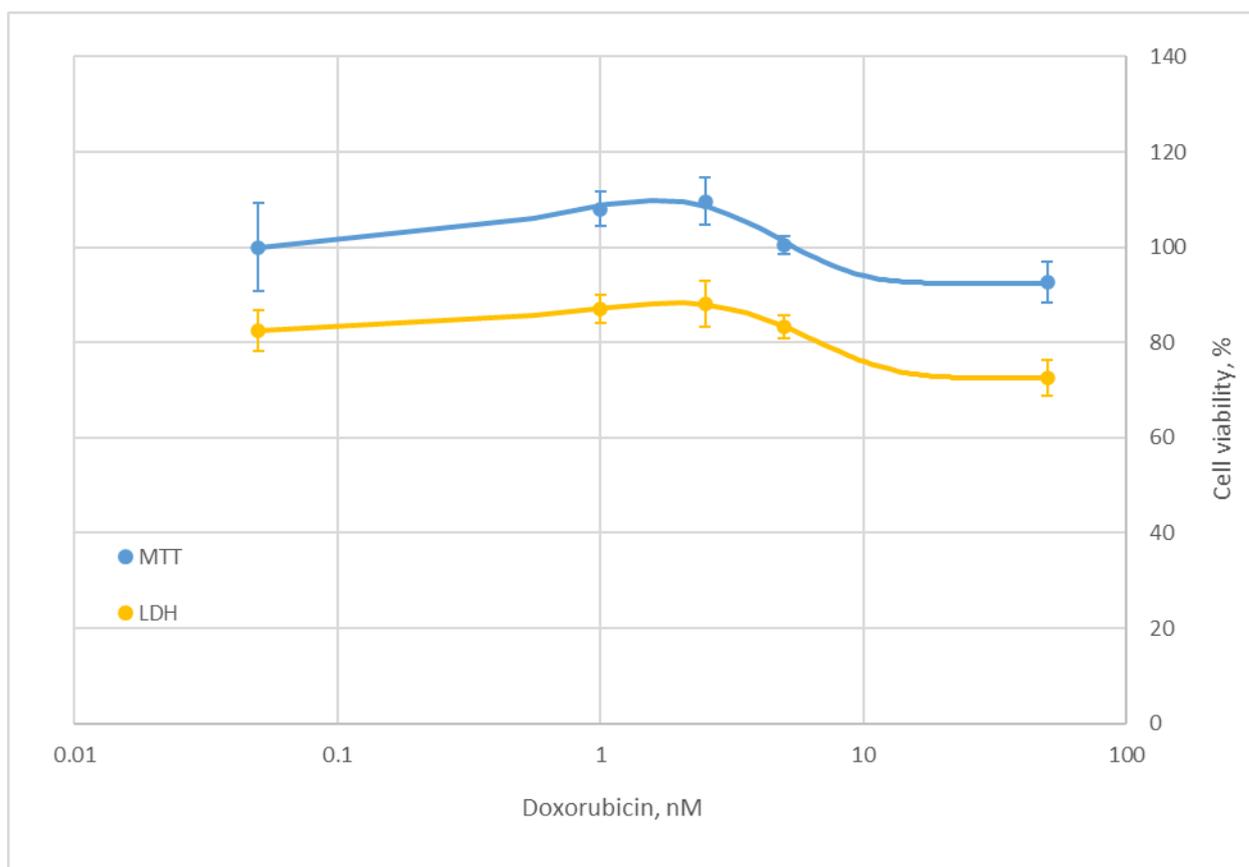


Figure 4. BT474 cell viability vs. doxorubicin concentration.

This, 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine in the concentrations larger than 0.6 μ M had a significant cytotoxic effect for a breast cancer cell line as demonstrated by both tests. This suggests that the test compound has two order higher cytotoxic concentrations vs. doxorubicin; however, clear therapeutic range makes it a suitable candidate for further studies.

Table 2. Summary cytotoxic efficacy of 2-(morpholin-4'-yl)-4,6-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine and doxorubicin

Agent	Minimum cytotoxic concentration, μM			
	For BT474		For fibroblasts	
	MTT test	LDH test	MTT test	LDH test
2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine	0.6	0.7	2.0	3.6
Doxorubicin	Outside the test range	0.007	-	-

Conclusion

1. 2-(Morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine possesses a cytotoxic activity for breast cancer cells with a smaller effect on fibroblasts and blood mononuclear cells.
2. A preliminary therapeutic range of this compound is 0.6 - 2.0 μM .
3. 2-(Morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine is a promising candidate for further anti-tumor efficacy studies.

The data obtained makes a basis allowing to conduct further studies of 2-(morpholin-4-yl)-4,5-bis(2'',2'',2''-trinitroethoxy)-1,3,5-triazine which, in a case of their success, could become an active substance of a novel chemotherapy for breast cancer treatment.

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