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Research Article

An effective way for targeting EGFR-mediated carcinogenesis: an in vitro study

Viktoria A. Pakina[1], Evgeniya Z. Iksanova[1], Evgeniya V. Shikh[1], Oksana M. Tumutolova[2], Karen K. Arutiunian[2], Irina V. Kargina[2], Kirill D. Blinov[1], Fedor P. Pilgaev[2], Anna A. Epishkina[3,4], Dmitrii S. Blinov[5], Evgeny V. Grebenkin[3,4], Ekaterina V. Blinova[1,4]

1. Sechenov University, 2 Bolshaya Pirogovskaya St., Bldg 4, Moscow 119435 Russia
2. N.P. Ogarev National Research Mordovia State University, 68 Bolshevistskaya St., Saransk 430005 Russia
3. LLC "UNIM", 11 Ordzhonikidze St., Bldg 1A, Moscow 115419 Russia
4. National Research Nuclear University MEPHI, 31 Kashirskoe Shosse, Moscow 115409 Russia
5. Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology, 1 Samora Machela St., Moscow 117997 Russia

Corresponding author: Ekaterina V. Blinova (bev-sechenov@mail.ru)

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Abstract

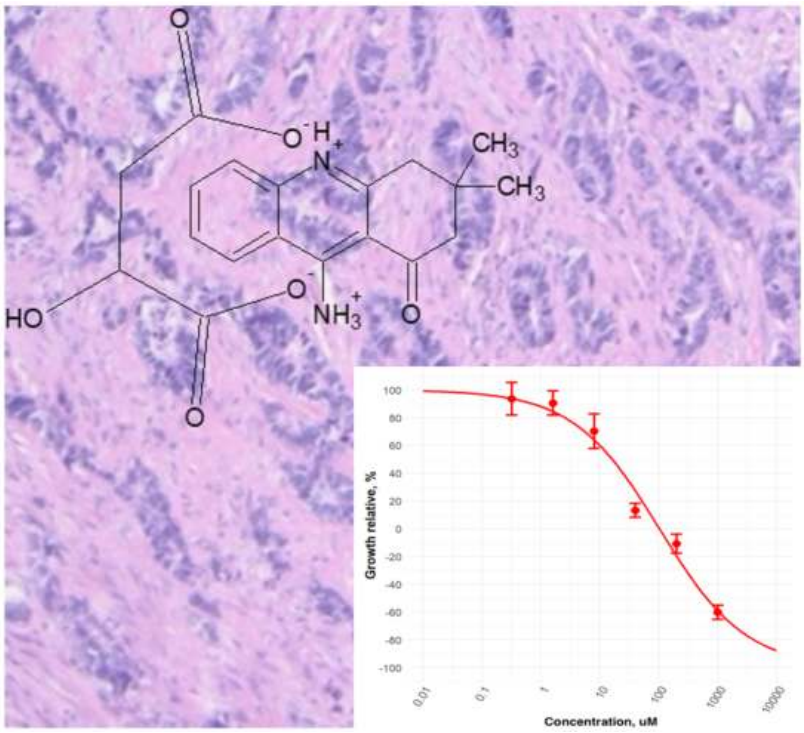
Introduction: EGFR-activating overexpression or somatic mutations are common in different human cancers. In this regard, the search for promising ways to control the carcinogenic transformation of tumor cells and the progression of malignant tumors expressing EGFR seems to be one of the most promising and developing areas of modern molecular pathology and pharmacology.

Material and Methods: An antitumor activity of a novel compound, a pyridine carboxylic acid derivative LHT-17-19, was studied. The molecule was developed and synthesized at the Department of Chemistry, Drug Design and Technology of All-Russian Research Center for Biological Active Compounds Safety (Russia). The study was carried out in cell cultures of stomach cancer (Hs746T, AGS and MKN1) and patient-derived organoid (PDO) model of breast cancer (BC) expressing wild-type EGFR.

Results: It was shown that LHT-17-19 induced concentration-dependent cytotoxicity of EGFR-expressing gastric cancer cells of all the aforementioned cultures. Pathomorphological, immunohistochemical and molecular validation of BC organoids derived from ductal breast carcinoma cells of a 68-year-old patient was done. PDOs were established as ER-negative, PR-negative, Her2/neu-negative, EGFR-positive with 35% of the Ki-67 expression index. In addition, the tumor cells translocation was resulted in a loss of ER expression and PDOs molecular pattern conversion towards a more aggressive triple negative type. PDOs incubation with 0.5-60.0 μM LHT-17-19 was accompanied not only by inhibition of their growth and proliferation, but also by significant cytoreduction.

Conclusion: Thus, in two-dimensional and three-dimensional tumor cell cultures, the possibility of controlling the oncogenic expression of EGFR with the acridone compound 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxybutanedivacate (LHT-17-19) was shown.

Graphical abstract



Explanation: Cytoreductive effect of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxybutane against triple negative EGFR-expressing breast cancer cells.

Keywords

cytotoxicity, EGFR-mediated carcinogenesis, LHT-17-19, patient-derived organoid, pyridine compound, tumor cell cultures

Introduction

Malignant neoplasms continue to occupy a leading position in the structure of mortality throughout the world, second only to cardiovascular diseases. In terms of incidence, stomach cancer ranks 7th in the world (Ferlay et al. 2020), while breast cancer (BC) is the most common cancer in women. The most aggressive form of BC is suggested to be triple negative molecular subtype (TN) with hypo-expression of estrogen (ER), progesterone (PR) receptors and human epidermal receptor type 2 (Her2) (Goldhirsch et al. 2013). Due to its distinct molecular phenotype, TN BC demonstrates a high rate of resistance to endocrine or targeted therapy. Hence, the efficacy of traditional postoperative adjuvant chemoradiotherapy against TN BC is low, so it is of the great importance to identify molecular targets and develop effective treatment strategies with the prospect of further clinical implementation.

Epidermal growth factor receptor (EGFR/ErbB1) is a receptor tyrosine kinase of the ErbB family of proteins (ErbB1-4). EGFR undergoes homo- or hetero-asymmetric dimerization in response to ligand stimulation, which subsequently leads to autophosphorylation of EGFR at key tyrosine residues in its intracellular domain, which in turn activates downstream signaling cascades regulating cell growth (Roskoski 2014; Sigismund et al. 2018).

EGFR-activating overexpression or somatic mutations are common in different human cancers (Lin et al. 2014). Clinicopathological and *in vitro* studies have shown that EGFR expression has prognostic significance in several types of tumors such as BC (Farooqui et al. 2015; Abba et al. 2020) and gastric cancer (Yun et al. 2012; Xia et al. 2019). Overexpression of EGFR is associated with increased tumor cell survival, metastasis, invasion, chemotherapy resistance, and poor prognosis (Mendelsohn et al. 2001; Weihua et al. 2008). To overcome chemoresistance and block/inhibit EGFR activity, both monoclonal antibodies and small molecule tyrosine kinase inhibitors have been developed as therapeutic options for EGFR-dependent cancers (Mendelsohn et al. 2001; Roskoski 2014; Sigismund et al. 2018). In this regard, the search for promising ways to control the carcinogenic transformation of tumor cells and the progression of malignant tumors expressing EGFR seems to be one of the most promising and developing areas of modern molecular pathology and pharmacology.

Acridine derivatives are a well-known source of many antitumor drugs (Dudina et al. 2018). The scientific team of the All-Russian Research Center for Biological Active Compounds Safety (Russia), during an extensive search for new chemical structures with low molecular weight and antitumor properties, has selected promising dihydroacridone derivatives with various amino- and carbonic acid moiety. Quantitative structure-activity analysis of the chemical structures, carried out using opensource PASS® software, has shown that the most active molecule with laboratory code LHT-17-19 possesses a wide range of antitumor activity with a predicted score above 0.87 and a prognostic EGFR inhibitory property (Pa>0.9). Equally advantageous is the fact that the derivative, 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxybutanediovate, can be easily synthesized in the laboratory equipment.

The aim of this study was to evaluate the antitumor properties of the novel acridone derivative LHT-17-19 in wild-type EGFR-expressing *in vitro* settings.

Materials and Methods

Ethics

The protocol of this study included the use of living tissues and cells of patients for scientific purposes, and therefore the research protocol was submitted for consideration to the Local Ethics Committee of the First Moscow State Medical University named after I.M. Sechenov Ministry of Health of Russia (Sechenov University), and approval was received at the committee meeting dated June 15, 2023 (meeting minutes No. 11-23).

EGFR-expressing cancer cell cultures

The following human gastric cancer cell lines were used in this study, as listed in Table 1. The MTT assay was used to measure cellular metabolic activity as an indicator of tumor cell viability, proliferation, and cytotoxicity. This colorimetric assay is based on the reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT from Merck Sigma-Aldrich, Germany) to purple formazan crystals by metabolically active mitochondrial reductase cells.

To evaluate the ability of compound LHT-17-19 to effectively inhibit EGFR kinase activity on various EGFR-positive tumor cells from human gastric cancer cultures expressing wild-type EGFR kinase (amount of cells – 10^5/mL), the cells were treated with increasing doses of compounds (from 0.5 to 10 μM) for 24 hours, and the phosphorylation status of EGFR was assessed as a marker of its kinase activity using Western blotting. To knock down EGFR in the original cells and in the corresponding cells treated pharmacologically with the compound LHT-17-19, 500 ng of EGFR siRNA was used (catalog number EHU076761, manufacturer Merck Sigma-Aldrich, Germany) in 12-hole format.

EGFR-expressing organoid model

To form the organoids, fresh tumor tissue from a 68-year-old patient with bilateral breast cancer who had not previously been exposed to chemotherapy, confirmed morphologically and immunohistochemically (IHC), was used. A tissue sample with a volume of ~120 mm^3 was obtained during a surgical operation performed at the oncology clinic of The First Moscow State Medical University named after I.M. Sechenov Ministry of Health of Russia (Sechenov University) and was divided into three equal parts of ~40 mm^3 each one. One part of the sample was fixed and then used for pathomorphological examination. For morphological validation, sections of the original tumor sample and cultured breast cancer organoids were stained with hematoxylin and eosin, and immunohistochemical staining was also performed with the following antibodies: rabbit monoclonal antibodies to the estrogen receptor (ER), human progesterone receptor (PR), anti-Her2/neu and anti-Ki67 antibodies (DAKO, Agilent Technology, USA). To determine IHC expression of EGFR, the following protocol was used: in the first stage, endogenous peroxidase was blocked with a peroxidase blocking solution (DAKO, USA) for 5 minutes. Sections were then incubated with primary rat anti-EGFR antibodies (clone EGFR.113, 1/200 dilution, Novocastra Laboratories Ltd., Newcastle, Tyne and Wear, UK) for one hour at room temperature. The reaction was visualized using an EnVision staining kit (DAKO, USA). Sections were counterstained with hematoxylin. The second fragment was frozen (to a temperature of -90°C) and subjected to molecular analysis. The third piece of tumor tissue was mechanically separated into small pieces (about 1 mm^3) using microsurgical scissors from World Precision Instruments (USA) and immediately placed in MACS tissue storage solution from Miltenyi

Table 1. Summary of EGFR-expressing human gastric cancer cell lines used in the present study

Cell culture	Source	Cultivation medium	Additionally
AGS (ECACC, catalogue number 89090402)	European Cell Culture Collection	RPMI 1640 (Life Technologies, Darmstadt, Germany) with the addition of 2 mM L-glutamine (Life Technologies, Darmstadt, Germany)	10% fetal bovine serum (FBS) Sera Plus (PAN-Biotech, Germany) containing penicillin and streptomycin (Merck Sigma-Aldrich, Germany) at concentrations of 100 IU/mL, 100 μg/mL, respectively. Incubation in CO_2 incubator at 37°C.
MKN1 (catalogue number RCB1003 and catalogue number RCB1062)	Cell bank RIKEN BioResource Center (Tsukuba, Japan)		
Hs746T (LGC Standards GmbH, Wesel, Germany, catalogue number ATCC HTB-135)	Cell Biology Collection ATCC	Modified Dulbecco's Medium (DMEM) with the addition of GlutaMAX™-I, 4500 mg|L D-glucose and sodium pyruvate (Life Technologies, Darmstadt, Germany)	

Biotec (Germany). Samples were stored at 40°C for no more than 8 hours until pathological confirmation of the histological type of the tumor and immunohistochemical (IHC) phenotyping of its molecular identity. The scheme of the formation of an organoid tumor-like model of EGFR-positive breast cancer is shown in Figure 1. The model did not require precise cell counting in each piece of cancer tissue assigned to an organoid cultivation.

Experimental intervention

In this work, we studied compound 9-aminium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxy-butanediovate (laboratory number LHT-17-19), obtained in the Department of Chemistry, Technology and Analytical Control of LLC "All-Russian Research Center for Biological Active Compounds Safety " (LLC "AURC BACS", Staraya Kupavna, Moscow region) and kindly provided by the head of the team of authors – Professor S.Ya. Skachilova (Kudryavtsev et al. 2022).

Statistical analysis

Data were presented as continuous variables characterized by Mean ± SD. Normality of the variables distribution was checked by graphical method. Difference between two groups was assessed using one-sided t-test with significance level of 0.95. STATA software version 17 (StataCorp. LLC, USA) was used for statistical analysis.

Results

The results of the study demonstrated that incubation of EGFR-expressing gastric cancer cells Hs746T, AGS and MKN1 with various concentrations of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxy-butanediovate was accompanied by development of concentration-dependent cytotoxicity (Fig. 2). Even the lowest concentration studied inhibited the viability of Hs746T and AGS gastric cancer cell cultures. However, in the case of MKN1 culture cells, the lowest concentration studied did not cause inhibition of cell viability; on the contrary, only when the concentration of LHT-17-19 was increased to 1 μM, was the formation of a cytotoxic effect of the compound observed. In this case, even the next order of concentration caused complete death of tumor culture cells.

A study of the effect of experimental exposure on the activity of the intracellular driver of oncogenesis using Western blotting demonstrated a decrease in the level of the phosphorylated form of receptor tyrosine kinase in all three cell cultures of EGFR-expressing gastric cancer with different levels of suppression of enzyme activity in different cultures. The measurement was carried out in cells exposed to 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-butanediovate at concentrations ranging from 0.0001 to 1000 μM (Fig. 3).

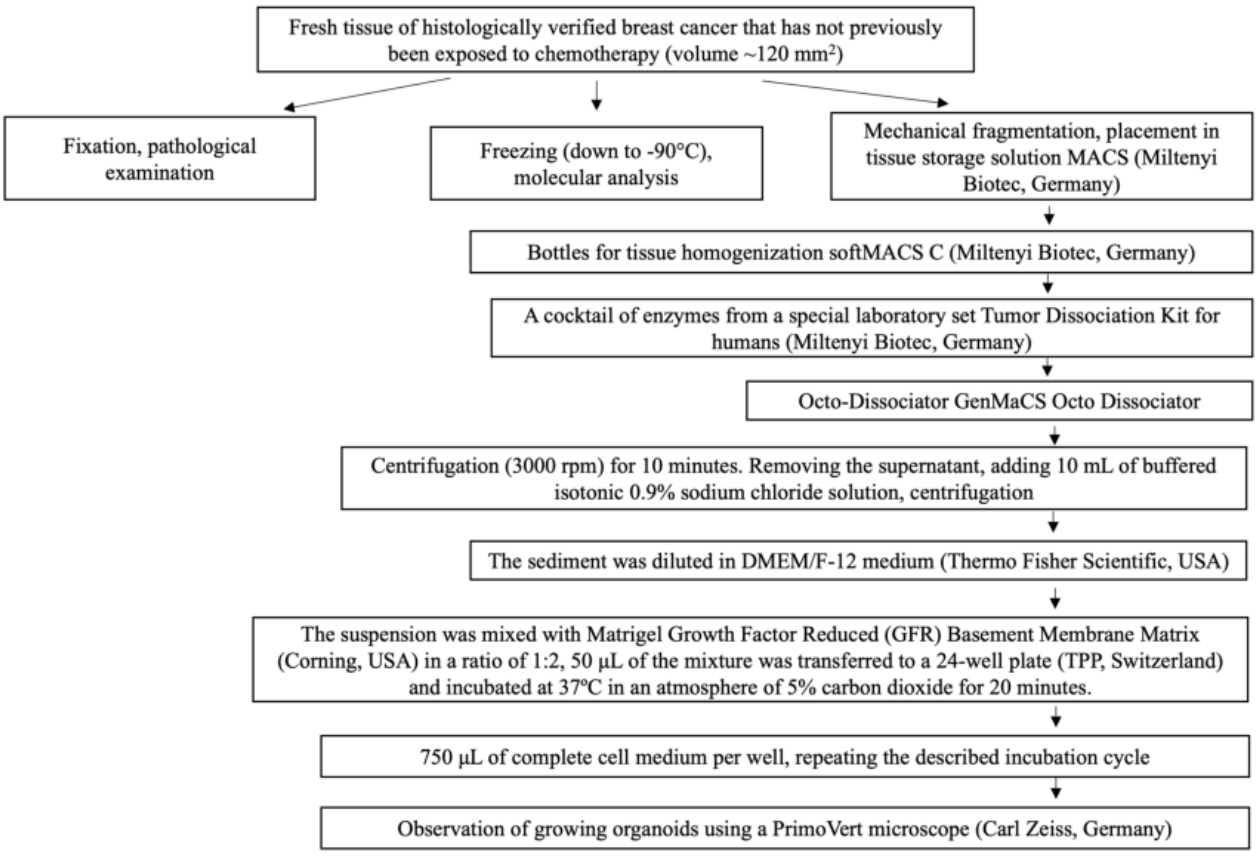


Figure 1. Scheme of the formation of an organoid tumor-like model of EGFR-positive breast cancer.

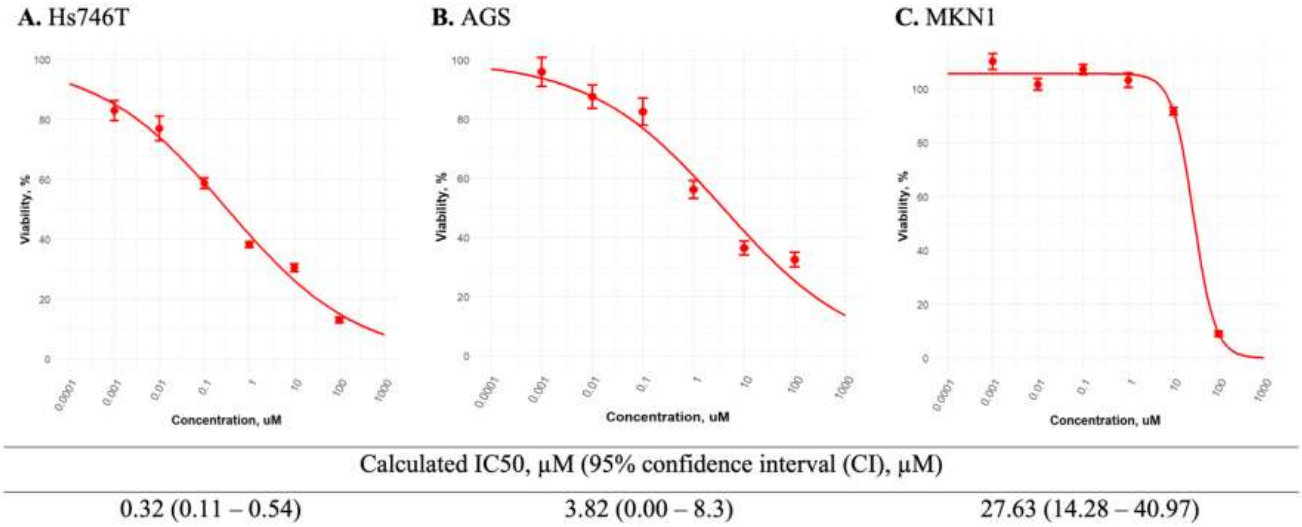


Figure 2. Cytotoxic effect of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH against gastric cancer cells Hs746T (**A**), AGS (**B**) and MKN1(**C**).

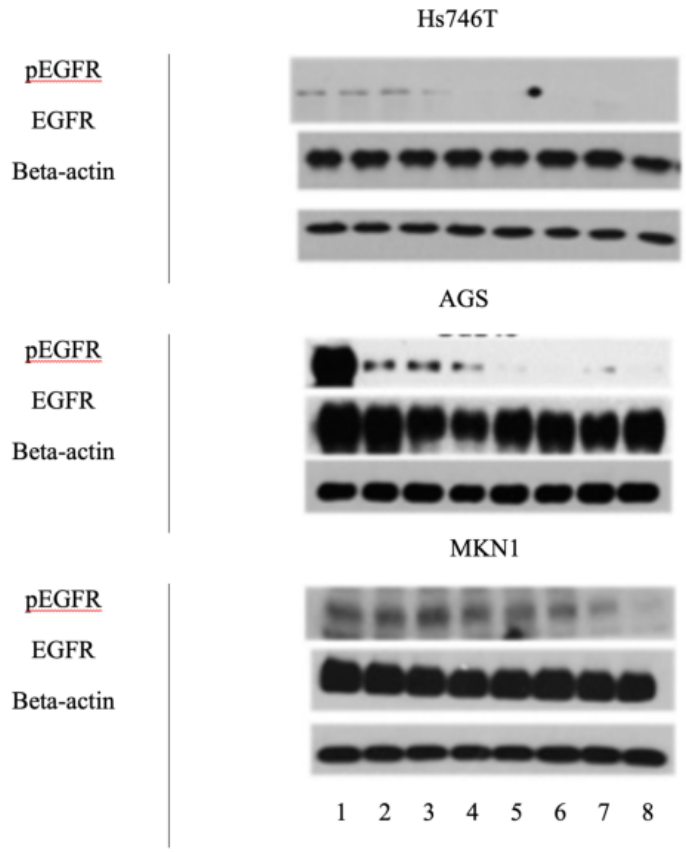


Figure 3. LHT-17-19 inhibits EGFR activity depending on its concentration in gastric cancer cell cultures: 1, 2, 3, 4, 5, 6, 7, 8 – compound concentrations in the range from 0.0001 to 1000 µM.

The antitumor properties of the molecularly targeted drug LHT-17-19 were assessed in a patient-derived organoid (PDO) model of breast cancer expressing wild-type EGFR. Histological examination using standard hematoxylin and eosin staining revealed that the original tissue sample corresponded to the morphological structure of ductal adenocarcinoma of the mammary gland (Fig. 4A). At the same time, the pathological microstructure of the organoids completely reproduces the morphological version of the original tumor sample (Fig. 4C). Light, round or irregularly shaped foci in the center of organoid structures represent foci of necrosis of tumor tissue due to the lack of vascularization of organoids (avascular structures).

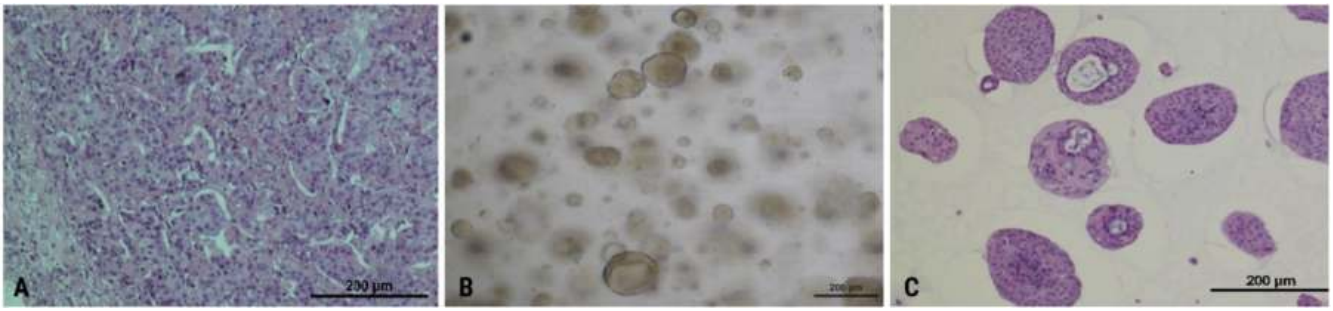


Figure 4. Microphotographs of the initial tumor sample of a 68-year-old patient (**A**), organoid cultures after 7 days of incubation in a nutrient medium (**B**, **C**): A and C – staining with hematoxylin and eosin; B – native drugs in transmitted light; ×200.

Pathological immunohistochemical analysis of intraoperative material (Fig. 5) showed that the presented breast cancer cells were characterized as ER-positive, PR-negative, Her2/neu-negative, EGFR-positive with a Ki-67 expression index of 40%. Expression of progesterone receptors, Her2/neu, and EGFR from original breast cancer tissue was maintained in the organoids. The Ki-67 expression index of organoid cells was also close to that of primary tissue (35%). However, it was found that the expression status of the estrogen receptor was not preserved in the organoids.

At the next stage, an analysis of the expression of the EGFR gene in the cells of the original tumor tissue and grown organoid cultures was carried out (Fig. 6). It was found that in the cells of the original tumor tissue, the expression of the receptor tyrosine kinase gene is 27±4%. In the cells of frozen organoid samples, the expression of the target gene was 31±5% (p=0.35 when compared with

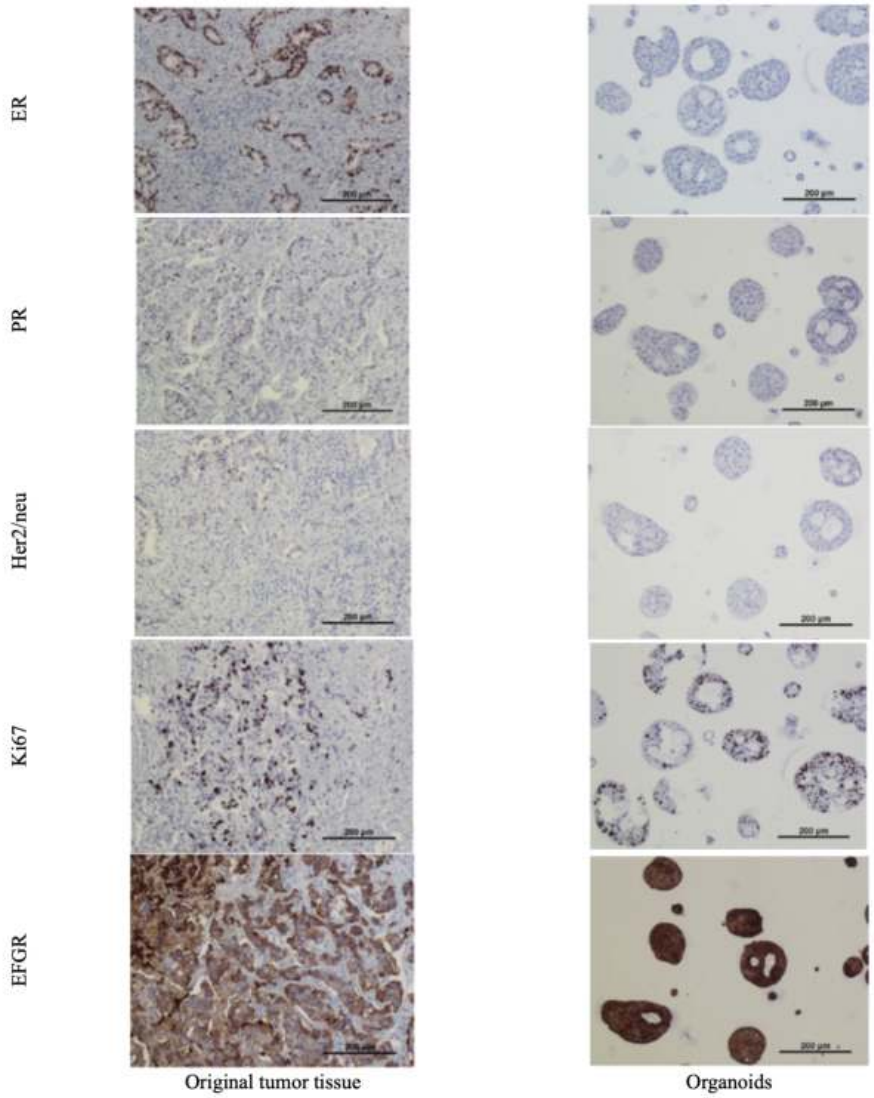


Figure 5. Expression of breast cancer cell markers in the original tumor tissue and organoids, IHC, ×200.

expression in the original tumor tissue), which also indicates that the organoid cells retained the EGFR molecular pattern of expression of "maternal" breast cancer cells.

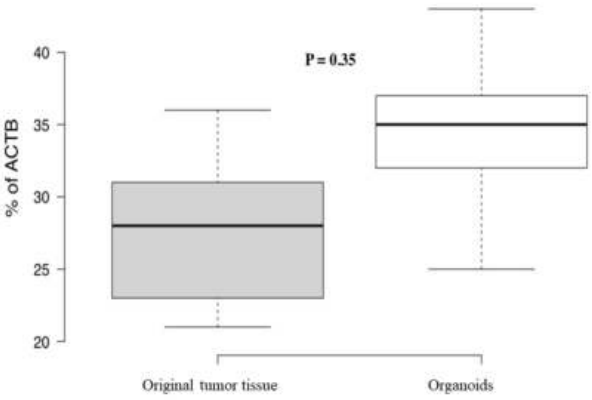


Figure 6. Expression of the EGFR gene in cells of the original tumor tissue and grown organoid cultures (the significance of the differences was assessed after checking the normality of the distribution using a two-sided Student's t-test).

The results of pathomorphological, immunohistochemical and molecular genetic studies allowed us to consider the organoid model of breast cancer validated as ER-negative, PR-negative, Her2/neu-negative, EGFR-positive with a Ki-67 expression index of 35%, which corresponds to the molecular profile of the triple negative EGFR-expressing breast cancer.

After a seven-day incubation of the organoid culture with 0.5-60.0 μM LHT-17-19, depression in the growth of organoid bodies was observed. Increasing the concentration of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxybutane divate to 250 and 1000 μM led to a significant decrease in the size of the organoids (Fig. 7). Cytoreduction indicated not only inhibition of proliferation, but also the formation of a cytotoxic effect of the compound. The calculated organoid growth inhibition index (GI50) was 0.32 μM (95% CI 0.11–0.54 μM).

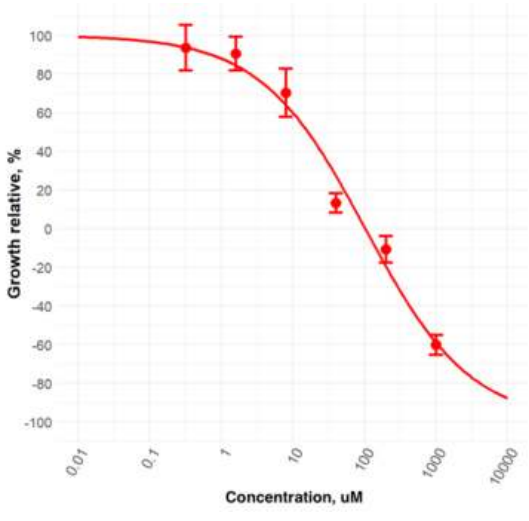


Figure 7. Cytotoreductive effect of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxybutanediovate against triple negative EGFR-expressing breast cancer cells.

Discussion

Epidermal growth factor receptor (EGFR) is a known driver of tumor development and progression. EGFR tyrosine kinase modulates the growth and differentiation of epithelial cells through phosphorylation of intracellular substrates (Sigismund et al. 2018). Kinase inhibitors are considered an effective basis for treatment strategies for lung cancer, pancreatic cancer, breast cancer and others (Arteaga and Engelman 2014). Chemically, they originate from various organic structures such as 4-quinazolinomine (erlotinib hydrochloride, gefitinib), 2-butenamide (afatinib), 2-propenamide (osimertinib), etc. (Carbone 2011, Sachs et al. 2018). In this regard, the class of acridine derivatives attracted the attention of our research group. In laboratory conditions, a fairly simple synthesis of the LHT-17-19 molecule was carried out, which was a salt of 9-amino-3,3-dimethyl-3,4-dihydroacridine-1-(2H)-OH and L-2-hydroxybutanedioic acid. The addition of a carboxylic acid residue increases the solubility of the molecule in water and allows it to be used as an aqueous solution.

When performing docking studies, 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH showed high affinity for the EGFR kinase domain (PDB ID: 1M17) with a dG value of -7.9 kcal/mol, EDoc -5.45 kcal/mol and Ki 101.24 μM due to the formation of π-σ bonds between the aromatic nuclei of the 1,2,3,4-tetrahydroacridine-1-OH fragment with the amino acid residues Leu820, Leu694 and Val702. In addition, the alkyl and π-alkyl complex is stabilized by the interactions of the methyl groups at position 3 and the 1,2,3,4-tetrahydroacridine-1-OH fragment with the amino acid residues Lys721, Met742, Ala719, Leu820, and Val702 (Deryabina et al. 2022).

The most important element of modern personalized cancer therapy is the search and validation of relevant biological models that reproduce, under conditions as close as possible to real ones, the pathological process in all its complexity and multi-level diversity.

To effectively solve the scientific problems, it was decided to focus on two cell models of EGFR-associated oncogenesis – two-dimensional cell cultures of human gastric cancer and a three-dimensional organoid tumor-like culture of breast cancer.

We have demonstrated that incubation of EGFR-expressing gastric cancer cells Hs746T with various concentrations of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxy-butanedionate was accompanied by the development of concentration-dependent cytotoxicity. It is worth noting that even the lowest concentration studied caused inhibition of cell viability. Incubation of EGFR-expressing AGS gastric cancer cells with increasing concentrations of 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxy-butanediovate (LHT-17-19) also resulted in development of concentration-dependent cytotoxicity against tumor cells. In the case of MKN1 cell culture, it was possible to achieve the cytotoxic effect of the compound only when the concentration of LHT-17-19 was increased to 1 μM.

Pathomorphological and immunohistochemical analysis of intraoperative material showed that the presented breast cancer cells were characterized as ER-positive, PR-negative, Her2/neu-negative, EGFR-positive

with a Ki-67 expression index of 40%. Expression of progesterone receptors, Her2/neu, and EGFR from parental breast cancer tissue was maintained in the organoids. The Ki-67 expression index of organoid cells was also close to that of primary tissue (35%). However, it was found that the expression status of the estrogen receptor was not preserved in the formed organoids. However, this phenomenon was previously observed by a group of researchers led by Hans Clevers, one of the pioneers in the study of patient-derived organoids (Kopper et al. 2019). They also showed that even ER-negative tumors can generate ER-positive organoids (Lindström et al. 2012). It is especially worth noting that the membrane expression status of estrogen receptors is not a constitutive phenotypic feature of tumor cells. Numerous studies have shown the flexibility of estrogen receptors throughout the evolution of tumor progression (Lo 2010). Moreover, estrogen receptor-b can be activated by several small molecular weight molecules, including 3,30-diindolylmethane (Nikulin et al. 2020).

The results of pathomorphological, immunohistochemical and molecular genetic studies allowed us to consider the organoid model of breast cancer validated as ER-negative, PR-negative, Her2/neu-negative, EGFR-positive with a Ki-67 expression index of 35%, which corresponds to the molecular profile of the triple negative EGFR-expressing breast cancer. Incubation of organoids in the presence of 0.5-60.0 μM LHT-17-19 was accompanied not only by inhibition of their growth and proliferation, but also by significant cytoreduction.

Conclusion

Thus, in two-dimensional and three-dimensional tumor cell cultures, the possibility of controlling the oncogenic expression of EGFR with the acridone compound 9-ammonium-3,3-dimethyl-3,4-dihydroacridine-1(2H)-OH L-2-hydroxybutanedivacate (LHT-17) was shown.

Conflict of interest

The authors have declared that no competing interests exist.

Funding

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Acknowledgements

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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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Author contributions

- **Viktoria A. Pakina**, Cand. Sc. (Med), Docent, PhD, Department of Life Safety and Disaster Medicine, Sechenov University, Moscow, Russia; e-mail: shapo-viktoriya@mail.ru, **ORCID ID** https://orcid.org/0000-0002-0509-5737. Individual contributions: carrying out experiments, data validation, and writing the manuscript.
- **Evgeniya Z. Iksanova,** M.D., postgraduate student, Department of oncology, Sechenov University, Moscow, Russia; e-mail: ixanovaez@yandex.ru, **ORCID ID** https://orcid.org/0000-0002-9098-1214. Individual contributions: methodology, data processing, and writing the manuscript.
- **Evgeniya V. Shikh,** D. Med. Sc, Professor, Head of Department of Clinical Pharmacology and Propaedeutics of Internal Diseases, Sechenov University, Moscow, Russia; e-mail: chih@mail.ru, **ORCID ID** https://orcid.org/0000-0001-6589-7654. Individual contributions: conceptualization of the study and data validation.
- **Oksana M. Tumutolova,** Cand. Sc. (Med), Docent, PhD, Department of Obstetrics and Gynecology, N.P. Ogarev National Research Mordovia State University, Saransk, Russia; e-mail: tumutolov@mail.ru, **ORCID ID** https://orcid.org/0000-0002-8809-6507. Individual contributions: visualisation and writing the manuscript.
- **Karen K. Arutiunian,** M.D., postgraduate student, Department of oncology, N.P. Ogarev National Research Mordovia State University, Saransk, Russia; e-mail: Dr.karen@mail.ru, **ORCID ID** https://orcid.org/0000-0002-4797-596X. Individual contributions: carrying out experiments and writing the manuscript.
- **Irina V. Kargina,** M.D., postgraduate student, Department of oncology, N.P. Ogarev National Research Mordovia State University, Saransk, Russia; e-mail: ir.cargina2014@yandex.ru, **ORCID ID** https://orcid.org/0009-0007-2643-8368. Individual contributions: visualisation and experimental study.
- **Kirill D. Blinov,** graduate student, Sechenov University, Moscow, Russia; e-mail: pyrk2@yandex.ru, **ORCID ID** https://orcid.org/0009-0002-7195-2191. Individual contributions: carrying out experiments and writing the manuscript.
- **Fedor P. Pilgaev,** V.D., Cand. Sc. (Vet), Docent, Department of Morphology, Physiology and Veterinary Pathology, N.P. Ogarev National Research Mordovia State University, Saransk, Russia; e-mail: pilgaev.fiodor@yandex.ru, **ORCID ID** https://orcid.org/0000-0002-2036-7657. Individual contributions: experimental study.
- **Anna A. Epishkina,** Cand. Sc. (Med), Docent, PhD, Department of fundamental medicine of National Research Nuclear University MEPHI Pathologist of LLC «UNIM», LLC ”UNIM”, National Research Nuclear University MEPHI, Moscow, Russia; e-mail: afina-nn@mail.ru, **ORCID ID** https://orcid.org/0000-0002-7824-7949. Individual contributions: morphological part of experiments and writing the manuscript.
- **Dmitrii S. Blinov,** D. Med. Sc, Professor, Doctor of medical science, head of molecular and clinical pharmacology department, Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology, Moscow, Russia; e-mail: dmitriy.blinov@fccho-moscow.ru, **ORCID ID** https://orcid.org/0000-0002-8385-4356. Individual contributions: conceptualization of the study, data validation, statistical analysis, and manuscript draft review.
- **Evgeny V. Grebenkin,** Cand. Sc. (Med), Senior Lecturer of the Department of fundamental medicine of National Research Nuclear University MEPHI of National Research Nuclear University MEPHI; pathologist of

LLC ”UNIM”, Moscow, Russia; e-mail: grebenkin_urolog@mail.ru, **ORCID ID** https://orcid.org/0000-0002-4990-6722. Individual contributions: writing the text of the manuscript.

- **Ekaterina V. Blinova,** D. Med. Sc, Professor, Head of Department of fundamental medicine, Sechenov University, National Research Nuclear University MEPHI, Moscow, Russia; e-mail: bev-sechenov@mail.ru, **ORCID ID** https://orcid.org/0000-0003-0050-0251. Individual contributions: conceptualization of the study and data validation.