Xiangpu Li [1], Alexandra Bulygo [2], Dziyana Charkas [3], Katsiaryna Verameyenka [4]

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[1–4] Department of Genetics, Faculty of Biology, Belarussian State University, Minsk, Republic of Belarus

OBTAINING PRIMARY CELL CULTURES AND DEVELOPING PRIMERS FOR ANALYSING THE BIOLOGICAL EFFECTS OF PHENAZINES

The article presents the results of experimental work on obtaining primary cultures of eukaryotic cells of the Rattus norvegicus Wistar line from various tissues. The results of studies on the selection of marker genes for analysing the biological effect of phenazines on eukaryotic cells are presented, as well as bioinformatic studies on the development of primers for real-time PCR in cells before and after treatment with phenazines. It was possible to generate lines of primary cultures of liver, skin, bone marrow and spinal cord cells of the R. norvegicus Wistar line. In addition, primers for the most important selected marker genes of rat and human were developed for the subsequent analysis of the biological effects of phenazines.

Keywords: phenazines; marker genes; cell culture; primers.

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Сянпу Ли [1], А. В. Булыго [2], Д. Д. Черкас [3], Е. Г. Веремеенко [4]

[1–4] Кафедра генетики биологического факультета, Белорусский государственный университет, Минск, Республика Беларусь

ПОЛУЧЕНИЕ ПЕРВИЧНЫХ КУЛЬТУР КЛЕТОК И РАЗРАБОТКА ПРАЙМЕРОВ ДЛЯ АНАЛИЗА БИОЛОГИЧЕСКИХ ЭФФЕКТОВ ФЕНАЗИНОВ

В статье представлены результаты экспериментальной работы по получению первичных культур эукариотических клеток Rattus norvegicus линии Wistar разных тканей. Приведены результаты исследований по выбору маркерных генов для анализа биологического действия феназинов на эукариотические клетки, а также биоинформатических исследований по разработке праймеров для проведения ПЦР в реальном времени в клетках до и после обработки феназинами. Удалось создать линии первичных культур клеток печени, кожи, костного мозга и спинного мозга R. norvegicus линии Wistar. Также были разработаны праймеры для ключевых выбранных маркерных генов крысы и человека в целях последующего анализа биологических эффектов феназинов.

Ключевые слова: феназины; маркерные гены; культура клеток; праймеры.

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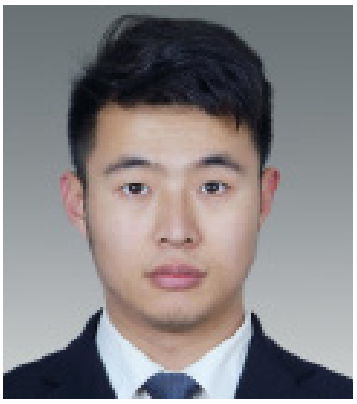
Авторы:

[1] **Сянпу Ли** – аспирант кафедры генетики биологического факультета, БГУ.

lixiangpu3@gmail.com

Authors:

Xiangpu Li – Postgraduate Student of Depart-ment of Genetics of Faculty of Biology, BSU.



[2] **Александра Валерьевна Булыго** – студентка кафедры генетики биологического факультета, БГУ.

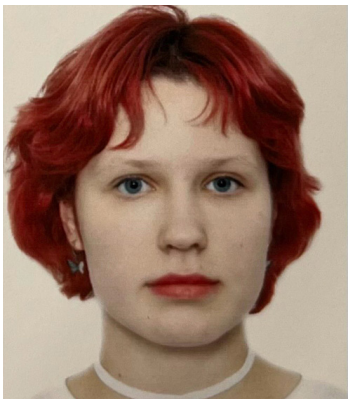
abulygo33@gmail.com

Alexandra Bulygo – student of Depart-ment of Genetics of Faculty of Biology, BSU.

[3] **Диана Дмитриевна Черкас** – студентка кафедры генетики биологического факультета, БГУ.

dziyanacharkas@gmail.com

Dziyana Charkas – student of Depart-ment of Genetics of Faculty of Biology, BSU.



[4] **Екатерина Геннадьевна Веремеенко** – кандидат биологических наук, доцент кафедры генетики биологического факультета, БГУ.

https: //orcid.org/0000-0002-7530-8427

veremeenkokatya@yandex.ru

Katsiaryna Verameyenka – PhD in Biology, Associate Professor of the Department of Genetic, Faculty of Biology, BSU.

Oncological diseases are currently one of the most pressing medical and social problems. Despite the intensive development of drugs to treat oncology, this group of diseases is still one of the most common causes of death in the world. The main problem in the development of effective anti-tumour drugs is still our superficial knowledge of the molecular genetic and epigenetic mechanisms of the occurrence of this group of diseases and of the formation of drug resistance during chemotherapy. Furthermore, it cannot be denied that many modern antitumour drugs are quite toxic to normal body cells and are therefore often poorly tolerated by patients. Therefore, it is important to develop new, more specific antitumour drugs and to deepen the understanding of the mechanisms of carcinogenesis. Phenazine antibiotics have already proved quite effective as antimicrobial agents against phytopathogens and some human and animal pathogens [5; 7]. In recent years, active research has been carried out to identify the antitumour activity of phenazines [1; 6]. It is known that some types of phenazine compounds can cause the development of oxidative stress in forms that are sensitive to them. Presumably, this is the basis for the antimicrobial effect of phenazines [2; 8]. However, the spectrum of biological effects of phenazines on the lineage of malignant cell cultures can only partly be explained by the oxidative effect. At the same time, most modern studies focus on the demonstration of antitumour activity against different types of tumour cells and neglect the clarification of the molecular basis of the cytotoxic effects and the effect of phenazines on normal cells.

The aim of this study is to obtain primary cultures of normal *R.norvegicus* Wistar cells in order to use them as controls for experiments to investigate changes in the expression profile of marker genes under the influence of phenazines and without them, and to select marker genes whose expression could serve as an indicator of the state of the cell.

In the first phase of the work, primary cultures of epithelial cells, bone marrow cells and neural tissue cells were obtained.

To obtain a primary liver cell culture, the liver extracted from *R.norvegicus* was washed in 70 % alcohol solution, cut into 5 cm pieces and kept in an antibiotic solution in a CO_2 incubator for 40 minutes.

The connective tissue capsule of the organ was then removed and the parenchyma fragmented into 0.5–1 cm pieces. The extracted explants were placed in a culture flask pre-incubated with calf embryo serum and mixed with DMEM culture medium. Cultivation was carried out for 3 days under constant cytological control. After cell migration from the explants and their fixation on the surface of the flask, the explants were removed and the cells were left to obtain a sufficient amount of biomass. The results are shown in *Figure 1*.

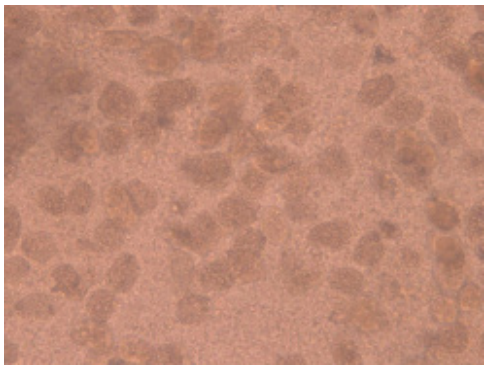


Fig. 1. R.norvegicus liver cell culture.
Inverted microscope Mshot MSX2-G (Mshot, China). Objective L Plan FI 40x/0.65 (Mshot, China).

The femurs and tibias of *R.norvegicus* were used to obtain a primary bone marrow cell culture. After collection, the bones were washed with 70 % ethyl alcohol, placed in a laminar flow bonnet, and the bone epiphyses were removed. The cavity of each bone was washed with 1 ml RPMI 1640 medium using a sterile syringe to remove blood cells. Subsequently, 2 ml of DMEM medium was used to extract bone marrow cells (*Figure 2*).

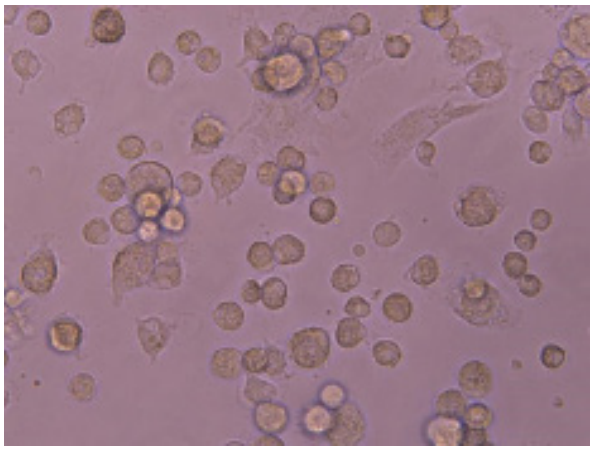


Fig. 2. Culture of marrow cells of *R.norvegicus*.
Inverted microscope Mshot MSX2-G (Mshot, China). Objective L Plan FI 40x/0.65 (Mshot, China).

To obtain a primary skin cell culture, skin from the ear of R. norvegicus, pre-treated with 70 % ethanol, was fragmented, washed in PBS buffer and incubated in a solution with DMEM nutrient medium and a mixture of collagenase and elastase at a concentration of 30 mg/ml for 2 days. After the time had elapsed, the liquid and several fragments were transferred to a culture flask. As a result, a primary rat skin culture was obtained, represented by various cell types (*Figure 3*).

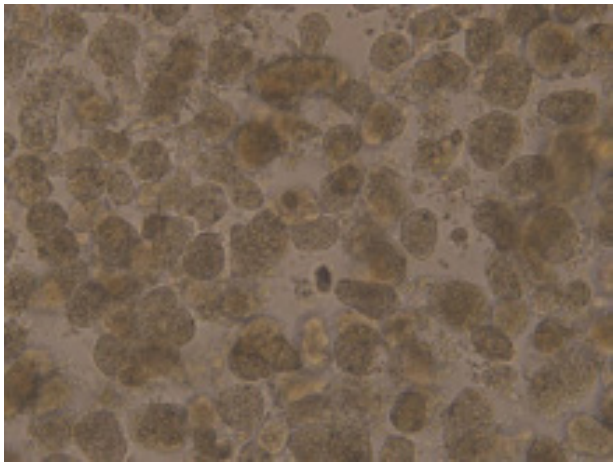


Fig. 3. R.norvegicus skin cell culture.
Inverted microscope Mshot MSX2-G (Mshot, China). Objective L Plan FI 40x/0.65 (Mshot, China).

To obtain a spinal cord cell culture, the spinal cord was extracted and fragmented from the spine of *R.norvegicus*. The resulting explants were cleared of connective tissue membranes, and the nervous tissue was transferred to a culture flask with DMEM nutrient medium.

At the next stage of the work, based on literature data and the results of proteomic analysis performed in our laboratory (data not shown), genes of the cytoskeleton, cell adhesion and associated vesicular transport were selected as molecular markers for monitoring changes in cells after treatment with phenazines. Such changes often cause the initiation of malignancy or its progression. Genes encoding proteins that are part of ribosomes were also selected for analysis. Ribosomal proteins were given priority due to their involvement in protein synthesis and stress signaling, and literature data link their dysregulation with chemotherapy resistance and cancer progression [3; 4].

To analyze the expression level of the described genes, primers were created for subsequent amplification. Primers were designed using the Primer-BLAST and UNAFold electronic resources.

The criteria for creating primers were:

- Location: the primers were selected in such a way that the sequence of the forward and reverse primers were located at the boundaries of 2 adjacent exons separated by an intron of 500–1000 bp, the exons had to be present in most of the presented gene transcripts;
- Size of the amplified product: the expected size of the PCR product was in the range from 150 to 300 bp;
- Melting temperature of primers: the minimum temperature was 52□, the maximum was 55□, and the optimal temperature was 53.5□;
- The length of primers was selected in the range of 15–20 bp.

All primers were checked for the formation of secondary structures, homo- and heterodimers, for which the UNAFold web service (DINAMelt service) was used. A check was also performed via BLAST (blastn, RefSeq RNA database) to assess potential non-specific annealing sites, which can lead to depletion of primers when they bind at unintended sites.

The obtained primer sequences and their melting temperatures for such organisms as *Homo sapiens* and *R.norvegicus* are presented in *Table 1*.

Table 1

Primers for analysis of gene expression levels under the influence of phenazines

Gen	*Species*	*Forward primer sequence*	*Reverse primer sequence*
CDH1	human	CGAACTATATTCTTCTGTGA	AGATTGATTTTGTAGTCACC
CDH1	rat	CAAGAACTTCTGCTAGACTT	CATCCTTCAAATTTCACTCT
CDH2	human/rat	TCAGGTCTGATAGAGATAA	ATGTCAATGGGGTTCTC
CDH3	human	GCTAACACTGATTGATGTC	GTCATATGTATCCTGCTTCA
CDH3	rat	AACATATCCATCATTGTGAC	AACATATCCATCATTGTGAC
PCDH1	human	GCAGAAATCGAATACACATT	ATCTTGATGAGTCACTAGC
PCDH1	rat	TACTCCGACTACAGCTATC	ATAATAACTGTGCTGTGATG
PTK2	human/rat	CTCCAGAGTCAATCAATTTT	AATCTTTCCCCATTTTCAAT
ITGAV	human/rat	AGTGTACCCTAGCATTTTA	CTTGAGTTTATCCAAAAGAAG
ITGB3	human/rat	GTGTGGAGTGTAAGAAGTT	TCATAGTACTGGAATCTGAC
MCAM	human/rat	ATCTGGTACAAGAATGGC	AGTAAAACTGGGCATCT
KRT18	human	CATGCAAAGCCTGAAC	TAAAGTCATCAGCAGCAA

KRT18	rat	AGACTAGAGAGCAAAATCC	GTCTCATACTTGACTCTAAA
KRT19	human	CAACGAGAAGCTAACCAT	CAATCCTGGAGTTCTCAAT
KRT19	rat	AATGAGAAGATCACCATGC	ACTATCTTGGAGTTCTCAAT
DES	human	AACATTTCTGAAGCTGAG	CTCATCAGGGAATCGTT
DES	rat	ACACCTAAAGGATGAGATG	GTCTTGATCATCACTGTC
VCL	human	CAACTCCGACTAACAGA	CAACACCTATACCCACC
VCL	rat	CAGACCTTGAACAGCTA	ATTGCCCTTGCTAGAC
FLNC	human	GGAGCCCTCTGAAGT	TTGTCGGTGATGTTGG
FLNC	rat	AAATATGTCATCACCATCC	GGATGACCAAGTTGAAAG
MYH11	human	AAGAAAGACACAAGTATCAC	CATGTAGTAAAAGATGTGGA
MYH11	rat	AAGAAAGACAGCAGCAT	AGACTTTTCCAGAAGATATG
LMNA	human	GCATCACCGAGTCTG	ACCCTCCTTCTTGGTA
LMNA	rat	CCGAAGTTCACCCTAAA	TCATTGTCCTCAACCAT
LMNB1	human	CCTGGAGACGGAGAA	ATCAGATTCCTTCTTAGCAT
LMNB1	rat	GACAAAAAGAGTTTAGAGGG	CATTGATCTCCTCTTCATAC
AP1B1	human	GAAGGATATTCCCAATGAG	TTCAGGGACAGCTCTA
AP1B1	rat	AAATGACTGACTCAAAATACT	CATAGTTCATCAAGTACAGG
VAPB	human	AAACACAAGTTTATGGTTCA	AAGAACTCAGAGACTTAGAC
VAPB	rat	GTTACAGCCTTTCGATTAT	TATTTCTACATCATGTGGTTT
RPSA	human/rat	TACCTACCATTGCGCTGTGT	CCATGGGTGTTCACGGGAAAT
RPL10A	human/rat	AGCACTGTGACGAGGCTAAG	GACTCTGAGGCCAAAAACGC
RPL4	human/rat	AGCCGCTTCCCTCAAGAGTA	TCGAAGGGCTCTTTGGATCTC
RPL6	human/rat	GGTGACAAGAACGGCGGTA	CACGTGCTGACTGAAGGGTT
RPL7	human/rat	AAATTCGAATGGCGAGGATGG	TTCGAACCTTTGGGCTCACTC
RPL7A	human/rat	AGAAAGTGGTGAATCCCCTGT	TATAGCGGGGCCATTTCACAA
RPL8	human/rat	GGCCAGTTTGTGTATTGCGG	CAGCTTCACACGGGTCTTCT
RPL9	human/rat	GTGTATGCTCACTTCCCCATCA	TCTGGGCTTGAGATACTGAAC
RPL10	human/rat	TGATCCCCAGAAGGACAAGCG	GGCCTTAGCCTCGTCACAGT
RPL12	human/rat	CTGGCCCCCAAGATCGG	GCCTGTCTGTTCTGAATGGT
RPL13	human/rat	GGCATTCACAAGAAGGTGGC	GGGTGGCCAGTTTCAGTTCT
RPL15	human/rat	GCCAAGATGGGTGCATACAAG	GCACCCTTAGGAACTGGGC
RPL17	human/rat	AACTGCTCAGGCCATCAAGG	CACTCTTTTTGGGCCACCGA
RPL18	human/rat	AACTGATGATGTGCGGGTTC	ACAGTGCCACAGCCCTTAG
RPL18A	human/rat	ATCTGGCTGCGCTATGACTC	ATCTGAATGGAGTGGGCTCG
RPL19	human/rat	CCGAATGCCAGAGAAGGTCA	TCCATGAGAATCCGCTTGTT
RPL21	human/rat	GAGTTGTTCCTTTGGCCACATA	ACACTTGTGGGGCATTCCTT
RPL22	human/rat	AAAGCTTGTGGTGAAGGGGG	AAAGGCACCTCGGATGTCAC
RPL27	human/rat	CTACCCCCGCAAAGTGACAG	CGGGCCTTGCGTTTAAGAG

RPL30	human/rat	AGTGGGAAGTACGTCCTGGG	GCCACTGTAGTGATGGACACC
RPL27A	human/rat	TGCATCACCACCGGATCAA	TTCACCCGTGTCTGTTCACT
RPL29	human/rat	ACATGGCCAAGTCCAAGAAC	TCGTATCTTTGTGATCGGGG
RPL34	human/rat	AAATCTGCATGTGGTGTGTGC	AGGCCCTGCTGACATGTTTC
RPLP0	human/rat	GGTCATCCAGCAGGTGTTCG	TCAGCAAGTGGGAAGGTGTA
RPLP1	human/rat	CCCTCATTAAAGCAGCCGGT	ACATTGCAGATGAGGCTCCC
RPS4X	human/rat	CTATGACACCAAGGGTCGCT	ACCTTGATGAGGGGATCGGG
RPS5	human/rat	GAGACCCCAGACATCAAGCTC	TGAGCTTCTTGCCGTTGTTG
RPS6	human/rat	GATACTACAGTGCCTCGCCG	GCAGGACACGTGGAGTAACA

The developed primers and the obtained primary cultures of normal eukaryotic cells will be further used to analyze the effect of phenazines on non-malignant cells, which is critical for assessing their toxicity and potential for further use as a substance for antitumor drugs.

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