STUDY OF MOLECULAR BACKGROUND OF NON-SMALL CELL LUNG CANCER ON CISPLATIN AND ERLOTINIB TREATMENT

Ph.D. Thesis

EDIT FÜLÖPNÉ KISS

University of Pécs
Faculty of Pharmacy
Department of Pharmaceutical Biotechnology

Theoretical Medicinal Sciences
Leader of doctoral school: Prof. Dr. Szekeres Júlia, M.D., Ph.D, DSc.
Leader of program: Prof. Dr. Berki Tímea, M.D., Ph.D, DSc.
Leader of project: Prof. Dr. Pongrácz Judit Erzsébet, Ph.D, DSc.

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Medical School

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INTRODUCTION

Non-small cell lung cancer

Of all the tumorous diseases, lung cancers are the most common. Unfortunately, Hungary remains among countries with the highest incidences and mortality rates of lung cancers. Most lung cancers develop from the epithelial cells that line the airways. One of the two major types of lung cancer is small cell lung cancer (SCLC) accounts 10-15% of all cases and significantly associated with cigarette smoking. The vast majority (80-85%) of lung cancers belong to non-small cell lung cancer (NSCLC) that can be further classified into adeno (AC)-, squamous cell (SCC) -, and large cell (LCC) carcinoma subtypes. The incidence of AC has been steadily increasing in recent years and currently this is the most common subtypes of NSCLC, especially among women and non-smokers. Adenocarcinomas are histologically heterogeneous peripheral masses that metastasize early while squamous cell carcinomas are rather centrally located endobronchial masses and metastasize late in the disease progression. The least common large cell carcinomas are poorly differentiated peripheral masses associated with early metastases. In the initial stages of lung carcinogenesis most of the patients are asymptomatic, but at the time of diagnosis, in most cases, an advanced, non-operable tumor can be identified. Based on patient data from Hungary in 2019, 44% of newly registered patients were diagnosed with the most advanced stage of the disease (Stage IV).

Treatment of lung cancer

Despite the improvements in diagnosis and therapy made during the past few decades, the prognosis for patients with lung cancer is still unsatisfactory. The primary treatment for resectable and operable stages of the disease (Stage I -IIIA) is surgery which provides the best option for long-term survival. Besides surgery additional adjuvant and neoadjuvant treatments can improve success rate of resection. Although surgical resection offers a good chance of recovery for patients diagnosed at an early stage, in many cases it is not applicable. In the case of advanced age, poor general condition, or severe comorbidities, radiotherapy is recommended, but it results in a lower chance of survival compared to surgical procedures. For patients with stage IV chemotherapy is the only way to treat the disease. The standard first-line chemotherapy option for NSCLC is platinum-based combination therapy. Among the platinum derivatives, cisplatin (cis-diammine-dichloro-platinum (II)) and carboplatin are the most widely used agents in treatment of NSCLC. Cisplatin exerts its cytotoxic properties by reacting with DNA where primarily interacts with the N7-sites of purine residues to form DNA-DNA inter- and intrastrand crosslinks. DNA adduct formation ultimately results in inhibition of DNA replication and transcription which consequently leads to apoptosis. However, due to the development of molecular

diagnostic methods in recent decades, subgroups of NSCLC can be further classified at molecular level by identifying oncogenic driver mutations. In addition to histological classification of lung cancers, the new molecular classification has significantly changed therapeutic decisions. Although traditional cytotoxic chemotherapy remains the treatment of choice for many malignancies, targeted therapies provides personalized treatment strategies based on the identification of driver mutations. NSCLC, especially lung adenocarcinomas, can be further sub-classified by their genetic mutation profiles where gene mutations of KRAS and EGFR are the most common genetic alterations. Activating EGFR mutations usually occur within exon 18 and 21, which result in enhanced sensitivity for EGFR tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib and erlotinib. Through competitive inhibition of EGFR-TK these small molecule inhibitors lead to inhibition of cell proliferation and apoptosis. Although KRAS mutation frequency is the highest in the Caucasian population, no treatment option is available yet in KRAS positive lung cancer patients.

Role of pro-inflammatory cytokines in carcinogenesis

Cytokines are secreted low-molecular-weight proteins that mediate cell-to-cell communication and regulate of immune system. Interleukin-6 (IL-6), a member of the cytokine family, is a low molecular weight glycoprotein. It is a soluble mediator with a pleiotropic effect on immune response, hematopoiesis, inflammation, and oncogenesis. As a multifunctional cytokine plays an important role in the development of effector T cells, the differentiation of activated B cells into antibody-producing plasma cells, the differentiation of CD8 + T cells into cytotoxic T cells, promotes megakaryocyte maturation in the bone marrow increasing platelet numbers in the circulation. Besides its role in immune regulation IL-6 also plays an important role in the development of tumors. In many cases, high levels of IL-6 can be observed in the tumor microenvironment where it promotes tumorigenesis by enhancing tumor cell survival, proliferation, migration, and the process of angiogenesis and metastasis. Chin Hao Chang et al. reported that elevated plasma IL-6 levels in patients with advanced NSCLC serve as a prognostic marker of survival and high IL-6 levels are associated with a decreased chemotherapeutic response. It was also demonstrated that elevated IL-6 expression plays a central role in augmented chemoresistance in lung cancer cell lines inducing increased expressions of Bcl-2, Mcl-1, Bcl-xl anti-apoptotic proteins and MDR-associated protein ABCG2 via activation of ATM/NF-κB pathway. In rat tumor cells, IL-6 reduced cell sensitivity to chemotherapeutic agents such as paclitaxel and cisplatin by activating the PI3K/AKT and STAT3 pathways.

ABC transport proteins

Resistance to anticancer agents is still the major problem in cancer therapies. In most cases, drug resistance develops during the treatment (acquired resistance). Major mechanisms underlying tumor cell drug resistance include reduced cellular drug uptake and increased drug efflux which latter promoted by enhanced upregulation of energy-dependent efflux transporters. The major cause of the cellular resistance is proteins belonging to the ABC transport family. Three multidrug efflux pumps of ATP-binding cassette (ABC)-superfamily are known to be responsible for chemoresistance; P-glycoprotein (ABCB1), MRP1 (ABCC1) and ABCG2 (BCRP). One possible mechanism for the development of resistance to cisplatin is the increased efflux transport of the platinum derivative caused by the overexpression of ABC transport proteins. High levels of ABCC1 mRNA have been linked with resistance to cisplatin in human lung carcinoma cell lines. Further studies confirmed the role of ABCC2 in the efflux transport of cisplatin. Upregulation of multidrug resistance protein-5 (MRP5, ABCC5) was also associated with cisplatin resistance in human embryonic kidney cells. Moreover, it was found that expression of ABCG2 is a predictive factor of poor clinical outcome in advanced NSCLC patients treated with standard platinum-based combination chemotherapy. However, multidrug resistance (MDR) associated with ABC proteins can be effectively inhibited by tyrosine kinase inhibitors. It has been shown that most EGFR-targeting TKIs, including erlotinib are substrates both of ABCB1 and ABCG2. Erlotinib reverses ABCB1, ABCG2 and ABCC10-mediated MDR through inhibition of their drug efflux functions resulting an increase in intracellular drug accumulation and thus reversal of MDR phenotype.

Further investigation of these processes may play a significant role in better understanding the molecular mechanisms induced by treatment and in therapeutic solutions for the treatment of lung carcinoma.

AIMS OF THE STUDY

1. Can our 3D lung tumor aggregates generated from human patient samples serve as an *in vitro* model for study of lung carcinomas? Can be suitable for *in vitro* therapeutic screening?

2. How does the sequence of the treatment affect response of tumor cells to the treatment and the expression of inflammatory cytokines?

3. Does the presence of IL-6 alter the response of tumor cells to treatment?

4. Is there any correlation between tumor cell viability and drug transporter protein levels regarding IL-6?

5. What is the role of IL-6 and IL-8 in the regulation of tumor cell migration and metastasis formation?

METHODS

Primary lung cancer tissues

Human lung tissue samples were collected during lung resections at the Department of Surgery, University of Pécs, Hungary. The project was approved by the Ethical Committee of the University of Pécs. Patients had given written consent to provide samples for research purposes. All collected samples were treated anonymously. A total of 21 samples were collected and grouped based on mutation analysis. All of the samples were lung adenocarcinoma.

Primary tumor cell isolation from tissue samples

Solid tumor tissues were resected, and viable tumor areas were selected by a certified lung pathologist. Tissue samples were placed and sliced into sterile MACS® Tissue Storage Solution then digested using a GentleMACS Dissociator according to the manufacturer's recommendation. (Miltenyi Biotec, Bergisch Gladbach, Germany) Briefly, solid tumor tissues were digested (60 min, at 37 °C) in RPMI 1640 supplemented with an enzyme mix provided by the manufacturer. Cells were pelleted, resuspended in RPMI 1640, passed through a cell strainer, and then centrifuged. The pellet was resuspended in DMEM. Cells were cryo-preserved using Cryo-SFM according to the manufacturer's recommendation (PromoCell, Heidelberg, Germany) and stored at -80 °C until used.

Primary tumor cell isolation from pleural fluid

After aspiration of tumor-derived fluid (pleural fluid) accumulating between layers of the pleura fluid was placed in a 250 ml conical-centrifuge tube and centrifuged at 1500 rpm for 10 minutes. Cell pellet was filtered through a 70 μm filter then layered on the top of Ficoll gradient and centrifuged at 2000 rpm for 20 min. Cells obtained in the interphase were harvested and washed in sterile PBS two times at 1000 rpm.

Cell cultures

KRAS-mutant A549 (p.*G12S* c.34G > A) human lung adenocarcinoma cell line (American Type Cell Culture Collection, Rockville, USA) was grown in DMEM (Lonza, Basel, Switzerland) supplemented with 10% FBS, 1% L-glutamine, 2% penicillin/streptomycin, 1% HEPES, 1% non-essential amino-acids and 1% PBS/beta-mercaptoethanol. EGFR-mutant PC-9 (exon19del E746–A750) human lung adenocarcinoma cell line (Sigma-Aldrich, St. Louis, USA) was maintained in RPMI 1640 containing 10% FBS, 1% L-glutamine and 2% penicillin/streptomycin. Primary human lung fibroblasts (NHLF) were cultured in FGM-2 according to the manufacturers' recommendations. All the above cell cultures were incubated at 37°C in humidified atmosphere containing 5% CO_2.

In vitro three dimensional (3D) lung aggregates

Three-dimensional lung aggregate tissues were generated from NHLF, A549, PC-9 and primary patient-derived lung adenocarcinoma cells. NHLF and lung adenocarcinoma cells were mixed in 1:1 ratio and a total of 30,000 cells/well were dispensed onto a low-attachment 96-well U-bottom plate (Corning, New York, USA). Cell suspensions were sediment at 600 g for 10 min and cultured at 37 °C and 5% CO_2 in mixed DMEM:FGM-2 or RPMI:FGM-2 media at 1:1 ratio, respectively.

Recombinant proteins and drugs

Drug dilutions were performed from stock solutions of 1 mg/ml cisplatin (Accord Healthcare, Ahmedabad, India) and 5 mM erlotinib (Selleckchem, Houston, USA). Drugs were added to cells at final concentration of 30 nM cisplatin, and various concentrations (1 nM, 10 nM, 100 nM and 1 μM) of erlotinib for 48 h. The choice of erlotinib optimal concentration was determined using a cell viability assay. Purified recombinant IL-6 and IL-8 proteins (R&D Systems, Minneapolis, USA) were dissolved in 1% BSA in PBS and used at a final concentration of 100 ng/ml for 48 h.

Cell viability assay

CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, USA) was used to evaluate cytotoxicity after drug treatment. For the experiments NHLF, A549, PC-9 and primary patient-derived lung adenocarcinoma cells were used to create 2D and 3D cell co-cultures. Co-cultures were seeded into 96-well plates, after 24 h incubation 2D or 3D cell cultures were treated for 48h with the following concentrations of drugs: 100 nM erlotinib, 30 nM cisplatin, 100 ng/ml IL-6. After incubation for 48 h at 37 °C, 100 µl of CellTiter-Glo reagent were added and luminescence measured with EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, USA). Each experiment was performed in triplicates for each concentration and repeated three times ($n = 3$).

RNA isolation and cDNS synthesis

Total RNA was extracted using NucleoSpin RNA II isolation kit according to manufacturer's protocol (Macherey-Nagel, Düren Germany) with on-column DNase digestion. RNA concentration was measured by Nanodrop (Thermo Fischer Scientific, Waltham, USA). One microgram of total RNA and random hexamer primers were used to generate cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, Waltham, USA).

Quantitative RT-PCR

qRT-PCR-s were carried out using the SensiFAST™ SYBR® Hi-ROX Kit (BioLine, London, UK). Amplifications were done on a StepOnePlus system (Thermo Fischer Scientific, Waltham, USA). Gene expression was analysed with StepOne software, using the housekeeping gene ß-actin as reference standard. The relative quantities (RQ) were calculated using the 2^{-ddCt} method.

Flow Cytometric Bead Array (CBA) cytokine assay

Production of pro-inflammatory cytokines IL-6 and IL-8 were quantified in cell culture medium after treatment using CBA Human IL-6 and IL-8 Flex Set Assays (BD Biosciences, San Jose, USA) according to the manufacturer's instructions. For the experiments NHLF, PC-9 and primary patient-derived lung adenocarcinoma cells were used to create 3D cell co-cultures. After 48h culture medium of 3D aggregate cell cultures were collected. The samples were diluted four times in the kit's assay buffer. Diluted samples were added to fluorescent cytokine capture bead suspension and incubated for 1 hour in dark. Then human inflammatory cytokine-phycoerythrin detection reagent was added to each samples and incubated for 2 hours in dark. Measurements were carried out using

BD FACSCanto II flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) and analysed by FCS Express V3 software.

Functionality test of drug transporters

Functional activity of ABC transporter proteins were done using eFluxx-ID® Green multidrug resistance assay kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturers' protocol. Briefly, 3D aggregates generated from NHLF and PC-9 cells were enzymatically digested using Accumax solution (Sigma-Aldrich, St. Louis, USA) and single cell suspension were mixed with diluted drug transporter inhibitor molecules, incubated for 5 min at 37 °C, then all samples were incubated with diluted eFluxx-ID® Green dye for 30 min at 37 °C. After 30 min, propidium-iodide was added to all sample tubes and were measured immediately with FACS Canto II flow cytometer (BD, Biosciences, Immunocytometry Systems, Erembodegen, Belgium). Appropriated controls (stained and unstained) were included into procedure. The results are calculated as multidrug resistance (MDR) activity factor values (MAF).

Scratch assay

A549 and PC-9 lung adenocarcinoma cells were grown to 90% confluence in 24 well plates (Corning, New York, USA) and wound was created in each culture by scratching the cellular monolayers. Fresh medium supplemented with cisplatin (30 nM) or erlotinib (100 nM) in the presence or absence of 100 ng/ml IL-6 or IL-8 was added to the cell culture, respectively. Wound healing was monitored by the decrease of gap area taking images with EVOS light microscopy (Thermo Fisher Scientific, Waltham, USA) at regular intervals and the gap area was quantified using ImageJ software.

Wound healing assay

For drug toxicity screening of cisplatin and erlotinib 3D Wound Healing Bioassay was used (Greiner Bio-One GmbH, Frickenhausen, Germany). A549 and PC-9 human lung adenocarcinoma cells were cultured on T-25 flasks until they reached 80% confluence, then treated with 200 µL NanoShuttle-PL overnight at 37 ºC/5% CO_2. After 24 h incubation single cell suspensions were made using trypsin/EDTA solution and cells were seeded to the 6-well repellent plate at a density of $1.2x10^6$ cells/well. A 6-well levitation plate was placed on the top of the plate to leviate the cells and induce ECM formation for 5 hours. After incubation we collected the cells and added to 24-well repellent plate at a concentration of $2x10^5$ cells/well. A 24- well ring drive was placed below the

well plate for 15 minutes to allow cells to aggregate into the magnet's ring shape. Then, the cells were treated with 30 nM cisplatin and 100 nM erlotinib. Finally, pictures were made on all wells every 6 hours for 24 hours using EVOS® FL Imaging System.

Statistical analysis

Statistical analysis was performed with IBM SPSS version 20 software. Data are presented as mean ± standard error of mean (SEM), and statistical analysis was performed using independent samples t-test and oneway ANOVA test with Bonferroni correction. $p < 0.05$ was considered as significant.

RESULTS

Drug sensitivity of primary human lung adenocarcinomas in *in vitro* tissue cultures

To investigate the drug response of primary lung AC-s with various mutation background *in vitro*, 3D aggregate cultures were formed from primary cancer cells, after which the cultures were exposed to cisplatin (30 nM) or erlotinib (100 nM). Similarly to our expectations patients with exon 19 deletions or exon 21 substitution (L858R) in the EGFR gene were responsive to TKI (erlotinib), while samples with KRAS mutation or wild-type (WT) KRAS/EGFR genes were not sensitive to erlotinib. Additionally, *in vitro* drug sensitivity assay confirmed that cisplatin was more cytotoxic to cells with WT KRAS/EGFR and KRAS mutations and was least effective in those with EGFR mutations. Our results indicate that *in vitro* drug sensitivity assays performed in 3D aggregate cultures replicate the clinically proven drug response and provide a suitable *in vitro* model for prediction drug sensitivity.

Expression of IL-6 and IL-8 in primary human lung adenocarcinomas

Pro-inflammatory IL-6 and IL-8 cytokine expressions in EGFR mutant, KRAS mutant and WT KRAS/EGFR primary adenocarcinoma samples were measured at mRNA level. Quantitative analysis highlighted a notable difference in the two main mutation status. While higher IL-6 mRNA levels was associated with EGFR mutation, higher IL-8 mRNA levels were detected in KRAS mutant samples.

Cisplatin and erlotinib treatment alters IL-6 and IL-8 expression

To test whether mono-, or combination treatment of cisplatin and erlotinib affect IL-6 and IL-8 cytokine expression, 3D aggregate cultures were generated using EGRF mutant PC-9 cell line. After 48h treatment cytokine mRNA and protein

levels were measured. Both of IL-6 and IL-8 production was significantly increased at mRNA level after cisplatin mono-treatment or cisplatin and erlotinib combination treatment. In contrast, erlotinib alone resulted in decreased mRNA and protein levels. Interestingly, we found that erlotinib was able to reduce cisplatin-induced IL-6 and IL-8 protein secretion.

The role of IL-6 in tumor cell survival

As IL-6 molecule showed higher levels in EGFR mutant samples, further investigations were carried out toward IL-6. To investigate the role of IL-6 in tumor cell survival, 3D aggregate cultures were generated using EGFR mutant PC-9 cell line then were pre-treated with human recombinant IL-6 prior to drug treatments. IL-6 pre-treatment increased cell survival in all treatment conditions, with significantly higher values after cisplatin monotherapy, cisplatin pre-treatment followed by erlotinib treatment and their co-administration.

The role of IL-6 in multidrug resistance

As results above indicated that IL-6 is a positive regulator of cell survival, we studied whether tumor cell viability could be associated with increased levels and activities of drug transporter proteins. Based on the literature expression of multidrug transporter proteins on tumor cells contributes to their resistance and correlates with decrease in response to various chemotherapy drugs. To investigate expression of ABC transporters at mRNA level, 3D aggregate cultures were generated using EGRF mutant PC-9 cell line then were pre-treated with human recombinant IL-6 prior to drug treatments. ABCG2 and ABCC1 expressions were increased by IL-6 pre-treatment in all treatment condition, while ABCC5 mRNA levels showed a decreased tendency. Further analysis also tested whether IL-6 affects activity of ABC transporter proteins. Although IL-6 alone reduced ABCG2 activity, IL-6 pre-treatment followed by cisplatin sequential treatments led to increased activity of ABCG2 transporter. In contrast, ABCC1 activity was slightly decreased compared to the untreated control.

Role of pro-inflammatory cytokines in tumor cell migration

Presence of pro-inflammatory cytokines plays a significant role in the regulation of tumor progression and metastasis. To test whether the presence of cytokines can modulate cellular migration, scratch assays were performed in the presence or absence of the two drugs and/or cytokines. The addition of IL-6 (100 ng/ml) significantly reduced the gap area by inducing cellular proliferation and migration in the KRAS mutant A549 cultures. This was also the case if the cultures were treated with cisplatin or erlotinib in the presence of IL-6. IL-8 showed a similar effect on gap closure in the KRAS mutant cell lines. In contrast, addition of IL-6 had no significant effect on EGFR mutant PC-9 cells. PC-9

cultures responded to IL-6 during erlotinib treatment when added IL-6 promoted gap closure. More interestingly, IL-8 inhibited gap closure in the PC-9 cell line and even more so in the presence of erlotinib. Cisplatin was unable to slow down gap closure in the PC-9 cell cultures and in the presence of cisplatin, however added IL-6 and IL-8 accelerated the process.

SUMMARY

1. *Can our 3D lung tumor aggregates generated from human patient sample serve as an in vitro model for study of lung carcinomas? Can be suitable for in vitro therapeutic screening?*

Two-dimensional (monolayer) cell cultures are still a dominant method in many biological studies, however development of cell and tissue culture techniques provides new and improved methods using three-dimensional (3D) cell culturing techniques. As their structure and complexity are closer to real tumor microenvironment, such 3D tissue cultures provide an accurate *in vitro* model for many malignancies and drug testing. Using our methodology, we were able to demonstrate that *in vivo* patient data and *in vitro* drug sensitivity tests provide highly similar results. In our study, we have shown that primary tumors with activating EGFR mutation were the most responsive to tyrosine kinase inhibition while cisplatin was only effective in WT KRAS/EGFR samples or in the presence of KRAS mutation. Our results prove that *in vitro* drug sensitivity assays performed in 3D aggregate cultures replicate the clinically proven drug response and provide a suitable *in vitro* model for prediction drug sensitivity.

2. *How does the sequence of the treatment affect response of tumor cells to the treatment and the expression of inflammatory cytokines?*

Sequential administration of cisplatin and erlotinib revealed different therapeutic responses on tumor cell viability. Our results show that primary use of cisplatin in combination treatment drastically reduced tumor cell viability, whereas primary use of erlotinib was more favourable for tumor cell viability. Further investigations also revealed that sequence of drug administration play a key role in the expression of inflammatory cytokines. In the case of EGFR mutation, cisplatin treatment further increased the production of IL-6, which is important in the process of lung carcinogenesis and is responsible for tumor cell proliferation and migration. In contrast, erlotinib could inhibit cisplatin-induced IL-6 secretion. These observations highlight the importance of treatment sequence that may contribute to the inhibition of tumor cell migration and thus metastasis formation.

3. *Does the presence of IL-6 alter the response of tumor cells to treatment?*

In cell viability studies, we detected that IL-6 pre-treatment induces cell survival in all treatment conditions. Our results show that IL-6 is a positive regulator in the process of tumor progression that may cause failure of drug treatment.

4. *Is there any correlation between tumor cell viability and expression levels of drug transporter proteins regarding IL-6?*

In parallel with the increase in tumor cell viability, we found that IL-6 is able to increase the mRNA level of multidrug transporter proteins ABCG2 and ABCC1. In functional analysis, IL-6 alone decreased AGCG2 activity, but in combination with cisplatin and erlotinib sequential treatments IL-6 led to increased ABCG2 activity. These results support our hypothesis that expression of efflux transporters in tumor cells can be altered by the inflammatory microenvironment through IL-6 in a manner that may lead to tumor progression.

5. *What is the role of IL-6 and IL-8 in the regulation of tumor cell migration and metastasis formation?*

According to our results, we concluded that IL-6 and IL-8 are positive regulators of cell motility in the KRAS mutant A549 cultures where addition of IL-6 and IL-8 significantly reduced the gap area by inducing cellular migration. However, in the case of EGFR mutant PC-9 cells the opposite effects of IL-6 and IL-8 were observed. PC-9 cells migrated faster in the presence of IL-6, while presence of IL-8 inhibited cell migration. Thus we hypothesize these cytokines are able to regulate cell migration and metastasis formation in an oncogenic mutation-dependent manner.

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Academic note

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