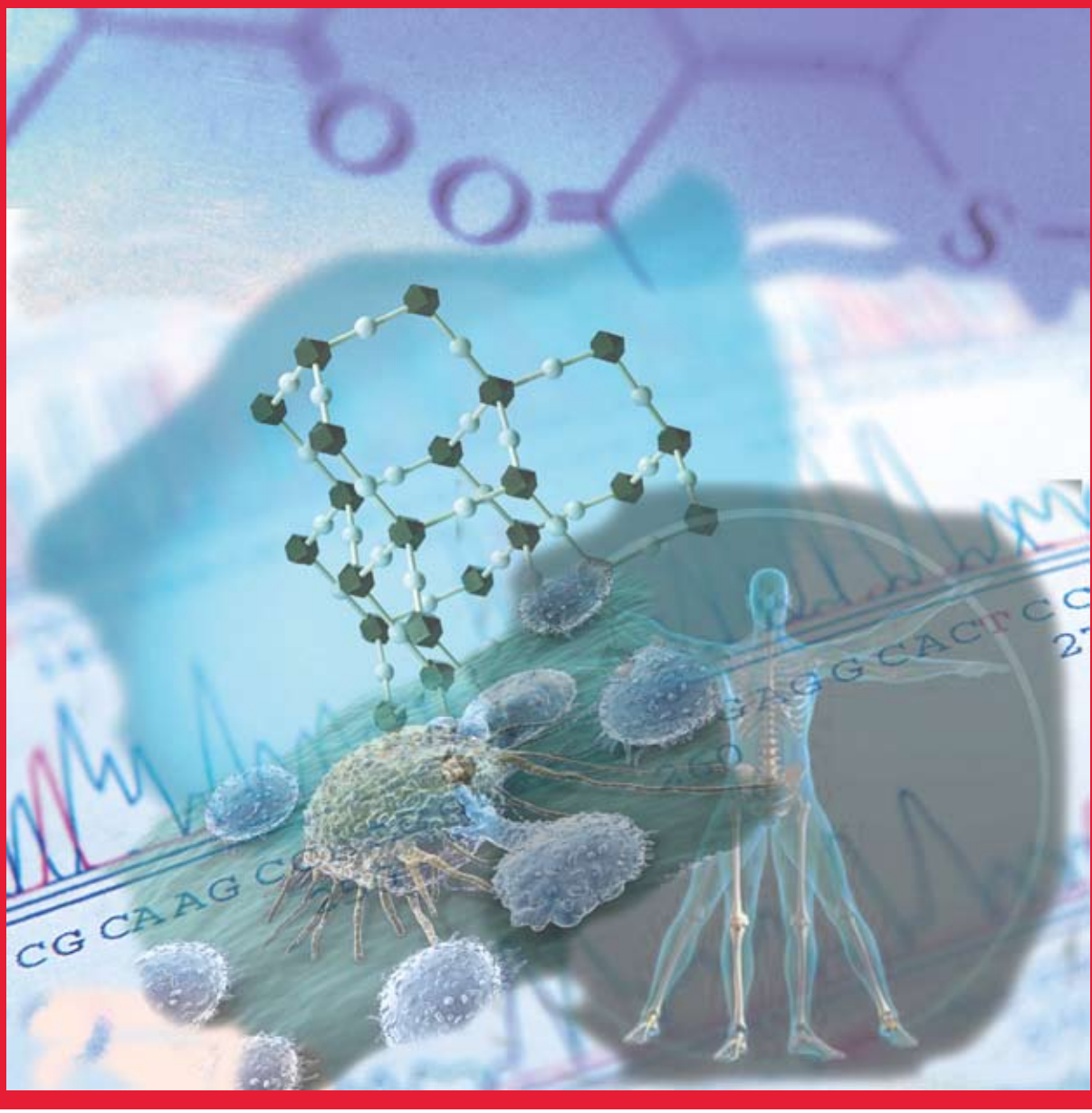
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New approaches to targeted drug delivery to tumour cells

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Basic approaches to the design of targeted drugs for the treatment of human malignant tumours have been considered. The stages of the development of these approaches have been described in detail and theoretically substantiated, and basic experimental results have been reported. Considerable attention is paid to the general characteristic of nanopharmacological drugs and to the description of mechanisms of cellular interactions with nanodrugs. The potentialities and limitations of application of nanodrugs for cancer therapy and treatment of other diseases have been considered. The use of nanodrugs conjugated with vector molecules seems to be the most promising trend of targeted therapy of malignant tumours.
The bibliography includes 122 references.

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I. Introduction

The idea that selective regulation of physiological processes is possible both at the level of the whole organism and at the level of organs and individual cells has been developed and specified in parallel with the general development of biological science. The German physician and researcher Paul Ehrlich has formulated the concept of an ideal drug that would act like a magic bullet, damaging nothing in the human body but finding and hitting germs in it.[1]

In 1899, Ehrlich became the director of the Royal Institute for Experimental Therapy in Frankfurt and formulated his scientific dream — to find drugs that, due to their tropism (selectivity) to specific tissues, will act only on the affected cells without damaging healthy cells. This idea, because of its beauty and clarity of purpose, over the years found its strongest supporters; however, after a century of efforts, such a drug has not been designed thus far. At the same time, the results of relevant studies, analysis of the errors and the development of new approaches in pharmacology have demonstrated that the creation of such drugs is possible, and the first preliminary results have been already obtained. For example, the first targeted drug Herceptin, which is specific to the tumour marker HER2/neu associated with a variety of malignant tumours, has been successfully administered to patients in hospitals around the world in recent years. The total number of different targeted drugs based on antibodies as vector molecules has already reached several dozens. This topic has been addressed in some reviews.[2–5] The present review mainly focuses on those aspects of targeted drug design that have not yet received adequate coverage in the literature but have great prospects for development of this kind of therapy.

A team of researchers at the Russian Research Centre of Molecular Diagnostics and Therapy has achieved significant results in using receptor-mediated endocytosis systems for selective inhibition of tumour cells without noticeable toxicity to healthy cells. In this review, preliminary results of relevant research are reported and discussed in detail.

The discovery of the most important component of the signal transduction chain — cyclic adenosine 3′,5′-monophosphate (cAMP) — made by Earl Sutherland in the late 1950s overturned the concept of regulation of biochemical processes in the cell.[6] This discovery was the basis for the creation of a holistic concept of secondary messengers, which is now rightfully considered to be fundamental in the cell and physicochemical biology. The significance of

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this concept for the development of molecular medicine is also discussed in this article.

II. Second messenger concept — on the way from general to targeted action

II.1. Secondary mediators (messengers) — the history and state-of-the-art of the problem

Once it has been accepted by the scientific community that large signalling molecules — ligands — unable to pass through the cell membrane interact with specific receptors and enzyme systems located on its outside, study of intracellular signal transduction mechanisms has attracted the attention of many research laboratories worldwide. Figure 1 shows that the number of reviews dealing with second messengers presented in the PubMed search engine has steadily increased until now, and the total number of articles exceeds 34 thousand.

It is a common knowledge that intracellular cascade reactions begin with formation or activation of second messengers — low-molecular-mass biologically active compounds transducing a signal to intracellular effectors. More than ten such compounds have been described. These are cyclic nucleotides cAMP and cyclic guanosine monophosphate (cGMP); products of inositide exchange — inosine triphosphate (IP_3) and diacylglycerol (DAG), as well as Ca^{2+} ions; polynucleotide oligoA; nitrogen monoxide, arachidonic acid exchange products; some other lipid compounds. Key stages of signal transduction mediated by second messengers, common for all known regulatory systems, are as follows: ligand–receptor–effector enzyme–second messenger–modulated enzyme component–physiological response (Fig. 2).

Key features of second messengers are their versatility and trigger properties: they trigger protein phosphorylation and activation of proteins by calcium ions or other factors. This leads to cell response — secretion, induction of various genes, proliferation, *etc*. Diverse molecular structures (for example ion channels) and multistep cascades of enzymatic reactions can act as effectors.

Cyclic adenosine 3′,5′-monophosphate is produced by the reaction of epinephrine with the liver cell membrane and then activates phosphorylase to give glucose. The discovery of cAMP initiated a new stage in modern biology and medicine. Currently, cAMP is crucial for describing the mechanisms of action of many biologically active compounds. It has been comprehensively covered in various reviews.[8–10] The intracellular level of cAMP is maintained by the joint action of adenylate cyclase and phosphodiesterase: the former catalyzes the transformation of adenosine triphosphate (ATP) into cAMP, and the latter destroys cAMP to adenosine monophosphate (AMP) through hydrolysis (PP_i is pyrophosphate).

$$\mathrm{ATP} \xrightarrow{2\,Mg^{2+}} \mathrm{cAMP} + PP_i + H^+$$

$$\mathrm{cAMP} + H_2O \longrightarrow \mathrm{AMP} + H^+$$

Key mechanisms of intracellular signal transduction have been well studied.[11]

For outstanding achievements in the study of second messengers, several Nobel Prizes in physiology or medicine have been awarded in different years: E Sutherland (1971), E Fischer and E Krebs (1992), A Gilman and M Rodbell (1994), L Ignarro, R Furchgott and F Murad (1998), P Greengard (2000) and other scientists.[12] In our country, at the Department of Biochemistry, Faculty of Biology, Moscow State University, under the guidance of Academician S E Severin, Professor V A Tkachuk and Professor E S Severin, the mechanism of action and regulation of cAMP metabolism enzymes and calcium-binding proteins have been studied, and articles and monographs addressing second messengers have been published.[13]

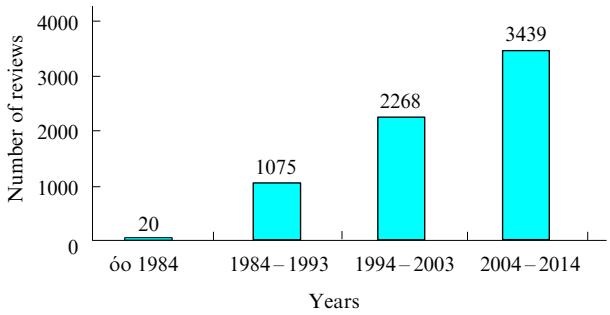


Figure 1. Growth of the number of reviews dedicated to second messengers (the PubMed search engine data are summarized by decades).

In subsequent years, the works of Russian researchers enabled the creation of the concept of second messengers as applied to clinical practice and molecular foundations of targeted drug therapy.[7] However, the progress achieved in understanding molecular and cellular principles of signal transduction was insufficient to ensure targeted regulation of biochemical processes in the cells and the body as a whole. In our opinion, the origin of this failure is polyvariance of each stage of the signal transduction chain and the selectivity of signalling (Fig. 3).

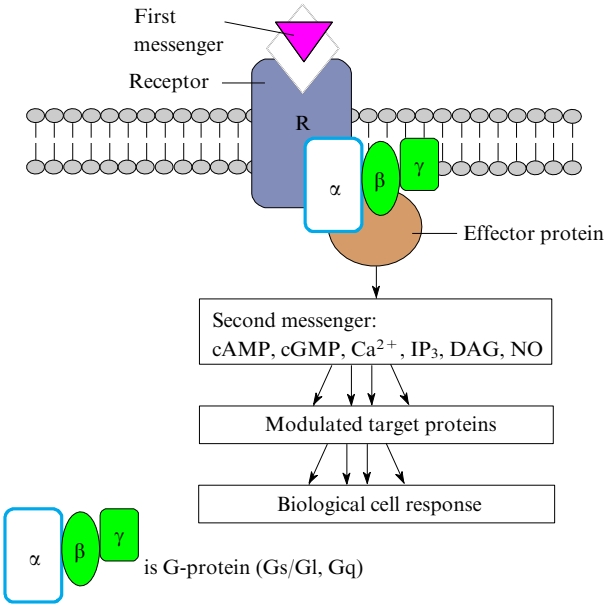


Figure 2. General scheme of modern concepts concerning the functioning of second messengers.[7]

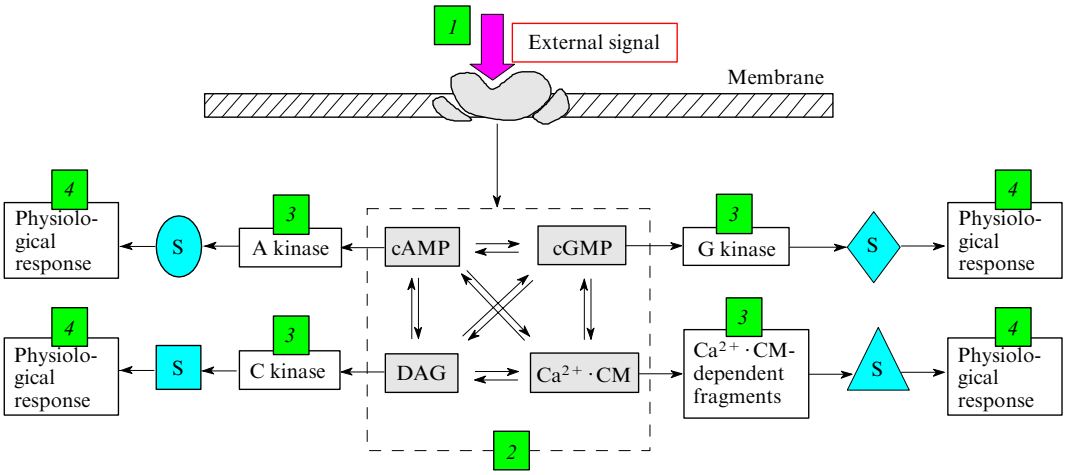


Figure 3. Four stages of selective signal transduction in a cell. CM is calmodulin; S is substrate specifically bound to an enzyme.

The selectivity (tropism) of a compound is its ability to affect cells, tissues and organisms of a definite type and ignore other objects, even if these are in contact with the former. The selectivity of a process is a set of conditions under which these effect occur. The higher the selectivity of antibiotic action, the more precisely it targets an infectious agent or a tumour cell and the lower its side effects. The higher the selectivity of the process, the more rigorous the conditions (pH, the presence of enzyme systems and other components of microenvironment) under which this process will occur along the specified path.

There are three key parameters responsible for the possibility of manifestation of the selective action of a substance:

— selective uptake, distribution and removal from different cells, tissues and organs;

— selective binding to specific membrane-bound and free biologically active endogenous compounds of the body;

— selective interaction with cytological structures existing in cells of definite types.

The selectivity of signalling in the cell manifests itself even at the first stage of binding of a protein ligand to the receptor of the adenylate cyclase complex at the cell membrane surface. Any structural changes in the protein ligand and changes in the charge and conditions of the microenvironment weaken this binding. Through feedback cAMP-dependent phosphorylation, cAMP can control its synthesis;[7] therefore, the external signal does not always lead to an increase in the level of second messengers and may cause no cell response. The activation of the second messenger system may lead to the activation of an alternative signalling path not only through cAMP but also through cGMP, diacylglycerols, calcium-binding proteins (for example, Ca^{2+}-calmodulin-dependent kinases) *etc*.

Upon signal transduction to the next stage, selection and inhibition of the enzyme–substrate complex formation are repeated, and the more complicated and diverse the enzymatic reaction cascade, the higher the selectivity of a given process. Thus, because of the complexity of the multicomponent and multilevel signal transduction system responsible for the protection of the cell and the organism as a whole from external impact, it is impossible to select a one-component drug that would lead to the desired physiological effect even if this substance has the exact binding site on the cell membrane. It is worth noting that all major achievements of molecular physiology are associated in some way with studying the cell surface receptors. The effect of specific ligands on definite receptors can be examined, predicted and used for practical purposes, rejecting, however, the mechanistic idea of the effect on a living organism based on the principle 'one drug–one effect'.

II.2. Receptor portrait of a cell as a basis for developing selective drugs

The cell membrane receptors are divided into two types. Receptors of the first type provide ion transport and are called ionotropic. These are membrane channels opened or closed by binding to the ligand. The resulting ionic currents modulate intracellular ion concentrations, which can indirectly lead to the activation of intracellular messenger systems. Receptors of the second type are associated with the direct activation of second messenger systems. A change in the spatial structure of these receptors upon binding to the ligand triggers a cascade of biochemical reactions and ultimately leads to a change in the functional state of the cell. All variety of receptors for different ligands present on the membrane of a specific cell constitutes its receptor portrait determining the physiological activity of the cell. Studying the receptor portrait of a cell and its changes makes it possible to elucidate the molecular causes of various diseases.[14] Impact on some molecular components of the cell is responsible for the targeted action of pharmaceuticals.

Paul Ehrlich's postulate about the selective action of drugs on selective targets (1905) was not recognized during his lifetime. However, nowadays it is precisely the search for binding sites to specific ligands, as well as for compounds that act competitively as a receptor inhibitor or activator, that serves as a methodological basis of the search for targeted drugs.

In particular, at the Russian Research Centre of Molecular Diagnostics and Therapy, parameters of the main human alveolar macrophage receptor systems in normal lungs and in chronic lung inflammation have been examined.[14,15] It has been demonstrated by the ligand dissociation constants as an example that the profiles of physiological parameters of receptors in norma lungs and chronic lung inflammation are essentially different (Fig. 4). Whereas the creation of the individual receptor portrait of

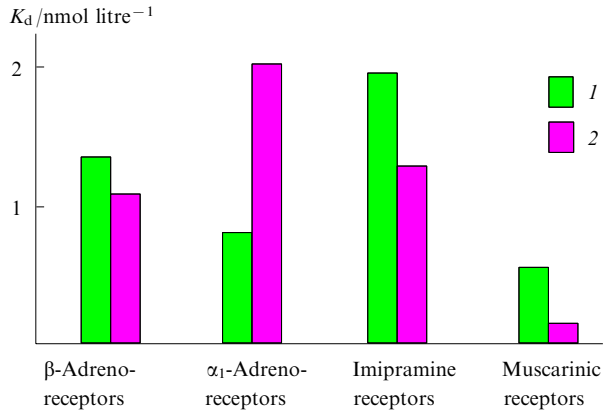


Figure 4. Binding parameters (K_d /nmol litre^{-1}) of receptors of the human lung alveolar macrophages with ligands in normal lungs (*1*) and chronic lunf inflammation (*2*).[15]

the human being as a whole is an issue of the distant future, the possibilities to obtain the receptor portrait, or receptor status, of individual organs and evaluate its changes depending on the course of a pathological process in target organs are actively studied in Russia [16] and abroad.[17]

Currently, the significance of the functions of membrane receptors and their spatial localization make these molecules the main targets in design of drugs for a wide range of diseases, including cancer. Comparison of the enzyme activity of the phosphorylation system in normal cells and in human tumour cells has shown that they differ substantially. At the same time, it has been revealed that the trends in the activity of the kinases under consideration have common features for different tumour cells.[18] Further comparative studies of the receptor portrait of tumour cells and healthy tissues have enabled the formulation of approaches to the development of candidate targeted antitumour drugs, which is discussed in detail below.[19]

III. Nanopharmacology as a priority area of pharmaceutical science and industry

Particles of high-molecular-mass compounds $\leqslant$100 nm in diameter are referred to as nanoparticles (nanocontainers, nanoliposomes, nanocapsules, *etc.*). Their relevance to modern medicine is due to the fact that up to 95% of newly created drugs of 'traditional' form exhibit unfavourable pharmacokinetic parameters and have pronounced side effects. Traditional medicines are delivered by the blood to all cells of the human body, and this is responsible for many side effects of drugs. Only a small amount of the drug administered intravenously reaches its destination in the affected organ of a patient. Moreover, stable resistance to the majority of new drugs is developed rather quickly (3 to 5 years), so that they cease to show the therapeutic effect. At the same time, most organelles and biologically and physiologically active substances of the human body, animals and plants are nanosized, which predetermines their high biochemical and pharmacological activity. This provides a theoretical basis for the development of nanopharmacology and a new branch of pharmacy — nanopharmacy.

Creation of a new generation of drugs and new nanotechnologies is a very expensive and lengthy process. At the

Table 1. Advantages and disadvantages of nanosystems used for drug delivery.

Nanocarrier	Advantages	Disadvantages	Ref.
Dendrimers, nanoemulsions	carrier of hydrophobic components, rapid absorption, lack of toxicity, long shelf life	rapid release, low stability under acidic conditions	26
	Nanoparticles		
Nanospheres	high surface-to-volume ratio, regulated drug release	low stability of some drugs, expensive production	27, 28
Nanocapsules (including PLGA nanocapsules)	the use of natural polymers, such as polysaccharides or proteins, enhances biocompatibility and biodegradability	large scatter in drug content in particles; purification is required after the synthesis of nanocapsules	27
Solid lipid nanoparticles	increase in the drug solubility in water, prolonged release from carriers, reduction of drug toxicity, rapid development of the pharmaceutical formulation	risk of recrystallization and low drug content in particles	29
Liposomes	ability to encapsulate water-soluble drugs in the liposome cavity and fat-soluble drugs in the lipid bilayer, possibility of targeted action, high stability of drugs at high water content, possibility of commercial production	rapid release, short shelf life	30
Lipid-based lyotropic liquid crystals	strictly specified structure, low toxicity	insufficient knowledge, possibility of spontaneous transition from one phase to another with premature drug release	31
Nanofibres	large surface area and porosity, possibility of commercial production, ability to transport thermosensitive drugs, high gas permeability	solubility of biopolymers limits their use in electrospinning (method of preparation of nanofibres)	32, 33

same time, the development of nanocarriers for drug delivery has received increasing attention. The requirements for carriers are extremely high: they should be inert or, at least, slightly toxic to the organism. They must also be non-immunogenic or poorly immunogenic. The structure of the carrier should be stable enough to go through all the barriers, including the blood – brain barrier, if the target is a brain tumour, and to retain its integrity until it reaches its final destination — the target cell. At the same time, this structure should release the active drug precisely in a target cell.[20] To ensure the 'correct' release of a substance, the process should be controlled. Control is accomplished by regulating ambient conditions (temperature, pH, presence of specific enzymes), time and other factors.

By their physicochemical nature, nanocarriers can be divided into three groups: inorganic, organic and hybrid. A large number of reviews and monographs on the use of various nanocarriers in the pharmaceutical industry have been published (see, *e.g.*, Refs 21 and 22).

The development of each of nanomaterials is a promising field of nanomedicine; however, a more detailed discussion of them is beyond the scope of this paper. Methods of synthesis, surface modification of materials, toxicity, physicochemical properties and applications of nanocarriers in biomedicine have been addressed in a large number of articles (see, *e.g.*, Refs 23 – 25). Advantages and disadvantages of nanosystems used for delivery of biologically active substances are presented in Table 1.

We believe that, in the context of biosafety and toxicity for humans, polymers based on lactic and glycolic acids are rather promising, which is due to their unique biocompatibility.[34] The degradation products of these polymers are completely metabolized in the Krebs cycle to carbon dioxide and water or are naturally cleared from the body. Poly(lactic-co-glycolic acid) (PLGA)-based materials have been approved by the U.S. Food and Drug Administration (FDA) for use in drug delivery systems, as degradable sutures, *etc*.[35] Biodegradable PLGA nanoparticles are capable of retaining different compounds: low-molecular-mass compounds, proteins, peptides and plasmid DNA (for protection from premature degradation).

Submicron size of spherical PLGA capsules contributes to their deep penetration into the tissue through the blood capillaries and epithelium. Drug release from nanoparticles occurs by the following mechanisms: desorption from the surface, diffusion through the polymer matrix and degradation of the matrix. When the drug is adsorbed on the particle surface, its release is considered to be a gradual process.

IV. PLGA particles as nanocarriers of pharmaceuticals

IV.1. Methods of fabrication of PLGA particles and properties of the copolymer

Poly(lactide-co-glycolide) copolymers are synthesized by copolymerization of cyclic dimers of lactic and glycolic acids to form nanospheres. Lactic acid (2-hydroxypropionic acid) exists in the L and D isomer forms, as well as the racemate. The product obtained in fermentation processes contains up to 99.5% of L isomer and 0.5% of D isomer. Lactic acid is represented by hygroscopic colourless crystals, readily forms a cyclic dimer, lactide, which also exists in optically active L and D forms and as the inactive racemate, and can polymerize to form high-molecular-mass polymers.[36]

Table 2. Biodegradation periods of PLGA nanoparticles.[a]

Polymer	Biodegradation period /months
Polylactide	18 – 24
Poly-DL-lactide	12 – 16
Polyglycolide	2 – 4
PLGA (50 : 50)	2
PLGA (85 : 15)	5 – 6
PLGA (90 : 10)	2 – 3

[a] The ratio of lactic and glycolic acid residues is parenthesized.

The resulting copolymers can have different molecular masses and structures of macromolecules, which makes it possible to vary the degree of interaction between macromolecules, as well as the particle biodegradation rate (Table 2); biodegradation occurs through natural decomposition in the body with participation of available enzymatic systems.[37]

Thus, PLGA copolymers can have various molecular weights and structure and are able to encapsulate molecules of any size.

The polymer is soluble in most solvents, including chlorinated hydrocarbons, tetrahydrofuran, acetone and ethyl acetate. In water, PLGA decomposes through hydrolysis of ether bonds. For this reason, the parameters describing the polymer powder (molecular mass, moisture content) can vary with time. The effect of these properties of the polymer on the rate of drug release from the polymer matrix has been widely studied. Gradual release of the incorporated drug occurs through diffusion from the polymer matrix, biodegradation of the polymer matrix, or a combination of these mechanisms.[37]

The amount of a drug in a nanoparticle (in PLGA and other polymeric nanoparticles) depends on some factors, such as the method of preparation, additives (stabilizers, bioadhesive substances), the nature of a pharmaceutical and polymers, their solubility and pH of a medium. The amount of a drug in nanoparticles can be increased by varying the synthesis conditions. Depending on the preparation process and physicochemical properties of drug molecules and the carrier, the drug can be either encapsulated into the carrier or adsorbed onto its surface.[38]

If the encapsulated substance is fat-soluble, a simple emulsion method is usually used. A polymer and a drug are dissolved in a volatile water-immiscible organic solvent, as a rule, in methylene chloride or ethyl acetate. The solution containing the drug and the polymer is added dropwise to an aqueous solution of a surfactant under stirring. Surfactants are used to stabilize the emulsion and maintain a relatively small size of the particles formed. The resulting emulsion is sonicated for crushing the dispersed phase. After evaporation of the organic solvent, the nanoparticles were washed and collected by centrifugation and then dried or lyophilized.

In the case of encapsulation of water-soluble substances (proteins), a double emulsification method is used. An aqueous solution of a drug substance is added to a polymer solution in an organic solvent having the aforementioned properties. The resulting primary emulsion is added dropwise to a solution of the surfactant and then the above

procedure is used. There are various modifications of the procedure of obtaining nanodrugs. For their production on the industrial scale, the nanoprecipitation method is used: a solution of a polymer, a pharmaceutical and a surfactant in a water-miscible solvent (acetone) is added dropwise to an aqueous solution which is not a salting-out agent for the drug and the polymer. In these methods, there is a high probability of denaturation, oxidation and aggregation of peptides and proteins; therefore, it is advisable to use stabilizers, such as polysorbate, mannitol, proteins (albumin), *etc*.[39]

IV.2. Application of PLGA particles in medicine and biology

Application of PLGA particles in experimental and clinical medicine and biology is rather diverse. They are used as long-acting drugs for treatment of the hormone imbalance and diseases causing these disorders. A rather large number of pharmaceuticals based on PLGA particles are available in the international pharmacological market (Table 3).

As transport systems, PLGA particles are also used to deliver plasmid DNA to the cells, the DNA being thus protected from enzymatic degradation. The ability of the particles to penetrate into tissues depends on the size of the encapsulated plasmid DNA: the optimal size is 1000 – 10 000 base pairs.

V. Treatment of cancer and other diseases with the use of PLGA particles

PLGA particles loaded with bioactive substances, such as cytokines, hormones and chemotherapeutic agents, are capable of reacting with tumour tissues with high affinity. In addition, the surface of the particles has functional groups that can be conjugated with fluorescent, magnetic and radioisotope labels or other means of visualization. These drugs have a much higher tropism for tumour cells as compared with the healthy cells due to the specific histological structure of tumours: high density of blood capillaries, poor endothelial differentiation, friability and chaotic distribution of cellular components, increased permeability of the vascular endothelium, the lack of functionally active lymph capillaries resulting in the accumulation of tissue fluid. All this leads to the retention of macromolecules in the tissue fluid of tumour tissues and to the accumulation of nanoparticles. For drugs, such as docetaxel and doxorubicin, a significant increase in efficiency of PLGA nanoforms as compared with the free drugs has been demonstrated (Fig. 5).[19, 40]

For visualization of the tumour tissue, the signal from the target area should be noticeably stronger than the signal from the surrounding tissues. Classical methods for tissue visualization, such as ultrasound, magnetic resonance imaging and others, are applicable only in case of sufficient involvement of this tissue in the pathological process. Nanoparticles with incorporated substances contrast to these methods can significantly improve the sensitivity of diagnosis of tumour lesions.[41]

Using PLGA-nanoparticles can also increase the efficiency of doxorubicin delivery to the brain glioma tissues; in its common form, doxorubicin does not penetrate the blood – brain barrier.[42]

Application of nanosomal (nanoparticle-based) preparations containing cytostatics and chemotherapeutic agents makes it possible to overcome drug resistance of tumour cells (multidrug resistance, MDR). The main mechanism of resistance is the reverse transport of cytostatics from tumour cells. The currently known molecular carriers of drug compounds, which are called transporters, can be divided into two large groups: SLC transporters (solute carriers) and ABC transporters (ATP-binding cassette).

P-glycoprotein (P-gp) belongs to the ABC transporters. It was discovered in 1976 by Juliano and Ling,[43] who found its enhanced expression on the membrane surface of tumour cells showing multiple resistance to anticancer drugs. More recent studies[44] have demonstrated that P-gp is present not only in tumour cells but also in normal tissues (small intestine, liver and kidneys) where it is responsible for removal of exogenous toxins from the

Table 3. Selected commercially available drugs based on biodegradable PLGA particles.

Drug name	Drug substance	Manufacturer	Application
Decapeptyl	triptorelin	Ipsen	female infertility, ovary stimulation for *in vitro* fertilization
Nutropin Depot	Ssmatotropin	Genetech	growth retardation of different genesis
Risperdal Consta	rispiredone	Jannsen	treatment and prevention of exacerbations of schizophrenia and schizoaffective disorder
Somatuline	lanreotide	Ipsen	acromegaly, carcinoid tumours, gastroenteropancreatic endocrine tumours, acute pancreatitis
Sandostatin	octreotide	Novartis	
Trelstar	triptorelin	WatsonPharma/Pfizer	prostate cancer (hormone-dependent)
Zoladex	goserelin	AstraZeneca	
Eligard	leuprolide	Sanofi-Aventis	
Gliadel	carmustine	MGI Pharma	chemotherapy of brain, head and neck tumours
Atridox	doxycycline	Tolmar	periodontitis
Atrisorb D	doxycycline	Tolmar	
Arestin	minocycline	OraPharma	
Lupron Depot	leuprolide	Abbott	endometriosis, uterine fibroids, precocious puberty, hypogonadotropic amenorrhea, prostate cancer
Decapeptyl Depot	triptorelin	Ferring	
Vivitrol	naltrexone	Cephalon	treatment of alcohol dependence

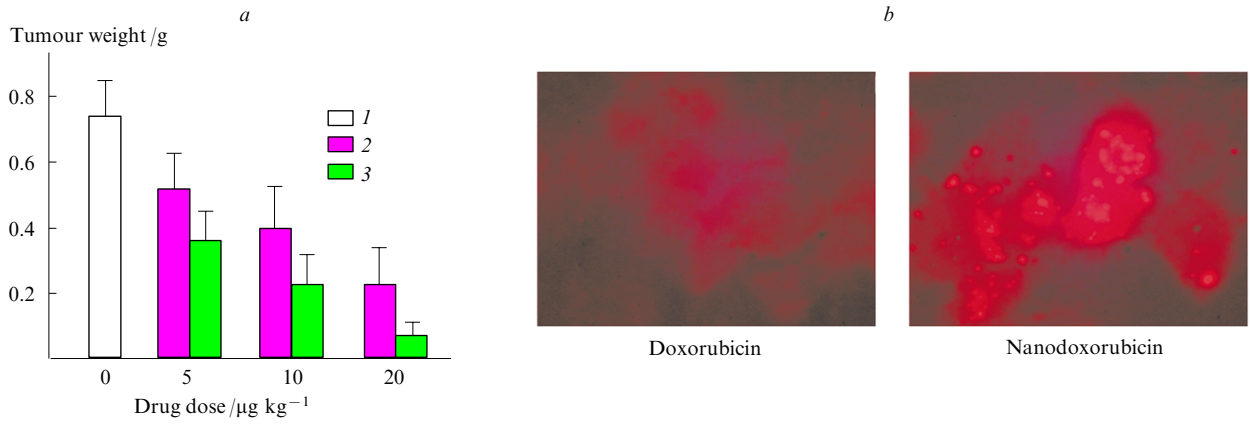


Figure 5. Enhancement of the efficiency of chemotherapeutic drugs loaded in PLGA particles as compared with the common form; (*a*) *in vivo* effect of docetaxel and nanodocetaxel on the model of the BalbC mouse mammary adenocarcinoma (inoculation of Ca755 cells); (*1*) control, (*2*) docetaxel, (*3*) nanodocetaxel;[40] (*b*) doxorubicin and nanodoxorubicin uptake by MDR cells of human adenocarcinoma MCF-7Adr *in vitro* (the uptake efficiency was assessed from the doxorubicin fluorescence intensity, see text).[19]

blood. Currently, P-glycoprotein is considered the major factor contributing to the resistance of tumours to chemotherapeutic agents.[45]

P-Glycoprotein is a glycoprotein with a molecular weight of ~170 kDa composed of two identical moieties, each of which contains six hydrophobic transmembrane segments and a protein with a molecular mass of 190 000 (Fig. 6). P-Glycoprotein is highly expressed by MDR tumour cells, which leads to a decrease in the intracellular concentration of an anticancer drug, so that the therapeutic effect is reduced or abolished. It has been proved that the administration of anticancer drugs increases the mRNA level of P-gp by direct amplification of nuclear translocation of various transcription factors. Antineoplastic drugs also affect the processes of DNA methylation and histone acetylation, which are associated with the interaction of transcription factors with the promoter region, thus determining the mRNA level of P-glycoprotein.[45] Direct addition of P-gp inhibitors to pharmacological formulations also leads to MDR suppression, but their use is limited by the high toxicity, deteriorating patient's condition, as bad as it is.

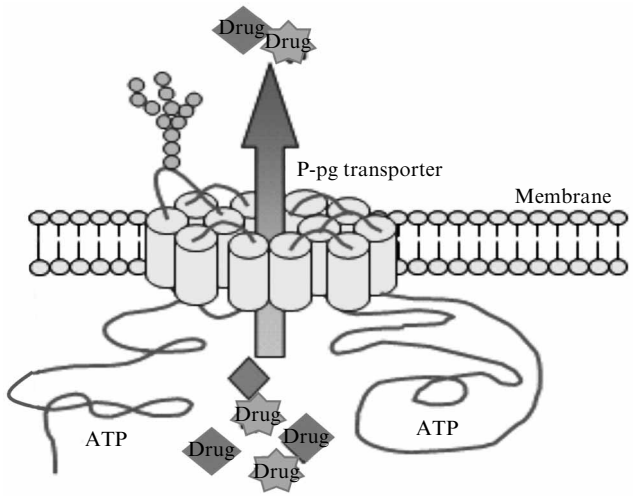


Figure 6. Structure of the P-gp transporter (see text).
Development of systems based on nanoparticles for targeted drug delivery through the blood–brain barrier.

Comparative evaluation of the effect of doxorubicin and nanodoxorubicin on MDR MCF-7Adr cells of breast adenocarcinoma has shown that the the nanoform on MDR-cells is significantly more efficient than the common form of the drug.[46]

A multifold enhancement of the efficiency of the nanosomal form of some anticancer drugs as compared to the normal form has been demonstrated for both the cell cultures and animal tumour models. *In vitro* cytotoxicity tests of docetaxel and nanodocetaxel on the Ca755 cells of mouse mammary adenocarcinoma have shown that the cytotoxic activity of docetaxel nanocompositions with respect to Ca755 tumour cells is 2.5 times higher than the activity of the free drug.[47]

In vivo studies of the average lifetime of DBA/2 mice inoculated with ascites tumour (lymphoid cell line P388) have shown a significant increase in lifetime (22.2%) and a decrease in tumour size (60%) in the group of mice injected with nanodaunorubicin in a dose of 7.5 mg kg^{-1} as compared with the group receiving standard daunorubicin. The *in vivo* nanodaunorubicin toxicity is statistically significantly lower than that of daunorubicin. When administered intravenously into the tail vein of Black VL57G line mice in a dose of 5 mg kg^{-1}, the standard drug caused swelling of the injection site followed by necrosis of the tails, which was almost not observed in the groups of mice treated with nanodaunorubicin.[48]

When a drug is delivered in nanoparticles, its uptake by the cell occurs through non-specific endocytosis, which is a general biological mechanism for the uptake of the active particles from the environment.[49] This significantly alters the pharmacokinetics and pharmacodynamics of the drug (Fig. 7); however, the presence of numerous systems for elimination of toxic substances from the cell reduces the efficiency of drugs.

Despite the effectiveness of nanoparticles containing cytostatics in the treatment of various cancers, their therapeutic effect on brain cancer is limited by the

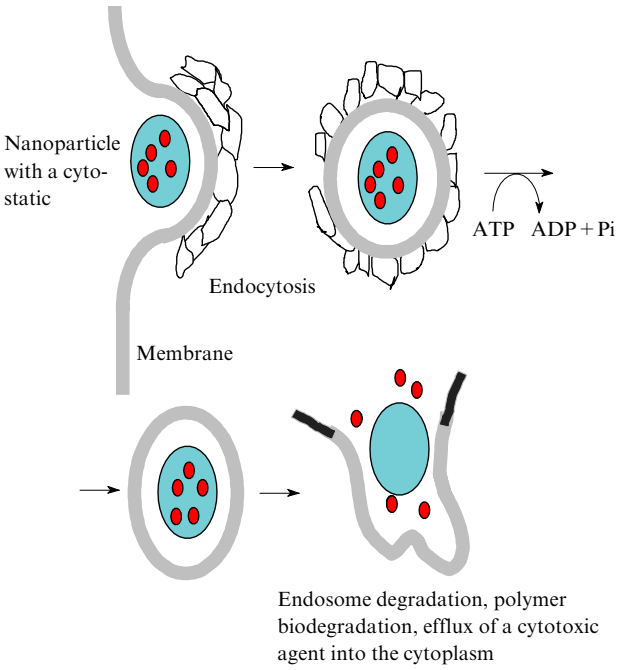


Figure 7. Delivery of the drug nanoform into the cell by non-specific endocytosis.

blood–brain barrier. It has been shown that the drug delivery to the brain unable to cross the blood–brain barrier can be achieved by colloidal delivery systems based on nanoparticles,[50] wherein the polysorbate modifies the nanoparticle surface and, thus, affects the drug efficiency (Fig. 8).

In recent years, PLGA nanoparticles have also been used to treat some other diseases primarily associated with bacterial infections. The results of research have shown that, owing to their natural biodistribution, polymeric particles provide passive directed transport of bound antibiotics into macrophages, which is responsible for the high efficacy of these drugs in the treatment of infections whose pathogenesis involves the accumulation of pathogens in macrophages.[52]

Despite advances in the synthesis of new generations of antibiotics, there is a critical situation in the treatment of tuberculosis and sepsis because of the appearance and spread of multidrug-resistant strains of intracellular bacterial infections. As a result, experts note the rise in the incidence of tuberculosis and acute and chronic diseases of the kidneys and lungs. At the Russian Research Centre of Molecular Diagnostics and Therapy, in collaboration with the Central Research Institute of Tuberculosis, Russian Academy of Sciences, works are carried out with the aim to create new forms of antimicrobial drugs rifampicin, rifabutin, cycloserine and lomefloxacin incorporated into PLGA-based nanoparticles.[52, 53]

The possibility of controlled drug release reduces toxic and allergic complications and makes these dosage forms good candidates for the treatment of various infections, including tuberculosis.

VI. Targeted nanodrug delivery — the most important task of nanopharmacology

VI.1. Challenges of targeted drug delivery and approaches to its realization by means of nanodrugs

Despite advances in surgical and therapeutic treatment in oncology, there is a great number of unresolved problems that require development of new approaches. One of the major challenges is to achieve the therapeutic drug concentration in the tumour tissue and retain it for a long period of time. Even at high concentrations of cytotoxic agents in the blood and prolonged course of treatment, inefficient penetration of drugs into tumour tissues results in the survival of some of tumour cells. In addition, the use of high doses of drugs required to maintain complete remission is the cause of severe systemic side effects.

Directed transport of drugs with the use of specially designed targeted delivery systems is a good alternative for increasing the therapeutic efficacy of the drug and reducing the incidence of systemic toxicity of anticancer agents.

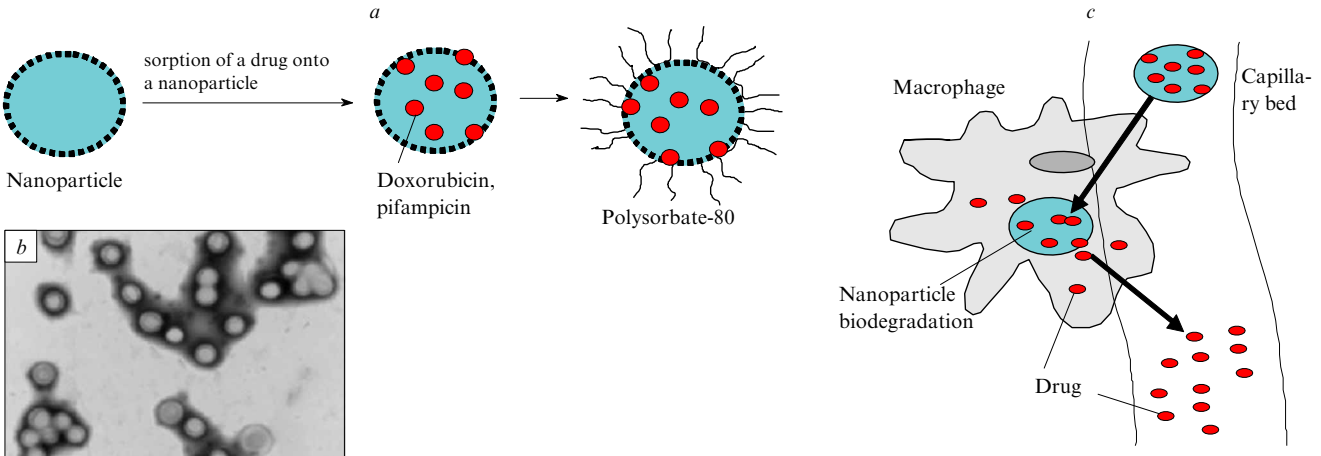


Figure 8. Method for increasing the nanodrug efficiency that ensures the penetration through the blood–brain barrier.
(*a*) Fabrication of PLGA nanoparticles; (*b*) electron microscopy image of PLGA nanoparticles;[51] (*c*) capture of nanoparticles by a macrophage, biodegradation and drug release.

VI.2. Methods of targeted drug delivery in nanopharmacology

The most pronounced interest in the development of targeted drugs is shown by oncology. In 2000, Hanahan and Weinberg,[54] based on molecular biological studies, determined basic hallmarks of malignant tumours, which dictate the strategy of targeted therapy. First of all, these traits are self-sufficiency in growth signals and insensitivity to growth-inhibitory signals. In tumours with the elevated content of dividing cells, apoptosis is suppressed, and, therefore, histological examinations reveal abnormal mitoses and binucleate cells-monsters, as well as signs of incomplete apoptosis. Angiogenesis stimulated by the tumour cells leads to the formation of a vast network of blood vessels having features of immaturity and differentiation of the vascular wall components. In most cases, tumour cells have the ability to invade and metastasize. Manifestation of each of these hallmarks is associated with overexpression of target molecules, whose binding to a chemotherapeutic agent enables its selective accumulation at the locus of actively dividing tumour cells. To ensure the fulfilment of all stages of drug action, targeted delivery agents should have some well-defined properties: they should have receptors for directional movement toward the goal, have the ability to pass through cell membranes, release the content in exactly the right place at exactly the right time and be nontoxic. The greatest efforts of researchers are currently focused on the development of anticancer drugs of this type.[40, 55]

New targeted drugs are bifunctional molecules, which consist of a target domain coupled to a therapeutic component. Linkers that connect the two parts of the drug largely determine its pharmacokinetics, but the success of the target action depends, first of all, on the properly selected vector, the component that directly binds to the target. The target of the target domain should be localized in the tumour, and the therapeutic moiety can be an antitumour antibiotic, a cytostatic agent, a plant or bacterial toxin, an enzyme, an antisense oligonucleotide and others. For drug delivery, the nanosized carriers described above are used. This approach can significantly reduce the numerous side effects that accompany chemotherapy, which noticeably extends the range of application of targeted drugs.

VII. Receptor-mediated endocytosis as the basis for development of effective drugs for targeted therapy

The process of targeted drug transport is based on the transfer of a toxicant (covalently or noncovalently bound

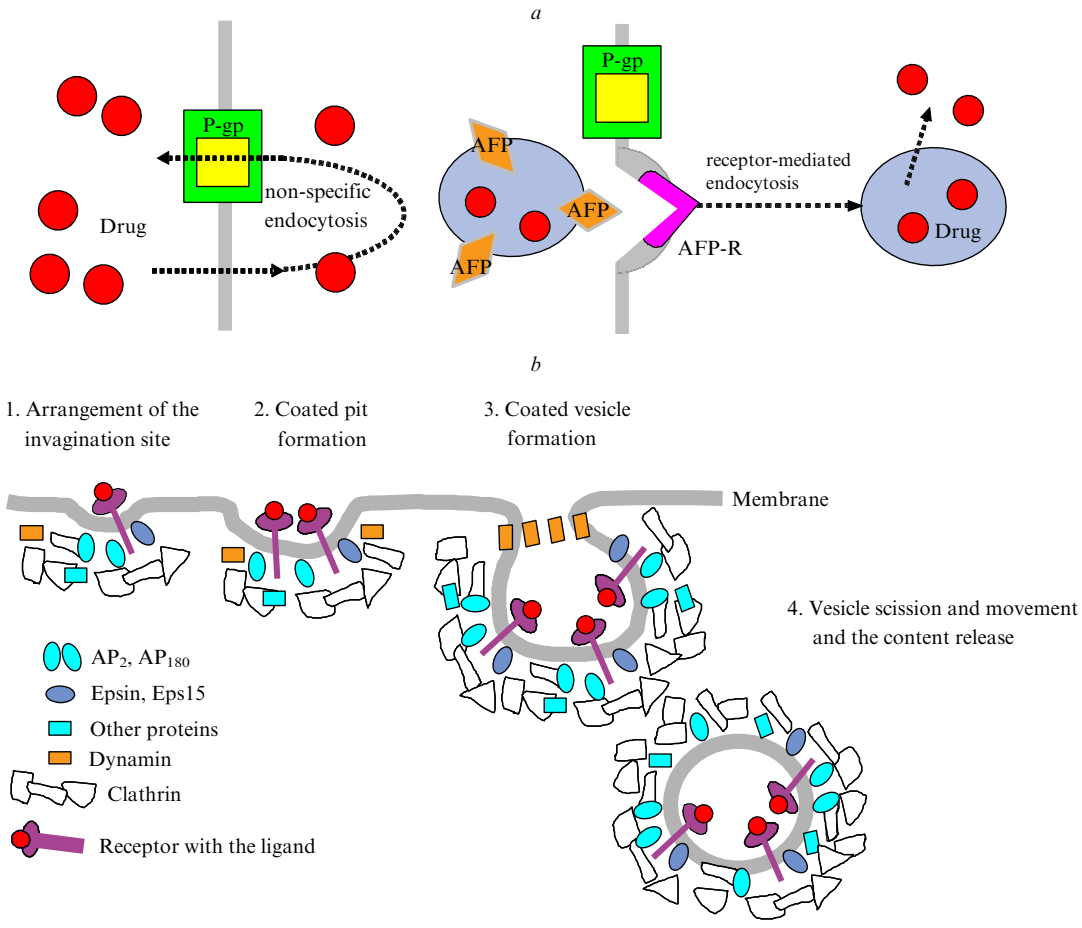


Figure 9. Receptor-mediated endocytosis as a mechanism to overcome tumour cell resistance.
(*a*) Function of the P-gp transporter in drug delivery through non-specific and receptor-mediated endocytosis; (*b*) sequence of stages of receptor-mediated endocytosis.

to a vector molecule) into a target cell as a result of receptor-mediated endocytosis. Clathrin-dependent receptor-mediated endocytosis is a complex mechanism that is used by cells for selective internalization of proteins and allows cells to capture large amounts of specific ligands without absorption of a respectively larger volume of the extracellular fluid. The processes of receptor-mediated endocytosis have been covered in many reviews (see, *e.g.*, Refs 56–59); therefore, the process is presented in the most general terms, and a more detailed description of the molecular and cellular mechanisms is beyond the scope of this review.

It is generally known that receptors in the plasma membrane have high affinity for certain substances, and liquids and extraneous solute molecules that do not bind to the receptors are hardly absorbed. This ensures the effective delivery of specific molecules into the cell. Vacuoles formed in endocytosis appear in the plasma membrane covered, on the cytoplasmic side, with a fibre material, membrane protein clathrin (Fig. 9). Preliminarily, clathrin-coated pits are formed in the membrane, which can account for up to 2% of the surface of some cells. By means of coated pits accommodating the corresponding receptors, a number of substances are internalized, such as low-density lipoproteins, growth factors, hormones, transport proteins (transferrin, albumin, and alpha-fetoprotein) and others. Clathrin binds to integral receptor proteins on the cytoplasmic side through adapter proteins. Adapter proteins bind to phospholipids of the plasma membrane and to the target amino acid sequences of internalized proteins.[60] Three clathrin molecules are associated to form triskelion. Triskelions form a loose three-dimensional net on the inner surface of the pit. Further pit invagination leads to the formation of an endocytic vacuole, or a vesicle, coated on the interior side with polygonally packed clathrin. Such a vacuole, also called 'coated vesicle', transfers, into the cell, a complex of transmembrane receptors binding to the ligands of the absorbable material. Study of the mechanisms of clathrin-dependent receptor-mediated endocytosis enables the creation of systems of targeted drug delivery to the tumour cells. For example, the presence of somatostatin receptors on the membrane of tumour vascular endothelial cells has allowed Severin *et al.*[61] to achieve significant progress in experimental studies on creation of targeted drugs — conjugates of a somatostatin analogue with camptothecin, methotrexate, paclitaxel and doxorubicin, and their high antitumour activity has been proved in experimental animal models.

To date, it has been shown that targeted drugs, which are vector protein conjugates with doxorubicin, have cytotoxic activity against several cell lines with the multidrug resistance phenotype. Since the drug is enclosed in a polymer nanosized capsule, which promotes the penetration through the blood–brain and blood–neural barriers,[62] its transfer as a conjugate into the cells through the receptor-mediated endocytosis mechanism does not activate the P-gp-mediated transport (Fig. 10 *a*).

Intracellular drug release leads to the accumulation of drug in the cell and, accordingly, to the increase in the cytotoxic activity of doxorubicin. Conjugates of vector molecules with antibiotic esperamicin significantly enhance

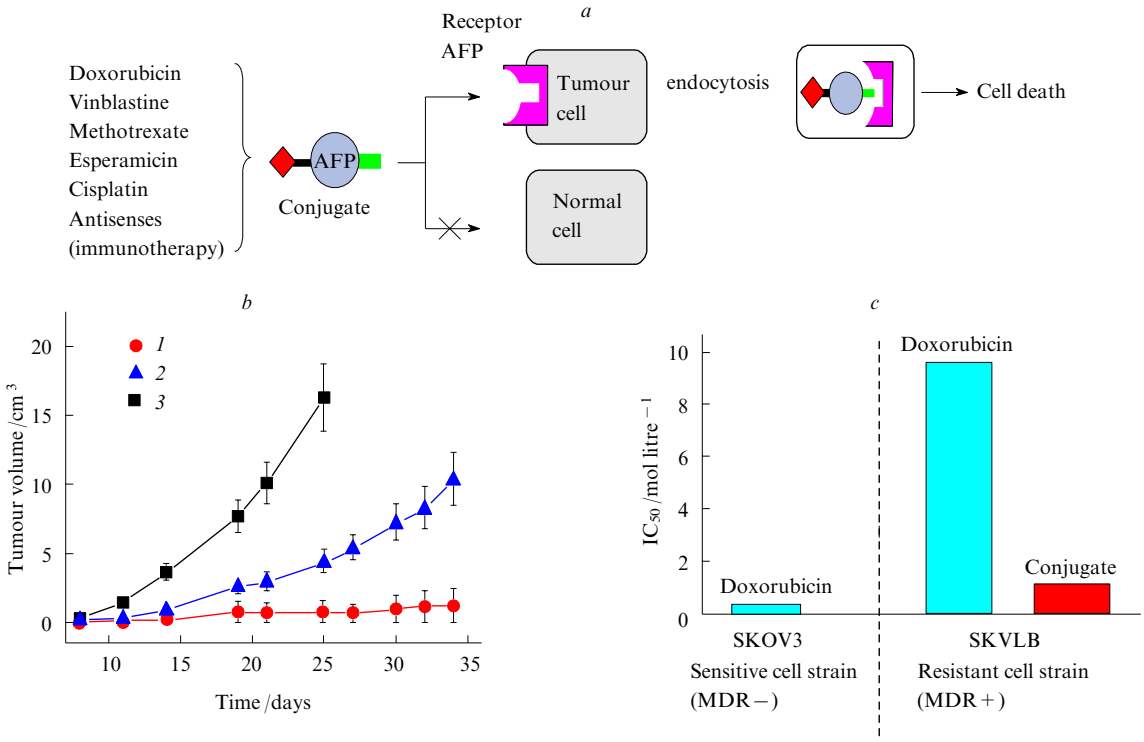


Figure 10. Enhancement of the antitumour activity of a drug through its conjugation with alpha-fetoprotein.
(*a*) General scheme of interaction of the AFP–drug conjugate with tumour cells; (*b*) comparative *in vivo* efficacy of the AFP–esperamicin conjugate (*1*) and standard esperamicin (*2*) against mouse lymphocytic leukemia P388 solid tumour; (*3*) control;[64] (*c*) overcoming of the MDR of human ovarian carcinoma cells *in vitro* using the AFP–doxorubicin conjugate.[40]

its antitumour activity and even lead to the complete recovery of some animals[63] (Fig. 10 *b*). Importantly, no toxic effect on blood lymphocytes has been noted.

Alpha-fetoprotein (AFP), belonging to the class of human tumour-specific antigens, is among the most promising proteins that can be used for targeted delivery in targeted cancer therapy. When used as a vector, AFP molecules gave highly active conjugates with various cytostatics.[19, 65, 66]

VIII. Human alpha-fetoprotein and possibilities of its application for targeted drug delivery

VIII.1. Discovery and study

The history of the discovery and study of human alpha-fetoprotein is a bright page in Russian and world science. In 1944, Swedish biochemist Pedersen found a massive protein fraction in the calf serum completely absent in the blood of adult animals. The protein was named fetuin (from Lat. *foetus* meaning fetus). In continuation of these studies, Bergstrand and Czar in 1956 and 1957, comparing the serum composition of human fetus and adults, found a protein fraction specific to fetal serum and called it alpha-fetoprotein. It has later turned out that AFP is not a fetuin analogue; however, this case shows that sometimes misconception in science can open a paradoxical way to the truth. Since AFP was only detectable in the embryo, it has been believed that it is synthesized in the placenta, which is removed after delivery, along with specific proteins synthesized by it. No relation of AFP to tumours has been supposed. In the same period of time, a group of Russian scientists working in the laboratory of L A Zilber, studied specific tumour antigens, in the hope to find the protein of the alleged virus in tumours. The group of biochemists under the leadership of G I Abelev (Avenirova, Engelhardt *et al.*) has developed immunochemical approaches that are widely used up to now in biology, virology, oncology and many other areas. In comparison of the protein composition of normal liver and liver cancer cells by the immunodiffusion method, a special method — immunofiltration — has been developed. This method was used to identify and isolate a hepatoma protein, absent in the liver, and to obtain strictly specific antibodies to it. It was the first accurately identified specific tumour antigen. Simultaneously, the appearance of proteins in normal development was studied. For control, antibodies to the hepatoma antigen were taken. Surprisingly, it was found that the fetal liver contained a larger amount of the hepatoma antigen than the liver tumours. In tissue culture, hepatoma cells also synthesized the same antigen and released it into the medium. Thus, it has been shown that the fetal liver synthesizes and secretes into the blood, along with other proteins, the fetal-specific protein (referred to as alpha-fetoprotein), and it is the main component of fetal serum. Shortly after birth, the AFP synthesis stops for rest of the life; however, if a malignant tumour appears in the liver, it resumes the AFP synthesis and secretion into the adult blood. As first suggested by Abelev in 1963, the appearance of AFP in the blood of an adult body is evidence for the presence of the tumour; *i.e.*, AFP is its specific marker.[67] Then, Astrakhan biochemist Tatarinov has discovered a protein similar to AFP in the blood of patients with liver cancer. This was a decisive step on the way to diagnosis of liver cancer in humans.[68] There was registered the scientific discovery 'Phenomenon of synthesis of fetal-specific protein by malignant tumours', and G I Abelev, S D Perova and N I Khramkova (Kuprina) were recognized as its authors (registration No. 90, priority dates May 25, 1960; January 30, 1963; October 4, 1966).[69] Later, one more paper by Abelev has been published in *Advances in Cancer Researches*.[70] A year earlier, British physician J B Smith published a paper in another journal.[71] The same author together with D Todd published in 1968 a paper in *Lancet*;[72] therefore, the priorities for the discovery of AFP are disputed to date.

In March 1968, Abelev and Tatarinov, together with an employee of the International Agency for Research on Cancer A Tuins, visited health centres of Senegal, Ivory Coast, Uganda and Kenya with lectures and demonstrations of the AFP-test. The results were absolutely convincing. An agreement was reached on the collection of blood sera of patients and sending encoded samples for independent testing in three laboratories — to Professor Uriel in Villejuif (France), Tatarinov in Astrakhan (Russia) and Abelev in Moscow. In addition, it was agreed on a business trip to Dakar (Senegal) of Abelev's colleagues to detect AFP. The results convincingly showed the effectiveness of the method. AFP test gave no false-positive reactions: in patients with non-oncological diseases, this protein was not

Table 4. Dynamics of accumulation of data on the abundance of the AFP receptor in human tumours of different types.

Tumour	Year	Country	Ref.
Breast cancer	1984	France, Spain	76, 77
	1993	Canada	78
	1994	Canada	79
	2001	Russia	80
	2002	Korea	81
	2003	Russia	82
	2005	Russia, United States	75, 83
Ovarian cancer	1981	Japan	84
	2001	Russia	85
	2003	Russia	86
Lung cancer	1985	Japan	87
	1994	Canada	88
	2001	Russia	84
Prostate cancer	1996	Ireland	89
	2007	Japan	90
	2009	Russia	91
Hepatocellular cancer	1995	Japan	92
	2005	Russia	
Colorectal cancer	1991	United States	93
	1996	Russia	94
	2002	Korea	81
Stomach cancer	2005	Canada, Russia	74, 75
Cholangiocellular carcinoma	2005	Russia	75
Lympholeucosis (T- and B-cell forms)	1979	United States	95
	1989	France	96
	2001	Russia	80
Bladder cancer	1977	Iraque	97
	1996	Greece	98

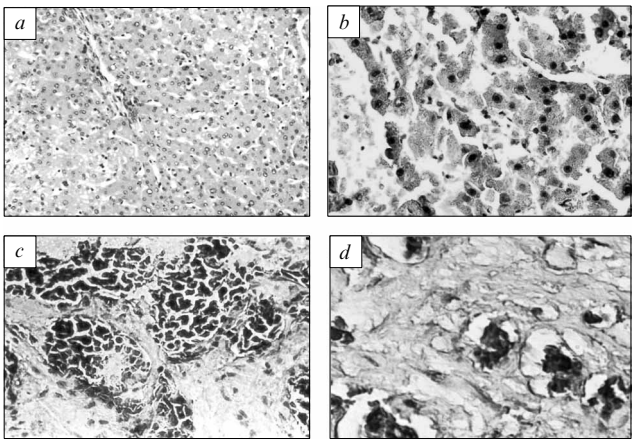


Figure 11. Immunohistochemical staining of tissues with anti-AFP receptor monoclonal antibodies in sections of liver (*b*), breast (*c*) and the stomach cancer (*d*).[46]
(*a*) Control section of the normal liver tissue. Background staining with hematoxylin and eosin; magnification, 400x.

revealed. This test was positive in 62% of cases and doubtful in 8% of cases of verified hepatoma. [73]

In 1973, the latex agglutination test for the evaluation of AFP in serum was developed in Tatarinov's laboratory; this test has been used in screening studies in patients with various liver diseases.

Later, other tumour-specific proteins have been discovered, but interest in alpha-fetoprotein is kept up to date. Simultaneously with Russian researchers, foreign authors, such as J Uriel, R Moro, M P Laderoute and M J Villacampa, have continued studying the distribution of AFP receptors; in some cases, the time of these publications [74] coincided with the time of Russian publications [75] (Table 4).

It has been shown that, in the cells of normal or benign tumour tissues, the expression of the AFP receptor (AFP-R) is almost absent. Receptor expression has been detected in tumour cells of different origin, which has led to the suggestion that AFP-R can act as a universal cancer marker. At the Russian Research Centre of Molecular Diagnostics and Therapy, anti-AFP monoclonal antibodies have been obtained and visualization techniques for different types of tumour tissues have been elaborated.[99] Immunohistochemical staining reveals AFP-R in sections of liver, stomach and breast cancer (Fig. 11) and other tumours.[86]

VIII.2. Structure of alpha-fetoprotein

Alpha-fetoptotein is a glycoprotein with a molecular mass of 69 000 composed of one polypeptide chain containing ~600 amino acid residues (a.a.r.) and ~4% carbohydrates. In the primary structure of AFP, three rather conservative domains are distinguished. Each of them consists of ~195 a.a.r. Limited proteolysis of AFP with pepsin leads to formation of three polypeptides with molecular masses of 38, 32 and 27 kDa. Further proteolysis of the fragments with molecular masses of 38 and 32 kDa gives products with molecular masses of 26, 23 and 12 kDa. The fragment with a molecular mass of 23 kDa corresponds to the N-terminal moiety of AFP (12–204 a.a.r.) and the fragment with a molecular mass of 26 kDa, to the C-terminal moiety of AFP (363–591 a.a.r). These data enabled the suggestion that the N-terminal (domain I) and C-terminal (domain III) moieties of the AFP molecule are stable AFP domains having definite functions. The central moiety of the AFP molecule (domain II, ~170 a.a.r.) is rather labile and is readily subjected to proteolysis.[100] The AFP molecule contains various functional sequences and binding sites:

— EGF-like segment (domain I);
— insulin-like segment (domain I);
— actin-binding site (domain II);
— bilirubin-binding site (domain II);
— estrogen-binding site (domain II);
— laminin-like segments (domain II);
— milk casein-like segment (domain II);
— growth-inhibitory peptide (domain III);
— segment similar to class II histocompatibility antigens (domain III);
— plasminogen-activating site (domain III);
— AFP receptor-binding site (domain III).

There are also three segments distributed over the entire chain of the AFP molecule:

— apoptosis-inducing sequences (domains I, III);
— fatty acid binding sites (domains I, II, III);
— kinesin-like segments (domains I, II, III).

A unique feature of the AFP structure is the presence of polypeptide motifs that mediate adhesion functions. AFP shares this feature with the family of extracellular matrix proteins — collagen, fibronectin, laminin, vitronectin, thrombospondin, *etc*. — playing a key role in embryogenesis.

Structural analysis by combined dark-field electron microscopy and circular dichroism method has confirmed the existence of the U-shaped structure of AFP with three regions of mass density: one at the top and two along the arms. On the basis of these data, a three-domain spatial organization of the AFP molecule has been proposed (Fig. 12).

Domains I and III have a rigid, compact tertiary structure and are linked through the flexible proteolytically labile domain II, whose conformation behaves as a molten globule and, thereby, promotes the binding of AFP to ligands or other proteins. The site for binding to the receptor on the surface of tumour cells is in domain III. This polypeptide was detected and identified by Moro *et al*.[78] Despite the stability in solution, AFP is rather conformationally mobile and can change its conformation under the influence of some factors, such as high concentrations of hydrophobic ligands (estrogens and fatty acids) and pH change.[101]

The AFP receptor is expressed in rapidly dividing cells, be they embryonic or neoplastic, and ensures receptor-mediated endocytosis of AFP. In the postnatal period, this protein is synthesized only in the process of carcinogenesis. The AFP receptor is also an oncofetal protein; *i.e*., in the postnatal development period, it is expressed mainly on the tumour cell surface.[102] This fact enables the use of AFP as a vector molecule for the targeted delivery of chemotherapeutic agents to malignant cells.

Alpha-fetoprotein penetrates into cells *via* receptor-mediated endocytosis.[103, 104] A receptor with a molecular mass of 62–67 kDa is considered the classical AFP-R, which has been identified on the surface of fetal cells, as well as on the surface of monocytes, stimulated lymphocytes and tumour cells of the reproductive system. The AFP

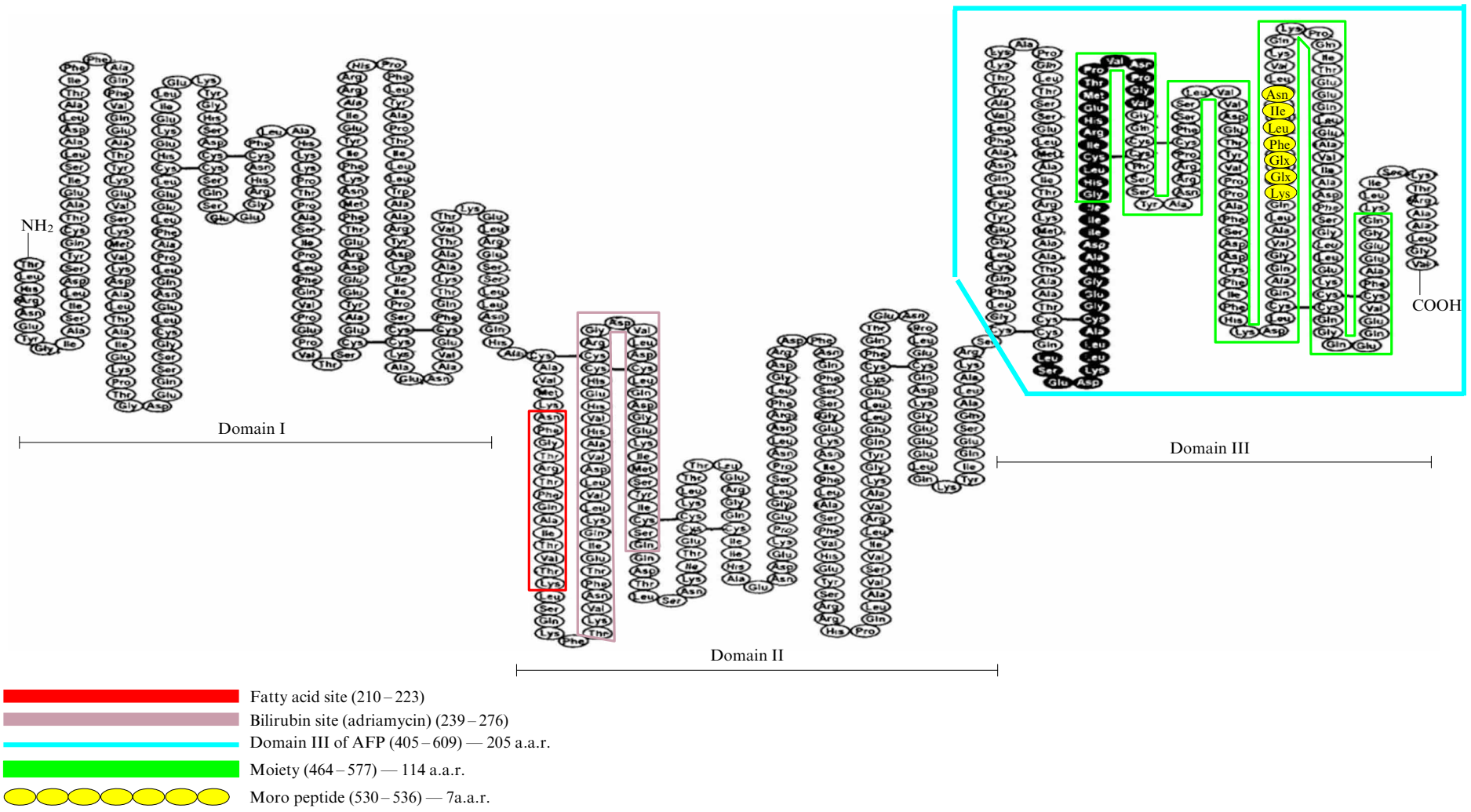


Figure 12. Structure of alpha-fetoprotein.[99]

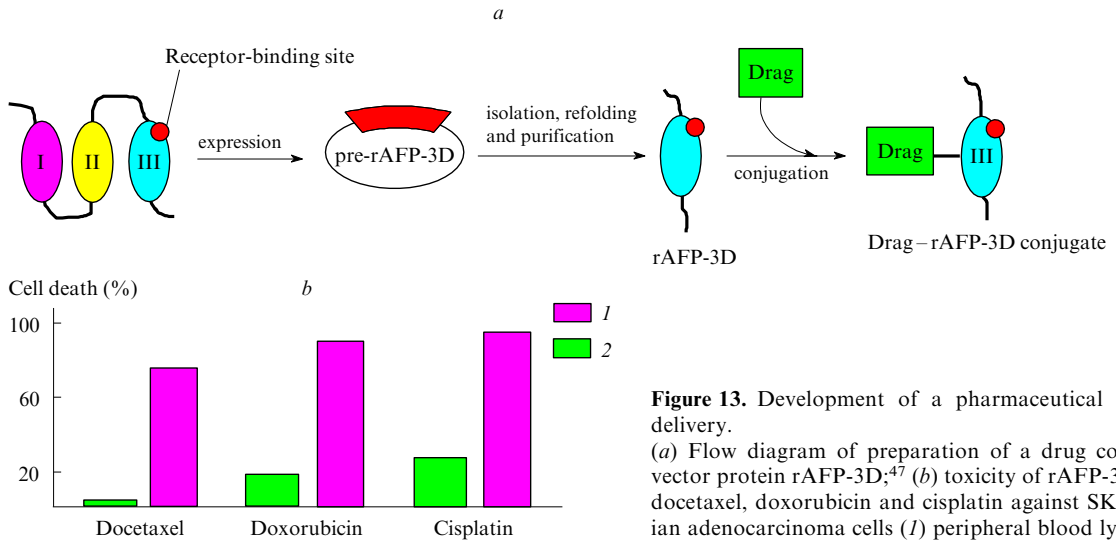


Figure 13. Development of a pharmaceutical for targeted drug delivery.
(*a*) Flow diagram of preparation of a drug conjugated with the vector protein rAFP-3D;[47] (*b*) toxicity of rAFP-3D conjugates with docetaxel, doxorubicin and cisplatin against SKOV3 human ovarian adenocarcinoma cells (*1*) peripheral blood lymphocytes (*2*).[65]

receptor is part of a larger complex in which proteins are glycosylated and surrounded by lipids.[105]

At the Russian Research Centre of Molecular Diagnostics and Therapy, ^{125}I-labelled human alpha-fetoprotein has been intravenously injected in DBA2 mice inoculated with mouse lymphoid tumour (line P388, 1×10^6 tumour cells in 0.1 ml of 0.9% NaCl), and its distribution in tissues and organs of an animal has been studied. It has been shown that the maximum accumulation of ^{125}I-labelled AFP occurs primarily within 5 hours after administration and persists in the liver, intestines and blood of intact mice for at least 3 days.[101] Radiolabelled AFP can be used most successfully to identify tumours of the hematopoietic system, lungs, brain, muscles and kidneys.[106]

It has been shown that the natural AFP conjugates with cytostatics inhibit tumour cell growth *in vitro* [103, 107, 108] and *in vivo*.[99] In particular, AFP conjugates with docetaxel, doxorubicin and cisplatin have a statistically significant specific effect on SKOV3 adenocarcinoma cells (Fig. 13).[65]

The antitumour activity of the AFP conjugate with esperamicin against P388 tumour (pre-B cell lymphoma) is significantly higher in comparison with the esperamicin active substance when administered intravenously in the same doses and the same mode of administration: in the animals treated with the AFP conjugate with esperamicin, tumours develop much more slowly, and complete tumour regression and significant increase in lifetime are observed in 30% of animals.[81] One of the limitations to the use of cisplatin in chemotherapy regimens is its strong negative impact on the hematopoietic system, manifested in the decline in the number of all blood cells. In this context, decreasing the toxicity of the conjugate for peripheral blood lymphocytes has important practical significance.[109]

As described above, the AFP receptor-binding site of tumour cells (Moro peptide [78]) is in domain III of the AFP molecule. Isolation of alpha-fetoprotein from abortion material or amniotic fluid is associated with technical difficulties and ethical issue; therefore, for its industrial production, it was necessary to develop the technology for the production of recombinant protein. Researchers at the Russian Research Centre of Molecular Diagnostics and Therapy have successfully solved the problem of producing recombinant AFP (rAFP) and its biologically active fragments.[40, 55, 110, 111]

Conjugates of chemotherapeutics with rAFP effectively overcome barriers of resistance to anticancer drugs, as has been found in studying the cytotoxic effect on the chemotherapy-resistant SKVLB line cells and on resistant MCF-7Adr75 subline cells of human breast adenocarcinoma MCF-7Adr75.[112] Studies have also shown that rAFP is bound and internalized by tumour cells, but is not translocated into lymphocytes.[113]

It has been proved that the conjugate of the C-terminal domain of rAFP with cisplatin has a higher *in vitro* antitumour activity as compared to the native drug substance.[65] Russian researchers have shown that recombinant domain III of AFP (rAFP-3D) does not differ from the full-length protein by its ability to bind to and penetrate into cancer cells.[65, 82, 114, 115] This has enabled the conclusion that that recombinant protein fragments based on C-terminal domain III of AFP can be used for targeted drug delivery.[65, 82, 114, 115]

The doxorubicin conjugate with rAFP-3D has shown the high efficacy against doxorubicin-sensitive cell line SKOV3 and against resistant cell line SKVLB; at the same time, the toxicity to human peripheral blood lymphocytes is low.[115]

A serious drawback of conjugates of vector proteins and peptides is the small amount of molecules of a cytotoxic drug that can be attached to the peptide without affecting its ability to bind to its receptor. To solve this problem, new approaches are currently developed at the Russian Research Centre of Molecular Diagnostics and Therapy: a cytotoxic drug is incorporated in biodegradable polymeric nanoparticles to which vector molecules are attached. Using the receptor-mediated endocytosis in combination with controlled intracellular drug release can significantly enhance the antitumour efficacy of chemotherapeutic drugs, while their toxic effect is reduced. Figure 14 shows the scheme of preparation of a new dosage form through the formation of the conjugate of a nanoparticle containing a drug with rAFP-3D. N-Succinimidyl 3-(2-pyridyldithio)propionate was used as the cross-linking agent. The rAFP moiety interacting with receptors on the tumour cell surface was used as a vector molecule to deliver various chemothera-

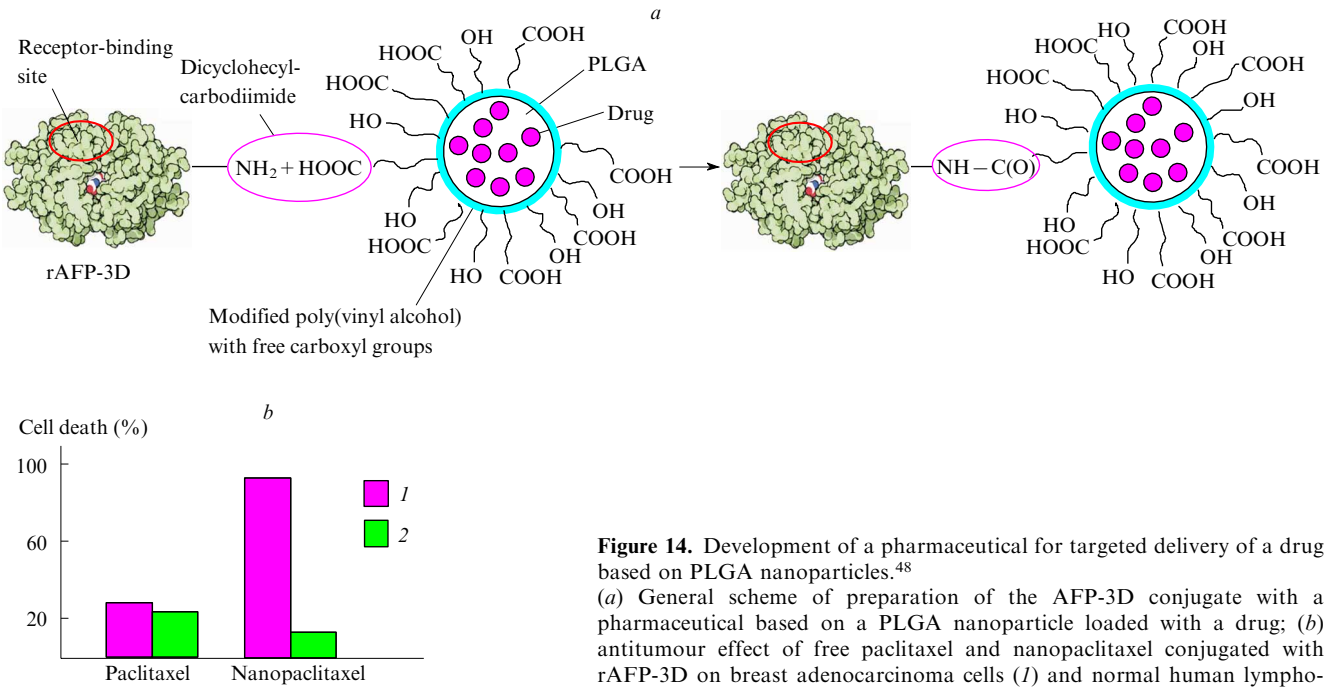


Figure 14. Development of a pharmaceutical for targeted delivery of a drug based on PLGA nanoparticles.[48]
(*a*) General scheme of preparation of the AFP-3D conjugate with a pharmaceutical based on a PLGA nanoparticle loaded with a drug; (*b*) antitumour effect of free paclitaxel and nanopaclitaxel conjugated with rAFP-3D on breast adenocarcinoma cells (*1*) and normal human lymphocytes (*2*).

peutics. The antitumour activity of the drug with a vector molecule was several times higher than that of the nanodrug without vector molecules in experiments on resistant cells of human breast adenocarcinoma. The level of accumulation of the drug in the tumour cells was significantly higher as compared to its level in lymphocytes.[116]

The level of binding to receptors and subsequent endocytosis of the nanoparticles in tumour cells was studied using fluorescent-labelled PLGA-doxorubicin nanoparticles (PLGA-Doxo-rAFP-3D), in which the free carboxyl group of the polymer has been preliminarily bound to doxorubicin (dicyclohexylcarbodiimide serving as a cross-linking agent). The receptor binding level and subsequent endocytosis of the nanoparticles was carried out *in vitro* on MCF-7 human breast carcinoma cell line, characterized by the high expression of AFP receptors (~310 000 AFP-R per cell).[117] Flow cytofluorometry has shown that, upon the incubation of PLGA-Doxo-rAFP-3D with MCF-7 cell line at 4 °C, particles bind to the cells, and the binding is specific, since it is partially blocked by an rAFP-3D excess.[55]

IX. Further prospects for the development of targeted antitumour drugs

Further progress in the design of dosage forms based on polymeric carriers depends on the development of improved carriers. They should contain vector ligands providing selective binding of the nanoparticles to the tumour cells or endothelial cells of blood vessels that supply the tumour with nutrients and oxygen. The use of vector nanocarriers for creating drugs with optimized properties will protect drugs from degradation and improve their pharmacokinetics due to their accumulation in the tumour.[118] A technology to produce high-performance targeted vector nanocontainers has been created. The development of this kind of drugs was started in Russia[66, 119] and abroad.[120] Various substances specifically interacting with receptors on the tumour cell membranes are used as the vectors in them.

However, the use of vector nanocontainers for antitumour therapy optimization does not obviate the problem of the fight against cancer stem cells (CSC). They are highly resistant to anticancer drugs, because of a high level of expression of ABC-transporter genes. Even when it is possible to cope with the cells of the primary tumour burden, CSCs survive during chemotherapy and are responsible for the subsequent recurrence and metastasis of tumours. In addition to some anticancer drugs, ABC-transporter substrates are fluorescent dyes Hoechst 33342 and rhodamine 123. After incubation of the cells with these dyes, the flow cytofluorometry study reveals CSCs as populations of unstained or weakly stained cells. The ability of stem cells to weakly incorporate dyes is used as a surrogate marker for identification of 'normal' and cancer stem cells.[121]

Currently, active search is underway for new drugs that could selectively affect not only the primary tumour burden cells, but also the CSCs and could increase their sensitivity to anticancer drugs. The results of recent studies performed by Moskaleva and Severin[122] suggest that the ionophore polyether antibiotic salinomycin has selective toxicity against CSC, is an active inhibitor of protein P-gp and, thus, is capable of overcoming multidrug resistance of tumour cells. At the same time, normal lymphocytes and healthy prostate cells were significantly more resistant to salinomycin than tumour cells.

X. Conclusions

It is obvious from the above material that the main task of nanopharmacology in the near future is to create selective anticancer nanodrugs conjugated with a vector molecule that has a specific binding site on the tumour cells. To solve this problem, various nanocarriers should be created,

screening of their toxicity, biological and immunological compatibility with the human body should be performed, and their stability *in vitro* and *in vivo* should be evaluated. It is necessary to assess the effectiveness of the inclusion of the active substance, determine physicochemical properties (surface charge, size, *etc.*), the delivery selectivity, the ability to penetrate through natural barriers, and evaluate the possibility of controlled release or active lysis under the action of enzyme systems of the body cells exactly in the predetermined locus. It is necessary to elaborate parameters for determining the exact dose of the drug contained in nanocapsules, and to explore the pharmacodynamics and pharmacokinetics of nanodrugs since they are very different from the commonly used dosage forms.

Using one of the domains of the recombinant alpha-fetoprotein as a vector for drug delivery to tumour cells holds great promise; however, this activity should be accompanied by studies of the mechanisms of receptor binding on the tumour cell membranes, which, in its turn, requires further study of structural parameters of AFP receptor molecules.

Design of drugs based on the above mechanisms will enable targeted delivery of chemotherapeutic agents to tumour cells with reduction of the total toxic action. The results obtained and the algorithms developed can be used in therapy of other diseases requiring a local treatment of the human body cells.

References

1. P Ehrlich, in *The Collected Papers of Paul Ehrlich in Four Volumes Including a Complete Bibliography. Chemotherapy* Vol. III (Ed. F Himmelweit) (London: Pergamon Press, 1960) p. 95
2. E Martinelli, R De Palma, M Orditura, F De Vita, F Ciardiello *Clin. Exp. Immunol.* **158** 1 (2009)
3. S M Deev, E N Lebedenko *Acta Naturae* **1** (1) 32 (2009)
4. L G Zhukova *Prakt. Onkol.* **11** 182 (2010)
5. O L Polanovsky, E N Lebedenko, S M Deyev *Biochemistry (Moscow)* **77** 227 (2012) [*Biokhimiya* **77** 289 (2012)]
6. M V Nesterova, L P Sashchenko, V Yu Vasiliev, E S Severin *Biochim. Biophys. Acta: Enzymol.* **377** 271 (1975)
7. E S Severin, E L Muizhnek, S E Severin *Kontseptsiya Vtorichnykh Messendzherov: ot Fundamental'nykh Osnov k Klinicheskoi Praktike* (Concept of Second Messengers: from Foundations to Clinical Practice) (Moscow: Dimitreid Grafik Grupp, 2005)
8. R K Sharma, T Duda *Front. Mol. Neurosci.* **7** 56 (2014)
9. K McCormick, G S Baillie *Curr. Opin. Genet. Dev.* **27** 20 (2014)
10. W Wei, R Graeff, J Yue *World J. Biol. Chem.* **5** 58 (2014)
11. B Klinger, N Blüthgen *Biochem. Soc. Trans.* **42** 770 (2014)
12. J A Beavo, L L Brunton *Nat. Rev. Mol. Cell Biol.* **3** 710 (2002)
13. E S Severin, M V Nesterova *Adv. Enzyme Regul.* **20** 167 (1981)
14. E S Severin, M V Savvateeva *Acta Naturae* **3** (1) 20 (2011)
15. T Y Kondratenko, I V Zacharova, N V Kuzina, V Yu Katukov, E S Severin, Z Ch Kornilova, M I Perelman *Biochem. Mol. Biol. Int.* **29** 123 (1993)
16. I V Kolyadina, I V Poddubnaya, G A Frank, D V Komov, A S Ozherel'ev, A I Karseladze, V D Ermilova, Ya V Vishnevskaya, N P Makarenko, R A Kerimov, K A Teterin, S M Banov *Sovrem. Tekhnol. Med.* (4) 48 (2012)
17. B P Head, H H Patel, P A Insel *Biochim. Biophys. Acta: Biomembr.* **1838** 532 (2014)
18. S E Severin *XLV Bakhovskie Chteniya* (The XLVth Bakh Lecturing) (Moscow: Nauka, 1991)
19. A V Godovannyi, E A Vorontsov, N V Gukasova, N V Pozdnyakova, E A Vasilenko, N G Yabbarov, S E Severin, E S Severin, N V Gnuchev *Nanotechnol. Russ.* **7** (1–2) 76 (2012) [*Ros. Nanotekhnol.* **7** (1–2) 77 (2012)]
20. O Kammona, C Kiparissides *J. Controlled Release* **161** 781 (2012)
21. J Liu, P S Ayyaswamy, D M Eckmann, R Radhakrishnan *Heat Mass Transf.* **50** 315 (2014)
22. J Verma, S Lal, C J Van Noorden *Int. J. Nanomed.* **9** 2863 (2014)
23. Y-T Liao, C-H Liu, J Yu, K C-W Wu *Int. J. Nanomed.* **9** 2767 (2014)
24. Yu S Dzyazko, Yu M Volfkovich, V E Sosenkin, N F Nikolskaya, Yu P Gomza *Nanoscale Res. Lett.* **9** Art. No 271 (2014)
25. H Lee, M-Y Lee, S H Bhang, B-S Kim, Y S Kim, J H Ju, K S Kim, S K Hahn *ACS Nano* **8** 4790 (2014)
26. R Adjonu, G Doran, P Torley, S Agboola *J. Food Eng.* **122** 15 (2014)
27. Y Li, Y Tan, Z Ning, S Sun, Y Gao, P Wang *Carbohydr. Polym.* **86** 291 (2011)
28. C E Mora-Huertas, H Fessi, A Elaissari *Int. J. Pharm.* **385** 113 (2010)
29. M Fathi, M R Mozafari, M Mohebbi *Trends Food Sci. Technol.* **23** 13 (2012)
30. B D Isailović, I T Kostić, A Zvonar, V D Đorđević, M Gašperlin, V A Nedović, B M Bugarski *Innov. Food Sci. Emerg. Technol.* **19** 181 (2013)
31. Y Chen, P Ma, S Gui *BioMed Res. Int.* **2014** ID 815981 (2014)
32. F Kayaci, T Uyar *Food Chem.* **133** 641 (2012)
33. N Okutan, P Terzi, F Altay *Food Hydrocolloids.* **39** 19 (2014)
34. F S T Mirakabad, K Nejati-Koshki, A Akbarzadeh, M R Yamchi, M Milani, N Zarghami, V Zeighamian, A Rahimzadeh, S Alimohammadi, Y Hanifehpour, S W Joo *Asian Pacif. J. Cancer Prev.* **15** 517 (2014)
35. P Gentile, V Chiono, I Carmagnola, P V Hatton *Int. J. Mol. Sci.* **15** 3640 (2014)
36. M I Shtil'man *Polimery Mediko-Biologicheskogo Naznacheniya* (Polymers of Biomedical Destination) (Moscow: Akademkniga, 2006)
37. I G Kuznetsova, S E Severin *Razrab. Registr. Lek. Sredstv* [4 (5)] 30 (2013)
38. A Orchel, K Jelonek, J Kasperczyk, P Dobrzynski, A Marcinkowski, E Pamula, J Orchel, I Bielecki, A Kulczycka *BioMed Res. Int.* **2013** ID 176946 (2013)
39. *Nanotherapeutics. Drug Delivery Concepts in Nanoscience* (Ed. A Lamprecht) (Singapore: Pan Stanford Publ., 2009)
40. G A Posypanova, N V Gorokhovets, V A Makarov, L V Savvateeva, N N Kireeva, S E Severin, E S Severin *J. Drug Target.* **16** 321 (2008)
41. A V Sosnov, R V Ivanov, K V Balakin, Yu A Fedotov, Yu M Kalmakov, D L Shobolov *Kachestv. Klin. Praktika* (2) 4 (2008)
42. A S Khalanskii, S E Severin, S E Gel'perina, Z S Smirnova, I N Skidan, I Kroiter *Ros. Bioterapevt. Zh.* (3) 56 (2004)
43. R L Juliano, V Ling *Biochim. Biophys. Acta: Biomembr.* **455** 152 (1976)
44. A T Fojo, K Ueda, D J Slamon, D G Poplack, M M Gottesman, I Pastan *Proc. Natl. Acad. Sci. USA* **84** 265 (1987)
45. T Kobori, S Harada, K Nakamoto, S Tokuyama *J. Pharm. Sci.* **125** 242 (2014)
46. V Kaur, T Garg, G Rath, A K Goyal *J. Drug. Target.* (2014) (PubMed ID 25101945)
47. Patent RF 2317102 (2008)
48. E S Severin, G A Posypanova *Ros. Fiziol. Zh.* **97** 553 (2011)
49. *Biologicheskaya Khimiya* (Biological Chemistry) (Ed. S E Severin) (Moscow: GEOTAR-Media, 2013)

50. J Kreuter, D Shamenkov, V Petrov, P Ramge, K Cychutek, C Koch-Brandt, R Alyautdin *J. Drug Target*. **10** 317 (2002)
51. F-Y Cheng, S P-H Wang, C-H Su, T-L Tsai, P-C Wu, D-B Shieh, J-H Chen, P C-H Hsieh, C-S Yeh *Biomaterials* **29** 2104 (2008)
52. I G Kuznetsova, E A Vorontsov, S L Kuznetsov, G G Barsegyan, S E Severin *Vopr. Biol., Med. Farm. Khim.* (8) 48 (2013)
53. A A Naidenova, G V Sukoyan, E A Vorontsov, S L Kuznetsov, N V Gukasova, M S Ryabtseva, G G Barsegyan, I V Bocharova, O V Demikhova, E S Severin, V V Erokhin, S E Severin *Nanotekhnol. Okhrana Zdorov'ya* **4** (3) 23 (2012)
54. D Hanahan, R A Weinberg *Cell* **100** 57 (2000)
55. Patent RF 2451509 (2012)
56. N V Popova, I E Deyev, A G Petrenko *Acta Naturae* **5** (3) 62 (2013)
57. H T McMahon, E Boucrot *Nat. Rev. Mol. Cell Biol.* **12** 517 (2011)
58. C Godlee, M Kaksonen *J. Cell Biol.* **203** 717 (2013)
59. S E Severin, E M Pal'tseva, S V Konstantinova, E S Severin *Mol. Meditsina* (6) 3 (2009)
60. S E Severin, G A Posypanova, V Yu Katukov, I I Shmyrev, Yu M Luzhkov, G K Gerasimova, O S Zhukova, G N Vorozhtsov, O L Kaliya, E A Lukyanets, E S Severin *Biochem. Mol. Biol. Int.* **43** 1081 (1997)
61. S E Severin, E Yu Moskaleva, G A Posypanova *Priroda* (12) 71 (2013)
62. P Vlieghe, M Khrestchatisky *Med. Res. Rev.* **33** 457 (2013)
63. E S Severin, A V Rodina *Usp. Biol. Khim.* **46** 43 (2006) [a]
64. S E Severin, G A Posypanova, A I Sotnichenko, E Yu Moskaleva, N B Fel'dman, M I Grigor'ev, E S Severin, R V Petrov *Dokl. Akad. Nauk* **366** 561 (1999) [b]
65. A V Godovannyi, M V Savvateeva, A I Sotnichenko, N S Yabbarov, O V Klimova, N V Gnuchev, S E Severin *Mol. Meditsina* (1) 44 (2011)
66. N G Yabbarov, G A Posypanova, E A Vorontsov, O N Popova, E S Severin *Biochemistry (Moscow)* **78** 884 (2013) [*Biokhimiya* **78** 1128 (2013)]
67. G I Abelev, S D Perova, N I Khramkova, Z A Postnikova, I S Irlin *Transplantation* **1** 174 (1963)
68. Yu S Tatarinov, I V Assekritova, V N Masyukevich, S D Perova *Terapevt. Arkhiv* (8) 47 (1967)
69. *Nauchnye Otkrytiya Rossii. Gosudarstvennyi Reestr Otkrytii SSSR*; http://ross-nauka.narod.ru/03/03-090.html
70. G I Abelev *Adv. Cancer Res.* **14** 295 (1971)
71. J B Smith *Med. Clin. North Am.* **54** 797 (1970)
72. J B Smith, D Todd *Lancet* **292** 833 (1968)
73. G T O'Konor, Yu S Tatarinov, G I Abelev, Zh Uriel' *Vestn. Akad. Med. Nauk SSSR* (3) 3 (1971)
74. Ric Moro, J Tcherkassova, E Song, G Shen, Raf Moro, R Schmid, X Hu, A Kummer, C Chen *IVD Technol.* June 1 (2005); http://www.ivdtechnology.com/article/new-broad-spectrum-cancer-marker
75. M B Nitsvetov, E Yu Moskaleva, G A Posypanova, O V Makarova, V A Stepanov, K A Rogov, I A Koromyslova, A V Karaulov, S E Severin, E S Severin *Immunologiya* **26** 122 (2005)
76. J Uriel, M-J Villacampa, R Moro, J Naval, C Failly-Crgpin *Cancer Res.* **44** 5314 (1984)
77. M J Villacampa, R Moro, J Naval, C Failly-Crgpin, F Lampreave, J Uriel *Biochem. Biophys. Res. Commun.* **122** 1322 (1984)
78. R Moro, T Tamaoki, T G Wegmann, B M Langenecker, M P Laderoute *Tumor Biol.* **14** 116 (1993)
79. M P Laderoute, L M Pilarski *Anticancer Res.* **14** 2429 (1994)
80. S E Severin, A V Rodina, M B Nitsvetov, D V Astahov, N A Kovalenko, E Yu Moskaleva, G A Posypanova, O N Popova, V K Sologub, I A Koromislova, A V Karaulov *Russ. J. Immunol.* **6** 249 (2001) [*Ros. Immunol. Zh.* **6** 249 (2001)]
81. B-C Song, D J Suh, S H Yang, H-C Lee, Y-H Chung K-B Sung Y S Lee *J. Clin. Gastroenterol.* **35** 398 (2002)
82. E S Severin, A V Karaulov, E Yu Moskaleva, A V Rodina, S E Severin, G A Posypanova, O N Popova, L V Sladkova, I A Koromyslova, M B Nitsvetov *Mol. Meditsina* (2) 57 (2003)
83. G Arpino, R Laucirica, R M Elledge *Ann. Intern. Med.* **143** 446 (2005)
84. M Furumoto *Pathol. Res. Pract.* **173** 12 (1981)
85. M B Nitsvetov, A V Rodina, E Yu Moskaleva, G A Posypanova, O N Popova, V K Sologub, I A Koromyslova, S E Severin *Biomark. Environ.* **4** (1/2) 51 (2001)
86. M B Nitsvetov, E Yu Moskaleva, G A Posypanova, O V Makarova, V A Stepanov, K A Rogov, O N Popova, V K Sologub, I A Koromyslova, A V Karaulov, S E Severin, E S Severin, in *Materialy 3-i Nauchnoi Konferentsii i Shkoly-Seminara dlya Molodykh Uchenykh 'Belki-Markery Patologicheskikh Sostoyanii'* (Proceedings of the Scientific Conference and School-Seminar for Young Scientists 'Protein as Markers of Pathological Conditions', Astrakhan – Moscow, 2003) p. 14
87. N Kimura, L Ghandur-Mnaymen *Tohoku J. Exp. Med.* **145** 23 (1985)
88. M Laderoute, D Willans, T Wegmann, M Longenecker *Anticancer Res.* **14** 1233 (1994)
89. M J Duffy *Eur. J. Intern. Med.* **18** 175 (2007)
90. M Kuriyama *Nihon Rinsho Geka Gakkai Zasshi (J. Jpn. Surg. Assoc.)* **54** 1631 (1996)
91. Patent RF 2348042 (2009)
92. S Saitoh, K Ikeda, I Koida, Y Suzuki, M Kolayashi, A Tsubota, Y Arase, K Chayama, N Murashima, H Kumada *Cancer* **76** 1139 (1995)
93. C Esteban, M Geuskens, J Uriel *Int. J. Cancer* **49** 425 (1991)
94. S E Severin, E Yu Moskaleva, G A Posypanova, I A Koromyslova *Tumor Target.* **2** 299 (1996)
95. K Suzuki, T B Tomasi Jr *Immunology* **38** 539 (1979)
96. J M Torre, J Laborda, J Naval, N Darracq, M Calvo, Z Mishal, J Uriel *Mol. Immunol.* **26** 851 (1989)
97. E A Alsabti *Oncology* **34** 78 (1977)
98. D Pectasides, D Bafaloukos, F Antoniou, L Gogou, N Economides, J Varthalitis, M Dimitriades, P Kosmidis, A Athanassiou *Am. J. Clin. Oncol.* **19** 271 (1996)
99. M B Nitsvetov, A V Rodina, E Yu Moskaleva, G A Posypanova, O N Popopva, V K Sologub, I A Koromyslova, I I Shmyrev, A I Sotnichenko, D V Zabolotnev, A V Karaulov, S E Severin *Vopr. Biol., Med. Farm. Khim.* (3) 19 (2001)
100. V A Chereshnev, S Yu Rodionov, V A Cherkasov, N N Malyutina, O A Orlov *Al'fa-Fetoprotein* (Alpha-Fetoprotein) (Ekaterinburg: Ural Branch of the Russian Academy of Sciences, 2004)
101. V Yu Kanevsky, L P Pozdnyakova, O A Aksenova, S E Severin, V Yu Katukov, E S Severin *Biochem. Mol. Biol. Int.* **41** 1143 (1997)
102. G I Abelev *Immunologiya* (3) 4 (1994)
103. S E Severin, E Yu Moskaleva, I I Shmyrev, G A Posypanova, Yu V Belousova, V K Sologub, Yu M Luzhkov, R Nakachian, J Andreani, E S Severin *Biochem. Mol. Biol. Int.* **37** 385 (1995)

104. E Yu Moskaleva, G A Posypanova, L I Mikhailova, V K Sologub, I I Shmyrev, S E Severin, in *Materialy Mezhdunar. Konf., Posvyashchennoi Pamyati Akademika A A Baeva* (Proceedings of the International Conference Dediated to the Memory of Academician A A Baev, Moscow, 1996) p. 96
105. G J Mizejewski *Exp. Biol. Med.* **226** 377 (2001)
106. S E Severin, V N Kulakov, E Yu Moskaleva, E S Severin, I I Slobodyanik, T P Klimova *Vestn. Ros. Acad. Med. Nauk* (4) 11 (2012)
107. Patent RF 2404810 (2009)
108. Patent RF 2071351 (1996)
109. Patent RF 2431639 (2011)
110. Patent RF 2448116 (2012)
111. O A Sharapova, N V Pozdnyakova, D K Laurinavichyute, M S Yurkova, G A Posypanova, S M Andronova, A N Fedorov, S E Severin, E S Severin *Russ. J. Bioorg. Chem.* **36** 696 (2010) [*Bioorg. Khim.* **36** 760 (2010)]
112. M B Nitsvetov, E Yu Moskaleva, L V Sladkova, A V Rodina, G A Posypanova, O N Popova, V K Sologub, A V Karaulov, S E Severin, E G Severin, in *1-ya Vseros. Nauchno-Prakt. Konf. 'Bioterapiya Raka' (Sb. Tezisov Dokladov)* [The First All-Russian Scientific and Practical Conference 'Biotherapy of Cancer' (Abstracts of Reports), Moscow, 2002] p. 73
113. A A Terentiev, N T Moldogazieva *Tumor Biol.* **34** 2075 (2013)
114. G A Posypanova, Doctoral Thesis in Biological Sciences, National Research Centre 'Kurchatov Institute', Moscow, 2013
115. N G Yabbarov, G A Posypanova, E A Vorontsov, S I Obydenny, E S Severin *J. Controlled Release* **168** 135 (2013)
116. G Dubovik, A V Godovannyi, E A Vasilenko, in *VI Mizhnarodna Konferentsiya Molodikh Uchenikh 'Biologiya: vid Molekuli do Biosferi'* (The VIth International Conference of Young Scientists 'Biology: from Molecule to Biosphere', Kharkiv, 2011) p. 138
117. S S J Leong, A P Middelberg *Protein Sci.* **15** 2040 (2006)
118. A Accardo, L Aloj, M Aurilio, G Morelli, D Tesauro *Int. J. Nanomed.* **9** 1537 (2014)
119. A V Bereznikova, G A Posypanova, V A Makarov, O V Antipova, E S Severin *Vopr. Biol., Med. Farm. Khim.* (5) 15 (2012)
120. J Peng, X Qi, Y Chen, N Ma, Z Zhang, J Xing, X Zhu, Z Li, Z Wu *J. Drug Target.* **22** 428 (2014)
121. P Liu, X-D Zhou *Nan Fang Yi Ke Da Xue XueBao (J. South. Med. Univ.)* **34** 453 (2014)
122. E Yu Moskaleva, S E Severin *Mol. Meditsina* **6** 28 (2012)

[a] — *Biochemistry (Moscow) (Biol. Chem. Rev.) (Engl. Transl.)*
[b] — *Dokl. Biochem. Biophys. (Engl. Transl.)*