ORIGINAL ARTICLE

A novel dihydroacridine derivative targets epidermal growth factor receptor-expressing cancer cells *in vitro* and *in vivo*

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ABSTRACT

Small molecules are considered a source of novel medicines targeting carcinogenic intracellular pathways including epidermal growth factor receptor (EGFR) signaling. The main goal of the study is to assess whether LHT-17-19 could be considered an effective target molecule against EGFR-expressing tumor cells *in silico*, *in vitro,* and *in vivo*. This was an *in vivo*, *ex vivo,* and *in vivo* experimental study. LHT-17-19 affinity to EGFR's kinase domain was assessed by the ligand's molecular docking. EGFR-expressing Hs746T human gastric cancer cell culture and patient-derived organoid (PDO) model of EGFR-positive breast cancer (BC) were used for *in vitro* assessment of the molecule anticancer property. IC_{50} and GI_{50} indexes were estimated using MTT- and MTS-based tests, respectively. Anticancer activity of LHT-17-19 against EGFR-expressing mutant lung carcinoma was studied on patient-derived xenograft (PDX) model established in 10 humanized BALB/c male mice. Continuous variables were presented as a mean ± standard deviation. Intergroup differences were assessed by two-way *t*-test. Kaplan–Meier's curves were used for survival analysis. High affinity of LHT-17-19 for the EGFR kinase domain with dG score −7.9 kcal/mol, EDoc-5.45 kcal/mol, and Ki 101.24 uM was due to intermolecular π-σ bonds formation and the ligand intramolecular transformation. LHT-17-19 induced anti-EGFR-expressing gastric cancer cells cytotoxicity with IC_{50} 0.32 μM (95% confidence interval [CI] 0.11–0.54 μM). The derivative inhibited growth of EGFR-expressing BC PDO with GI_{50} 16.25 μM (95% CI 4.44–28.04 μM). 2 mg/kg LHT-17-19 intravenously daily during 7 days inhibited PDX tumor growth and metastatic activity, prolonged animals' survival, and eliminated EGFR-mutant lung cancer cells from residual tumor's node. LHT-17-19 may be considered a molecular platform for further search of promising molecules, EGFR-expressing cancer cell inhibitors.

Key words: Breast cancer, cell culture, dihydroacridine derivative, epidermal growth factor receptor, molecular docking, patient-derived organoid

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INTRODUCTION

Anticancer chemotherapy remains one of the main interventional options against malignancies. Despite

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the broad clinical implementation of immunobiological agents, small molecules still play a pivotal role in almost all anticancer treatment protocols. They are also considered a source of novel molecules targeting carcinogenic signaling pathways.[1]

Epidermal growth factor receptor (EGFR) is a well-known driver of tumor's development and progression.[2] EGFR modulates epithelial cell growth and differentiation through phosphorylation of intracellular substrates.[3] In pathological settings, it is involved in oncogenic transformation and tumor growth acceleration of many neoplasms.[4,5] EGFR inhibitors have been representing a reliable strategy for anticancer chemotherapy.[6] At the same time, tumor response is compromised by the development of early resistance, generally associated with *EGFR* mutations.[7] Acridine derivatives are well-known source for many anticancer medications.[8,9] Qualitative structure – activity analysis of novel dihydroacridine derivatives conducted recently using PASS® software has revealed one obtaining general anticancer activity with prediction score 0.87, and prognostic EGFR inhibitory property (P_a> 0.9) (patent RU 2704262 C1). Nonless advantageous is the fact that this derivative, 2(S)-2-hydroxy-butanedioic acid-9-amino-3,3-dimethyl-3,4-dihydroacridine-1(2H)-one (1:1) (laboratory code LHT-17-19), could be synthetized relatively simple in laboratory settings.

These new findings have inspired us to initiate a study of the novel molecule. The main goal of the study was to assess whether LHT-17-19 could be considered an effective target molecule against EGFR-expressing tumor cells *in silico*, *in vitro*, and *in vivo*.

SUBJECTS AND METHODS

Molecular docking of LHT-17-19

For receptor-oriented flexible docking, the open-source Autodock 4.2 package was used. We used the MGL Tools 1.5.6 software (The Scripps Research Institute, USA) for prepare ligands. We used crystallographic structures of the active centers of EGFRK receptor macromolecule from the Protein Data Bank (PDB ID: 1M17). The receptor map was prepared using AutoGrid software. Water molecules, ions, and ligands were removed from PDB ID: 1M17. Visual analysis of ligand–receptor complexes was done by Discovery Studio Visualizer. Binding affinity (dG score, kcal/mol), free energy of binding (EDoc, kcal/mol), and coefficient of interaction (Ki, uM) were calculated.

Bio-ethic statement

All the study procedures met the requirements of international regulations for human rights and laboratory animal treatment. The study protocol was reviewed and approved separately by Sechenov University Independent Ethic Committee on June 3, 2021 (record No 2021/06-04) and National Research Nuclear University MEPHI on June 18, 2021 (record No 132). Informed consent for the possibility of using tumor samples for scientific purposes was voluntarily signed by the human female and male.

Epidermal growth factor receptor-expressing cancer cell culture

For *in vitro* experiments, we used human EGFR-expressing gastric carcinoma cell line Hs746T (cat. No HTB-135), purchased at ATCC Cell Biology Collection (USA).[10] After being thawed, the cell culture was cultivated in Dulbecco's Modified Eagle Medium, containing high glucose concentration (4500 mg/L), sodium pyruvate, and GlutaMAX-I (Wuhan Servicebio Technology Ltd., China). Hs746T cells were incubated in the presence of 0.001, 0.01, 0.1, 1.0, 10.0, and 100.0 μM of Phosphate-buffered saline (PBS)-dissolved LHT-17-19 during 24 h. Cells' viability was assessed by MTT-test as previously described.[11]

Organoid model of epidermal growth factor receptor-expressing breast cancer

Anticancer property of LHT-17-19 was assessed on the patient-derived organoid (PDO) model of EGFR-expressing breast cancer (BC). For PDO establishment, fresh ~120 mm^3 tumor tissue sample of 68-year-old female with bilateral chemotherapy-naïve morphologically validated BC was obtained during surgery at Oncology Clinical Hospital of Sechenov University (Moscow). BC sample was mechanically divided into small pieces using microsurgical scissors and immediately placed in MACS tissue storage solution manufactured by Miltenyi Biotec (Germany). Samples were stored at 40°C for no longer than 8 h until histological and immunohistochemical (IHC) confirmation of the tumor type. After that, PDOs were established as previously described.[12] Growing BC organoids were observed using PrimoVert microscope (Carl Zeiss, Germany) and recultivated biweekly. PDO cell viability was assessed after 7 day-long presence of LHT-17-19 using MTS-based test, CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, USA) as previously described.[12] LHT-17-19 was added in the incubation medium daily at 0.5, 2.0, 9.0, 60.0, 250.0, and 1000.0 μM concentrations in separate series.

Xenograft animal model of epidermal growth factor receptor-expressing lung carcinoma

Heterotopic patient-derived xenograft (PDX) animal model of EGFR-expressing lung carcinoma (LC) was established in humanized 10-week-old BALB/c nu/nu male mice as previously described.[13] The animals were purchased at the Specific pathogen free (SPF) Animal Breeding Facility of the Russian Academy of Sciences (Pushchino, Moscow Region) and maintained under natural daylight conditions at 22°C temperature and 55%–65% humidity with food and water *ad libitum*.

The graft tissue samples were obtained from surgically removed EGFR-expressing tumor of 71-year-old male with chemotherapy-naïve nonsmall cell LC at Oncology Clinical Hospital of Sechenov University (Moscow). The 0.5–0.8 mm tumor particles were inoculated *sub cutis* at the lateral surface of the left thigh of the animal. The grafting cycles were repeated trice, and after histological, IHC, and molecular validation, the tumor samples were inoculated to 10 mice. PDX carriers were randomly allocated into two experimental groups: control and LHT-17-19 (n = 5 in each group). Mice of the control group were intravenously administered with PBS, whereas the animals of the other group were treated with 2 mg/kg LHT-17-19 (1.25% of LD_{50} index determined previously in mice) during 7 days since the xenograft volume had advanced 180–200 mm^3. Animals were surveyed until death; the tumors were harvested and examined; remote metastasis was counted. During the experiment, we controlled animals' pain syndrome using murine facial scale.[14,15] Mild-to-moderate pain was treated by intragastric 100 mg/kg ketoprofen (cat. No K1751, Merck Sigma-Aldrich, Germany) twice daily. Mice with severe uncontrolled pain were euthanized by isoflurane.

Morphological examination and immunohistochemical

4 μm-thick tissue sections were processed through routine hematoxylin and eosin staining and IHC. For immunophenotyping of BC samples and PDO's validation, we used rabbit monoclonal anti-human estrogen receptor, progesteron receptor, anti-Her2/neu, and anti-Ki67 antibodies (DAKO, Agilent Technology, USA). Rat monoclonal anti-EGFR antibody (clone EGFR.113, dilution: 1/200, Novocastra Laboratories Ltd., UK) was used for ICH assessment of EGFR expression status of both BC and LC tumor tissue as described previously.[10] All sections were assessed independently by two pathologists. In case of the result divergence, the supervisor pathologist rereviewed the sections, and the final decision was made up.

Epidermal growth factor receptor gene mutation detection

DNA was isolated from the paraffin-embedded tumor tissue using QIAamp® DNA FFPE Tissue Kit (cat. No 56404, Qiagen GmbH, Germany) according to the manufacturer's protocol. The Therascreen® EGFR Pyro Kit was used to sequence-based detection and quantification of mutations

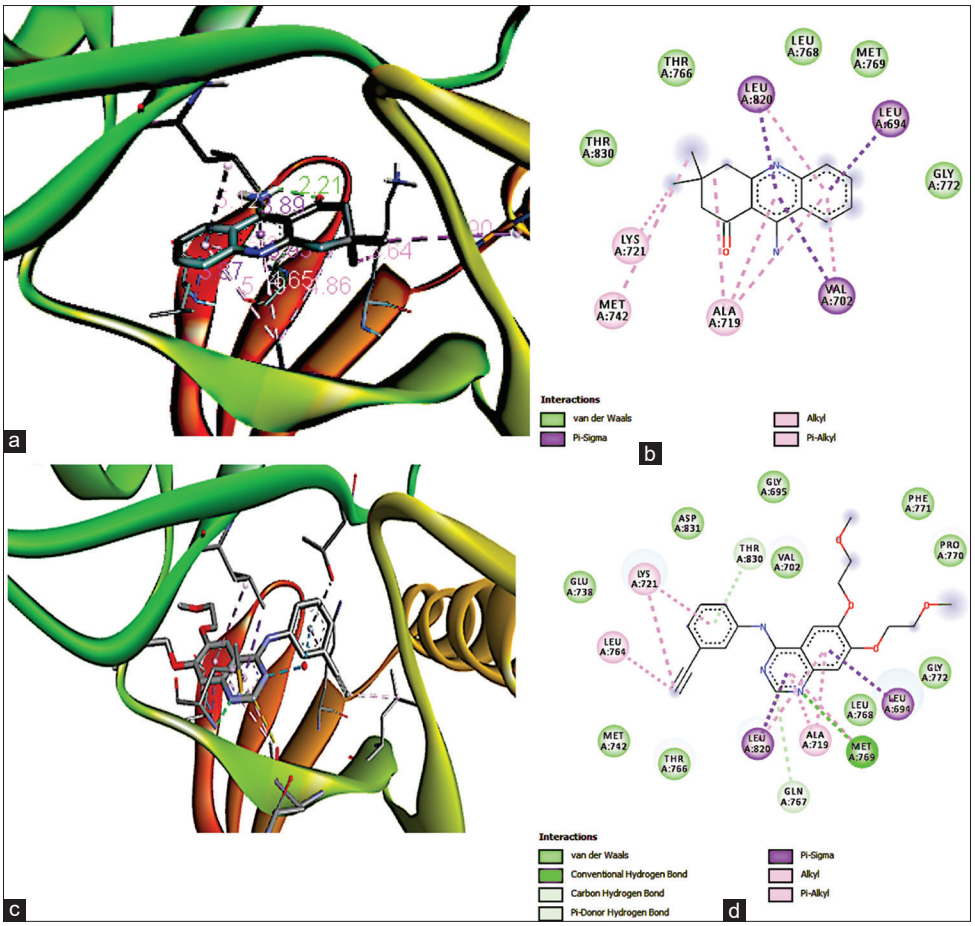

Figure 1: Spatial superposition (a and c) and molecular interactions (b and d) of LHT-17-19 and erlotinib in complex with kinase domain of Epidermal growth factor receptor (Protein Data Bank ID: 1 M17): colors mark different types of molecular interaction (Green – Van der Waals bond; bright green – Conventional hydrogen bond; light green – Carbon and pi-donor hydrogen bonds; violet – π-σ bond; purple – alkyl and π-alkyl bonds)

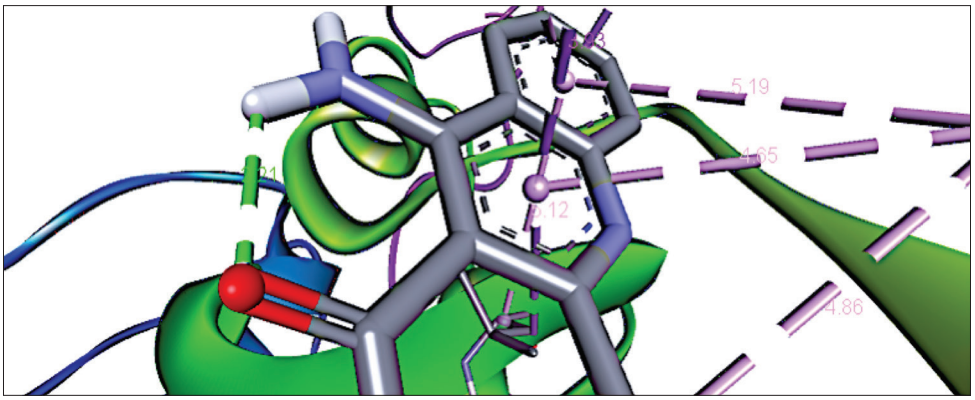

Figure 2: Formation of the intramolecular hydrogen bond in LHT-17-19

in exon 18 (codon 719), exon 19 (deletion), exon 20 (codons 768 and 790), and exon 21 (codons 858 and 861) of the EGFR gene (cat. No. 971480, Qiagen GmbH, Germany) using 10 ng of genomic DNA isolated from tumor tissue. *EGFR* gene mutations were detected by real-time quantitative polymerase chain reaction.

Statistical analysis

Normality of the data distribution was checked with Kolmogorov's test. Continuous variables were presented as a mean ± standard deviation. Intergroup differences were assessed by two-way *t*-test. Kaplan–Meier's curves were used for survival analysis. Differences in survival time were estimated by long rank tests. For the statistical analysis, STATA version 17 software was used (StataCorp. LLC, College Station, TX, USA).

RESULTS

LHT-17-19 showed a high affinity [Figure 1a and b] for the kinase domain of EGFR (PDB ID: 1M17) with dG score −7.9 kcal/mol, EDoc −5.45 kcal/mol, and Ki 101.24 uM. It was comparable with qualitative characteristics of erlotinib [Figure 1c and d]– Kinase domain interaction: dG score −7.5 kcal/mol, EDoc −4.38 kcal/mol, and Ki 611.59 uM. This complex was formed due to π-σ bonds between the aromatic nuclei of the 1,2,3,4-tetrahydroacridine-1-one fragment with the amino acid residues of Leu820, Leu694, and Val702 [Figure 1a]. In addition, the alkyl and π-alkyl complex is stabilized by interactions between the methyl groups in position 3 and the 1,2,3,4-tetrahydroacridin-1-one fragment with the amino acid residues of Lys721, Met742, Ala719, Leu820, and Val702 [Figure 1b]. It should be noted that the docking of LHT-17-19 to the active site of the tyrosine kinase was accompanied by the occurrence of the intramolecular hydrogen bond between an oxygen of the carbonyl atom and a hydrogen proton of the amino group with an atomic distance of 2.21 Å [Figure 2].

As shown in Figure 3, incubation of EGFR-expressing Hs746T gastric cancer cells with LHT-17-19 led to

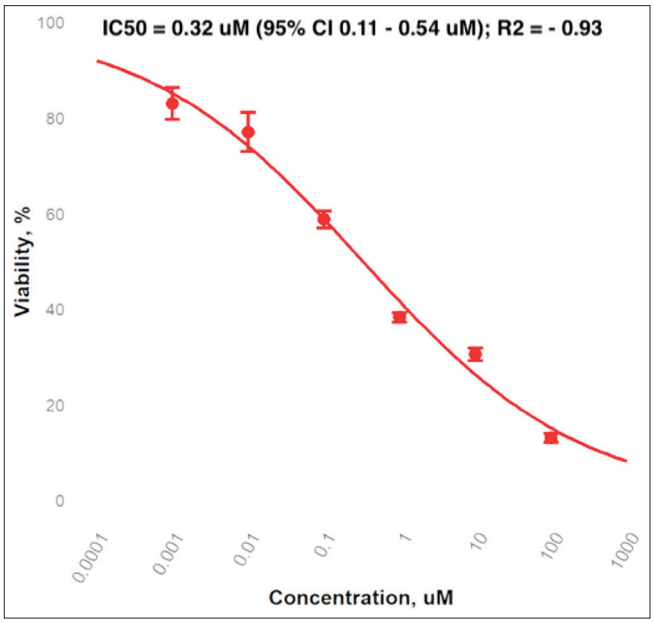

Figure 3: Viability of the epidermal growth factor receptor-expressing Hs746T gastric cancer cells 3 h after an incubation with LHT-17-19 ($n = 3$ for each concentration). CI: Confidence interval

concentration-dependent cytotoxicity with IC_{50} = 0.32 μM (95% confidence interval [CI] 0.11-0.54 μM; R^2 = −0.93, P = 0.005). It is worth noting that even the lowest concentration induced depression of the cells' viability.

EGFR-expressing BC PDOs were established and morphologically validated [Figure 4a-c]. Their 7-day long incubation with 0.5–60.0 μM LHT-17-19 was followed by depression in PDO's growth. Escalation of LHT-17-19 concentration to 250 and 1000 μM led to substantial reduction in PDO's size [Figure 4d]. Estimated GI_{50} was 16.24 μM (95% CI 4.44–28.04 μM; R^2 = −0.92, P = 0.005).

Before the experimental treatment, the morphology of the PDX LC node corresponded to adenocarcinoma of the acinar structure, G II. Its desmoplastic reaction was weak to moderate. EGFR intense of full membrane staining was found in more than 10% of tumor cells. However, given that there

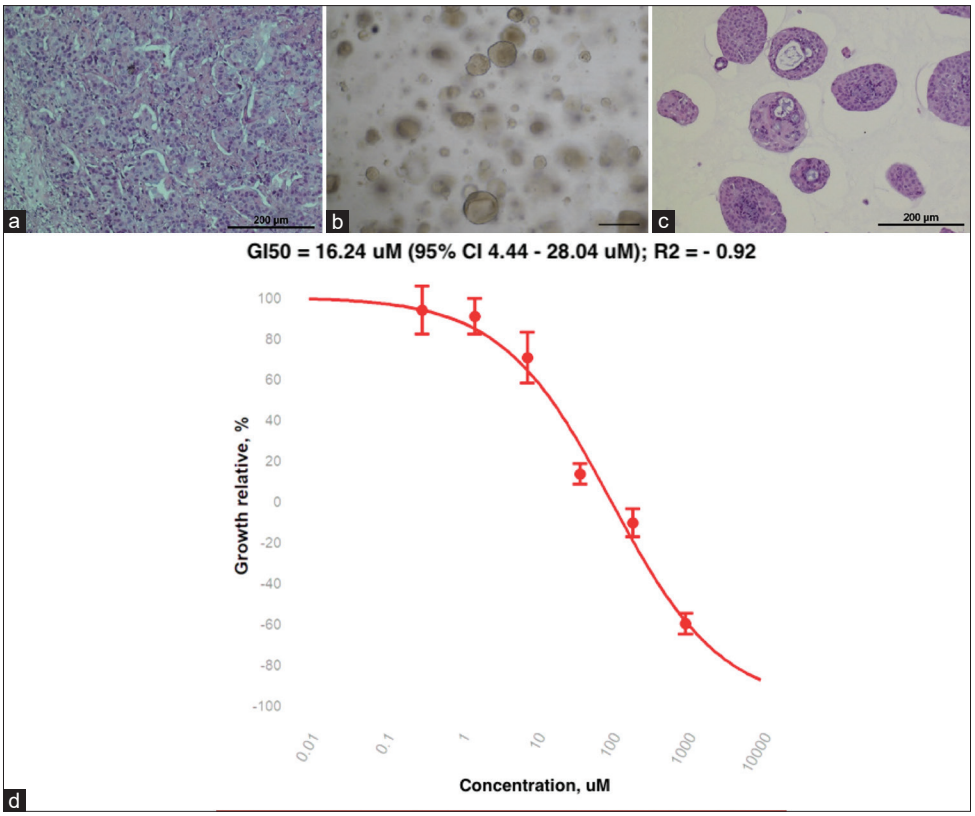

Figure 4: Sections of maternal breast cancer (BC) sample (a), and 7-day BC patient-derived organoid (PDOs) (b and c): (a and c) H and E; (b) Native culture; ×200; (d) Inhibition of the BC PDO's growth by LHT-17-19 (n = 3 for each concentration). CI: Confidence interval

was currently no single-validated scale for assessing the level of EGFR antibody expression, the results were confirmed by qrPCR. An activating mutation, a deletion in exon 19 of the *EGFR* gene (Del19) was detected. After the experimental treatment, the residual tumor showed a decrease in the number of cancer cells up to 30% with dystrophic changes, accompanied by fibrotic foci. The treated tumor showed an absence of EGFR membrane staining [Figure 5] without pathological insertions in exon 19 of *EGFR* gene.

LHT-17-19 administration led to the decrease of the number of remote brain and lung metastases to 22.5 ± 3.4 versus 88.6 ± 7.8 in the control group (P = 0.001) [Figure 6a]. The tumor doubling time increased to 26.5 ± 4.4 in the LHT-17-19 treatment group versus 10.7 ± 2.7 in the control (P = 0.005) [Figure 6b and c]. Therapeutic effect resulted also in the prolongation of the laboratory mice's median survival to 31 days versus 25 days in the control (log-rank test = 5.441, P = 0.02) [Figure 6d].

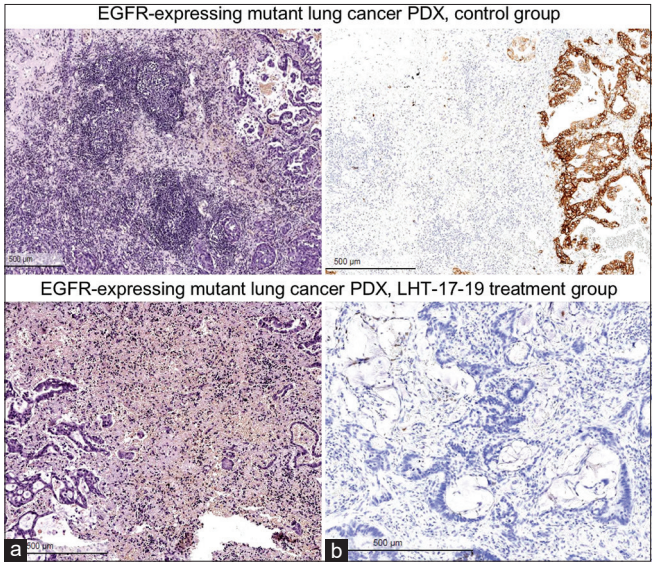

Figure 5: Sections of epidermal growth factor receptor (EGFR)-expressing lung carcinoma patient-derived xenograft's sample in control group and 35 days after experimental treatment of 2 mg/kg LHT-17-19 intravenously daily: (a) hematoxylin and eosin staining; (b) anti-EGFR ICH staining; ×500. EGFR: Epidermal growth factor receptor, PDX: Patient-derived xenograft

DISCUSSION

Experimental pharmacological study of a salt of 9-amino-3,3-dimethyl-3,4-dihydroacridine-1-(2H)-one and L-2-hydroxy-butanedioic acid has been conducted. During the molecular docking experiments, we have found out that a stable inhibitory complex between LHT-17-19 and a binding pocket of EGFR is formed due to the emerging of electrostatic and hydrophobic bonds, which are suggested to assist conformational posing of small molecules in

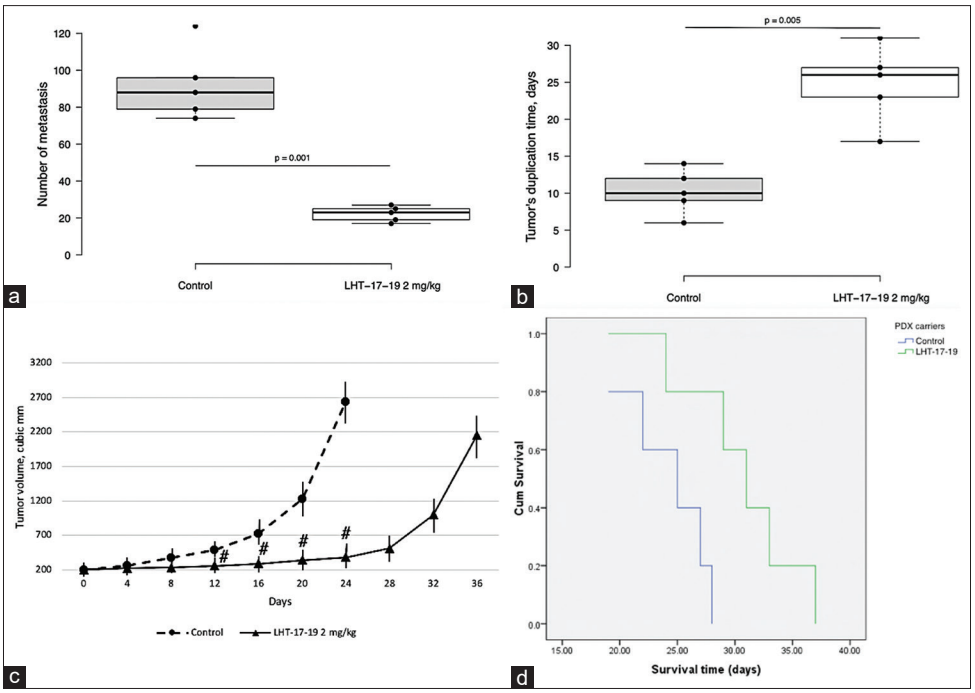

Figure 6: Number of the remote metastasis (a), time of the tumor's volume duplication (b) and its volume growth (c) of the epidermal growth factor receptor-expressing lung carcinoma patient-derived xenograft (PDX) with the mutated genotype treated with LHT-17-19 ($n = 5$ in each group): #$P < 0.05$ when compared with the control, two-way *t*-test; (d) Kaplan–Meier survival curves plotted for the control PDX carriers and the mice treated with intravenous 2 mg/kg LHT-17-19 daily for 7 days ($n = 5$ in each group)

a macromolecular binding pocket.[16] In addition, this complex is stabilized by the intramolecular modification of the LHT-17-19 and C – H…O type of hydrogen bond development.

Three laboratory approaches have been chosen to test LHT-17-19 biological activity. In accordance with the first of them, incubation of wild-type EGFR-expressing gastric cancer cell culture in the presence of the substance has led to concentration-dependent cellular toxicity, with an IC_{50} index similar to that of erlotinib.[17] In a more complex setting of the PDO model, LHT-17-19 not only dose-dependently inhibits the organoid's growth, but induces cytotoxicity. *In vitro* results are supported by animal experiments in the PDX model of EGFR-expressing nonsmall cell-mutated LC. Intravenous 7-day-long LHT-17-19 administration has led to tumor growth inhibition, suppression of metastasis, and an increase in the tumor carriers survival accompanied by an elimination of EGFR-expressing cells in the residual tumor node.

We clearly recognize some important limitations of our study. Thus, using only one crystallographic model of the EGFR kinase domain for docking experiments has not been informative enough to answer an important question whether the inhibitory complex is also formed with mutant form of the driver. No less important it has been to clarify molecular subtypes, and EGFR-expressing status in particular, of residual cellular populations of gastric cancer cell culture and BC PDOs after their incubation with different concentrations of LHT-17-19. Meanwhile, all aforementioned issues will be clarified in our upcoming research.

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Conflicts of interest

There are no conflicts of interest.

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