Part 1

Derinat® is an immunomodulatory agent, hemopoiesis stimulator, regenerative and reparative agent

Derinat® (deoxyribonucleic acid sodium salt (PDRN) is an eukaryotic polydeoxynucleotide (PDRN) with a molecular weight of up to 500 kDa derived from salmon milt. Considering that a weight of one nucleotide is about 345 Daltons, the number of nucleotides in the molecule of a parent substance is estimated to be 1500, that is the Derinat® molecule consists on the average of 750 base pairs. It is suggested in the available literature that a mechanism of action of Derinat® on immunocompetent cells can be mediated by Toll-like receptors (TLR) 9. Derinat® predominantly includes a short chain and a medium chain of DNA ended with CpG nucleotide motives on the average at least in 50 % of cases.

At the Research Institute of Experimental Biology and Medicine of Burdenko Voronezh Medical University, a nucleotide composition of DNA fragments composing Derinat® was determined by an electrophoretic method in a block of agarose gel using tris-acetate-EDTA (TAE) buffer. Evaluation of the main drug substance of Derinat® showed three types of DNA fragments: long – 40 %, medium – 40 %, and short – up to 20 %. In general, quantitative ratios between different types of nucleotides in fragments corresponded to distribution described using Chargaff's rule. Evaluation of a nucleotide composition of the end sites of DNA fragments in the composition of Derinat® showed that short fragments end with CpG unmethylated dinucleotide motifs in 25 % of cases. Medium fragments end with CpG unmethylated dinucleotide motifs in 15–20 % of cases, while long fragments have a CpG unmethylated motif at the end site in 5 % of cases [1].

Unmethylated CpG motifs of DNA are ligands for TLR9 (TLR9, CD289) - membrane proteins from a Toll-like receptor group which provide the innate immune response performance. Unmethylated CpG motifs are prevalent in bacterial and viral DNA. They are specifically recognized by TLR 9 receptors in the innate immune response system. [1] Such CpG motifs in eukaryotic DNA are harboured inside of its structure and are mainly contained in so-called CpG islands. A size of these CpG islands is about 0.5 to 5 thousand nucleotide pairs. Their frequency is 1 per 100 thousand nucleotide pairs. All housekeeping genes and 40 % of tissue-specific genes contain CpG islands.

Characteristics of CpG islands:

- size is 0.5 to 5 thousand nucleotide pairs
- frequency is 1 per 100 thousand nucleotide pairs
- commonly contain a CpG motif (1:16)
- no cytosine methylation

In the process of Derinat manufacturing, DNA molecules contained in salmon sperm homogenates are fragmented using ultrasound processing, unmethylated CpG motifs are exhibited and become available for exposure on TLR 9 cells expressing this receptor. It is obvious that methylation of ODN CpG motifs is not possible outside a living organism, so eukaryotic unmethylated CpG motifs are available for pharmaceutical use. DNA methylation is a dynamic process which changes with a development of tissue differentiation and ageing. Hypomethylation was found in old tissues of salmon, mice, rats, cows, and humans. It is particularly well presented in cerebral cells, liver, small intestine mucous membrane, heart, spleen, and T-lymphocyte tissues. [1]

A high-frequency TLR9 and NF-kB and MyD88 receptor-dependent pathway of a stimulatory signal transduction to immunocompetent cells determines a wide spectrum of therapeutic prospects of the medicinal product. As shown by Japanese investigators Mami Noda et al., Derinat®

decreased intracellular synthesis of reactive oxygen species, COX-2 expression in cells. Derinat blocks classic voltage-gated class C calcium channels, canonical / (TRPCs) according to calcium channel visualization [7]. Treatment with Derinat has antiinflammatory and antioxidant effects on ischemia reperfusion (IR) injuries of skin in model pressure ulcers (PU) in mice. Skin injury and oedema on the back of mice with mild pressure ulcers were reduced after treatment with Derinat. Immunohistochemistry and biochemistry showed that Derinat suppressed IR-induced oxidative injury, that is accumulation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and related inflammatory factors, such as cyclooxygenase 2 (COX-2) and IL-6 receptor (IL-6R) in the back skin of mice with model PUs. We also confirmed that a ratio of extracellular signal-regulated phosphorylated/unphosphorylated kinase (Erk) to mitogen-activated protein kinase p38 (MAPK) increased after IR which was attenuated by Derinat. Then we compared the effects of Derinat® with those of the salmon DNA and other therapeutic agents, prostaglandin E1 (PGE1) and basic fibroblast growth factor (bFGF), on the development of pressure ulcers using mice with model severe pressure ulcers. The effects of Derinat® and salmon DNA were consistent with the effects of PGE1 and bFGF [8]

Derinat barrier anti-inflammatory effect is associated with a stimulation of TLR9 plasmatic dendritic cells. TLR9 stimulation in dendritic cells having a lymphoid progenitor causes their differentiation (under the action of IL-10, TNF β, prostaglandin E2) into dendritic cells. Dendritic cells secrete a lot of type α and β IFN-1 and promote a differentiation of Th 0 into Th 2. B-cells are differentiated under Th 2 action, in plasmatic cells secreting Ig G2, IgG4, IgM. [3,5]

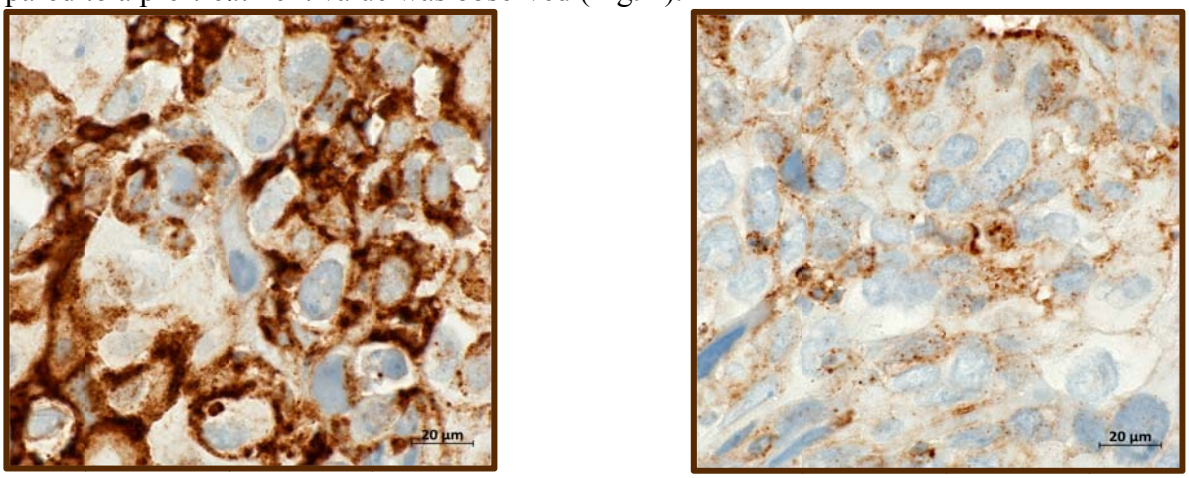
Presently, administration of Derinat® as a TLR9 agonist is the most prospective method to reduce toxicity of antitumor therapy such as chemotherapy and radiotherapy. Hematologic toxicity reduction during Derinat® administration is observed at all key hematopoiesis stages: myelopoiesis (granulocytopoiesis, erythropoiesis, and thrombocytopoiesis) and lymphopoiesis. Granulocytopoiesis is activated via the synthesis of the granulocyte-macrophage colony-stimulating factor (GM-CSF) by macrophages stimulated by Derinat® TLR9 agonist.

In 2017, our company Immunoleks Pharmaceutical Plant initiated a clinical study titled Strategy of Coping with Radioresistance of Stage IIB-IVA Squamous Cervical Cancer with “Derinat® Toll-Like Receptor 9 Agonist” to show a reduction in tumour radioresistance and its possible use in cervical cancer radiotherapy.

The first results demonstrated by immunohistochemistry of tumour bioptates evidence a significant reduction in the PD L-1 synthesis by tumour tissue both under Derinat systemic administration, and its local administration in the process of radiotherapy. Toxicity of such therapy is extremely low which is associated with specific pharmacodynamics of the medicinal product.

Several studies showed that radiotherapy in combination with TLR 9 agonist (sodium deoxyribonucleate) CpG-ODN was capable of reducing tumour radioresistance. It increases a number of cells in the most sensitive phase of the cell cycle G2 \ M and suppresses expression of the PD-L1 death receptor ligand by inhibiting its synthesis via the NF-κB signaling pathway. We supposed that TLR9 agonist (sodium deoxyribonucleate) in combination with radiotherapy could have a better antitumour activity and modulate the PD-L1 expression by tumour cells.

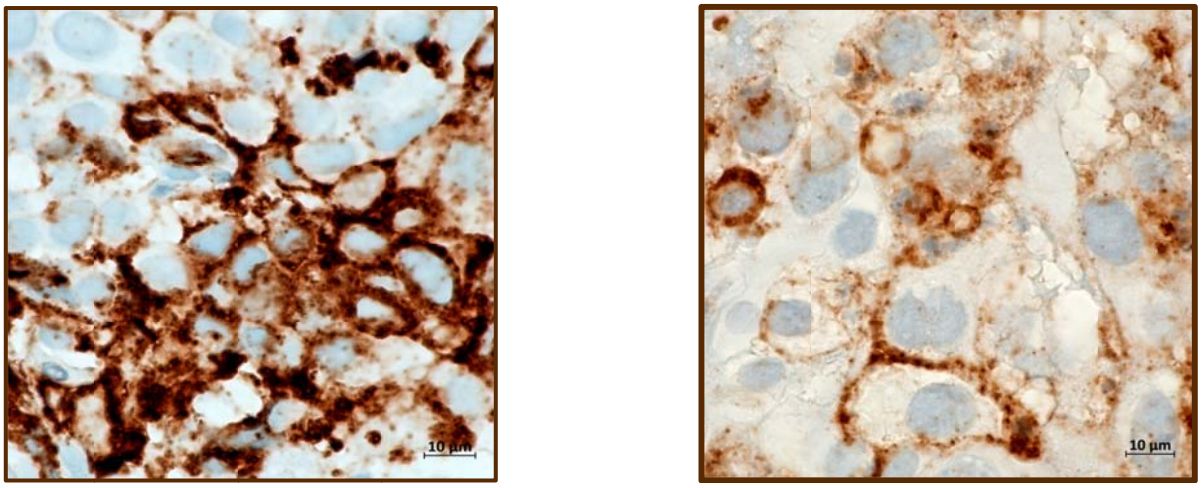
A statistically significant 2-fold reduction of the PD-L1 death receptor ligand expression compared to a pre-treatment value was observed (Fig. 1).



before immunotherapy after immunotherapy

Figure 1. PD-L1 Ligand Expression by Squamous Cervical Cancer Cells

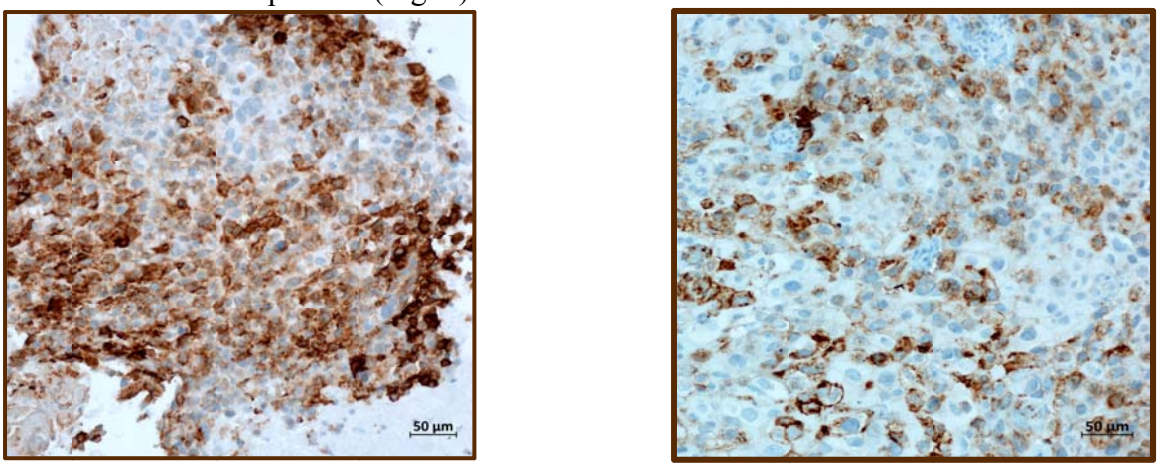
An average frequency of the PD-L1 expression in the sodium deoxyribonucleate group was 12.7 % before treatment and 6.1 % after treatment (Fig. 2).



before immunotherapy after immunotherapy

Figure 2. PD-L1 Ligand Expression by Squamous Cervical Cancer Cells

A frequency of the PD-L1 expression in the sodium deoxyribonucleate group was reduced by 18 % at the first biopsy relative to the pre-treatment values and by 5 % in the group of patients who did not receive the medicinal product (Fig. 3).



before immunotherapy after immunotherapy

Figure 3. PD-L1 Ligand Expression by Squamous Cervical Cancer Cells

We suppose that the immunotherapy strategy targeted at a reduction of the PD-L1 synthesis in tumour tissue will potentiate the effect of anti-PD-L1 antibodies such as pembrolizumab approved by FDA for patients with recurrent or metastatic cervical cancer which progressed during or after chemotherapy. There are also some current studies which can provide more evidences that the PD-1/PD-L1 pathway is a therapeutic target in cervical cancer.

Besides, Derinat® being a polydeoxynucleotide (PDRN) is also an agonist of A2 adenosine receptors (A_2AR). Stimulation of these receptors is a key factor promoting to wound healing and neovascularisation. Understanding of molecular interactions between A_2AR activation induced by Derinat® and inflammation can provide effective instruments to improve healing in some diseases.

Due to its chemical structure, the compound is freely circulated in plasma, distributed in tissues based on their blood supply and degraded by non-specific plasmatic or membrane-bound DNA nucleases which make its active oligo- and mononucleotides available for biological activity. Derinat® can be considered as a pro-drug which provides active deoxyribonucleotides for binding of purinergic receptors. A released adenosine acts on adenosine A_2A receptors by using 3.7-dimethyl-1-propargyl xanthine, the A_2AR selective antagonist, which neutralises Derinat® effects. Besides, nucleotides derived from Derinat® can also act via a rescue pathway, an effective energy-saving metabolic pathway for the nucleic acid synthesis.

Bases and nucleotides from PDRN at some point integrated into the DNA of injured and hypoxic cells contribute to the DNA formation and reactivate the cell proliferation and growth. Thus, Derinat® can not only act on adenosine receptors, but also can promote the cell proliferation providing new building blocks for cells via a rescue pathway, and particularly due to this double action administration of Derinat® may be more preferable compared to A_2AR agonists. Thus, these data encouraged investigators to suggest a hypothesis that Derinat® can be a therapeutic instrument for inflammation control via A_2AR stimulation when it is involved in the rescue pathway in the DNA synthesis in injured or hypoxic cells, promotes the DNA formation and reactivates the cell proliferation and growth.

Currently, there are completed and ongoing worldwide 6 clinical studies of the efficacy of medicinal products with the PDRN-containing drug substance similar to Derinat®: 1 polydeoxyribonucleotide (PDRN) for regeneration of rotation wrist cuff, 2. PLACENTEX® efficacy and safety in patients with sclerodermia, 3. Placentex® polydeoxyribonucleotide (PDRN) for treatment of diabetic foot ulcers, 4. Effects and safety of PDRN epidural administration compared to placebo in spinal stenosis, 5. A study of Placentex® rehabilitation effects on elbow (lateral) epicondylitis, 6. Defibrotide in patients with veno-occlusive disease of the liver. [9]

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Part 2
Derinat® Clinical Efficacy Experience

A stepwise study of Derinat® mechanism of action using advanced research technologies contributed to the clinical effects of the medicinal product becoming quite explicable.

The main schemes of Derinat® administration in different nosological forms have been currently developed and tested. A positive result of Derinat® administration by many healthcare professionals in practice is based on combined immunomodulatory and regenerative effects of the medicinal product in a complex therapy of patients with diseases in a pathogenic mechanism of which immune homeostasis disorders play a key role: recurrent chronic respiratory diseases, recurrent acute and chronic infection processes (both of bacterial, and viral ethiology), postoperative pyoinflammatory complications (in surgery, stomatology, urology, gynaecology, dermatovenerology). Besides, Derinat® proved itself as a haematopoiesis stimulator in oncological patients receiving radiotherapy and chemotherapy.

Derinat® (2.5 mg/mL) has been widely used by healthcare professionals as a pathogenetic component of a complex treatment of acute respiratory viral infections in both adults and children. Exposure to Derinat® (2.5 mg/mL) solution on the upper respiratory mucosal membrane causes TLR-9 stimulation in dendritic cells increasing their potential to effect the T-cell differentiation and to promote the formation of Type 2 T-helpers which induce the differentiation of B-cells into plasmatic cells secreting IgG and IgM. Mucosal epithelial cells stimulated by Derinat® (2.5 mg/mL) via TLR-9 enhance a secretion of sIgA performing both a direct barrier function and a function of the macrophagal phagocytosis activation. Thus, a stimulation of dendritic cells and macrophages by Derinat® through TLR-9 induces an activation of both a humoral and cellular arm of the immune response of the upper respiratory mucosal membrane. The effect of Derinat® administration can be noted on the first day of therapy: rhinorrhea resolves, nasal congestion disappears, swelling and hyperemia of palatine tonsils are reduced much faster and more effectively than in standard therapy schemes. Such effect was observed in administration of the medicinal product in both adults and children since birth [1,2,10,21].

A combined administration of Derinat ® (1.5 mg/mL) with different physiotherapeutic methods (laser, electrophoresis, biostimulation) of treatment in surgery as add-on to postoperative therapy in patients who underwent surgery for pyoinflammatory abdominal diseases showed a high efficacy of such treatment approach. A proinflammatory activity of the immune system in such patients was reduced and did not interfere with the recovery: more favourable trends in healing and reparative and restorative processes were predominant; quick regression of the wound site hyperemia and swelling, reduced pains, reduced asthenization and anxiety level, shortened duration of patient hospitalization (by 2-3 days), and no rise in the cost of treatment [4].

Derinat® (15 mg/mL) showed a high efficacy in a complex therapy of chronic pelvic inflammatory diseases in women (endometriosis, endometritis, adenomyosis) [3,12,17].

Derinat® (15 mg/mL) administration in a complex treatment of chronic bacterial prostatitis in the acute stage helps reduce disease symptoms in the criteria such as “pain and discomfort” and “quality of life” and decreases the frequency of late relapses The obtained results demonstrate a high efficacy of sodium deoxyribonucleate administration in a complex treatment of patients with chronic bacterial prostatitis in the active stage and allow for recommending this medicinal product for a wide use in urological and andrological practice [11].

Given a cytoprotective and regenerative effect of Derinat® (15 mg/mL) when it is systemically administered as both an immunoreparative agent and Toll-like receptor 9 agonist

(TLR9, CD289), we consider that it is reasonable to use this drug product for restoration of urinary bladder tissues damaged during radio-way excision. The highest expression of TLR9 is noted particularly in macrophages, and they get the most sensitive to stimulation by agonists, such as Derinat® (15 mg/mL). Regeneration of the urinary bladder epithelial tissue is accompanied by the release of fibrinogen from the vessel lumen in the intercellular compartment and by the synthesis of fibrin which forms a structure for the cell proliferation. Administration of agonists promotes TLR9-dependent accumulation of macrophages at the sites of tissue damage, stimulation of endothelial cell growth factor (VEGF) synthesis at the alteration region, angiogenesis acceleration, and epithelium defect closure [12,20], thus explaining the changed urinary sediment. A combination of conformal EBR with intramuscular Derinat® (15 mg/mL) reduces severity of Grade 2 and 3 radiation reactions, promotes uncomplicated disease course according to RTOG, has minimal effects on hematopoiesis, and minimizes inflammatory changes in the urothelium [8]

A potential to increase tumour radiosensitivity has a high practical value in oncology. The effects of the innate immunity mechanism induction leading to the increased sensitivity of tumour tissue to radiotherapy not only directly but also indirectly via activation of native immunocompetent cells, in the tumour microenvironment as well, were found in the mechanisms of Derinat® (15 mg/mL) immunotropic effects. Examination of pathomorphological markers of reduction in cervical tumour radioresistance on radiotherapy and with addition of Derinat® (15 mg/mL) [19] to schemes of cervical cancer chemoradiotherapy has a high potential to stimulate an increased expression of TLR9 among immunocompetent cellular components of the stroma.

A steadily increasing incidence of acute and recurrent respiratory infections (rhinitis, sinusitis, tracheitis, bronchitis, pleuritis, pneumonia, and COPD) in the world revealed an interaction between a severity level of inflammation and abnormal parameters of the immune response. From this very point of view, Derinat® administration is prospective in complex therapy of respiratory disorders. The results of Derinat® (15 mg/mL) administration in practice showed benefits of its use and a diverse efficacy in both adult patients and children with acute and infectious respiratory diseases [6,10].

Derinat® (15 mg/mL) has a special place in the complex treatment of severe tuberculosis having, in the whole, a favorable effect on the infectious process when it is included in the combination ethiopathogenetic therapy. The action of Derinat® (15 mg/mL) is based on activation of both cellular and humoral arms of the immunity enhancing regeneration of the damaged lung tissue [5,13,15].

Administration of Derinat® (15 mg/mL) in combination with a standard therapy of COPD promotes a significant improvement of the patient quality of life compared to patients receiving only a standard therapy with glucocorticosteroids as evidenced by a more significant reduction in symptoms of dyspnoe, cough, and the amount of produced sputum; improved clinical picture of a disease as evidenced by positive changes in the immunological status (increased T-cell count - particularly CD8-lymphocytes, decreased B-cell count, increased expression of the CD4 antigen) was noted [6].

The complex treatment of patients (aged 18 to 55 years) with a diagnosis of risk class I–II (PSI) community-acquired pneumonia had led to a more productive restoration of the pulmonary tissue structures, normalized the most of the immune status parameters such as CD3, CD8, and CD22, normalized concentrations of the proinflammatory cytokines such as IL-1, IL-8, and IL-10, and also allowed for reduced a duration of the patient's temporary disability when Derinat® (15 mg/mL) was used in combination with a standard therapy compared to the results obtained in patients receiving only a standard therapy [6]. During a study in school-aged children

(aged 7-16 years), a clinical efficacy of the medicinal product was determined which was characterised by a reduced disease duration, accelerated reparation of inflammatory changes in the lungs in rhinocytogram, decreased count of neutrophils and neutrophil destructive forms, decreased levels of the proinflammatory cytokines such as IL-8 and TNF-α. As a result, all this was manifested in enhanced adaptation resources of the respiratory immunity of children, restored epithelial lining of the upper respiratory airways and, thus, potentiated innate immune response [14]. Similar effects of Derinat 15 mg/mL were noted in the group of adult patients (30–40 years) with a diagnosis of moderate community-acquired pneumonia: concentrations of IL-8, IL-4, TNF-α, phagocytic index, parameters of the spontaneous NBT test, AHP, and catalase activity were normalized [16].

The recent events related to the COVID-19 pandemic in the world made healthcare professionals face challenges associated not only with a reversal of clinical symptoms of infection or arrangement of epidemic control measures, but also with a necessity of the remedial treatment and rehabilitation of patients who recovered from this disease. Lung damage, psychosomatic disorders, and immune system exhaustion present a serious barrier to recovery of patients with COVD-19-associated pneumonia. Derinat® (15 mg/mL) included in the remedial treatment and rehabilitation of patients with COVID-19 helps restore the pulmonary tissue, corrects induced immunosuppression and its somatic manifestations via stimulation of regeneration factors. A significant improvement of the quality of life of persons who recovered from COVID-19, recovery (up to normalization) of the pulmonary function indices, decreased volume of the inflammation area in the pulmonary tissue – all of that reduces a temporary disability duration for patients with COVID-19 and makes Derinat® (15 mg/mL) an indispensable element in a complex of rehabilitation measures on mitigation of risks related to disability of patients who had recovered from COVID-19 [7,9,18].

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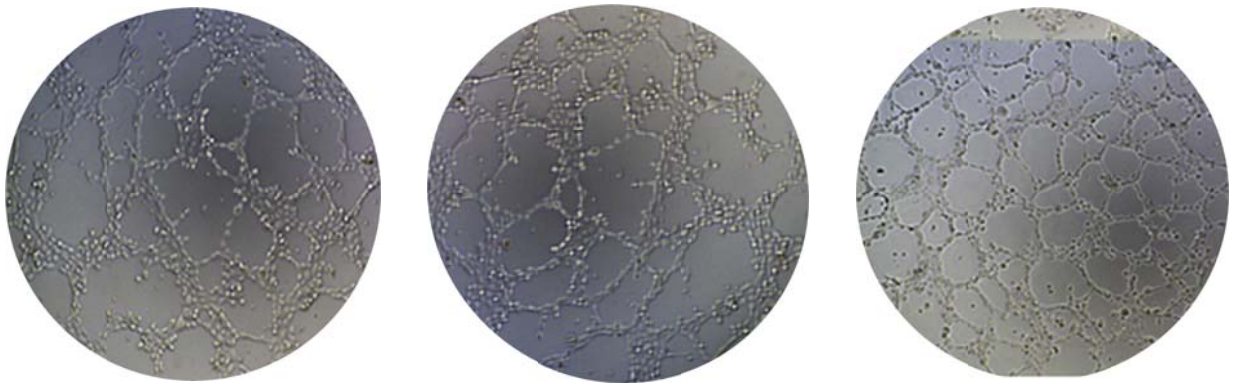
Part 3
Prospective Areas of Derinat® Efficacy Studies

Studies of Derinat® evaluating the effect of Derinat as a cultural medium component on the proliferative activity, migration activity, vascular tube formation, and determination of angiogenesis factors in supernatants of EA.hy926 endothelial cell cultures conducted at the Federal State Budgetary Research Institution D.O. Otta Research Institute of Obstetrics, Gynecology and Reproductive Medicine in 2020 confirmed its regenerative efficacy (Fig. 4).



Baseline *Placebo* *Derinat*

Before the study 72 hours after the study initiation

Figure 4. Derinat effects on EA.hy926 epithelial cell culture

72 hours after the study initiation, it was confirmed that Derinat® stimulated the endothelial cell proliferation (single-layer squamous epithelium): it promoted activation of stem cell mitotic division and differentiation, stimulated migration activity by increasing the number of endothelial cells migrated to the damage site. Besides, Derinat® stimulates vessel formation via branching angiogenesis. The data on the evaluation of the drug substance effect on proliferative and migration activity of endothelial cells are consistent and support the results of the evaluation of the drug substance effect on the potential of endothelial cells to form vessels. Thus, branching angiogenesis is characterised by vessel formation via lateral vascular sprouting or connection of the existing vessels. Angiogenesis of this type requires stimulation of the endothelial cell proliferation and formation of new growth points of the endothelium that is stimulation of the endothelial cell migration activity, which is consistent with the previous results of the evaluation of Derinat® efficacy. Besides, it was found that Derinat® stimulated an autocrine regulation of the endothelial cell proliferation: the endothelium destruction elements should be PRR-stimulators of intrinsic mitotic activity, but it does not occur and their function is fulfilled by Derinat®. The active substance of Derinat® is sodium deoxyribonucleate – this substance has effects on endothelial cells by interacting with a Toll-like receptor 9 (TLR9) found in endothelial cells. In the literature, there are evidences that endothelial cells express the pattern-recognizing receptor (PRR), including Toll-like receptors (TLR), which are activated in response to stimuli in the blood flow, including pathogens and damage signals. The endothelial cells tested with Derinat® also express TLR9. A Toll-like receptor 9 is a membrane intracellular protein localized in the endoplasmatic reticulum, lysosomes, and endosomes. A TLR9 ligand is presented by the DNA specific sites. Via pinocytosis and endocytosis processes, endotheliocytes are capable of uptaking the DNA specific sites which can interact with TLR9. It is a ligand-receptor interaction between TLR9 and sodium deoxyribonucleate in the composition of Derinat® on which a mechanism of action of this drug substance on the endothelial cells is based. Thus, Derinat® has the following effects: acceleration of

proliferative activity of squamous endothelial cells (endotheliocytes, mesotheliocytes, ependyma) not increasing a synthesis of signal proteins and proinflammatory substances, thus reducing risks of further development of uncontrolled inflammatory reaction at the damage site; a selective action on angiogenesis (predominantly capillary), thus limiting the excessive effects of the proinflammatory factors which could lead to more chaotic processes returned as contradictory properties – enhanced destruction and inflammation at the damage site; autocrine effects on the capillary and alveolar endotheliocytes excluding hyperproduction of cytokines and enhanced activity of circulating neutrophils.

Quite interesting therapeutic prospects appear when Derinat® is considered as a protective and regenerative agent when included in the treatment scheme of the increased epithelial permeability syndrome. (IEPS). The IEPS is based on a 3-component pathology: pre-epithelial, epithelial and subepithelial. A damage of one of these levels can cause quite a serious pathology in the human body: gastrointestinal (intestinal permeability disorders), cardiovasculare (IHD, stroke) and other visceral system diseases (rheumatic diseases, bronchopulmonary abnormalities). The IEPS has a special place in psychiatric disorders (neuroses, psychotic disorders, schizophrenia) and nervous disorders (epilepsy, Alzheimer's and Parkinson's diseases); in geriatric practice (frailty syndrome, inflammaging, age-related intestinal permeability and microbiota).

Thus, having a pronounced regenerative and cytoprotective effect, Derinat® can take the lead in clinical schemes for treatment of nosologies caused by the pathological increased epithelial permeability syndrome.