Magyar és angol nyelvű tananyagfejlesztés
a munkaerőpiaci szereplők bevonásával és az angol nyelvű képzés fejlesztése
a Debreceni Egyetem Gyógyszerésztudományi Karán.

TÁMOP-4.1.2-08/1/A-2009-0006

Selected chapters of Biopharmacy

A kiadásért felel a:

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PREFACE

As pharmacy students prepare to take Biopharmacy examination and final exams before graduation, it is imperative that they have the most up-to-date and relevant information concerning the practice of modern pharmacy available to them. The information explosion is such that knowledge and information changes daily and dozens of new drugs are marketed each year. The amount of information that any student in pharmacy must master is significant and doubles every 2 to 3 years. Although information in the life sciences is expanding rapidly, human brain capacity is not. This discrepancy creates an increasing challenge for book and lecture notes writers, teachers, and especially students. We have been forced to think harder in deciding what facts and concepts are important or essential for our students related to Biopharmacy. Based on these facts this study guide has attempted to summarize selected chapters of modern Biopharmacy.

My thanks and appreciation go to my associates, the faculty of the University of Debrecen Medical and Health Science Center, Department of Biopharmacy (Dr. Andrea Treszl, Dr. Armin Buglyo, Dr. Aniko Heitz, Sara Csiha) and other faculty members of the Health Science Center (Dr. Zita Steiber, Dr. Rudolf Gesztelyi) who participated in preparing this lecture notes, and to Dr. Zsuzsanna Szabo for her coordination of the preparation of the chapters. I am especially grateful to all these colleagues and experts who made major contributions to individual sections. Finally, I wish to thank to Lawrence M. Brown, PharmD, Ph.D., Associate Professor, University of Tennessee Health Science Center College of Pharmacy Department of Pharmaceutical Sciences, Memphis, TN, U.S.A. for his outstanding editorial work.
PHARMACOKINETIC BASES OF BIOPHARMACY

Introduction

Place of biopharmacy within the pharmacy disciplines
Practicing medicine is inconceivable without the use of pharmacons; molecules that are able to substantially influence the living organism even when used in small concentrations (Most of the pharmacons used in the medicinal practice are pharmaceuticals. The distinction of these two terms generally used as synonyms is justified by the fact that the term pharmacon is a purely theoretical term, whereas the term pharmaceutical is also a legal category). The pharmacons’ properties and general aspects of their use are described by pharmacology that may be divided into two subdisciplines: pharmacokinetics and pharmacodynamics. Pharmacokinetics investigates the effect living organisms exert on the pharmacon, and pharmacodynamics assesses the pharmacon’s effect on the living organism. Pharmaceutical technology deals with the manufacturing procedures, vehicles and other supplements that are needed to turn a pharmacon into a pharmaceutical form that may be used with a specific purpose at a specific location or will result in a drug releasing system. Biopharmacy is a relatively new discipline, (the term was created by Levy and Wagner in 1961), that deals with the behavior of even more complex pharmaceutical forms and drug releasing systems in living organisms.

Although biopharmacy is closely related to pharmaceutical technology, the therapeutic value of a pharmaceutical preparation (or dosage regime) is determined by the active agent(s)’ distribution in the body and the time course of the change in concentration; both of which topics are covered by pharmacokinetics. First the major pharmacokinetic principles will be discussed.

1. Qualitative pharmacokinetics

Didactically, pharmacokinetics may be divided into qualitative and quantitative parts. Qualitative pharmacokinetics deals with the processes of the living organism that determines the fate of the pharmacon in the organism. The main processes influencing pharmacons are usually discussed using the acronym ADME. ADME describes the pharmacon’s fate in the body starting from absorption, distribution, and leading up to metabolism and excretion. If
the pharmacon’s fate is followed from its liberation from the pharmaceutical form then the acronym LADME is used. Sometimes we may come across the term LADMER that includes even the response given to the pharmacon, naturally this already falls out of the topic of pharmacokinetics. Collectively metabolism and excretion are called elimination, because a specific molecule either changes or leaves the body unchanged. Elimination of the metabolite, therefore, is not a distinct elimination step; rather it is only the release of the derivate of the eliminated pharmacon from the body. It should be noted however that pharmacokinetic elimination doesn’t necessarily lead to the end of the effect as active metabolites or the permanent changes elicited by the pharmacon (e.g. irreversible inhibition of an enzyme or a receptor, alteration of the DNA) may prolong the effect. Collectively distribution and elimination are called disposition. Qualitative pharmacokinetics is dealt with in a different chapter of this study notes. Now we discuss some details about the basic components of this LADME system.

1.1. Absorption, routes of administration

Absorption refers to the process of drug movement from the site of administration to the systemic circulation. Obviously, there is no absorption when the drug is given intravenously. Ideally, we want the drug to be present only at the site of its biological action. In reality, however, this happens rarely. The bioavailability shows what percentage of the drug reaches the systemic circulation in unchanged form. It depends on the formulation, the administration route, and the hepatic first pass metabolism; it is 100% if the drug was given intravenously and less in any other cases. Bioavailability is a very important pharmacokinetic parameter, which is necessary to know in order to calculate the dosage of a certain drug preparation.

The administration route determines the onset of action, the blood concentration to be reached and the length of time the drug remains in the body. It may be (A) oral, (B) parenteral and (C) topical. Oral administration means that the drug is taken by mouth and is swallowed. It is absorbed from the gastrointestinal tract into the circulation and carried to the site of action by the blood. Preparations for oral administration are tablets, capsules, solutions, suspensions, emulsions… etc. Parenteral administration means that the drug enters the vascular system without passing through the gastrointestinal system. Examples include vaginal tablets and creams, inhalational solutions, or by intramuscular or intravenous injections. The advantage of topical administration is that the site of action is the same as the
site of application e.g. the skin or mucous membranes; to achieve a local effect. Creams, ointments, powders, solutions, sprays are administered this way.

The choice of the administration route depends on (1) what kind of effect (local or distant) is required, (2) how fast the effect should be evoked, (3) how long of an effect is desirable, (4) how convenient the administration is supposed to be, and (5) what physicochemical, pharmacological and toxicological properties the drug has. Of course, the most convenient route for the patient is oral, yet several substances are broken down by gastric acid (e.g. peptide hormones like insulin, some penicillins), so their parenteral administration is inevitable. In case of emergency care a prompt action is required, so inhalation and intravenous administrations are preferred. In patients with liver or kidney failure it is advised to choose a route, which is less challenging and use the topical way if possible. If the patient is poorly cooperative, sustained release depot preparations can be given under the skin or intramuscularly, to release the drug continuously and maintain a constant blood level.

The transport of drugs across biological membranes

The penetration of drugs through biological membranes is greatly influenced by their lipid/water distribution, which is characterized by the partition coefficient. The more polar or ionized the molecule, the smaller the partition coefficient. If ionization is cut down by the local pH, the unionized (apolar, lipophilic) molecule easily diffuses through the membranes.

Drugs can cross the biological membranes by different methods (1) Passive diffusion is available for apolar, lipophilic substances, with big lipid/water partition coefficient (e.g. inhalation anesthetics). (2) Filtration may help the transport of small (molecular weight is less than 100), and water soluble molecules (e.g. urea). Filtration rate is determined by the pressure difference between the two sides of the membrane, molecular size and membrane pore size. (3) Some drugs are transported across the membranes by active transport mechanism, against their concentration gradient, which is an energy demanding process. The protein carriers that take the drugs are specific for endogenous substances, and can carry only the drugs with similar size and structure. They are saturable, and may act as a site of drug interaction. (4) The facilitated diffusion mechanism also uses carriers, but work further down the concentration gradient and does not require energy. (5) Highly charged molecules (e.g. those containing quatermeric N) may form ion pairs with the opposite charge.
and become apolar, to then cross the membranes with passive diffusion. (6) Extreme large molecules, antibodies, radionuclide-carriers, pegylated substances wrapped up in liposomes can enter the cells by endocytosis, and be released from the liposomes within the intracellular space.

1.2. Drug distribution

During drug development it is very important to know where and how the drug is distributed in the human body. They need answers for questions like how the drug reaches its target, how long does it take to develop an effect and for how long its biological effect is sustained. All this information is used when designing the most appropriate dose, the route of administration, and formulation.

Most drugs are water soluble and reach their target via the bloodstream. In the blood they are usually carried by plasma proteins, which determine the extent and rate of their ability to cross the biological membranes and achieve an effective extravascular concentration. The ionization of the drug, depending on local pH, and relative solubility of the drug in lipids and water are also important factors, which determine drug distribution.

Drugs are distributed within the body compartments. They can be defined as follows: total body water is approximately 60% of the total body weight (~0.5 l/kg), for example 40-45 l for a person weighing 70 kg. Total body water is divided into intracellular (40% of body weight; 25-35 l) and extracellular compartments (20% of body weight; 10-20 l). Certain parts of the extracellular compartment are the plasma (4% of body weight; 2-3 l) and the interstitial compartment (16% of body weight; 10-12 l). Drug distribution has a major influence on the drug’s effect, it often described as the volume of distribution ($V_D$) and the distribution coefficient. The volume of distribution is defined as a virtual volume, which would be required to keep the whole amount of the administered drug in the body at the same concentration as it was in the plasma, right after intravascular administration. Here is the formula to calculate the volume of distribution:

$$V_D = \frac{\text{amount of drug in the body}}{\text{plasma concentration}}$$

Higher distribution volume means that the drug is likely to accumulate in the extracellular compartment. For example: the $V_D$ value of salicylates is 12 l/70kg, while it is 13000 l/70 kg for chloroquin. The latter high figure suggests that chloroquin reaches a very high concentration in peripheral tissues, which is necessary for its biological effect. In the case of
chloroquin, however, a high loading dose has to be given, to reach the effective drug concentration in the blood as well. Since the drug is rapidly removed from the blood and accumulated in the tissues, it takes longer to get back into the circulation. An effective plasma concentration is also important for the antimalaric effect of chloroquin.

The **distribution or partition coefficient** refers to the distribution of the drug between two different compartments (usually lipid and water). If the drug is highly lipophilic (e.g. general anesthetics), it readily diffuses into the body fat. At the same time, its concentration in the body water compartments is relatively low. In that case the volume of distribution may increase dramatically.

**Plasma protein binding**

Most drugs bind to specific proteins (e.g. albumin, globulins, lipoproteins, glycoproteins, transferrin) in the plasma. There is a **dynamic equilibrium** between the **free** and the **protein-bound fraction** of the drug; although only the free fraction can escape the circulation and reach the site of action. The equilibrium is maintained when the drug molecules dissociate from the plasma proteins, in order to replace the free molecules that have left the circulation, and thus the ratio between the **free** and the **protein-bound fraction** of the drug remains constant. The plasma proteins also act like a storage for drug molecules, making the drug’s effect more prolonged. Since the **binding affinity** of the drugs can be different, they might displace each other from plasma proteins, which often lead to **pharmacokinetic interactions**. If the free fraction is relatively small (<5%, e.g. oral anticoagulants like warfarin), even a little change in protein binding can cause severe alteration of the free drug concentration as well as the pharmacodynamic effect.

**1.3. Metabolism**

The human body has an urge to eliminate any extraneous materials. In order to fast clear them away, the body transforms these molecules and makes them less effective and more water soluble. This process is called **drug metabolism**. In rare cases, the metabolites of the drug are more potent than the original molecule (e.g. the so-called **prodrugs** or more toxic (e.g.
ethanol is metabolized to acetaldehyde and acetic acid). Some drugs do not undergo metabolism.

The metabolic transformations are classified as **phase I** and **phase II reactions**. Drugs may undergo both types of metabolism, or are altered only in phase I or II reactions, and then they are excreted from the body (**excretion**). The phase I reactions alter the drugs chemical structure, resulting a more polar metabolite, which is easier to be excreted. These chemical reactions usually involve oxidation, reduction, hydrolysis, hydration or isomerization. In the phase II reactions the molecules are conjugated to endogenous molecules, like glucuronic acid, certain amino acids, glutathione, or with sulfate, methyl, and acetyl groups. The conjugated molecules are water soluble, and easily excreted by the urine.

The phase I reactions are catalyzed principally by the microsomal **cytochrome P450 (CYP)** **mono-oxygenase enzyme system** of the liver. They use molecular oxygen and NADPH as a cofactor. They catalyze several reactions like aromatic oxidation (e.g. phenylbutazone), aliphatic oxidation (phenobarbital on side chain), epoxidation (vitamin K), N-dealkylation (diazepam, ephedrine), O-dealkylation (codeine), S-dealkylation, N-oxidation (imipramine), S-oxidation (chlorpromazine), dehalogenation (halothane), and alcohol oxidation. Certain substrates (e.g. the barbiturates, carbamazepine, rifampicin, hypericin) can increase enzyme activity; these are called **enzyme inducers**. When the patient takes an enzyme inducer together with other drug(s), it may increase the breakdown of the other drug(s), resulting in a shortened biological effect. It has serious consequences, if the patient is on certain medications, such as oral anticoagulants, oral antidiabetics, or oral antiepileptics. The enzyme inducers can increase their own metabolic rate, which is typical of the barbiturates, leading to drug tolerance. If the patient is taken off the enzyme inducer, the CYP enzymes are no longer induced, which would cause a sudden increase in the effect of the co-administered drugs. In such cases, in order to avoid major adverse reactions, under- or overdose, therapeutic drug monitoring is required.

Some drugs, however, have an opposite effect on the metabolic enzymes, they inhibit their activity and are called **enzyme inhibitors**. Such drugs are e.g. cimetidine, ketoconazole, and erythromycin. They are also substrates of the microsomal CYP enzymes, which bind them with high affinity, and thus inhibit the other drugs to bind to the metabolizing enzymes and promote an unwanted long-lasting effect.
The microsomal **mono-oxygenase enzyme system** of the liver is not the only enzyme system which plays a part in drug metabolism. There are **other oxidizing enzymes** like the alcohol dehydrogenase (has zero order kinetics!, e.g. for ethyl-alcohol, methyl-alcohol), aromatase, aldehyde oxidase, and amine oxidase. Less frequent modalities of metabolism are **reduction**, including azo reduction (e.g. sulfasalazine), nitroreduction (chloramphenicol metronidazole), keto reduction or epoxide reduction. Certain drugs are **hydrolyzed**; they are the esters (e.g. procaine, acetylcholine), amides (lidocaine), azides (isoniazid), or ring opening (coumarins).

The goal of the phase II reactions is to form water soluble conjugates. In these reactions the polar drug metabolite is conjugated with endogenous molecules, with the help of conjugating enzymes. The most common conjugating molecules are glucuronic acid (e.g. with digoxin), glutathione (paracetamol), glycine (salicylic acid), acetyl coenzyme A (clonazepam) or water (carbamazepine epoxide). The conjugated metabolites are usually inactive and easy to be eliminated. The conjugation process itself might carry some risk: in case of relative enzyme deficiency or oversupply of the drug the conjugation would slow down, resulting in the accumulation of potentially toxic metabolites. Such intoxication can happen when paracetamol is overdosed. Paracetamol in normally 95% glucuronidated and sulphated and 5% is coupled to glutathione. When the therapeutic dose is exceeded, the glutathione pathway becomes exhausted and hepatotoxic metabolites (naphthoquinones) are formed. The antidote is glutathione (some paracetamol preparations contain it) or other agents with sulfhydryl groups (e.g. acetylcysteine). If there is a relative deficiency in the glucuronyl transferase enzyme, either genetic (Crigler-Najjar syndrome type II, Gilbert’s syndrome) or acquired (hepatocellular damage), the chance of the accumulation of the toxic naphthoquinones are much higher. For patients with the above diseases it is recommended to avoid taking paracetamol.

In rare cases, the administered drugs are transformed to (more) powerful agents. They are called **prodrugs** or **soft drugs**. Without metabolism to the active agents these molecules are biologically ineffective. The reason to develop and formulate prodrugs involve low stability (e.g. levodopa, NO-donors), uneasy absorption (ACE inhibitors) or targeted effect (acyclovir, dipivefrine). Many drugs are transformed into several metabolites, which are also active yet less potent than the original molecule; they are called **active metabolites**. The active metabolites can sustain the drug’s effect.
The primary scene of drug metabolism is the liver, but there is reasonable enzyme activity in the lungs, intestines, kidneys and skin. The orally administered drugs are absorbed from the intestines to the mesenteric veins, which drain via the portal vein into the liver sinusoids. Many drugs (e.g. morphine, meperidine) are extensively metabolized when first passing through the hepatocytes, and thus a significant proportion of them never reaches the site of action. This so-called **first pass effect** has to be taken into consideration when choosing the appropriate dose and route of administration of a drug.

Drug metabolism is affected by many factors, including the patient’s age, sex, state of health, especially kidney and liver function, circulation and genetic background, as well as nutrition, smoking and other drugs taken. Numerous genetic alterations of the genes coding the metabolizing enzymes are known; such enzyme polymorphism would also have a great impact on the drug’s effect.

### 1.4. Excretion

Extraneous materials are excreted from the human body via urine, stool, sweat, saliva, or breast milk. The most common way is the excretion via urine, after the original drug was metabolized and became water soluble. There are some drugs, however, which are eliminated in unchanged form: e.g. digoxin, the cephalosporins, aminoglycosides, and gabapentin. Elimination, which includes metabolism and excretion, has a major impact on bioavailability and determines the rationale for dosing. In the case of impaired liver function it is suggested to avoid drugs with extensive hepatic metabolism; likewise patients with kidney failure should not be given drugs exclusively excreted through the kidney. In the lack of therapeutic alternative the dose has to be tailored to the individual, and blood concentration as well as therapeutic effect should be regularly monitored.

The rate of excretion is determined by both the drug and the human body. Many drugs are weak bases or acids, their dissociation and membrane penetration is primarily affected by the pH in the urine. Molecular size also limits filtration rate. If the drug is bound to plasma proteins, its excretion would be sustained, since only the free, unbound fraction of the drug can cross the biological membranes and be excreted.

The rate of excretion is described by the **clearance** (Cl). Clearance is a virtual volume of the plasma, which is cleared of a certain material per minute or hour. Another important property
of the drug is the **half life** \( (t_{1/2}) \), which gives the time taken to clear away 50% of the drug from the plasma. The half life is independent from the route of administration; it can be calculated as follows:

\[
t_{1/2} = \frac{0.693 \times V_D}{C_l}
\]

The plasma concentration of the administered drugs declines with time, mostly following first order, and rarely zero order kinetics. **First order kinetics** means that the change in concentration (\( dC \)) over a time interval (\( dT \)), thus the velocity of the reaction (\( dC/dT \)), is proportional to the concentration. The velocity decreases with the passage of time, as the concentration of unreacted substance decreases; a plot of \( C \) against time would yield a curve of progressively decreasing slope. After the time of \( 5 \times t_{1/2} \) has passed, 97% of the administered drug has been eliminated. Most drugs are eliminated according to first order kinetics. It is necessary to know the half life of the drug to decide at what intervals to administer the drug, to maintain adequate blood level.

**Zero order kinetics** means that the drug elimination is independent of the drug's concentration in the blood. Thus, the concentration of a drug decreases at a constant rate. Examples of drugs undergoing zero order metabolisms are phenytoin and ethanol. The human body can metabolize 7 g ethanol (100%) per hour, independent from the ingested amount. Individuals may differ in their ability to tolerate the central effects of ethanol but not in the rate of metabolism. In case of overdose, the elimination of such drugs may be speeded up by dialysis.

Phenytoin is eliminated by the so-called **Michaelis-Menten kinetics**. When blood concentration is below a certain value, the elimination follows first order, but above the limit it turns into zero order. The limit is usually resulted by the saturation of the metabolizing enzyme’s capacity. Administration of phenytoin after it has reached its limit concentration would lead to steep increase of blood levels and toxic effects. In order to prevent overdose and adverse reactions, the therapeutic drug monitoring of such drugs is required.

**Renal excretion**

Drugs are excreted to urine by glomerular filtration and/or tubular secretion. **Filtration** is determined by molecular size, polarity, and the hydrostatic pressure gradient between the renal artery and the Bowman’s capsule. Generally speaking, molecules below the weight of 5000 Da, not charged and not bound to proteins can freely pass to the renal tubules. In
contrast, molecules over the weight of 5000 Da, or electrically charged particles are excreted by **active tubular secretion**. Most of the fluids as well as the lipid soluble, non-ionized particles are **passively reabsorbed** from the filtrate, while the remaining fluid, urine, is passed through the collecting ducts and removed from the body. Ionization is greatly influenced by the urine pH.

Active tubular secretion means that the drugs are transported via an ATP-dependent mechanism from the plasma into the tubular fluid, by an anionic or cationic transport system. Organic acids (e.g. salicylic acid, the penicillins, sulfonic acids, and metabolites like glucuronide or sulphate conjugates) are usually excreted by **anionic transport**. Organic bases (e.g. morphine, meperidine, amiloride, histamine, or serotonin) are eliminated via **cationic transport system**. These are powerful mechanisms to remove the drugs, both working against concentration gradient, even when the plasma concentrations are virtually zero. Since the active tubular secretion decreases the free concentration of the drug in the plasma, it also promotes the dissociation of the drug from the plasma proteins. The active transport pumps, however, are saturable and similar molecules can compete for the transport, altering each other’s elimination kinetics (penicillin and probenecid).

**Passive reabsorption** from the renal tubules into the blood works down the concentration gradient of certain drugs. The rate of reabsorption is highly dependent on the lipophilic character of the drug and local pH. The dissociation of weak acids is set back at low pH, which means that the unionized molecules can freely cross the membranes. Since the pH is about 4 in the proximal and 8 in the distal tubule, weak acids tend to be reabsorbed passively in the proximal, while the weak bases are more readily reabsorbed in the distal tubules. Passive reabsorption delays drug elimination.

At the same time, the intentional change of urine pH can speed up the elimination of the drugs. **Acidification** (e.g. by ammonium chloride) would increase the removal of basic substances (e.g. cocaine, amphetamine); **alkalinization** (by bicarbonate) would promote the excretion of weak acids (e.g. barbiturates, aspirin). This principle is often used to treat drug overdose, usually combined with powerful diuretics such as furosemide, and is called **forced diuresis**.
**Biliary excretion**

The liver plays a prominent role in the elimination of drugs, being the organ at increased risk of drug toxicity. It has two lobes, and consists mainly of hepatocytes which form functional units (lobules). The liver produces bile, which is transported to the gallbladder via the intrahepatic and the extrahepatic biliary ducts, and then released into the duodenum. Several drugs and toxins are excreted via the same mechanism.

The orally administered drugs are absorbed from the intestines to the mesenteric veins, which drain via the portal vein into the liver sinusoids. Many drugs (e.g. morphine, propranolol, and lidocaine) are extensively metabolized when first passing through the hepatocytes. Drugs with high plasma protein binding (phenobarbital, warfarin) are slowly eliminated by the liver, since only the free fraction can bind to the metabolizing enzymes. Drugs that were administered parenterally also travel through the liver and are metabolized. Generally, the highly lipophilic and gross molecules are eliminated via the liver, while the small, water soluble drugs are eliminated via the kidney.

Drugs excreted with the bile are released into the duodenum. The glucuronide conjugates often leave the body with stool or are hydrolyzed by the intestinal glucuronidase enzyme, and the free drug is reabsorbed into the blood. This process, which delays the elimination of the drugs and protracts their effect, is called **enterohepatic reabsorption**. It is characteristic of the benzodiazepines, steroid hormones, or morphine. Since the glucuronidase enzyme is produced by intestinal bacteria, the enterohepatic reabsorption may be impaired aggressive by antibiotic treatment, which destroys these bacteria. The rate of enterohepatic reabsorption has to be taken into consideration when choosing the appropriate dose.

Drugs and other chemical substances may be **toxic** for the hepatocytes and cause their necrosis (e.g. paracetamol), inflammation (halothane, chlorpromazine), or hepatitis-like symptoms (isoniazid, methyldopa).

**Pulmonary excretion**

Gaseous (e.g. inhalation anesthetics) and volatile substances (alcohol, or volatile oils) are excreted via the lungs. Pulmonary excretion can be speeded up by increasing the respiratory rate or the heart rate and circulation; these methods are often used to regulate the depth of anesthesia. The bronchial secretory glands can excrete the expectorant salts, which have local stimulatory effect.
Other ways of excretion

Drugs and their metabolites might be excreted by the skin, which refers to the secretion by sebaceous or sweat glands. Alcohol, halogen substances, volatile oils, thiamine or sulfonamides are excreted in part by the skin, and it may cause a strange odor of the patient.

Certain drugs are accumulated in the saliva, which is not a real excretion, since the patient usually swallows his saliva. Drug accumulation in the saliva may serve a therapeutic benefit, e.g. in the case of the antibiotic clindamycin, which is often used to treat the aerobic and anaerobic infections in the oral cavity. An unpleasant taste, however, might render it more difficult to administer some drugs (e.g. metronidazole, ritonavir).

The highly lipophilic drugs and weak bases are easily diffused in the breast milk, where the pH is about 6.5. Carrier mediated transport mechanisms (anion and cation carriers) help the excretion of the anionic and cationic substances. Caffeine, theophylline, alcohol, nicotine, amphetamine and cocaine are excreted in the milk. Since breast feeding has a major physiological and psychological importance; drug abuse, smoking and drinking coffee should be avoided during this period. If medication of the mother is necessary, breast feeding has to be suspended. Some of the drugs are considered safe even during breast feeding; these should be taken right after the end of one feeding session.

2. Quantitative pharmacokinetics

The primary objective of quantitative pharmacokinetics is describing the change in the pharmacon’s concentration over time in a selected part or parts of the organism (generally where the given pharmacon is anticipated to develop its wanted and untoward effects). In view of the concentration-time curve rational dosage regime protocols may be formulated or optimized according to the therapeutic goal. In order to achieve this, appropriate analytical background is needed (a topic not covered in this chapter), as well as mathematical analysis and fitting the most parsimonious model to the measured and or calculated (or more often estimated) concentration data. The main goal of this chapter is to provide an approach that will be sufficient to understand or maybe even feel the complexity of the interaction between the living systems thriving for a dynamic equilibrium and chemical structures by introducing
the simpler mathematical models. Professional intuition may only be reached by having exact knowledge.

**Etymology**
By pharmacon (φαρμακον) ancient Greeks meant herbs, pharmaceuticals as well as poisons. Healing and wizardry have a common origin reflected by the fact that pharmacos (φαρμακος) meant both animal and man intended as placatory sacrifice (scapegoat). Logos (λογος) = speech, precept; bios (βιος) = living; pharmaclia (φαρμακια) = use of medication or poison; dynamis (δυναμις) = power, ability, authority; kinesis (κινηζις) = movement.

**2.1. Pharmacokinetic models**
Every pharmacokinetic calculation is based on the mathematical model describing the effect the living organism exerts on the pharmacon. This seems to be contradicted by the fact that the in the past most generalized calculations that were based on the fewest presumptions were called “model independent” methods. This contradiction may be resolved if we accept that in those cases “model independency” only meant the lack of presumptions inherent of more specific models. Presumptions for pharmacokinetic models may be predominantly physico-chemical in nature- these are the classic compartment (or chamber) models, or they may rest on the peculiarities of the tissue organization of living organisms e.g. in case of the physiological (or anatomic, or biologic) models.

The general (non-compartmental and non-physiological) models, as reflected by their name, are based on general assumptions; they contain no assumptions concerning the mechanisms of the pharmacon’s fate. Parallel to the rapid development of analytical methods and computers these models gradually became obsolete. They are not suitable for precise analysis, but they are cheap and allow quick orientation with respect to the most important pharmacokinetic characteristics of pharmacons, so in case of novel pharmacons computations based on general models are used initially. These are well utilized in cases when, due to the complex interaction between the living organism and the pharmacon (e.g. absorption from several sites, elimination at different locations, enterohepatic circulation), the relevant compartment or physiological models are difficult to handle.

Compartments of the compartment models are not necessarily the same as the tissue compartments they set to model. The advantage of compartment models however is that they describe the change in the concentration of the drug using mathematical formulations for the physico-chemical processes. Moreover they are somewhat flexible concerning the interactions
seen between the model living organism and the pharmacon (there are one-, two- or multi-
compartment forms, and additionally several different interrelations may be used to describe
the connections between the compartments). Computations based on the compartment models
are also called compartment analysis.

The main advantage of physiological models is that they reflect the real relations most
accurately. Their disadvantage however is that the mathematical descriptions they use are
many times empiric (lacking a more solid concept), and they need more hard to obtain data
(high demand for computations and analytical methods).

2.2. Classic compartment models

Volume of distribution (\(V_d\)) is the volume within the body where the pharmacon is present
(that is where its concentration is not zero). A compartment within this volume is where the
pharmacon’s concentration is the same at a specific time point (so the concentration in this
volume is homogenous). Theoretically a system contains as many compartments as many
concentrations are present within it. To simplify, we propose that within a compartment the
pharmacon is distributed immediately, furthermore we overlook the concentration gradient
driving the equilibration of pharmacons (in a dynamic equilibrium e.g. a pharmacon is
absorbed from some place while it is eliminated from another, thus there has to be a
concentration gradient even within the compartment, although this isn’t too large).

A. One-compartment open model

The pharmacon is homogenously distributed in the available space, and it can only leave via
elimination. (The term open refers to the fact that the pharmacon may enter and leave the
compartment. We will only focus on open models.) When assessing the living organism and
the pharmacon first we usually start by using the one-compartment model and we only extend
the model with other compartments if the measured parameters (e.g. the pharmacon’s
concentrations determined in the monitored compartment(s)) show poor correlation with the
values predicted by the model. The one-compartment model characterizes well for example
ethanol showing quick distribution in the body water and plasma expanders circulating in the
vessels after iv infusion (Fig. 1).
Figure 1: Schematic representation of the one-compartment open model

\( k_a \) and \( k_e \) are the rate constants for absorption and elimination.

**B. Two-compartment open model**

If there are two compartments, we have to make some decisions concerning the relationship between the two compartments and their relations to absorption and elimination. There are several possibilities; Fig. 2 shows two cases from these.

Figure 2: Schematic representation of two types of the two-compartment open models

*(top: mamillary model, bottom: caternary model)*; \( k_a \) and \( k_e \) are the rate constants for absorption and elimination, \( k_c \) and \( k_c' \) are the rate constants for entering and exiting a compartment. If absorption and elimination may be into/from several compartments, more types can be identified.

The most important representative of the two-compartment model is the mamillary model shown in Figure 2. This provides appropriate estimates for most pharmacons. Usually blood (or more exactly blood plasma) is the central compartment as this is where the pharmacon is absorbed to and from where it is eliminated from (mainly via renal and/or hepatic pathways). The peripheral compartment model(s) tissue compartment(s) that contain the pharmacon in a different concentration than that of the central compartment. The peripheral compartment
modifies the pharmacon’s time course of concentration in the central compartment (therefore by closely monitoring this latter the presence of the peripheral compartment may be identified).

The expansion of the two-compartment open models with additional compartment increases the possible number of models, but hinders implementation.

2.3. Kinetic order of the changes involving the pharmacons

Basics of reaction kinetics

The kinetics of every process influencing the fate of any given pharmacon (absorption, elimination, and transport between the compartments in a multi-compartment model) may be described using the same mathematical tools. Of the processes influencing the pharmacons, elimination can be assessed most easily using experimental tools.

As we previously discussed elimination is everything that (1) causes a change in the pharmacon’s primary chemical bonds (metabolism) or (2) clears the pharmacon from the living organism (excretion). The first is a chemical reaction; however in the second the transport process may also be viewed as a chemical reaction. The time course of change in the concentration of the reaction materials known as the rate (and closely relating to this the mechanism) of chemical reactions is described by reaction kinetics.

When using an empiric approach the equation expressing the rate of a chemical reaction contains the concentration of every reacting chemical powered to the exponent according to their stoichiometry (stoichiometric exponents), and the goal is to identify a k constant that if multiplied by the product of the powered concentrations will provide the reaction rate obtained experimentally (v):

\[ v = k \cdot c_1^{a} \cdot c_2^{b} \cdot ... \cdot c_n^{x} \]

The (gross) order of a chemical reaction is determined by the sum of the concentrations’ exponents determining the reaction velocity (a+b+...+x using the above equation as an example). Order of the reaction may be defined for a single constituent as well, in this case only the exponent of the given chemical’s concentration is taken into consideration (using the above example this would equal to a for chemical 1). The most important consideration from a practical point of view is for k constant, as this has to be invariable within the experimental conditions.
In the living organism pharmacons are metabolized in a chemically rich environment generally with the participation of several different molecules (usually present in a watery solution). Although theoretically only the first step is considered as metabolism (in which the structure of the pharmacon changes), metabolism is usually a chain reaction where the subsequent steps influence the rate of the first step, therefore the complete process should be considered.

Regardless the elimination of pharmacons may be approximated relatively simply due to the validity of the following principles:

1. The rate of a chain reaction (at least in the beginning of the process) is determined by the step with the smallest rate constant (rate limiting step). The kinetic order of the complete process is therefore well approximated by the order of the rate limiting step, so it is sufficient to deal with that.

2. If one of the reacting agents is present in such abundance that its concentration remains practically unaltered during the entire process, than it doesn’t contribute to the order of the reaction that is determined experimentally, therefore it may be omitted from the equation describing the rate of the reaction. This means for example that the concentration of water often present during metabolism may be left out of the calculations similar to the endogenous molecules that are present in high concentration (e.g. ATP, glucuronic acid).

3. The rate limiting step (similar to the other steps) is generally an enzymatic catalyzed reaction. The catalyst is retrieved unchanged at the end of the reaction therefore the enzyme may also be omitted from the equation describing the rate of the equation.

Summarizing, during the elimination of pharmacons (from the mathematical standpoint including their absorption and any other transport) only the rate limiting step of the chain reaction in which the pharmacon attaches to an enzyme (or a carrier) should be considered (while other molecules possibly also participating in the reaction are present in abundance compared to the pharmacon). Using these considerations we can say that the elimination (or absorption or transport) of pharmacons may be viewed as monomolecular process therefore the rate of the process is given by the product of the rate constant and the pharmacon’s concentration raised to the first power. In other words the elimination (and mostly all other processes) of pharmacons generally shows first order kinetics.
Elimination in the one-compartment open model

Due to the fact that elimination is the most readily investigated process of all processes involving the pharmacon, the principles of kinetics will be illustrated using that as an example. For the sake of simplicity -if not indicated otherwise- elimination will be discussed using a one-compartment open model.

Although the rate of elimination can be generally well estimated by the product of the elimination constant and the first power of the pharmacon concentration, the rate of elimination may be determined more precisely. Elimination (as an enzyme-catalyzed reaction) may be described using Michaelis-Menten kinetics, that is based on the law of mass action (more precisely, on the law of mass action described for equilibrium). Forerunner of the Michaelis-Menten equation is the Hill equation that is well-known in pharmacodynamics. The model of Michaelis and Menten also contains some presumptions, owing to this fact it is mathematically relatively simple. According to it, the rate of elimination \( v \):

\[
v = v_{\text{max}} \cdot \frac{c}{c + K_M}
\]

where: \( v_{\text{max}} \) is the maximal rate of elimination, \( c \) is the concentration of the enzyme’s substrate in the rate limiting step of the pharmacon’s elimination (in the simplest case this is the concentration of the pharmacon itself, but this is not necessarily true), \( K_M \) is the substrate concentration at which the rate limiting enzyme works at half-maximal rate (Michaelis-Menten constant). Using the above equation if rate is plotted as a function of concentration (using linear axes), a hyperbolic or saturation curve is obtained (Fig. 3).

![Figure 3](image-url)  
**Figure 3:** Rate of enzymatic elimination as a function of substrate concentration  
*(abbreviations are in the text).*
Although $K_M$ is the half-saturating concentration of the substrate for the rate-limiting enzyme, it describes the pharmacon’s concentration well in our case since the other processes barely contribute to the overall kinetics of complete elimination process.

The pharmacon’s rate of elimination in the $0 - K_M$ concentration interval may be simplified as follows since $K_M > c$ in this case:

$$v = v_{max} \cdot \frac{c}{c + K_M} = \frac{v_{max} \cdot c}{K_M} = \frac{v_{max}}{K_M} \cdot c = k_s \cdot c$$

here: $k_s = \frac{v_{max}}{K_M}$ is the rate constant for elimination. It should also be noticed that in the equation expressing the rate ($v = k_s \cdot c$) the first power of concentration occurs, so the elimination of pharmacons in the $0 - K_M$ concentration interval follows first order kinetics with good approximation. In this case the quantity of substrate is small when compared to the quantity (or more precisely the activity) of the enzyme, therefore substrate molecules (forming from the additionally added pharmacon dose) will readily find free enzymes so the rate of elimination will increase. According to this, Fig. 3 reflects that the rate-concentration function is approximately linear at concentrations below $K_M$; therefore the rate of elimination is roughly proportional to the concentration of the substrate (or the pharmacon in question).

The rate of elimination may be given in a simpler for concentrations higher than $4 K_M$ as well, using the assumption that $K_M < c$:

$$v = v_{max} \cdot \frac{c}{c + K_M} = v_{max} \cdot \frac{c}{c} = v_{max} = k_s \cdot c^0$$

So for concentration above $4 K_M$ the elimination rate constant equals to $v_{max}$, and the rate of elimination is always at maximum. It should be noted that lack of dependence on the concentration means the zero power of the concentration ($v = v_{max} = k_s \cdot c^0$), so elimination of pharmacons at concentrations exceeding $4 K_M$ shows zero order kinetics with good approximation. In this case substrate quantity greatly exceeds the quantity of the enzyme (or its activity), most of the enzyme molecules are occupied therefore it is difficult for the newly introduced (or produced) substrate molecules to find a free enzyme. This is reflected in Figure 3, where the rate-concentration function is approximately linear and runs almost horizontally showing that elimination became independent of the substrate concentration (or pharmacon in question).
It is not worth the effort to mathematically simplify the equation describing the rate of elimination in the concentration interval $K_M - 4K_M$ as this would yield a fractional order kinetic reaction (0-1 in this interval). In practice however this concentration interval is handled as either a first- or a zero order kinetic reaction depending on whether the therapeutically or toxicologically significant concentration is in the higher or lower range.

Although the elimination of pharmacons follow at least two kinds of (zero and first order) kinetics, in practice we see that elimination of most pharmacons is kinetically uniform; generally show first order kinetics, with only a limited scope of pharmacons that follow zero order kinetics (for example ethanol). The reason for this is that the concentration of most pharmacons has no significance in such a wide dosage range that would be lead to a change from first order kinetics to zero order kinetics. If a substance is efficacious in a concentration well below the $K_M$ value of the rate limiting enzyme (or transporter), the levels at $4K_M$ could be toxic or couldn’t develop (as these are incompatible with life). However if the concentration in interest is above the value of $4K_M$, concentrations below $K_M$ are generally ineffective, so these are not investigated.

Only a few special pharmacons are used over such wide concentration ranges where the change in kinetics may be seen in practice. For example acetylsalicylic acid is eliminated with first order kinetics when given in the indication of platelet aggregation inhibition (75-325 mg per day), however when given to ameliorate the symptoms of rheumatoid arthritis it may be given in a high dose (2-4 g), and is eliminated with zero order kinetics. For the sake of simplicity we disregard the fractional exponent kinetics in this case as well (although, for example, the dose range for the antipyretic and pain killer indication of acetylsalicylic acid – 0.5-2 g – would be best described using fractional exponents).

### 2.4. Classic compartment analysis

**First order elimination in a one-compartment open model with immediate absorption**

If the absorption of a pharmacon’s single dose given at $t_0$ time point is immediate into the compartment then the maximal $c_0$ concentration will evolve at $t_0$. Therefore in the case of first order elimination, the pharmacon’s $c_t$ concentration is described at any given $t$ time point by the following equation:

$$c_t = c_0 \cdot e^{-kt}$$

where: $e$ is the base of the natural logarithm. Since in the case of first order kinetics (and only first order kinetics) $k_e = \ln 2/t_{1/2}$ (with $t_{1/2}$ being the half life), the above relation is analogous to
the law of radioactive decay, so it may be expressed in the usual form of the latter (although the law of radioactive decay uses masses and not concentration, however these are equivalent if the volume is the same):

\[ c_t = c_0 \cdot 2^{\frac{-t}{T_{1/2}}} \]

Taking the logarithm of the above exponential equation, the time-dependency will become linear:

\[ \ln (c_t) = \ln (c_0) - k_e \cdot t \]

In the case of first order kinetics, \( k_e \) denotes the fraction of the pharmacon eliminated per time unit (so its dimension is \(1/\) time unit). If the volume of the compartment remains unaltered (so \( V_d \) remains constant), \( k_e \) provides the pharmacon’s mass eliminated per time unit as well as the proportion of the volume of distribution that is cleared during the time unit. According to this:

\[ k_e \cdot V_d = \text{clearance} \]

―Clearing‖ the distribution volume means that we look at that volume that would be cleared completely free of the pharmacon dissolved in it during the given unit of time, with the concentration of the pharmacon remaining unaltered in the remaining volume. This is the clearance that provides the proportion of the distribution volume “cleared” over time, so its dimension is volume/time unit. Since both \( k_e \) and \( V_d \) are constant, clearance is also constant in case of first order elimination. The product of clearance and \( c_t \) provides the mass eliminated during the time unit used (this is not constant as \( c_t \) is not constant). The advantage of clearance is that in the case of several processes (or places) for elimination the overall clearance may be calculated by summing up the clearance of the individual processes.

The following statements are true for first order elimination:

1. The proportion of eliminated concentration (and mass) is constant over time.
2. \( t_{1/2} \) is constant
3. Clearance is constant.
4. \( k_e \) is not dependent on \( c_0 \).
The above relationships may be used to calculate the concentration of a pharmacon with known parameters at any given time point, but it can also be used to determine the unknown kinetics of the pharmacons using experimental data. The following function illustrates the concentration-time curve of a pharmacon given in a single dose at a \( t_0 \) time point, if the absorption is immediate:

\[
\text{Figure 4: The concentration-time curve for a pharmacon given at } t=0 \text{ time point showing first order elimination kinetics with momentary absorption.}
\]

\[
The x and y axis are linear, the scale is arbitrary.
\]

By taking the logarithm of the pharmacon’s concentration, calculation will become simpler (this had greater relevance before the emergence of computers). Plotting the logarithm of the concentration (or using a logarithmic scale on the y axis) the concentration-time curve is more easily constructed:
Figure 5: The function shown in Fig. 4 when plotted semi-logarithmically.

A \( (t_1; c_1) \) and a \( (t_2; c_2) \) points of the function were highlighted (for details see the text).

Theoretically the concentration-time curve illustrated in Fig. 5 may be plotted using only two experimental concentration-time data pairs. This data is sufficient to compute the \( k_e \) elimination constant, and with use of this, any given point of the pharmacon’s concentration-time curve may be determined algebraically, since using a semi logarithmic illustration (Fig. 5) the \( k_e \) value equals to the slope of the linear concentration-time function, or more exactly it is -1 times the slope (see above the logarithmic form for the elimination of a pharmacon given in \( c_t \) concentration in case of first order kinetics):

\[
-tg\beta = k_e = \frac{\ln(c_1) - \ln(c_2)}{t_2 - t_1}
\]

In practice immediate absorption is mostly approximated by quick intravascular (iv. but intraarterial even more so) drug administration given in a bolus.
Zero order elimination in a one-compartment open model with momentary absorption

The concentration, $c_t$, of a pharmacon following zero order kinetics with immediate absorption at any given time point is described by the following equation:

$$c_t = c_0 - k_e \cdot t$$

This equation determines a linear function (when plotted using linear axes) the slope of which equals to -1 times $k_e$.

In the case of zero order elimination, $k_e$ determines the pharmacon concentration eliminated over time unit (so its dimension is concentration/time unit), therefore $k_e$ equals to the rate of reaction (that is both constant and maximal). According to this, the product of $k_e$ and $V_d$ gives the mass of eliminated pharmacon over a time unit that is constant (if $V_d$ is constant):

$$k_e \cdot V_d = \frac{n_{\text{eliminating}}}{t}$$

Due to the concentration independent loss of pharmacon, the proportion of the pharmacon’s mass and concentration increases over time during the process.

The following statements characterize zero order elimination:

1. The concentration (and mass) eliminated over a time unit is constant.
2. $t_{1/2}$ continuously decreases (therefore elimination is not characterized by this parameter).
3. Clearance continuously increases (therefore elimination is not characterized by this parameter either).
4. $k_e$ is not dependent of $c_0$ (here either).
The concentrations of the single dose of the immediately absorbed pharmacon at a $t_0$ time point may be illustrated using the following function in case of zero order kinetics:

![Graph](image)

**Figure 6:** The concentration-time curve for a pharmacon given at $t=0$ time point showing first zero elimination kinetics with momentary absorption. The $x$ and $y$ axis are linear, the scale is arbitrary. $c_0$ and $k_e$ values are the same as for the function shown in Fig. 4.

### 2.5. Calculations based on the general model

**Apparent volume of distribution**

Aside from a few pharmacons with special properties (e.g. plasma expanders that stay in the intravascular space), the volume of distribution of pharmacons may only be determined precisely using expensive methods (e.g. PET). For practical use the determination of the so called apparent volume of distribution is sufficient, that may be calculated as follows:

$$V_d = \frac{n_{\text{administered}}}{c_{\text{monitored}}}$$

where: $n_{\text{administered}}$ is the mass of pharmacon administered to the living organism, $c_{\text{monitored}}$ is the concentration of the pharmacon in a given compartment after distribution (unfortunately apparent volume of distribution is denoted by $V_d$ just the same as the volume of distribution is). If the investigated compartment is not specified otherwise then this compartment, in which the concentration of the pharmacon is monitored, is the blood plasma. It happens that instead of using $n_{\text{administered}}$, $m_{\text{administered}}$ (administered weight) is used, so in these cases this
former parameter should be divided by “plasma density” (a weight/volume quantity) instead of the concentration.

If the pharmacon shows a homogenous concentration distribution in the distribution volume (the simplest scenario for this is when the pharmacon is only present in the monitored compartment) then the apparent volume of distribution will be equal to the real volume of distribution. If the concentration distribution of the pharmacon is heterogeneous, the apparent volume of distribution may be smaller or larger than the real volume of distribution. If plasma is the monitored compartment then the compounds showing high lipid solubility will have an extremely high apparent volume of distribution, while pharmacons with strong plasma protein binding generally have a small apparent volume of distribution.

**Area under the curve for concentration-time curves (AUC)**

AUC is the area of the formation bordered by the concentration-time (usually plasma concentration-time) curve, and the x axis (given the x axis intersects the y axis at 0); shortly it is the area under the curve. AUC is the same as the integral of the concentration-time curve. Similar to the integral that may be definite or indefinite, the AUC may relate to a specified part of the concentration-time curve between any two \( c_1 \) and \( c_2 \) values (and the associated \( t_1 \) and \( t_2 \) values on the x axis) or the complete function (with the complete positive interval of the x axis ranging from \( t_0 \) to \( t_x \)). The advantage of AUC is that it characterizes the “weight” of the presence of the pharmacon in the body (or in the monitored compartment) using a single value; as AUC is higher if the pharmacon is present in a higher concentration and/or for longer time in the monitored compartment.

In the \( t_0 - t_n \) interval on the x axis (AUC\(_{0-n}\)) AUC may be approximated using the so called trapeze rule. This means that the function relating to the \( t_0 - t_n \) interval is divided into several sections in a way that the function within this section would be approximately linear. These quasi linear sections together with the associated sections of the x axis form a trapeze, the area of which may be computed as usual:

\[
AUC_{(x-1)-x} = \frac{c_{x-1} + c_x}{2} \cdot (t_x - t_{x-1})
\]

where: AUC\(_{(x-1)-x}\) is the area of the trapeze located on the x axis between points \( t_{x-1} \) and \( t_x \), \( c_{x-1} \) and \( c_x \) are the concentrations relating to \( t_{x-1} \) and \( t_x \) respectively. By adding the areas of the
trapezes we obtain $AUC_{0-n}$. The remaining area ($AUC_{n-\infty}$) may be approximated using the following equation:

$$AUC_{n-\infty} = \frac{c_n}{k}$$

where: $c_n$ is the last known concentration, and $k$ is the approximate negative slope (slope times -1) in the section past $c_n$. The sum of $AUC_{0-n}$ and $AUC_{n-\infty}$ provides the whole AUC ($AUC_{0-\infty}$).

In the case of first order elimination the relationship between the whole body clearance (WBC) and the whole AUC is as follows:

$$WBC = \frac{\eta_{administered}}{AUC}$$

Determination of AUC is compulsory in the assessment of novel pharmacons (and comparison to older ones).
References


1. DNA, RNA, protein

Proteins are the major macromolecular elements in the biological functions of the cell. The structure and function of proteins are determined by their amino acid sequence. The sequence of amino acids in a certain protein is defined by genes encoded in DNA. This encryption is deciphered with the help of different types of RNAs and translated into the appropriate amino acids. Understanding the function of DNA and RNA resulted in specific methods by which their structures – and hereby cellular functions – can be modified.

1.1. Chemical structure of DNA

Polynucleotides built from nucleoside monophosphates are linear polymers such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Fig. 7). The monomers in DNA molecules are purine (Adenine: A and Guanine: G) and pyrimidine (Cytosine: C and Thymine: T) bases. Independently of the origin of DNA, these four types of bases are always present, only their rate is different (e.g. in mammals A/T is 45-55%).

Nucleotides are joined together in DNA and RNA by phosphate ester bonds between the phosphate component of 3’ carbon group in one nucleotide and the 5’ OH group on the sugar component of the next nucleotide. These covalent phosphodiester bonds make the primary structure of DNA. One end of the nucleic acid molecule always has a free -OH group (called the 3’ end) and the other end of the molecule always has a phosphoric acid group (the 5’ end). By convention, the primary structure of a DNA (or RNA) molecule is reported from the 5’ end to the 3’ end.

The double-helix structure of DNA, which is one of the most significant models in modern biology, was published by F.H.C. Crick and J.D. Watson in 1953 [Watson 1953]. The double helix in DNA consists of two right-handed polynucleotide chains that are coiled about the same axis. The heterocyclic amine bases project inward toward the center so that the base of one strand interacts or pairs with a base of the other strand by hydrogen bonds: thymine - adenine pair interacts through two hydrogen bonds (T=A) and the cytosine-guanine pair interacts through three hydrogen bonds (C=G). This secondary structure of DNA is illustrated in Fig. 7. As a consequence of the helical structure, the negative charges are situated on the exterior surface of the DNA. These negative charges in the nucleus are compensated for by
proteins rich in basic amino acids (e.g. histones). The purine and pyrimidine bases of both strands are stacked inside the double helix, with their hydrophobic and nearly planar ring structures very close together and perpendicular to the long axis. In the model of Watson and Crick, the conformation of double helix is the so called B-DNA, the most common double helical structure where the double helix is right-handed with about 10–10.5 nucleotides per turn. B-DNA has two distinct grooves: a major groove and a minor groove. These grooves form as a consequence of the fact that the beta-glycosidic bonds of the two bases in each base pair are attached on the same edge [Watson 1953]. The edges of the bases are more accessible in the major groove. As a result, proteins like transcription factors that can bind to specific sequences in double-stranded DNA usually make contacts to the sides of the bases exposed in the major groove [Pabo 1984].
1) Nucleotides are joined together by phosphate ester bonds between the phosphate component of 3' carbon group in one nucleotide and the 5' OH group on the sugar component of the next nucleotide. 2) Polynucleotides built from nucleoside monophosphates are linear polymers (DNA and RNA). The monomers are purines (Adenine: A, Guanine: G) and pyrimidines (Cytosine: C and Thymine: T or Uracil in RNA). 3) The most common double helical structure is B-DNA, where the double helix is right-handed with about 10–10.5 nucleotides per turn and has two distinct grooves: a major groove and a minor groove.

During replication, the double helix unwinds and single-stranded DNA sequences will be available. This process is called denaturation or melting. DNA can be denatured by changing temperature, pH, or the salt concentration. In appropriate conditions, the two strands of DNA can renature. The case where DNA strands with different origin are bond together is called hybridisation. Hybridisation can occur between DNA strands or RNA strands, or between DNA and RNA.
DNA in prokaryotes is circular and attached to basic non-histone proteins. In eukaryotes, the combination of DNA and proteins (histones and non-histones) make up the contents of the nucleus, called chromatin. Chromatin with its supercoiled DNA-protein complex makes up the chromosomes (*Fig. 8.*). Chromatin is stabilized by electrostatic interactions between the negatively charged DNA and basic nuclear proteins. This supercoiled form, the quartary structure, makes possible for the DNA to fit into the nucleus.

*Figure 8.* From DNA to chromosome.

*In the nucleus, the 2 nm DNA double helix is subjected to at least two levels of coiling and forms 10 nm nucleosome which consists of eight histone proteins and about 160 base pairs. Multiple nucleosomes wrap into 30 nm fibres (solenoid) which are packed into loopes containing 60,000 base pairs each and condensed to form a chromosome.*
1.2. Chemical structure of RNA

The primary structure of RNA is a bit different from that of DNA:

– Bases in RNA are always bound to D-ribose by β-N-glycosidic bonds (while DNA contains the slightly different sugar deoxyribose).
– RNA has the nucleobase uracil instead of thymine (uracil and thymine have similar base-pairing properties), and sometimes rare bases or thymine.
– RNA usually forms single strands and the rate of purines and pyrimidines is variable. Certain parts of RNA are often single-stranded while other parts in the same RNA are double-stranded.
– RNA can be hydrolyzed in alkaline conditions while DNA remains stable [Gergely 1994].

Based on the role and structure, the following RNA molecules can be distinguished:

**Messenger RNA (mRNA)** encodes a chemical "blueprint" for a protein product and carries coding information to the sites of protein synthesis: the ribosomes. In mRNA, as in DNA, genetic information is encoded in the sequence of nucleotides arranged into codons consisting of three bases each. Each codon encodes for a specific amino acid, except the start and stop codons. In eukaryotes, mRNA is synthesized as a precursor (pre-mRNA) in the nucleus and contains non-coding intron sequences. Introns are cut out by special enzymes during the process called splicing. The mature mRNA is then transported from the nucleus to the cytoplasm where it is bound by ribosomes, and protein synthesis (translation) can start.

**Transfer RNA (tRNA)** carries a specific active amino acid to the growing polypeptide chain at the ribosomal site during translation. tRNA has a 3’ terminal site for amino acid binding and a three base region called the anticodon that can base pair to the corresponding codon region on mRNA. Each type of tRNA can be bound to only one type of amino acid, but because the genetic code contains multiple codons that specify the same amino acid, tRNA molecules with different anticodons may also transfer the same amino acid.

**Ribosomal RNA (rRNA)** interacts with tRNAs during translation and provides a mechanism for decoding mRNA into amino acids. More than one rRNA can attach at the same time to the same mRNA, thus several proteins can be translated from a single mRNA simultaneously [Lewin 2008].
In addition to mRNA, rRNA and tRNA, there are a number of regulatory, non-coding RNAs, e.g. microRNAs (miRNA). miRNA’s are post-transcriptional regulators that bind to complementary sequences on target mRNA transcripts, usually resulting in translational repression and gene silencing [Wu 2008]. Another type of regulatory RNAs is the group of small interfering RNA (siRNA), a class of double-stranded RNA molecules, 20-25 nucleotides in length. The most notable role of siRNA is its involvement in the process of RNA interference (RNAi), where it interferes with the expression of a specific gene [Sontheimer 2005]. Antisense RNAs usually downregulates the function of a given gene by forming a double helix with the mRNA, which is than recognized and degraded. Long non-coding RNAs (long ncRNAs, lncRNA) are generally considered as non-protein coding transcripts longer than 200 nucleotides. An example for ncRNAs is Xist, which inactivates one of the two chromosomes X in female mammals [Heard 1999]. However, mRNA contains special sequences (cis elements in the 5’ and 3’ non-translated regions) that influence its own function [Batey 2006].

1.3. Amino acids, peptides, proteins

Amino acids are the structural units that make up peptides and proteins. Based on their side-chains, the 20 basic amino acids can be classified into 4 groups: hydrophobic, neutral, acidic, alkaline. The abbreviations and charges of amino acids are listed in Table 1. Amino acids are linked with peptide bonds when the carboxyl group of one molecule reacts with the amino group of the other molecule, thereby releasing a molecule of water (Fig. 9).

![Peptide bond](image)

**Figure 9.** Peptide bond

*Amino acids are linked with peptide bonds when the carboxyl group of one molecule reacts with the amino group of the other molecule, thereby releasing a molecule of water.*
Peptides are composed of less than 50 amino acids, while a single protein can have even 2000 amino acids [Gergely 1994]. Besides the amino acids, proteins can contain other kind of molecules named cofactors. The primary structure of a protein is determined by its amino acid sequence, and the secondary structure is defined by patterns of hydrogen bonds between the main-chain peptide groups. Two main types of secondary structure are the alpha helix and the beta strand. Tertiary structure refers to the three dimensional structure of a single protein molecule, while quaternary structure is a larger assembly of several protein molecules or polypeptide chains, usually called subunits (Fig. 10).
## Table 1. Abbreviations and charge of the most important amino acids

<table>
<thead>
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<th>Amino acid</th>
<th>Three-letter abbreviation</th>
<th>One-letter abbreviation</th>
<th>Charge</th>
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<td>Leu</td>
<td>L</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
<td>Alkaline</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
<td>Neutral</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
<td>Neutral</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
<td>Neutral</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
<td>Hydrophobic</td>
</tr>
</tbody>
</table>

Proteins are involved in all cellular functions, can catalyze biological reactions (enzymes), can serve as structural elements (actin, intermedier filaments) or play an active part in
transportation (myosin) and movement (actin-myosin complex), and have a key role in cell signaling and communication between the cell and its environment.

The significance of peptides in molecular biology has been multiplied in the last decades:

a) the so called peptide antibodies can be produced in animals without the need of complex purification of the protein [Du 2001];

b) we are able to identify proteins based on peptide’s mass and sequence by mass spectrometry;

c) in clinical research, inhibitory peptides are used to influence the growth of different types of cancer.

![Image of protein structures](image_url)

**Figure 10.** Structure of proteins.

1) The primary structure of a protein is determined by its amino acid sequence. 2) Secondary structures are defined by patterns of hydrogen bonds between the main-chain peptide groups. Two main types of secondary structure are the alpha helix and the beta strand. 3) Tertiary structure refers to the three dimensional structure of a single protein molecule. 4) Quaternary structure is a larger assembly of several protein molecules or polypeptide chains.
References


BIOTECHNOLOGY IN PHARMACEUTICAL MANUFACTURING

Introduction
The United Nations Convention on Biological Diversity defines biotechnology as: "Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" [United Nations Convention on Biological Diversity]. Although not normally thought of as biotechnology, agriculture clearly fits the broad definition such that the cultivation of plants may be viewed as the earliest biotechnological activity. Modern biotechnology as suggested by the European Association of Pharma Biotechnology (EAPB) refers to those technologies that are necessary to discover, develop and bring new medicinal products to the market, and includes the modification of DNA, hereby the modification of protein. Genetic and tissue engineering, the two basic techniques in medicinal biotechnology, opened new perspectives in medicine, and they are essential in pharmaceutical production, pharmacogenomics, gene therapy and testing for familial disorders.

Figure 11. Generating recombinant DNA

1. A plasmid carrying gene(s) for antibiotic resistance, and a DNA strand, which contains the gene of interest, are cut with the same restriction endonuclease. 2. The opened DNA fragments have complementary "sticky ends" and can be ligated together with DNA ligase. 3. The resulted recombinant DNA is transfected into a bacterial cell, 4. which is than exposed to antibiotics. All the cells except those which have been encoded by the plasmid DNA are killed, leaving a cell culture expressing the desired protein encoded by the recombinant DNA.
Modern biotechnology has started with the discovery of restriction endonucleases which are able to open DNA at specific sites, and an optional nucleotid sequence that can be inserted with the help of DNA-ligase. With this method, DNA can be designed to encode for specific polypeptides or proteins. The resulting recombinant DNA or rDNA can be multiplied (cloned) in a plasmid and used as an expression vector to produce the desired protein. The most often used donor organisms are the bacterium *Escherichia Coli* (*E. coli*) or the yeast *Saccharomyces cerevisiae*, while certain proteins can be expressed only in insect or mammalian cells. The foreign protein is secreted to the culture media or accumulates inside the cell and is then extracted (*Fig. 11*). Developmental steps for a recombinant protein are summarized in *Table 2*.

The first drugs produced by biotechnology were antibiotics: penicillin became commercially available for the indication of human bacterial infections in 1940. The real breakthrough for medicine was the development of recombinant DNA technology: in 1977 Herbert Boyer produced human insulin in the laboratory [Genetech 1977]. In his process, the gene encoding for human insulin was inserted in *E. coli*. This was the first pharmaceutical generated by recombinant DNA technology and approved by the USA Food and Drug Administration (FDA) as Humulin in 1982. Until that time, insulin was extracted from cows and pigs. Although this insulin is highly similar to human insulin, it induces allergic reactions quite often; moreover its source is limited. Insulin made by biotechnology theoretically can be produced in unlimited quantity and it does not cause allergic side effects.

The next milestone in biotechnology was the cloning of tissue plasminogen activator (tPA) and announced also by Genentech Inc. in 1983. It was confirmed that tPA expressed in *E. coli* is suitable for catalyzing conversion of inactive plasminogen to active plasmin, the major enzyme responsible for clot breakdown and can be used to treat embolic or thrombotic stroke [Lapchak 2002]. However, this tPA had low activity and was shortly replaced by recombinant tPA manufactured by Genentech (rtPA, brand name: Activase) [Lapchak 2002]. rtPA exerts its effect more quickly, it is more efficient and has fewer side effects [Robertson 2010]. Since the production of the first rtPA, newer plasminogen activators have been developed by biotechnological methods for more efficient and safer inhibition of blood clotting. For example, the deletion of the first three domens in tPA prolongs plasma half-life in a significant manner; in order to decrease the risk of bleeding, a new, shorter chimeric tPA was designed which has a more efficient fibrin binding capacity [Bavami 2010].
Table 2. Developmental steps for a recombinant protein [Kayser 2004]

<table>
<thead>
<tr>
<th>I. Process development</th>
<th>II. Analytical steps</th>
<th>III. Pre-clinical/clinical test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning of the gene</td>
<td>Defining standars</td>
<td>Preclinical studies (2 mammalian species)</td>
</tr>
<tr>
<td>Construction of the expression vector</td>
<td>Bioassays</td>
<td>Toxicology in rodent species (acute, chronic, subchronic)</td>
</tr>
<tr>
<td>Transfection of the host cell</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
<td>Toxicology in nonrodent species (acute, chronic, subchronic)</td>
</tr>
<tr>
<td>Selection of stable clones</td>
<td>Western blot</td>
<td>Safety pharmacology (cardiovascular, respiratory, renal, gastrointestinal, CNS, depending on drug)</td>
</tr>
<tr>
<td>Optimization of expression, culture media selection</td>
<td>Capillary zone electrophoresis</td>
<td>Pharmacokinetic studies</td>
</tr>
<tr>
<td>Master cell bank</td>
<td>Reverse phase HPLC</td>
<td>Phase I. studies (healthy volunteers):</td>
</tr>
<tr>
<td>Working cell bank</td>
<td>Size-exclusion HPLC</td>
<td>Safety/Tolerance/Pharmacokinetic/Pharmacodynamic</td>
</tr>
<tr>
<td>Characterization and safety of cell bank</td>
<td>Product specific ELISA</td>
<td></td>
</tr>
<tr>
<td>Upstream procedures: fermentation process</td>
<td>Host-cell-protein ELISA</td>
<td></td>
</tr>
<tr>
<td>Downstream procedures: purification process</td>
<td>Residual DNA detection</td>
<td>Phase II. studies (patients): Safety, proof of efficiency, dose finding</td>
</tr>
<tr>
<td>Optimization of individual process steps</td>
<td>N-terminale sequencing</td>
<td></td>
</tr>
<tr>
<td>Stability and robustness of the process</td>
<td>C-terminal amino acid composition</td>
<td></td>
</tr>
<tr>
<td>Introducing of GMP</td>
<td>Peptide mapping</td>
<td>Phase III. studies: Controlled safety and efficacy in specific indications</td>
</tr>
<tr>
<td>Consistency batches</td>
<td>Total amino acid content</td>
<td>Multiple arms, vs. reference therapy or placebo controlled. Typically blinded studies and matched patient groups.</td>
</tr>
<tr>
<td>Validation</td>
<td>Carbohydrate analyses</td>
<td></td>
</tr>
<tr>
<td>Pharmaceutical development (formulation)</td>
<td>Molecular weight spectroscopy</td>
<td></td>
</tr>
<tr>
<td>Development of fill and finish</td>
<td>Detection of free sulfhydryl moieties</td>
<td>Serological assays to quantify drug and antidrug. Neutralizing antibodies</td>
</tr>
<tr>
<td>Stability studies</td>
<td>Circular dichroim spectroscopy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface plasmon resonance</td>
</tr>
</tbody>
</table>
This tPA is expressed in mammalian cells so the secondary structure of the protein is more similar to the original, its clearance is slower, and its anti-thrombotic activity is the same as of normal tPA.

1. Biological response-modifiers (biotherapy, immunotherapy)

Biological response modifiers (BRM) are substances that are able to trigger the immune system to fight against infections or indirectly affect tumors. Many of them can be found naturally in small amounts in the body but with the development of biotechnology BRMs can be produced in the laboratory in larger amounts. The use of BRMs spread quickly and they are essential in the treatment of certain cancers, autoimmune and inflammatory diseases and transplantation medicine. BRMs can act passively, for example, against cancerous cells by boosting the immune system, or they actively affect differentiation and cell division. Although BRMs have several favorable effects, the unwanted side effects have been multiplied because of their direct pharmacological and immunological actions [Murthy 2010]. Most BRMs are endogenous effector peptides or proteins including cytokines, growth factors, certain enzymes, coagulation factors, hormones and monoclonal antibodies.

Examples of biological response modifiers

1.1. Cytokines

Cytokines are small cell-signaling protein molecules responsible for intercellular communication; they harmonize the immune system and activate immune cells. Because of their central role in the immune system, cytokines take part in different immunological, inflammatory and infectious diseases. Virtually all nucleated cells are able to secret cytokines but for example interleukins (IL-1, IL-6) and tumor necrosis factor alfa are produced by endothelial/epithelial cells and macrophages [Boyle 2005]. The most important therapeutic recombinant cytokines are listed in Table 3.

In the treatment of different types of cancer, interferon and IL-2 are often used cytokines. They can directly kill tumor cells, stimulate the immune system and can combat side effects such as chemotherapy-induced thrombocytopenia [Weber 2004]. Interleukin plays a pivotal role in the immune system as it regulates the growth and function of different leukocytes. It can be used in the therapy of cancer, AIDS, or other immunodeficiencies and wound healing.
<table>
<thead>
<tr>
<th><strong>Table 3. Therapeutic recombinant cytokines</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Generic name</strong></td>
</tr>
<tr>
<td>Aldesleukin /IL-2/</td>
</tr>
<tr>
<td>Denileukin diftitox (IL-2 and Diphtheria toxin)</td>
</tr>
<tr>
<td>Interferon alfacon-1</td>
</tr>
<tr>
<td>Interferon alfa-n1</td>
</tr>
<tr>
<td>Interferon alfa-2a</td>
</tr>
<tr>
<td>Interferon alfa-2b</td>
</tr>
<tr>
<td>Interferon alfa-n3</td>
</tr>
<tr>
<td>Interferon beta-1b</td>
</tr>
<tr>
<td>Interferon beta-1a</td>
</tr>
<tr>
<td>Interferon gamma-1b</td>
</tr>
<tr>
<td>Oprelvekin (IL-11)</td>
</tr>
</tbody>
</table>

1.2. Growth factors

Growth factors regulate cell growth, proliferation, and differentiation. Epidermal Growth Factor (EGF) as a drug can help wound healing [Fernandez 2009], Insulin-like Growth Factor (IGF) stimulates tissue growth and it can be used in the therapy of growth deficiencies [Wyatt 2011]. Platelet-Derived Growth Factor (PDGF) helps building collagen during tissue regeneration [Rosen 2006], while Colony Stimulating Factor (CSF) – as a hormone of the immune system – regulates differentiation, growth and activity of white blood cells and can be used in immunodeficiencies. Hematological growth factors in the market are listed in Table 4 [Rogers 2004].
Table 4. Hematological growth factors in the market

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Effect</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Becaplermin</td>
<td>Regranex</td>
<td>Inhibition of platelets</td>
<td>Diabetic foot ulcers</td>
</tr>
<tr>
<td>(PDGF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darbepoetin alfa</td>
<td>Aranesp</td>
<td>Stimulates erythropoiesis</td>
<td>Chronic renal failure, chemotherapy-induced anemia</td>
</tr>
<tr>
<td>Epoetin alfa</td>
<td>Epogen,</td>
<td>Stimulates erythropoiesis</td>
<td>Chronic renal failure, chemotherapy-induced anemia</td>
</tr>
<tr>
<td></td>
<td>Procrit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filgrastim</td>
<td>Neupogen</td>
<td>Stimulates granulocyte proliferation and</td>
<td>Severe chronic neutropenia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>differentiation</td>
<td>(chemotherapy, bone marrow transplantation)</td>
</tr>
<tr>
<td>Pegfilgrastim</td>
<td>Neulasta</td>
<td>Pegilated form of Filgrastim, granulocyte</td>
<td>Chemotherapy-related neutropenia and infections</td>
</tr>
<tr>
<td></td>
<td></td>
<td>colony stimulating analogue</td>
<td></td>
</tr>
<tr>
<td>Sargramostim</td>
<td>Leukine</td>
<td>Granulocyte-macrophage colony stimulating</td>
<td>Myeloid reconstitution after autologous or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>factor, stimulates the immune system</td>
<td>allogeneic bone marrow transplantation, acute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>myeloid leukemia-induced neutropenia (clinical phase:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crohn's disease, autoimmune pulmonary alveolar proteinosis)</td>
</tr>
</tbody>
</table>

1.3. Enzymes

Severe combined immunodeficiency (SCID) is the most serious hereditary immunodeficiency in which both B-cells and T-cells are impaired due to a defect in one of several possible genes. One autosomal recessive form of SCID (X-SCID) is caused by a defective enzyme, Adenosine DeAminase (ADA), necessary for the breakdown of purines. Lack of ADA causes accumulation of dATP. This metabolite will inhibit the activity of ribonucleotide reductase, the enzyme that reduces ribonucleotides to generate deoxyribonucleotides. The effectiveness of the immune system depends upon lymphocyte proliferation and hence dNTP synthesis. Without functional ribonucleotide reductase, lymphocyte proliferation is inhibited and the immune system is compromised [Online Medelian inheritance]. ADA-deficiency in children can be treated by the delivery of the enzyme as a drug [Hersfield 1998].

The most diverse forms of chemical catalysts are produced by recombinant DNA technology; some of them are presented in Table 5.
Table 5. Recombinant enzymes used as drugs

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Effect</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alteplase</td>
<td>Activase</td>
<td>Plasminogen activator</td>
<td>Acute myocardial infarction, stroke, pulmonary embolism</td>
</tr>
<tr>
<td>Bivalirudin</td>
<td>Angiomax</td>
<td>Direct thrombin inhibitor</td>
<td>Instable angina</td>
</tr>
<tr>
<td>Dornase alfa</td>
<td>Pulmozyme</td>
<td>Hydrolyzes the DNA present in sputum/mucus of patients with cystic fibrosis and reduces viscosity in the lungs</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>Eptifibatide</td>
<td>Integrelin</td>
<td>Inhibition of platelets</td>
<td>Acute coronary syndromes</td>
</tr>
<tr>
<td>Imiglucerase</td>
<td>Carezyme</td>
<td>Analogue of human β-glucocerebrosidase</td>
<td>Type I Gaucher disease</td>
</tr>
<tr>
<td>Lepirudin</td>
<td>Refludan</td>
<td>Direct thrombin inhibitor</td>
<td>Instable angina</td>
</tr>
<tr>
<td>Reteplase</td>
<td>Retavase</td>
<td>Thrombolytic drug</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>Tenecteplase</td>
<td>TNKase</td>
<td>Thrombolytic drug</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>Tirobifan</td>
<td>Aggrastat</td>
<td>Inhibition of platelets</td>
<td>Acute coronary syndromes</td>
</tr>
</tbody>
</table>

1.4. Blood coagulation factors

Blood coagulation is a complex chemical process including several coagulation factors. Lack or deficiency of any of the coagulation cascade leads to severe anomaly. Hemophilia A is caused by a reduction in the amount or activity of factor VIII. People living with hemophilia require regular transfusions of clotting factors in order to maintain a normal blood clotting system. However, prior to 1992, there was no screening tool available to guarantee that donated blood products were HIV-free. Therefore, those hemophilia patients receiving untested and unscreened clotting factor prior to 1992 were at an extreme risk for contracting HIV via the very blood products that were saving their lives. Over 10,000 people living with hemophilia contracted HIV from the tainted blood supply [US Department of Health and Human Services]. Today not only recombinant factor VIII but IX and VII are also available for clinical use, thus making the treatment of Hemophilia B (factor IX) and factor VII deficiency safer [Pipe 2008, Table 6].
Table 6. Blood coagulation factors produced by recombinant DNA technology

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VII</td>
<td>NovoSeven</td>
<td>Hemophilia</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Bioclate, Recombinate, Kogenate, Helixate, ReFacto</td>
<td>Hemophilia A</td>
</tr>
<tr>
<td>Factor IX</td>
<td>BeneFIX</td>
<td>Hemophilia B</td>
</tr>
</tbody>
</table>

1.5 Hormones

The use of hormones produced by recombinant DNA technology is not a rare event in everyday medical practice (Table 7). Before the widespread utilization of biotechnology, Human Growth Hormone (hGH, somatotropin) was extracted from the pituitary gland of human cadavers, and used for the treatment of childhood growth disorders such as dwarfism. In some cases, this hormone from cadavers contained protein-associated infectious particles and caused Kreutzfeld-Jacob disease which is an incurable degenerative neurological disorder and invariably fatal. Today clean, recombinant hGH is available and has replaced the old, limited form which raised ethical issues as well. As hGH can strengthen bones and muscles, it is suitable for the treatment of severely underweight patients or those who suffer from certain nutrition disorders.

Before the production of recombinant human insulin, insulin was extracted from the pancreas of cows and pigs and often caused allergic reactions. Recombinant human insulin can be produced reliably, in large amounts without the risk of allergic side effects. Thanks to biotechnology, human insulin can be generated even in plants (Carthamus tinctorius, safflower) [From SemBiosys].

Assisted reproduction and in vitro fertilization would be out of the question without hormones produced by recombinant DNA technology.
Table 7. Some hormones used in the clinic and produced by recombinant DNA technology

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Effect</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choriogonadotropin alfa</td>
<td>Ovidrel</td>
<td>Interacts with the luteinizing hormone/choriogonadotropin receptor and promotes the maintenance of the corpus luteum during the beginning of pregnancy, causing it to secrete progesterone, so that it can sustain the growing fetus</td>
<td>Infertility</td>
</tr>
<tr>
<td>Follitropin alfa</td>
<td>Follistim, Puregon</td>
<td>Stimulates ovarian follicular growth</td>
<td>Ovulation problems</td>
</tr>
<tr>
<td>Follitropin beta</td>
<td>Gonal-F</td>
<td>Stimulates ovarian follicular growth</td>
<td>Ovulation problems</td>
</tr>
<tr>
<td>Human insulin</td>
<td>Humulin, Humalog, Novolin, Lantus</td>
<td>Insulin binds to its receptors and opens holes on the cell membrane for the glucose to enter into the cell</td>
<td>Insulin-dependent (type I) diabetes mellitus</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>Protopin, Serostim, Humatrope, Genotropin</td>
<td>Stimulates growth, reproduction and regeneration of cells</td>
<td>Childhood and adult growth hormone deficiency, Turner syndrome, AIDS wasting syndrome</td>
</tr>
<tr>
<td>Ganirelix</td>
<td>Antagon</td>
<td>Gonadotropin-releasing hormone antagonist, regulation of ovulation in assisted reproduction</td>
<td>Compensates for luteinizing hormone deficiency in the therapy of infertility</td>
</tr>
<tr>
<td>Glucagon</td>
<td>GlucaGen</td>
<td>Causes the liver to convert stored glycogen into glucose, increases blood glucose level</td>
<td>Hypoglycemia</td>
</tr>
<tr>
<td>Growth hormone releasing hormone</td>
<td>Geref</td>
<td>Triggers the release of stored growth hormone</td>
<td>Childhood growth hormone deficiency</td>
</tr>
<tr>
<td>Tireotropin</td>
<td>Thyrogen</td>
<td>Thyroid stimulating hormone</td>
<td>Thyroid cancer</td>
</tr>
</tbody>
</table>

2. Potential side effects of therapies with recombinant biological response-modifiers

Interferon

The most frequent adverse effects are flu-like symptoms: increased body temperature, feeling ill, headache, muscle pain, fatigue, dizziness, convulsion, hair thinning, and depression. Erythema, pain and hardness on the spot of injection are also frequently observed. IFN
therapy causes immunosuppression, in particular through neutropenia and can result in some infections manifesting in unusual ways [Bhatti 2007].

*Interleukin*

IL-2 can cause mood changes including irritability, confusion, insomnia or depression. Recombinant human IL-3 had stimulatory hematopoietic effects, flulike symptoms and fever. IL- 4 causes gastric ulceration, diarrhea, headache with nasal congestion, fluid retention, and arthralgia, in addition to constitutional symptoms such as anorexia, fatigue nausea, and vomiting.

*Erythropoietin*

Erythropoietin is generally well tolerated. Side effects can include chest pain, swelling due to retention of fluid, headache, fast heart beat, high blood pressure, increase in number and concentration of circulating red blood cells, shortness of breath, seizures, skin rash, pain in joints, diarrhea, nausea, fatigue, or flu-like syndrome after each dose.

*Tumor necrosis factor*

Tumor necrosis alpha may produce side effects such as malignancy, injection site reactions, infusion reactions, heart failure, demyelinating disease, infections, induction of autoimmunity.

*Colony stimulating factor*

The most common side effects while taking sargramostim are mild to moderate fever, weakness, headache, chills, nausea, diarrhea, and bone and muscle pain. Side effects less often seen are leg and arm swelling, shortness of breath, or a mild rash at the site of injection. Filgrastim is well-tolerated. The most common side effect in cancer patients receiving chemotherapy and patients with severe, chronic neutropenia due to cyclical chemotherapy, a birth defect or an unknown cause, is mild to moderate bone pain. Patients receiving filgrastim after bone marrow transplantation commonly experience nausea and vomiting. Patients preparing to donate bone marrow who receive filgrastim commonly experience muscle and bone pain.
3. Gene therapy

Recombinant DNA technology made it possible so that now we can think about the therapy of those diseases which could be treated only by directly modifying the genetic material. Although it was proposed in the 70s that exogenous "good" DNA could be used to replace the defective DNA in those who suffer from genetic defects, significant advance in gene therapy has been achieved only in the 90s, and approved drugs made by gene therapy is still not available.

The aim of gene therapy is to insert, alter, or remove genes within an individual's cells and biological tissues to treat disease. It is a technique for correcting defective genes that are responsible for disease development. The most common form of gene therapy involves the insertion of functional genes into an unspecified genomic location in order to replace a mutated gene; other forms involve directly correcting the mutation or modifying normal gene that enables for example a viral infection. Theoretically, it is suitable to correct genetic disorders, metabolic syndromes or neurological defects, kill cancer cells, modify the immune response or for immunization against pathogens. Target for gene therapy could be any of the body’s cells (somatic gene therapy) or germ line cells. In case of germ line gene therapy, germ cells are modified by the introduction of functional genes, which are integrated into their genomes. Therefore, the change due to therapy would be heritable and would be passed on to later generations. With germ line gene therapy, not only genetic diseases could be treated but also several serious ethical problems arise, thus at least for now somatic gene therapy remains as an option. Most of somatic gene therapy procedures manipulate cells \textit{ex vivo}: they take out cells from the patient’s body, treat them in sterile environment, than put them back. Another possibility is to introduce the gene-containing material directly \textit{in vivo} into the tissues to be treated, for example heart or brain.

\textit{Mutant genes can be corrected in different ways:}

a) functional gene is inserted into a non-specific place of the genome;

b) change the mutant gene with homologous recombination;

c) selective reverse mutation;

d) modification of the regulation of defective gene (enhancing or silencing).

The latest method includes antisense RNA and RNA interference techniques and will be discussed in a following chapter.
Today we know nearly 3000 disorders that are caused by a mutation in a single gene (for example hemophilia and muscular dystrophy). Gene therapy studies are in an advanced stage in the therapy of cardiovascular diseases, inherited blindness, diabetic retinopathy, macular degeneration; most recently successful gene therapy of HIV-infection has been published [Lalezari 2011].

3.1. Technical problems related to gene therapy

Short “half life” - DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. Problems with the insertion of therapeutic DNA into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving any long-term benefits and patients will have to undergo multiple rounds of gene therapy.

Immune response - Anytime a foreign object is introduced into human body, the immune system attacks the invader. The risk of stimulating the immune system in a way that reduces the effectiveness of gene therapy is always a potential risk. Furthermore, the immune system's enhanced response to invaders, it has seen before, makes it difficult for gene therapy to be repeated in the same patient.

Viral vectors – Viruses are the carrier of choice in most gene therapy studies but present a variety of potential problems to the patient: toxicity, immune and inflammatory responses, and gene control and targeting issues. In addition, there is always the fear that the viral vector, once inside the patient, may recover its ability to cause disease.

Multigene disorders - Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Many of the most commonly occurring disorders, such as heart disease, high blood pressure, arthritis, Alzheimer's disease and diabetes, are caused by the combined effects of variations in many genes. Such multifactorial disorders would be especially difficult to treat effectively using gene therapy.

3.2. Human vaccines made by recombinant DNA technology

Using rDNA technologies, a disease causing agent can be isolated, reduced to its basic components, its genetic makeup can be studied, and the agent can be modified so that it no longer causes disease but still induces a strong immune response. Vaccine development using rDNA technologies requires a thorough understanding of the disease agent, particularly the
antigens critical for inducing protection. In addition, it is important to understand the pathogenicity of the invader and the immune response of the host, to ensure that the vaccine induces the appropriate immunological reaction. Recombinant vaccines are designed to be safer, more efficient and effective and/or less expensive than traditional ones.

Recombinant vaccines fall into three basic categories: live genetically modified organisms, recombinant inactivated vaccines, and genetic vaccines [Ellis 1999].

The first recombinant vaccine was developed against Hepatitis B virus (HVB). The vaccine contains one of the viral envelope proteins, hepatitis B surface antigen (HBsAg). Recombinant HBV vaccines are safer than the attenuated type which through mutations can cause hepatitis or hepatic cancer. HBV vaccine is available in combination with Haemophilus influenzae vaccine (Comvax).

The first vaccine against cancer was approved in 2006 under the name Gardasil and it is used in the prevention of Human papillomavirus (HPV) types 6, 11, 16 and 18 [Lowy 2006]. HPV types 16 and 18 cause an estimated 70% of cervical cancers, and are responsible for many cancer cases of the genital tract; HPV types 6 and 11 cause an estimated 90% of genital warts cases. It does not treat existing infection, therefore, to be effective it must be given before HPV infection occurs. Gardasil cannot be added to patients on immunosuppressive therapy, during treatment with alkylating, cytotoxic or corticosteroid agents, or in immune deficiency. It is contraindicated in blood clotting disorders because it raises the risk of bleeding and hematoma formation.

**Table 8.** Recombinant vaccines on the market

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Immune response</th>
<th>Indication</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemophilus B/ Hepatitis B vaccine</td>
<td>Comvax</td>
<td>Haemophilus influenzae type b capsular polysaccharide covalently bound to an outer membrane protein complex of Neisseria meningitidis and Hepatitis B surface antigen (HBsAg)</td>
<td>Prevention of H. influenzae and Hepatitis B infections</td>
<td>Irritability, drowsiness, fever, loss of appetite, local reactions at the injection site</td>
</tr>
<tr>
<td>Hepatitis B vaccine</td>
<td>Engerix-B, Reombivax HB</td>
<td>Hepatitis B surface antigen (HBsAg)</td>
<td>Prevention of Hepatitis B infection</td>
<td>Irritability, local reactions at the injection site, fatigue</td>
</tr>
<tr>
<td>Human papillomavirus vaccine</td>
<td>Gardasil</td>
<td>Recombinant virus-like particles assembled from capsid proteins of HPV 6,11,16,18, it does not contain viral DNA</td>
<td>Prevention of HPV types 6, 11, 16 and 18</td>
<td>Local reactions at the injection site, fever</td>
</tr>
</tbody>
</table>


Clinical trials are ongoing with recombinant influenza vaccine but it is not available on the market. Recombinant influenza vaccines consist solely of hemagglutinin proteins produced by cell culture.

In the future, the development of recombinant vaccines will focus on bioavailability, increased efficiency and safety, and delivery methods will be made easier (oral administration). Trials are ongoing to develop vaccines against non-infectious diseases, first of all in autoimmune disorders and transplantation.

4. Monoclonal antibodies

The adaptive immune system is composed of highly specialized cells that provide the vertebrate immune system with the ability to recognize and remember specific pathogens and generate immunity, and to mount stronger attacks each time the pathogen is encountered. Most important elements of the adaptive immune system are lymphocytes (T and B-cells), but dendritic cells, antibodies and the complement system play also a key role in the immune response. B-cells respond to antigens presented by pathogens by producing large quantities of antibodies (immunoglobulin) which then neutralize foreign objects like bacteria and viruses. B-cells perform the role of antigen-presenting cells and eventually develop into memory B-cells (plasma cells) [Falus 1993]. Thus the main function of the proteins produced by plasma cells is the specific recognition of antigen bearing foreign particles (Fig. 12). Monoclonal antibodies (mAb) are produced by th1 descendants (clones) of a certain B-lymphocyte in a huge amount and are highly specific to a given epitope. Antibodies help immune cells to incorporate antigens, neutralize toxins produced by bacteria and can directly attack pathogens. Antibodies activate the complement-system and are indispensable in eliminating certain bacterial infections. While polyclonal antibodies (antisera) are produced by different B-cells and are specific to different epitopes of the same pathogen, monoclonal antibodies are monospecific and made by the clones of a single immune cell [Falus 1993]. In the clinic, monoclonal antibodies can be used. Besides therapy, antibodies can be used for highly specific detection of proteins, are important tools in diagnostics and are ultrasensitive analytical reagents [Hagemeyer 2009].
The variable region of the antibody gives the antibody its specificity for binding antigen, and includes the ends of the light and heavy chains. The constant region determines the mechanism used to destroy antigens. Based on their constant region structure and immune function, antibodies are divided into five major classes: IgM, IgG, IgA, IgD, and IgE.

Therapies with monoclonal antibodies are actually a kind of passive immunotherapy. This means that antibodies made in a lab (foreign antibodies) and introduced into the body of the patient will fight against the invaders. By introducing the antibodies, they will stimulate other elements of the immune system and boost immune response.

The concept of drug targeting was created by Paul Ehrlich more than 100 years ago. He conceived the idea of a molecule with specific affinity for a certain organ and which could be linked to a therapeutically active group and then selectively delivered to the organ in question, thus eradicating the disease. The vision of a “magic bullet” was thereby born. However, there was no concept of how to create “magic bullets” and they remained unexplored, therefore, for many decades. Based on the “magic bullet” theory, radiopharmaceuticals, immunocytokines, immunotoxins etc. can be attached to cancer specific monoclonal antibodies, and thus cancer cells can be killed specifically without destroying normal tissues (Fig. 13) [Zhang 2007]. Many laboratories study monoclonal antibody treatment for example in rheumatoid arthritis, multiple sclerosis and cancer. The first FDA approved monoclonal antibody was a murine IgG2a CD3 specific transplant rejection drug, OKT3 (also called muromonab), in 1986. Since then, several monoclonal antibodies have been approved (see Table 9.), and hundreds of therapies are undergoing clinical trials.
4.1. Production of monoclonal antibodies

In the beginning, monoclonal antibodies were produced by hybridoma technology. Hybridomas are able to produce specific antibodies of the same quality for a long time *in vitro* as cell culture or *in vivo* in animal models. Hybridomas are hybrid cell lines made by fusing a specific antibody-producing B-cell with a myeloma cell that is selected for its ability to grow theoretically indefinitely in tissue culture and for an absence of antibody chain synthesis. The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal. The product of hybridomas is suitable for the study of proteins’ presence and quantity, and for the delivery of active substances to specific targets [Roger 2006]. Today hybridomas have been mainly replaced by recombinant DNA technology, transgenic mice, and phage display libraries. Based on the production method, four main types of monoclonal antibodies exist: expressed in rodents, chimeric, humanized and human.

*Monoclonal antibodies produced in rodents (-omab)*

Initially, murine antibodies were obtained by hybridoma technology. However, the dissimilarity between murine and human immune systems led to the clinical failure of these antibodies, except in a few cases. A major problem associated with murine antibodies is the formation of complexes after repeated administration, which results in mild allergic reactions or even anaphylactic shock.

*Chimeric and humanized antibodies (-ximab, -zumab)*

In order to reduce immunogenicity, monoclonal antibodies must be “humanized”. With recombinant DNA technology, murine molecules can be engineered to remove immunogenic content and to increase their immunologic efficiency. This was initially achieved by the production of chimeric and humanized antibodies. Chimeric antibodies are built up of murine variable regions fused onto human constant regions. Gene sequences from the kappa light chain and the IgG1 heavy chain, results in antibodies that are approximately 65% human. This reduces immunogenicity, and increases serum half-life. Humanized antibodies are produced by grafting murine hypervariable amino acid domains into human antibodies. This results in a molecule of approximately 95% human origin and can produce antibodies in cell culture [Boado 2007].

*Human monoclonal antibodies (-umab)*

Human monoclonal antibodies are produced in transgenic mice or phage display libraries. By cloning human immunoglobulin genes into a rodent then vaccinating the animal against the
desired antigen, the animal immune system will produce human monoclonal antibodies [Hudson 2003].

4.2. Conditions potentially treatable by monoclonal antibodies

Autoimmune diseases

Monoclonal antibodies are used in different fields of immunology:

1) anti-TNF α antibodies as adalimumab and infliximab are used in rheumatoid arthritis, Crohn’s disease and ulcerative colitis [Rang 2003];

2) daclizumab and basilixumab inhibits IL-2 production of activated T-cells thus are suitable to prevent rejection of organ transplants;

3) omalizumab, an anti human immunoglobulin E (IgE) can help in the treatment of moderate to severe allergic asthma.

Cancer

Anti-cancer monoclonal antibodies can exert their effects through several mechanisms (Fig. 13):

1. In radioimmunotherapy, antibodies against a cell surface marker of cancerous cells are conjugated to radioactive isotopes (e.g. tositumomab in the treatment of radio-sensitive lymphomas). Because this type of monoclonal antibodies is produced in rodents, their immunogenicity is higher but promotes clearance from the body.

2. In antibody-directed enzyme prodrug therapy, monoclonal antibodies are attached to drug-activating non-toxic enzymes. This enzyme will transform a prodrug into a cytotoxic drug only in the targeted cells. The clinical success of this approach has been limited till now but holds great promise in future oncological treatments [Phrancis 2002].

3. Drug molecules or therapeutic agents can be linked to immunoliposomes and targeted directly against malignant cells. Tissue-specific gene delivery using immunoliposomes has been achieved in brain, and breast cancer tissue [Krauss 2000] but it has not been used routinely in the clinic, yet.
Figure 13. Antibody-based cancer therapy.
1. Naked monoclonal antibody 2. Monoclonal antibody conjugated to radionuclide, cytokine, toxin or liposome 3. Complex monoclonal antibody conjugated to biotinylated radioactive ligand through streptavidin [Carter 2001].
<table>
<thead>
<tr>
<th>Generic name</th>
<th>Brand name</th>
<th>Year of first approval</th>
<th>Source</th>
<th>Effect</th>
<th>Indication</th>
<th>Rout of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muromonab CD3</td>
<td>Orthoclone OKT</td>
<td>1986</td>
<td>Mouse</td>
<td>Targeted at the CD3 membrane protein on the surface of T cells</td>
<td>Acute, glucocorticoid resistant rejection of allogeneic renal, heart and liver transplants</td>
<td>Intravenous injection</td>
</tr>
<tr>
<td>Abciximab</td>
<td>ReoPro</td>
<td>1994</td>
<td>Chimeric</td>
<td>Glycoprotein IIb/IIIa receptor antagonist, platelet aggregation inhibitor</td>
<td>During and after high risk coronary artery procedures</td>
<td>Intravenous injection</td>
</tr>
<tr>
<td>Rituximab</td>
<td>Rituxan</td>
<td>1997</td>
<td>Chimeric</td>
<td>Against CD20 on the surface of B-cells</td>
<td>B-cell lymphomas and leukemias, transplant rejection</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>Daclizumab</td>
<td>Zenapax</td>
<td>1997 (European marketing authorization withdrawn in 2009)</td>
<td>Humanized</td>
<td>Against the alpha subunit of the IL-2 receptor (CD25) of T cells</td>
<td>Rejection in organ transplantation</td>
<td>Intravenous injection</td>
</tr>
<tr>
<td>Basilixumab</td>
<td>Simulect</td>
<td>1998</td>
<td>Chimeric</td>
<td>Against CD25</td>
<td>Rejection in organ transplantation</td>
<td>Intravenous injectionor infusion</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Remicade</td>
<td>1998</td>
<td>Chimeric</td>
<td>Against TNF-α</td>
<td>Psoriasis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, ulcerative colitis</td>
<td>Intravenous injection</td>
</tr>
<tr>
<td>Palivizumab</td>
<td>Synagis</td>
<td>1998</td>
<td>Humanized</td>
<td>Directed against an epitope in the A antigenic site of the F protein of RSV</td>
<td>Respiratory syncytial virus infection</td>
<td>Intramuscular injection</td>
</tr>
<tr>
<td>Drug Name</td>
<td>Trade Name</td>
<td>Year</td>
<td>Type</td>
<td>Mechanism</td>
<td>Indications</td>
<td>Route of Administration</td>
</tr>
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<td>---------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Gemtuzumab ozogamicin</td>
<td>Mylotarg</td>
<td>2000 (withdrawn in 2010)</td>
<td>Humanized</td>
<td>Anti-CD33 linked to a cytotoxic agent from the class of calicheamicins</td>
<td>Acute myelogenous leukemia</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>Campath-1H</td>
<td>2001</td>
<td>Humanized</td>
<td>Against cell surface protein CD52 on matured lymphocytes</td>
<td>Chronic lymphocytic leukemia, cutaneous T-cell lymphoma, T-cell lymphoma; bone marrow and renal transplantation</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>Humira</td>
<td>2002</td>
<td>Human</td>
<td>TNF-α inhibitor</td>
<td>Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, moderate to severe chronic psoriasis, juvenile idiopathic arthritis</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>Ibritumomab tiuxetan</td>
<td>Zevalin</td>
<td>2002</td>
<td>Mouse</td>
<td>B-lymphocyte specific anti-CD20 antibody conjugated to a radioactive isotope</td>
<td>B-cell non-Hodgkin lymphoma, myeloproliferative diseases</td>
<td>Intravenous injection</td>
</tr>
<tr>
<td>Omalizumab</td>
<td>Xolair</td>
<td>2003</td>
<td>Humanized</td>
<td>Selectively binds to human immunoglobulin E</td>
<td>Allergic asthma</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>Tositumomab, Tositumomab I131</td>
<td>Bexxar</td>
<td>2003</td>
<td>Mouse</td>
<td>Anti-CD20; I131 same antibody covalently bound to the radionuclide iodine-131</td>
<td>Follicular lymphoma</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>Efalizumab</td>
<td>Raptiva</td>
<td>2003 (withdrawn in 2009)</td>
<td>Humanized</td>
<td>CD11a subunit of lymphocyte function-associated antigen 1, immunosuppressant</td>
<td>Psoriasis</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Erbitux</td>
<td>2004</td>
<td>Chimera</td>
<td>EGFR inhibitor</td>
<td>Colorectal cancer, head and neck cancer</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>Drug</td>
<td>Brand Name</td>
<td>Year</td>
<td>Monoclonal</td>
<td>Target</td>
<td>Indication</td>
<td>Route</td>
</tr>
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</tr>
<tr>
<td>Natalizumab</td>
<td>Tsabri</td>
<td>2004</td>
<td>Humanized</td>
<td>Against cellular adhesion molecule α4-integrin</td>
<td>Sclerosis multiplex</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(withdrawn for breast cancer indication in 2010)</td>
<td></td>
</tr>
<tr>
<td>Ranibizumab</td>
<td>Lucentis</td>
<td>2006</td>
<td>Humanized</td>
<td>Binds to VEGF-A, anti-angiogenic</td>
<td>Macular degeneration</td>
<td>Intravitreal injection</td>
</tr>
<tr>
<td>Eculizumab</td>
<td>Soliris</td>
<td>2007</td>
<td>Humanized</td>
<td>Directed against complement protein C5</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>Certolizumabpegol</td>
<td>Cimzia</td>
<td>2008</td>
<td>Humanized</td>
<td>Anti-TNF-α</td>
<td>Chron's disease, rheumatoid arthritis</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>Golimumab</td>
<td>Simponi</td>
<td>2009</td>
<td>Human</td>
<td>Anti-TNF-α</td>
<td>Rheumatoid and psoriatic arthritis, active spondylitis ankylopoetica</td>
<td>Subcutaneous injection</td>
</tr>
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MONOCLONAL ANTIBODIES AND TARGETED THERAPY

Introduction

Targeted therapy is the result of about 100 years of research dedicated to understanding the differences between cancer cells and normal cells. To date, cancer treatment has focused primarily on killing rapidly dividing cells because one feature of cancer cells is that they divide rapidly. Unfortunately, some of our normal cells divide rapidly too (such as mucosal and hair cells), causing multiple side effects.

Targeted therapy is about identifying other features of cancer cells. Scientists look for specific differences in the cancer cells and the normal cells. This information is used to create a targeted therapy to attack the cancer cells without damaging the normal cells, thus leading to fewer side effects. Each type of targeted therapy works a little bit differently but all interfere with the ability of the cancer cell to grow, divide, repair and/or communicate with other cells.

There are different types of targeted therapies, defined in three broad categories. Some targeted therapies focus on the internal components and function of the cancer cell. These use small molecules that can get into the cell and disrupt the function of the cells, causing them to die. There are several types of targeted therapy that focus on the inner parts of the cells. The second variety target receptors that are on the outside or surface of the cell. This form of targeted treatment includes the monoclonal antibodies. Finally, antiangiogenesis inhibitors target the blood vessels that supply oxygen to the cancer cells, ultimately causing the cells to starve and die.

Researchers agree that targeted therapies are not a replacement for traditional therapies, but may best be used in combination. More research is needed to identify which cancers may be best treated in this way and to identify additional targets for more types of cancer.

Great progress in targeted cancer therapy was made with the discovery of receptors and the development of monoclonal antibodies (MAbs). As we mentioned, several MAbs are now approved for clinical use and are very effective against appropriate types of cancer.

These include Cetuximab® (erbitux), a derivative of the monoclonal antibody MAb 225, which binds to the epidermal growth factor(EGF) receptor and inhibits metastatic colorectal cancer, Herceptin® (trastuzumab) a humanized monoclonal antibody against EGF-R2 (HER-2) which is very successfully used against metastatic breast cancers overexpressing
HER-2, and Avastin® (bevacizumab), a recombinant monoclonal antibody targeting vascular endothelial growth factor (VEGF). Many cancers are resistant to treatment with monoclonal antibodies alone, but can be killed when a cytotoxic agent is attached to that antibody. Immunoconjugates of this kind are made by linking chemotherapeutic drugs, radioisotopes, enzymes or toxins to the antibody. Several recombinant immunotoxins exist, composed of antibody fragments fused to powerful bacterial toxins. In addition, various low molecular weight inhibitors of EGF-R tyrosine kinase, exemplified by gefitinib (Iressa) and erlotonib (Tarceva), have been developed, and can be used orally. However, tyrosine kinase inhibitors of this type are targeted only passively and not actively, as they are not attached to a carrier that binds to a specific target.

1. Monoclonal antibodies and tyrosine kinase inhibitors as targeted drugs

Trastuzumab (Herceptin) is a monoclonal antibody that interferes with the HER2/neu receptor (EGF receptors). The HER receptors are proteins that are embedded in the cell membrane and communicate molecular signals from outside the cell to inside the cell, and turn genes on and off. The HER proteins regulate cell growth, survival, adhesion, migration, and differentiation—functions that are amplified or weakened in cancer cells. In some cancers, notably some breast cancers, HER2 is over-expressed, and, among other effects, causes breast cells to reproduce uncontrollably. Trastuzumab is an antibody that binds selectively to the HER2 protein. When it binds to defective HER2 proteins, the HER2 protein no longer causes cells in the breast to reproduce uncontrollably. However, cancers usually develop resistance to trastuzumab. Trastuzumab reverses the effects of an overactive HER2 receptor. If the breast cancer doesn't have overactive HER2 receptors, trastuzumab will have no beneficial effect. Herceptin is given by infusion.

Erlotinib (Tarceva) is an epidermal growth factor receptor (EGFR) inhibitor. This drug follows gefitinib (Iressa), which was the first drug of this type. Erlotinib specifically targets the EGFR tyrosine kinase, which is highly expressed and occasionally mutated in various forms of cancer. It binds in a reversible fashion to the adenosine triphosphate (ATP) binding site of the receptor. For the signal to be transmitted, two members of the EGFR family need to come together to form a homodimer. These then use the molecule of ATP to autophosphorylate each other, which causes a conformational change in their intracellular structure, exposing a further binding site for binding proteins that cause a signal cascade to
the nucleus. By inhibiting the ATP, autophosphorylation is not possible and the signal is stopped. Tarceva is used to treat non-small cell lung cancer, pancreatic cancer and several other types of cancer. Tarceva is given in tablet form to be taken by mouth at least one hour before or two hours after eating.

**Bevacizumab (Avastin)** is a drug that blocks angiogenesis, the growth of new blood vessels. It stops tumor growth by preventing the formation of new blood vessels. Bevacizumab is a humanized monoclonal antibody that inhibits the function of a natural protein called vascular endothelial growth factor A (VEGF-A). VEGF-A is a chemical signal that stimulates angiogenesis in a variety of diseases, especially in cancer, and retinal proliferation of diabetes in the eye. VEGF is a cytokine (a small protein released by cells that have specific effects on the behavior of cells) which when it interacts with its receptors in the cell leads to new blood vessel formation or angiogenesis. Bevacizumab was the first clinically available angiogenesis inhibitor. Bevacizumab is produced in a mammalian cell (Chinese Hamster Ovary) expression system in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product. Bevacizumab is used to treat various cancers, including colorectal, lung, and kidney cancer, and eye disease. In certain renal (kidney) cancers, Bevacizumab improves the progression free survival time but not survival time. Bevacizumab is usually given intravenously every 14 days. In colon cancer, it is given in combination with the chemotherapy drugs. Bevacizumab is one of the most expensive drugs widely marketed.

**2. Rationale for the Concept of Delivery to Peptide Receptors**

This short chapter focuses on the concepts, descriptions and experimental and clinical evaluation of chemotherapeutic compounds linked to various hormonal peptides, developed for targeting (homing) to tumors expressing receptors for these peptides. Thus one of the many possible approaches to targeted cancer therapy is based on the finding that receptors for certain peptide hormones such as somatostatin, bombesin, growth hormone-releasing hormone (GHRH) and LHRH1 [now also known in genome and microarray databases as GnRH-1] are expressed on various tumors in higher concentrations than on most corresponding normal cells. Consequently, these peptide hormones and their analogs can be used as carrier vectors to deliver cytotoxic agents such as anthracycline derivatives directly to malignant cells. Various radionuclides also can be conjugated to the analogs of these diverse hormonal peptides.
These hybrid peptides conjugated to a cytotoxic agent bind to the receptors on the cell surface, are internalized by receptor-mediated endocytosis, and upon delivery to the cytoplasm, mediate cell death. The presence of peptide receptors on various tumors, can serve as a target for peptide ligands, and permit the implementation of the targeted therapy proposed by Paul Ehrlich more than 100 years ago. These specific approaches will be reviewed shortly below.

Most endocrine (hormonal) approaches to target cytotoxic agents and radionuclides to tumors are based on peptides. Antibodies are subject to nonspecific uptake by the liver and reticuloendothelial system whereas peptides are not. Liver and bone marrow toxicities are thus also dose limiting factors. Peptides are easier to design and synthesize and are much smaller than monoclonal antibodies, which because of their size may not reach the interior of large tumors. Thus peptides may be superior to monoclonal antibodies as targeting agents.

Targeted chemotherapy, which can be homed directly to tumor cells, represents a modern oncological strategy designed to improve the effectiveness of cytotoxic drugs while decreasing toxicity. This targeted approach increases the concentration of the drugs in tumor tissue and spares normal, non-cancerous cells from unnecessary exposure to the toxic effects of systemic chemotherapy. It is well documented that chemotherapeutic agents produce toxic effects. Various chemotherapeutic agents can cause myelosuppression and can also have gastrointestinal, cardiac, pulmonary, hepatic, renal, neurologic and other types of toxicities. The efficacy of systemic chemotherapy can also be restricted by multi-drug resistance (MDR) of tumor cells. Some cancers such as ovarian cancers, may quickly become refractory to further therapy and other neoplasias such as non-small cell lung cancer (non-SCLC), are intrinsically resistant to most chemotherapeutic agents. Dose escalations also may be limited by toxicity.

A more selective delivery of the chemotherapeutic agents to the primary tumors and their metastases, based on targeted chemotherapy, can potentially allow significant dose escalation while reducing the systemic toxicity. Targeting chemotherapeutic drugs directly to tumor cells could overcome MDR based on transmembrane efflux. Various findings indicate that targeted chemotherapy with cytotoxic peptide analogs can delay or suppress chemoresistance mediated by the gene MDR-1 and MDR-1 protein (MRP-1) efflux pumps. Collectively, the advances in the past 20 years have placed the concept of “magic bullets” on a firm foundation.
2.1. Cytotoxic Analogs of LHRH

**LHRH and its receptors**

Hypothalamic luteinizing hormone-releasing hormone-1 (LHRH1), also known as gonadotropin-releasing hormone-1, (GnRH1), is the primary link between the brain and the pituitary in the regulation of gonadal functions and plays a critical role in vertebrate reproduction. In addition, LHRH1 may be a growth factor in various tumors. Several LHRH1 agonists, such as triptorelin (Decapeptyl®), leuprolide (Lupron®), and goserelin (Zoladex®) have various important clinical applications in gynecology, urology, and oncology. The actions of LHRH and its analogs are mediated by high-affinity receptors for LHRH found on the membranes of the pituitary gonadotrophs and of multiple cancers. LHRH receptors are members of the rhodopsin-like family of seven-transmembrane-domain G protein-coupled receptors.

**Targeting of LHRH conjugates to tumors**

Tumoral receptors for LHRH have been detected on human breast, prostatic, ovarian, endometrial, and pancreatic cancers. More recently they have been detected in melanomas, non-Hodgkin’s lymphomas, and renal cell carcinomas. The expression of LHRH receptors in tumors has been exploited for an approach to targeted cancer therapy. Various toxins conjugated to LHRH and other chimeric proteins have been used for targeting to the LHRH receptors on tumors. Thus, LHRH and its analogs have been fused with or linked to bacterial and plant toxins to target and kill cancer cells expressing LHRH receptors. Thus, various compounds with anticancer activity can be produced by targeting LHRH receptors.

**Design and synthesis of targeted cytotoxic analogs of LHRH**

The design of cytotoxic LHRH analogs was based on the use of agonists or antagonists of LHRH as carrier molecules which were then linked to cytotoxic agents. Because the replacement of the Gly residue at position 6 of LHRH by various D-amino acids increases the stability of the analog to enzymatic degradation and results in potent analogs of LHRH with high binding affinity and biological activity, a D-Lys moiety at position 6 has been used for attachment of various cytotoxic compounds. It was found that even bulky molecules could be linked to the ε-amino group of the D-Lys^6 moiety without loss of the binding affinity to receptors of LHRH. Thus, cytotoxic LHRH hybrids in which diverse cytotoxic radicals were attached covalently to the D-Lys side-chain of the LHRH carrier were developed. These
cytotoxic compounds initially included cisplatin, methotrexate, and 2-(hydroxymethyl) anthraquinone. D-Melphalan, an alkylating nitrogen mustard derivative of D-phenylalanine, has also been incorporated at position 6 of LHRH. Early conjugates with [D-Lys\(^6\)] LHRH also included the DNA intercalating antibiotic, doxorubicin (DOX), a widely used anticancer agents with a broad spectrum of antitumor activity. The antiproliferative activity of DOX is due mainly to its ability to intercalate into DNA and break the strands of double helix by inhibiting topoisomerase II. As an example, cytotoxic analog, AN-152 in which DOX is linked to [D-Lys\(^6\)]LHRH, fully preserved the cytotoxicity of DOX and the binding affinity of the LHRH carrier. The production of free oxygen radicals by DOX has been linked to its cardiotoxicity, which is its dose limiting toxicity. The binding of cytotoxic LHRH analogs to receptors for LHRH on cancerous cells is followed by cellular internalization of the hybrid molecule and the release of the cytotoxic agent in the lysosomes.

The interaction with the LHRH receptors and the entry into cell cytoplasm of cytotoxic LHRH agonist, AN-152, containing DOX was already investigated in LHRH receptor-positive cancers. These processes were revealed by two photon laser-scanning microscopy of fluorophore-labeled AN-152 and confocal laser scanning microscopy using the autofluorescence of the doxorubicin (DOX) moiety in AN-152 in human ovarian and endometrial cancer cell lines. AN-152 entered only into LHRH receptor-positive cells while unconjugated DOX entered all cell types independently of their receptor status. A significantly higher fluorescence signal could be detected in the nuclei of LHRH receptor-positive cells after treatment with AN-152 was the case than with DOX. Accordingly, AN-152 was significantly more cytotoxic than DOX in these cells. Similar results were obtained in human estrogen-dependent MCF-7 breast cancer cells in vitro. Two-photon emission fluorophores were linked to the free amino group of the daunosamine moiety of AN-152 or to the epsilon amino group of the carrier [D-Lys\(^6\)] LHRH. The energy required for the excitation of these fluorophore tags allowed “real-time” optical tracking of the conjugate and the carrier in the different compartments of living MCF-7 cells. The labeled carrier peptide was localized mainly in the cytosols of MCF-7 cells and not in the nucleus, but the fluorescent label linked to AN-152 and coupled to the daunosamine nitrogen of DOX was found only in the nuclei. No entry of labeled AN-152 could be observed in LHRH receptor-negative UCI-107 human ovarian cancer cells.
References


In the regulation of gene expression antisense RNAs and RNA interference play a crucial role. Understanding these two mechanisms opened a new era in pharmaceutical therapy. Today, antisense RNAs are available as drugs, and many pharmaceuticals based on the mechanism of RNA interference are tested in clinical trials.

1. Antisense drugs: nucleic acids as therapeutic agents

Antisense oligonucleotides are short, single stranded RNAs or DNAs which can alter protein synthesis through the inhibition of gene expression (Fig. 14). Antisense oligonucleotides hybridize to targeted complementary nucleic acids and block their expression [Avidor 2003]. This mechanism can be used to design antisense drugs that link to specific DNA or RNA sequences. As we do not need to know the three dimensional structure of special protein binding site - as is the case of standard “anti-protein” drugs -, but theoretically it is enough to know the nucleic acid sequence of the target, designing active substances is much easier. Because for absolute specific coding of many target genes it is enough to use only 15-25 nucleotides, and the specificity of these agents is outstanding. If we change only one base in the sequence (mismatch), the inhibition of translation will dramatically decrease. If more nucleotides are exchanged, the whole inhibition will be arrested. Making the oligonucleotide sequence longer, specificity will be higher (but binding to alternative sites will be also more likely). Binding affinity can be increased with the use of more guanines and cytosines. The main problem related to antisense drugs is bioavailability. Because molecules made of natural oligonucleotides are very sensitive to nucleases, modified oligonucleotides are used. RNAs in the cell have given secondary and tertiary structure and there is correlation between the secondary structure of the RNA and the antisense activity and it is possible that the target region is hidden by other regions. Other problems are the delivery of antisense drugs, permeation and absorption because of the negatively charged phosphodiester chain. This negative charge can be neutralized for example by binding to cationic lipids that help to enter into the cell. Various tissue culture studies demonstrated that antisense oligonucleotides enter into cells via receptor-mediated endocytosis pathway [Tari 2001]. Stability of the antisense oligonucleotide-target sequence hybrid molecule is another problem. This can be improved by the use of modified nucleotides or sugars, and blocking the 3’ exonuclease activity or 3’ capping can also be effective [Ötvös 2004].
Table 10. Antisense drugs in the clinic and clinical studies

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Generic name</th>
<th>Approval/Clinal studies</th>
<th>Route of administration</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitravene</td>
<td>fomivirsen</td>
<td>1998</td>
<td>Intravitreal injection</td>
<td>Treatment of cytomegalovirus retinitis (CMV) in immunocompromised patients; inhibition of viral proteins</td>
</tr>
<tr>
<td>Mucagen</td>
<td>pegaptanib</td>
<td>2004</td>
<td>Intravitreal injection</td>
<td>Age-related macular degeneration; lowers VEGF level</td>
</tr>
<tr>
<td>Genasense</td>
<td>oblimersen</td>
<td>Phase III</td>
<td>Subcutaneous injection</td>
<td>Chronic lymphocytic leukemia, B-cell lymphoma, breast cancer; inhibits Bcl2 protein</td>
</tr>
<tr>
<td></td>
<td>mipomersen</td>
<td>Phase III</td>
<td>Subcutaneous injection</td>
<td>Cholesterol-reducing drug; inhibits the synthesis of apolipoprotein-B</td>
</tr>
<tr>
<td>AVI-6002,</td>
<td></td>
<td></td>
<td>Preclinical phase</td>
<td>Ebola, Marburg virus infection</td>
</tr>
<tr>
<td>AVI-6003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The target of antisense drugs can be any protein coding gene or mRNA blocking of which will result in therapeutic amelioration. Antisense drugs, for example, can inhibit the expression of essential viral or cancer proteins leading to the death of these cells. Antisense drugs available in the clinic or in clinical studies are listed in Table 10.
mRNA is single-stranded, and its sequence of nucleotides is called "sense". When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked.

**Mechanism of action of antisense agents**

Antisense agents can exert their effects through different mechanisms from RNA transcription to the translational steps of proteins:

**Blocking transcription at DNA level**

- DNA-like antisense molecule can bind to the major groove of the double-stranded DNA forming a triplex and prevent, for example, helicase enzymes to separate DNA strands and inhibits DNA replication. If the antisense oligonucleotide is bound to simple stranded DNA region, RNA-polymerase will be arrested and replication cannot be completed.

**Blocking transcription at mRNA level**

- DNA-like antisense oligonucleotide binds to RNA and forms DNA/RNA duplex which will be cut into small pieces by RNaseH.
- Antisense RNA hybridizes to exon-intron junction and stops splicing.

**Inhibiting mRNA transport**

- The poly (A) tail, which is important for the nuclear export, translation and stability of mRNA, is synthesized by polyadenylation and can be inhibited by antisense RNA, and both the transport and stability of mRNA are influenced.
Inhibiting translation

- Antisense RNA can block protein initiation factors, association of ribosomes or elongation of the protein by inhibiting the interaction between mRNA and ribosomes.

2. RNA interference (RNAi)

In 2006, Andrew Fire and Craig Mello got the Nobel Prize in Physiology or Medicine. They were investigating how gene expression is regulated in the nematode worm *Caenorhabditis elegans*. Injecting mRNA molecules encoding a muscle protein led to no changes in the behavior of the worms. Injecting antisense RNA also had no effect. But when they injected sense and antisense RNA together, they observed that the worms displayed peculiar, twitching movements. Similar movements were seen in worms that completely lacked a functioning gene for the muscle protein [Fire 1998]. After a series of simple but elegant experiments, Fire and Mello deduced that double-stranded RNA can silence genes, this RNA interference is specific for the gene whose code matches that of the injected RNA molecule, and RNA interference can spread between cells and even it is inheritable. It was enough to inject tiny amounts of double-stranded RNA to achieve an effect, meaning that RNA interference (RNAi) is a catalytic process [Fire 1998].

Soon it became clear that RNAi is a key mechanism in the regulation of gene expression and can be found in many eukaryotes. Main steps of RNAi have been described shortly (Fig. 15.). In the initiation step, input dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs) by the enzyme Dicer. In the effector step, siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3’ terminus of the siRNA, thus mRNA is silenced [Agrawal 2003].
Figure 15. Mechanism of RNA interference.

Input dsRNA is digested into 21-23 nucleotide small interfering RNAs by the enzyme Dicer. siRNA duplexes bind to a nuclease complex to form RNA-induced silencing complex (RISC). The active RISC then targets the homologous transcript by base pairing interactions, cleaves the mRNA, and mRNA is silenced.

RNAi is important in switching on/off genes. It was demonstrated that short (on average 22 nucleotides long) RNAs, called micro-RNA (miRNA) are generated in the nucleus. miRNA appears at transcription but are not converted into protein. miRNA molecules display a truly unique property: folding back on themselves to create a double-stranded structure known as a stem-loop (Fig. 16). This stem-loop shape flags down special enzymes in the nucleus that chop the miRNA molecules into smaller pieces and shuttle them out into the cell. Once here the enzyme Dicer further cuts the miRNA down, bringing it to its tiny working size. Finally, the miRNA binds to RISC and assumes a linear single-stranded shape and is ready to fulfill its function: degrade specific mRNAs and silencing genes [Scherr 2007]. miRNAs have important function in translational repression, during morphogenesis and maintaining the undifferentiated or partly differentiated stem cell pool [Carrington 2003].
RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RISC.

In plants, insects and nematodes, RNAi also functions as an innate defense response against viruses. siRNAs can originate from extensive secondary RNA structures in the viral RNA or from dsRNA viral replication intermediates and may be fully complementary to viral mRNA. After loading into RISC, siRNAs typically trigger cleavage of the mRNA. The fact that several animal viruses encode RNAi suppressor function may be evidence in favor of an RNAi-dependent mammalian immune response [Berkhout 2006].

Other function of RNAs can be the regulation of “jumping elements” (transposons). Transposons are sequences of DNA that can move or transpose themselves to new positions within the genome of a single cell and can create phenotypically significant mutations and alter the genome size. While copying themselves, transposons are single stranded at a certain point and can be targets for Dicer. In mammals, almost half the genome (45% to 48%) comprises transposons or remnants of transposons [Mills 2007].

RNAi has already become an important research tool in biology and it is predicted that will be used in many fields including clinical medicine.
3. Therapeutic uses of siRNA

Table 11. A comparison of various drug discovery attributes of small molecules and siRNAs

<table>
<thead>
<tr>
<th>Properties</th>
<th>Small drug molecule</th>
<th>siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Conformation driven, low to medium</td>
<td>Sequence driven, high</td>
</tr>
<tr>
<td>Potency</td>
<td>Variable</td>
<td>Typical pM</td>
</tr>
<tr>
<td>Number of accessible targets</td>
<td>500-1000</td>
<td>&gt;&gt;1000</td>
</tr>
<tr>
<td>Number of potential leads and backups</td>
<td>&lt; 2-3</td>
<td>&gt;&gt;10-100 (depending on target length)</td>
</tr>
<tr>
<td>Speed to lead molecules</td>
<td>2-4 years</td>
<td>2-8 weeks</td>
</tr>
<tr>
<td>Species cross reactivity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Manufacturing</td>
<td>Variable, can be complex</td>
<td>Rapid, calculable</td>
</tr>
</tbody>
</table>

Before siRNA may become a therapeutic option, many obstacles must be eliminated. These include steps required for lead selection, the use of chemical modifications to confer appropriate biopharmaceutic properties, the design of formulation that enable delivery to a target tissue, and screening of these products for safety, including assessments for potential off-target effects [Vaishnaw 2010]. The process of the latest is not clear but is a potential source of side effects including interferon response. In order to enter the cell, siRNA must come into contact with a lipid bilayer of the cell membrane, whose head groups are also negatively charged. This problem can be solved for example with transfection involving the use of packaging particles called liposomes to facilitate the cellular uptake of siRNA [Scherr 2007]. A comparison of various drug discovery attributes of siRNAs and small molecules are presented in Table 11.
3.1. Viral infections

The first demonstration of RNAi-mediated inhibition of a human pathogenic virus, respiratory syncytial virus (RSV), was reported by Bitko and Barik in 2001 [Bitko 2001]. RSV is a major cause of morbidity in infants, young children, and the elderly worldwide. Currently, there is no effective vaccine, and antiviral drugs to control infection are limited. RNA interference is a potent tool suitable to development of antiviral drugs. Using siRNA targeting the P gene of RSV (siRNA-P), RSV replication can be silenced both in vitro and in vivo [Zhang 2008]. Currently, they focus on siRNA-mediated inhibition of various viruses (Herpes simplex type 2, Human immunodeficiency virus type 1, Hepatitis-A, Hepatitis-B, influenza viruses, measles) in many laboratories. In early studies for the inhibition of HIV in primates both synthetic and promoter expressed small interfering RNAs (siRNAs) or expressed short hairpin RNAs (shRNAs) were used to demonstrate that this virus was susceptible to RNAi. In addition to targeting the virus itself, RNAi-mediated down-regulation of cellular targets that encode receptors required for viral entry also proved to be effective. The unique properties of RNAi as an anti-HIV agent have propelled development of RNAi-based gene therapy approaches for the treatment of HIV infection in humans [Rossi 2006]. However, targeting the virus directly represents a substantial challenge for clinical applications, because the high viral mutation rate will lead to mutants that can escape being targeted [Rossi 2006].

3.2. Cancer therapy

At least 74% of human genes are alternatively spliced, and wrong splicing can result in many inheritable disorders and different types of cancer [Johnson 2003]. The discovery of RNAi opened a new way in the inhibition of these splice-isoforms. The epidermal growth factor receptor-2 (Her2) proto-oncogene is amplified in 25 to 30 percent of human primary breast and ovarian cancers and this alteration is associated with disease behavior [Hynes 1994, Slamon]. Silencing Her2 by siRNA inhibits cell proliferation and induces apoptosis in breast cancer cells [Faltus 2004]. Moreover, it was published that in human cell lines there is a new splice variant (ΔHER2) with stronger transforming ability [Kwong 1998]. As it is suggested that this new isoform plays a regulatory role, siRNA targeted against ΔHER2 may provide a more efficient therapy in breast cancer [Gaur 2006].
3.3. Metabolic disorders

RNAi can be used to silence endogenous genes involved in the cause or pathway of metabolic diseases and holds considerable promise as a therapeutic approach, particularly those that encode so-called ‘nondrugable’ targets. High potency, specificity and chemical structure can help to avoid toxicity and side effects often seen with small molecules. Hepatic insulin resistance is a critical component in the development of type 2 diabetes mellitus. In many cases, insulin resistance in liver is associated with reduced expression of both major insulin receptor substrate (IRS) proteins, IRS-1 and IRS-2. By silencing IRS genes separately or together it was demonstrated that IRS-1 is important in glucose homeostasis while IRS-2 is important in lipid metabolism of the liver [Taniguchi 2005]. Valades et al [2006] designed a therapeutic, vector-based RNAi approach to induce posttranscriptional gene silencing of hepatic phosphoenolpyruvate carboxykinase (PEPCK), a key component of gluconeogenesis, using nonviral gene delivery [Gomez 2005].

It is predicted that RNAi will be used in diseases otherwise difficult to treat like depression, obesity or neuropathy.
References


STEM CELL THERAPY

An estimated 10 to 100 trillion cells make up the average adult human body. In different tissues, cells are differentiated to provide different functions. In the human body there are approximately 200 types of differentiated cells. The majority of them have a very limited proliferation ability and short life time (e.g. erythrocytes live for 120 days), even those differentiated cells that are still able to replicate, can renew only at most 50-60 times (Hayflick limit). In order to maintain the structure and function of tissues, dead cells have to be substituted; this is the role of stem cells.

1. Types of stem cells

Stem cells have the outstanding potential to replicate through mitosis and differentiate into diverse specialized cell types and self renew to produce more stem cells (Fig. 17). Their role is to substitute weakened or damaged cells, and regenerate defective tissues. In mammals, there are different stem cells grouped by their potency (i.e. the capacity to differentiate into specialized cell types). The only totipotent cells are the fertilized oocyte and the first 4 or so cells produced by its cleavage. These cells can produce any cells of the adult body (somatic cells) and any cells of the extraembryonic membranes. Real stem cells, i.e. those that can produce other stem cells by asymmetric division, are pluripotent [Schöler 2007, Mitalipov 2009]. Pluripotent cells (embryonic stem cells, embryonic germ cells, and embryonic carcinoma cells) are direct descendants of totipotent cells, and are able to differentiate into germ cells and all cell types of the three germ layers. Pluripotent cells can be isolated exclusively from embryonic or fetal cells, and can be cultured in vitro by inhibiting their differentiation. In the lab, pluripotent cells can be developed from already differentiated cells, and are called induced pluripotent cells (iPS) [Takahashi 2006]. Stem cells in the adult body are multipotent and can only differentiate into a limited number of types. Multipotent cells are for example bone marrow stem cells that give rise to all cells of the blood. Oligopotent stem cells – beside renewing themselves -, can generate only few, while unipotent stem cells only one cell type (e.g. stem cells in the muscle) [Schöler 2007].

2. Stem cell cycle

Theoretically, in all tissues that have the ability for self-renewal (e.g. cornea, hair follicle epithelium or hematopoietic cells) there is a stem cell population with strong potency for self-
renewal. By the mitosis of these stem cells, a special cell type called progenitor or transit amplifying (TA) cells are produced. Within this cell population young TA cells are able to proliferate many times, while more “matured” TA cells only few times. This aging of stem cells have been seen as unidirectional but accumulating evidence shows that, in certain circumstances, TA cells can have stem cell-like phenotype [Loeffler 1998, Lehrer 1987].

Figure 17. Stem cell differentiation.

*The only totipotent cells are the fertilized oocyte and the first 4 or so cells produced by its cleavage. These cells can produce any cells of the adult body (somatic cells) and any cells of the extraembryonic membranes. Real stem cells, i.e. those that can produce other stem cells are pluripotent. Stem cells in the adult body are multipotent or unipotent and can generate few or only one cell type, respectively.*

3. Therapy with stem cells

As the use of embryonic stem cells rise many ethical, moral and religious questions, research has focused on somatic stem cells derived from adult tissue samples rather than destroyed human embryos. Main types of somatic stem cells are summarized in *Table 12.*

Many disorders arise from damage to differentiated cells, for example type-I diabetes mellitus where the beta cells of the pancreas have been destroyed by an autoimmune attack and are not able to produce insulin; or blindness caused by damage to the cornea. In these cases, stem cells can replenish damaged cells and thus restore normal function of the given tissue. The isolation, *in vitro* generation and genetic modification of somatic stem cells are of great promise in biomedicine. Tissue engineering, a relatively new field of biotechnology is closely
associated with applications that repair or replace portions of or whole tissues using stem cells. The aim of this research is to generate tissues for example in order to facilitate cartilage and bone growth, or make heart valves, joints, vertebral disk or even whole organs which can compensate diseased tissue or organ functions. Stem cells also may have a fundamental role in gene therapy. Mutant genes could be changed with the wild type in stem cells than by the implantation of these fixed cells hereditary diseases could be cured. Main advantages of stem cell therapy are: human origin, significant proliferation potency, normal genetic structure, relatively stable, can be differentiated to many cell types, can be genetically modified.

**Table 12.** Types of somatic stem cells and their function

<table>
<thead>
<tr>
<th>Type of stem cell</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic stem cells</td>
<td>Found in the bone marrow and give rise to all the blood cell types [Muller-Sieburg 2002]</td>
</tr>
<tr>
<td>Mammary stem cells</td>
<td>Are source of cells for growth of the mammary gland during puberty and gestation [Liu 2005]</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>Can differentiate into a variety of cell types, including: osteoblasts, chondrocytes and adipocytes; can be isolated from teeth, cord blood; inhibit cell death, induce capillary growth, have immunosuppressive effect, stimulate endogenous stem cells [Phinney 2007]</td>
</tr>
<tr>
<td>Endothelial stem cells</td>
<td>Multipotent stem cells in bone marrow with the ability to differentiate into endothelial cells [Masters 2009]</td>
</tr>
<tr>
<td>Neural stem cells</td>
<td>Multipotent cells that generate the main phenotypes of the nervous system; when injected into the blood, differentiate into various cell types of the immune system [Altman 1965]</td>
</tr>
<tr>
<td>Olfactory stem cells</td>
<td>If they are given the right chemical environment, they have the same ability as embryonic stem cells to develop into many cell types; can be harvested with ease without harm to the patient [Murrell 2005]</td>
</tr>
<tr>
<td>Neural crest stem cells</td>
<td>Can generate neurons, Schwann cells, myofibroblast, chondrocytes and melanocytes [Sieber-Blum 2008]</td>
</tr>
<tr>
<td>Testicular cells</td>
<td>Multipotent stem cells in the testicles [Goosens 2006]</td>
</tr>
</tbody>
</table>

A number of stem cell therapeutics exist, but most are at experimental stages and/or costly, with the notable exception of bone marrow transplantation in acute myeloid and lymphoid leukemia, severe combined immunodeficiency or inherited or acquired autoimmune diseases. According to several researchers, neurological disorders like Parkinson’s or Huntington’s disease, type I diabetes mellitus, heart and muscle damage, blindness, deafness and certain
kind of cancer will be treatable in the near future by stem cell therapy. Stem cell types potentially suitable for the treatment of cardiovascular diseases are summarized in Table 13. Stem cells can be transplanted using autologous or allogeneic transplantation. Autologous stem cell transplantation requires the extraction of hematopoietic stem cells from the patient; allogeneic stem cell transplantation involves two people: the donor and the recipient. In case of allogeneic transplantation, donor stem cells must be tested for the same characteristics as in the case in other organ transplantation. For the therapy of hematological/immunological diseases, stem cells can be obtained from bone marrow, peripheral blood or umbilical cord blood. Bone marrow stem cells are removed from a large bone of the donor, typically the pelvis, through a large needle that reaches the center of the bone under spinal or general anesthesia. Stem cell yield can be boosted with injections of granulocyte-colony stimulating factor, serving to mobilize stem cells from the donor's bone marrow into the peripheral circulation then collected from the blood. Umbilical cord blood has a higher concentration of stem cells than is normally found in adult blood and can be separated right after birth without any risk to the child or the mother. However, the small quantity of blood obtained from an umbilical cord (about 50 ml) makes it more suitable for transplantation into small children than into adults. Stem cells separated from cord blood can be stored in liquid nitrogen in cord blood banks for a long period. Although many private cord blood banks exist, there are very few conditions that can be fixed with cord blood, and in the case of hematological diseases or cancer it is far from advantageous to be giving the defective blood back. In contrast, public cord blood banks have much more importance and their wide spread use hopefully will happen in the near future. Advantages of cord blood include: easy to collect, often more likely to provide a suitable match, graft-versus-host reaction is less likely and less severe, cord blood transplants are associated with lower risk of viral infections, cells are „newborn” with fewer potential damages than adult stem cells, and is stored frozen, ready to use. However, it usually takes longer for cord blood cells to engraft, and in case of autologous transplantation, for example in the treatment of leukemia, the risk of relapse is higher.
Table 13. Stem cell types potentially suitable for the treatment of cardiovascular diseases [Lian 2010]

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic stem cells</td>
<td>• Pluripotent, unlimited supply</td>
<td>• Social and ethical issues</td>
</tr>
<tr>
<td></td>
<td>• Autologous transplantation is possible</td>
<td>• Risk of graft-versus-host reaction, immunosuppression is needed for allogeneic transplant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Risk of tumor formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Limited supply of human oocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Proarrhythmic risk due to immature phenotype of derived cardiomyocyte</td>
</tr>
<tr>
<td>Skeletal myoblast</td>
<td>• Autologous transplantation possible, no need for immunosuppression, no risk of rejection</td>
<td>• Cannot differentiate into cardiomyocyte phenotype</td>
</tr>
<tr>
<td></td>
<td>• Can be expanded in vitro, resistant to ischemia and fatigue</td>
<td>• Lack of integration with host cardiomyocyte with arrhythmogenic potential</td>
</tr>
<tr>
<td>Bone marrow stem cells</td>
<td>• Autologous transplantation possible, no need for immunosuppression, no risk of rejection</td>
<td>• Limited differentiation to cardiomyocytes</td>
</tr>
<tr>
<td></td>
<td>• Possible induction of angiogenesis, pluripotent?</td>
<td>• Limited supply, need for in vitro culturing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Difficult to isolate and culture</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>• Autologous transplantation possible, no need for immunosuppression, no risk of rejection</td>
<td>• Limited differentiation to cardiomyocytes</td>
</tr>
<tr>
<td></td>
<td>• Possible induction of angiogenesis, pluripotent?</td>
<td>• Limited supply, need for in vitro culturing</td>
</tr>
<tr>
<td></td>
<td>• Allogeneic transplantation is possible</td>
<td>• Difficult to isolate and culture</td>
</tr>
<tr>
<td>Adult cardiac stem cells</td>
<td>• Phenotypically cardiomyocytes, no need for differentiation</td>
<td>• Very limited supply</td>
</tr>
<tr>
<td></td>
<td>• Can integrate with host cardiomyocytes</td>
<td>• Difficult to isolate and culture</td>
</tr>
<tr>
<td></td>
<td>• Autologous transplantation possible, no need for immunosuppression, no risk of rejection</td>
<td>• Immature phenotype, risk of proarrhythmia</td>
</tr>
<tr>
<td>Induced pluripotent stem cells</td>
<td>• Pluripotent, unlimited supply</td>
<td>• Risk of tumor formation</td>
</tr>
<tr>
<td></td>
<td>• Autologous transplantation is possible</td>
<td>• Risk of viral vectors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Proarrhythmic risk</td>
</tr>
</tbody>
</table>
The major problem with allogeneic stem cell therapy is graft-versus-host reaction. This can be prevented by autologous transplantation if healthy cells from the patient can be obtained. If neither allogeneic nor autologous transplantation is an option, somatic cell nuclear transfer is possible [Hemmat 2010]. In somatic cell nuclear transfer the nucleus of a somatic cell, which contains the organism's DNA, is removed and the rest of the cell discarded. At the same time, the nucleus of an oocyte is removed. The nucleus of the somatic cell is then inserted into the enucleated egg cell. At the stage of blastocyst, embryonic stem cells can be isolated and culture in vitro. Stem cells generated with this method than can be transplanted without the risk of rejection.

4. Embryonic and induced pluripotent stem cell lines

Human embryonic stem cell lines are cultures of cells derived from the epiblast tissue of the inner cell mass of a blastocyst or earlier morula stage embryos. They can be used in developmental research, pharmaceutical testing or in cell-based therapies. Embryonic stem cell lines can be established from embryos (Fig. 18.) or created by in vitro fertilization, but this raises serious ethical questions. Lately, induced pluripotent cells have been used in stem cell research. Induced pluripotent cells can be generated from adult cells by inducing expression of specific genes (Fig. 19.) [Takhashi 2006, 2007].

![Figure 18. Establishment of embryonic stem cell line.](image)

*Cells from the epiblast tissue of the inner cell mass of a blastocyst can be cultured in vitro and embryonic stem cells can be isolated. Isolated stem cells theoretically can be differentiated into different tissues or divide for unlimited time.*
Figure 19. Establishment of induced pluripotent stem cell lines.

By the insertion of transcription factors into adult somatic cell, cells can be reprogrammed to pluripotency than can be differentiated theoretically to any type of somatic cells.

5. Some further problems with stem cell therapy

Imprinted genes

Sperm and oocyte each contain certain genes that carry an "imprint" identifying them later in the fertilized egg as being derived from the father or mother, respectively. Creating an oocyte with a nucleus taken from an adult cell may not allow a proper pattern of imprinting to be established [Frost 2011].

Aneuploidy

In primates (in contrast to e.g. mice), the process of removing the nucleus causes molecules associated with the centrosome to be lost as well. Although injecting a donor nucleus allows mitosis to begin, spindle formation may be damaged, and the resulting cells fail to get the correct set of chromosomes [Schatten 2009].
Somatic mutations

It is possible that mutations that might be well-tolerated in a single somatic cell of the donor might turn out to be quite harmful when they become replicated in a clone of cells injected later into the patient [Schambaun 2010].

6. Ethical dilemmas

Human stem cell research raises sharp ethical and political controversies. The generation of pluripotent stem cell lines from oocytes and embryos is full with disputes about the onset of human personhood. Others think that embryos less than 14 days old cannot be considered as human beings and it is a big mistake to stop research that aims to save lives of already living people. Induced pluripotent stem cells can be generated without using human embryos or oocytes, thus bypassing some of the ethical issues that have limited the use of human embryonic stem cells [Parham 2009]. The other main ethical question associated with stem cell research is associated with the combination of embryonic stem cell and cloning technologies, leading to the generation of an embryo that is a genetic clone of the donor of the nucleus. The Prohibition of Human Cloning Act 2002 (Cth) prohibits all types of human cloning by any method while the Research Involving Human Embryos Act 2002 (Cth) allows for regulated use of a suitable number of excess embryos derived from assisted reproductive technology in approved research programs.
References


PREPARATION OF BIOLOGICAL MEDICINES

Introduction

Biological medicines are all those products whose active substance is a biological material. Biological agent is the material which made or extracted from a biological source. The spatial structure of the macromolecular active substances cannot be accurately determined by the current available methods. Determination of their quality requires a combination of physicochemical and biological methods, as well as for the manufacturing and checking production. Biological drugs may include plasma-derived medicines (human blood or plasma), vaccines, cell and gene therapy products.

The active substances of biological medicaments are macromolecules, which are mainly proteins, and most of which are currently produced by various biotechnological methods in different biological systems. The active molecules induce immunologic reactions in the organism. The presence of neutralizing antibodies frequently leads to the inhibition of the effect of the bio-pharmaceuticals. The European Medicines Agency (EMA) registers medicinal products including similar macromolecular active substances produced by biotechnological methods as biosimilar agents, since their chemical properties cannot be proven. The similarities and differences of their biological and immunological effects can be evaluated only in relative non-clinical and clinical trials. The frequent exchange of biological medicines increases the presence or occurrence of antibodies in the patients, therefore the substitution of biological drugs should be done only by the treating physicians if clinically necessary (1). This chapter tries to summarize the most important information about the production of the main biological medicaments.

1. Fermentation

The use of biotechnology in medicine has increased dramatically in recent years. Knowledge of DNA translation mechanisms has already given us the ability to induce expression of the fetal hemoglobin gene to improve oxygen carrying capacity in patients with sickle cell anemia (2). Today there are many fields of pharmaceutical production where we can meet with the achievements of Biotechnology. As a result of the advance of Biotechnology there are a lot of useful production, for example: antibiotics, steroid stocks, antisense RNA that inhibit HIV replication, and can be used in gene therapy respectively in pharmacogenomial investigations.
One of these biotechnological methods is Fermentation. During fermentation, microorganisms – like bacteria, fungi, algae, eucaryotic plant cell, and even mammalian cells – are able to breakdown carbohydrate nutrients. This creates primary and secondary metabolites, which prove to be useful for the human life. Therefore the industrial production of these metabolites is beneficial for biotechnology.

The Primary metabolites are such a biochemical end product which is essential for the microbes, and is directly related to the growth and energy production of the cell. Primary metabolites are for example: amino acids (for infusion solutions), organic acids (citric acid, gluconic acid, acetic acid, lactic acid), alcohols (ethanol, acetone-butanol).

In contrast, the product of secondary metabolites not essential for the microorganisms. These are usually produced to influence some change in living conditions (nutrient deficiency, mineral deficiency, stress effect). Secondary metabolites help the microbes to survive. Secondary metabolites produced by some microorganisms may give the microorganisms selective advantages over other microbes. Such a valuable by-product can be for example: antibiotics (penicillin, streptomycin, tetracycline, etc.), vitamins (riboflavin, B12, β - carotin), alcaloids.

Obviously, fermentation products such as the cells (biomass), proteins and oils (probiotics, SCP) in the ferment can be useful.

The technology itself can be virtually divided in two main phases. These are the upstream-processing and the downstream-processing.

The upstream-processing practically include the whole process of fermentation: from the preparation (inoculum preparation, microbe propagation, making medium, sterilization) to the end of the process. This is followed by processing of the product: this is the so-called downstream-processing. The extraction of the product can vary depending on the quality and the quantity of the product. In general, the first step in the extraction is the separation of solid and liquid phases that is the cell mass and the ferment. This procedure may happen with filtration (microfiltration) or with settling. For isolation there are several methods, such as extraction, adsorption, precipitation and membrane filtration. These techniques of isolation are also used in later phases. During purification, contaminants are removed from the medium.
2. Strain Development

During the selection of these organisms, the following should be kept in mind. The species in what intensity produce the requested compound; that the strain must be not pathogenic; do not produce substances that are too toxic; and the need to have some minimal physiological point of variability. First of all from the soil samples take a suspension then homogenize, and plate on agar disk. A few days after the incubation time, the enriched microbial colonies on other agar plates are bred in the optimal parameters of the species. This step is followed by the so-called screening phase. During this process, the strain that is selected is present insufficient quantity and quality for the product sought. This item is necessary because the feral strains from nature, in a high percentage of cases, do not produce in economically efficiency the industrial metabolites of interest. The microorganisms can be easy genetically modified, to simply create a viable mutant strain with mutagens (nitrosoguanidine, ethyl-methyl sulfonate) and UV irradiation. Digestion of the bacterial cell walls with lysozyme could easily create a protoplast culture that undergoes fusion with other protoplast cells. Thus, hybrid cell lines can be established. The advantage of cell lines, that they are more susceptible to mutations. The next station for the selected strain determines the most appropriate living conditions, and finally optimization between industrial conditions with setting parameters are needed to produce a metabolic product. The latter step is known as scale-up phase. At the ideal setting parameters, the strain starts producing the required metabolic products, which usually includes the appearance of the microbe in the ferment (Fig. 20.).
3. Maintenance of the strain

The microorganisms, used in biotechnology are extremely sensitive, so maintaining them requires due foresight. At the beginning of the 19th century microbiological research was developed on a large scale. This created the need to create a collection of strains, which main function is to keep alive the different strains of microbes, and make them available for other laboratories. The first of this kind of institute, the Centraalbureau voor Schimmelcultures Centrum (CBS, Baarn, The Netherlands), was founded in 1904. The main function of this institute was to maintain yeast and filamentous fungi strains. Today, the world's largest collection of strains is located in the NRRL (U.S. Department of Agriculture, Northern Regional Research Center, Peoria, Illinois), where around 80,000 strains are maintained. The number of microbial strains in the American Type Culture Collection (ATCC) is around 60,000.

**Figure 20.** Strain development process

From tracing of the microorganism to scale production
4. Composition of the medium in the process of fermentation

The different stages of fermentation processes for producing strains required a different growing media composition. Other nutriment conditions are required in the reproduction of microbes and others in the production of a metabolite. One of the main parameters is the appropriate rate of the carbon (glucose, starch, molasses) and nitrogen (ammonium, nitrates, proteins such as soybean meal, fish meal and amino acids such as corn jam) source. A defect in the proportion may lead to catabolite repression. In addition, the media should include the various macro- (S, P, Mg, K, Na, Ca) and microelements (Cu, Co, Mo, Mn) vitamins such as biotin, pantothenic acid, thiamine, riboflavin, and B12. As carbon sources may be used, various complex compounds can function not only as a source of carbon, but also as nitrogen, vitamins and trace elements resources. Such materials are a by-product of the manufacture of sugar like molasses (starch-based by-product of sugar production), and use more malt extract, cellulose and vegetable oils. One important criterion of this technology is the high degree of purity that is, except the producing strain not to be present other microbes in the ferment, that is why sterilization of the growth media is essential. This is usually done by disinfection (chemical sterilization), or physical means like heat, or radiation. It also may be done by filtering. The oxygenation with by aeration or with stirring and the addition of the appropriate composition of the medium can be an important parameter during this process. The suitable temperature and precise pH value requirement are important too. These values can be traced with sensors that are placed in bioreactors and, if necessary, easily modify the parameters.

5. Fermentation technologies

There are two basic categories of the bioreactors: liquid culture, which is common in industrial and solid phase fermentation (SSF: solid state fermentation). It is easier to monitor the SSF procedure.

Main types of fermentation techniques:

Liquid culture:

A. batch fermentation,
B. feed batch,
C. semi-continuous,
D. continuous
During batch fermentation, the nutrients needed for the reproduction of the microbes are present in limited amounts. During this process product and substrate inhibition may occur, the culture will decline, and the product can be easily extracted from the ferment. It is widely used closed, simple, inexpensive procedure. Primarily used for the production of alcohol.

The *batch fermentation* can be divided into four phases (*Fig. 21.*):

**A/ Lag phase:** the number of cells can be relatively constant, the microbes adapt to new environmental conditions.

**B/ Log phase:** the number of cells growing exponentially, resulting in growth of the cells.

**C/ Stationary phase:** the proportion of proliferating cells and dying cells is in balance because of a reduction of carbon and nitrogen sources, or because of toxic materials which appear in the ferment during the metabolic processes.

**D/ Dying phase:** the number of reproducing cells is rather small, however, the number of the dying cells is large. In this phase, there is a depletion of energy reserves.
Figure 21. Growth curve of microorganisms

A, Lag phase B, Log phase C, Stationer phase D, Dying phase. The number of organisms, represented in the time gets a semi-logarithmic growth curve.

The **fed-batch fermentation** is a new method for the substrate-product which inhibits the problem of batch fermentation. The needed nutrition for the production and reproduction of the microbial strain are permanently added during the fermentation process, in this way the stationary phase is stretched toward more yield.

**Semi-continuous** technology is a combination of batch and continuous fermentation. The feeding of nutrients is continuous, but the bioreactor is emptied from time to time in order to extract the product. In this way, higher yields can be achieved thanks to a well-defined production time period.

Characteristic of the **continuous fermentations**: the medium is continuously fed to the bioreactor and the product extraction is also continuous so that an equilibrium state is maintained. In contrast to the batch fermentation, it is more easily traceable.
6. Recombinant technology in the service of the pharmaceutical industry

Nowadays gene therapy enjoys increasing attention. It has a great potentiality to incorporate different genes, into the cells; furthermore it can replace or repair faulty genes, and also help to prevent hereditary diseases, as well as allow simple and effective treatment (cystic fibrosis, hemophilia). Moreover with gene therapy we can sensitize the tumor cells for the treatment, thereby these cells may become easily destroyed. In this way the cells can be made resistant to virus infection (HIV), and modified immune response by gene therapy methods makes the treatment of autoimmune diseases simpler.

In recent decades, the science of genetic engineering began to develop rapidly. As a result, hormones (insulin, calcitonin), anticoagulants (anticoagulant protein, hirudin), and even extinguishing agents can be easily produced (sub-unit vaccines, for example HBV DNA vaccines such as vaccinia and adenovirus vaccine) by cloning DNA sequences of bacterial and viral proteins.

In various bacteria (Escherichia coli, Bacillus subtilis) and yeast (Saccharomyces bayanus, Saccharomyces cerevisiae, Pichia pastoris) via recombination technology, with help of vectors or by directly building into the chromosome a variety of genes, then using fermentation, large quantities of valuable products for human therapeutics and the pharmaceutical industry can be produced.

The first medicinal products manufactured by this technology and licensed was r-insulin that was produced using E. coli. Today, the therapeutic effect produced by recombinant insulin production is approximately two tons per year. Previously, the insulin had been extracted from the pancreas of pigs and bovine, it was biologically active in the human body after enzymatic modification; however, the amino acid sequence did not correspond to human insulin. That is why, for sensitive patients r-insulin can trigger intense immune reactions.

During industrial scale production of recombinant human insulin the preservation of the same amino acid sequence is very important. The main problem with the production of recombinant insulin is that it was developed to be the mature form of the insulin. In human body under physiological conditions, insulin is produced in the form of pre-pro hormone. This form has an amino acid which does not constitute an integral part of the mature form. The N-terminal part of hormone found in the pre-phase that plays a role in the selecting of the protein. In the central region of the protein is located the pro sequence, which is responsible for the adequate packaging and creating of the final structure of the hormone. During maturation disulfide-bridges can be formed which consists of an A and B chain. During the first steps, in order to
create the recombinant insulin, researchers were focusing on the synthesis of DNA segments. These two chains of the DNA segments consisted of oligonucleotides were synthetised separately. These were ligated into separated expression vectors, in such a way that the gene responsible for coding the insulin were related to a β-galactosidase linked with the methionine. Then the vector were transformed into E. coli, thereupon the bacteria will be selected extracellulary as β-gal-insulin. After digestion of cells and protein purification, the treatment with CNBr can be done. Then the insulin can be easily split off from the β-galactosidase. Further purification steps occur after the A and B chains are mixed, and finally evolves a biologically active compound. At present the process is much simpler, both chain and the β-galactosidase can be found in a fusion protein from which the mature hormone is only requires one splitting step to create. This way can be produced in E. coli as pro-insulin, and after the transformations it can be put on the market. Furthermore in the pre-pro form of insulin, the hormone becomes active after the splitting. It could be produced in S. cerevisiae as Pro-insulin and then with replacement of the amino acids in the end of the chain it can become a marketable product.

Nowadays, in addition of r-insulin (recombinant insulin), this biotechnology has allowed the production of human growth hormone, hepatitis B vaccine and human erythropoietin. Recombinant DNA technology is based on in-vitro recombination, so called cloning. The purpose of the procedure is to get large amounts of pure and specific gene-sections.

The main steps of the process:

- Find the DNA fragment that encodes the desired gene, and isolate it.
- Insert the DNA fragment into the vector.
- Introduction of the vector into the cell, this can be transduction or transfection, it is dependent on the type of the vector.
- Transformation and production of the clones.
- Selection of the appropriate clone
- Amplification of the clone carrying bacteriophage.

7. Cloning vectors and types

We could achieve the insertion of the requested gene into the cell with the help of vectors. These vectors could be viral carriers (retroviral vectors, adenovirus vectors and adeno-associated vectors, etc), or non viral vectors (plasmids, lambda – bacteriophage, cosmids vectors, etc). There are some other known vectors, such as expression vectors, secretion
vectors, shuttle vectors; - that are capable of reproduction and protein expression in prokaryota, and also in eukaryotic cells (M13 phage sequencing and yeast synthetic chromosome).

**Non-viral vectors**

In the bacterial cells, there are naturally occurring mobile genetic elements called plasmids. Normally, it promotes the movement and the survival of the bacteria. It is one of the most commonly used carriers, because of its small size (3-20 kB); it is simply extracted from the bacteria with one-step affinity chromatography, and after transformation can be easily transferred back into another bacteria cell. The plasmids are double-stranded circular elements, whose replication is independent from the replication of the host cell. Another advantage of the plasmids is that, they are present in multiple copies in the cell. Such proteins coded by these plasmids will be expressed in higher amount. Plasmids can also carry resistance genes, which may fulfill the role of selection markers (Fig. 22). Its disadvantage is that, only a certain DNA size could be cloned by plasmids, due to the fact that excessively large DNA fragment may make the structure unstable.
Bacteriophages as vectors

One of the first phage used as a vector was the lambda - phage of the E. coli. This is a so-called moderate phage, which means that among the two alternative life-session the lysogen is the dominate one. The lambda-bacteriophage is incorporated into the bacterial chromosome as a pro-phage. It is indistinguishable from the bacterial DNA, as long as a mutation does not induce it. In this case the phage splits off the chromosome of the bacteria, and changes over to
lytic reproduction. Similarly, a plasmid vector with lambda-bacteriophage could clone larger DNA fragments (15 - 20kB). In addition, the bacteriophage binds to cell surface receptors; that is why with larger efficiency one could put in the cell the DNA fragment one desires to clone. Furthermore, well suited, for the creation of gene library.

The advantage of the cosmid-vectors is that, with them up to 40 kB insert installation is possible. The cosmids actually are nothing more than plasmids which are disguised as phages; in this way they combine the advantages of both cloning vector. Cosmids can be manufactured so that the - phage cos (sticky) ends cut off and are cloned in a plasmid vector. They are more stable and can be stored for longer than the two aforementioned vectors. The access of the cosmid into the cell is made via transduction, which operates more effectively than transfection. After the transduction, the cosmid that is placed into the cell works as a plasmid, and thus can be cleaned.

**Viral vectors and their role in gene therapy**

The success of viral vectors is that they have a strong promoter, high transduction efficiency, and moreover all prokaryota all eukaryotic cells can be used. Their drawback, however, is that their production is more circumstantial towards the non-viral carriers, because their production requires special laboratory conditions, and their application needs special security measures. These conditions are also very important because they are viral carriers for a large percentage of cases of human viral pathogens. For this reason, modified, defective viruses are used in gene therapy. Viral genome is responsible for the replication of the virus. If the genetical material responsible for its own replication of the virus is removed or the genetical material of the virus is very small (pox, herpes viruses) the foreign DNA can only be integrated to replace its own replication genes.

An important criteria regarding viral vectors is that their application do not change the basic function of the cells. Moreover they must have a stable genome. Their application in gene therapy can be important because of the frequent mutation of vectors. The most prevalently used viral vectors in gene therapy are adenovirus vectors, adeno associated vectors, and retroviral vectors. The adeno-associated viruses (parvo) or retroviruses are effectively integrated into the host cell genome, and thus are easily integrated into the foreign gene in the host cell's genetic material.

In the case of adenovirus vectors, the viral DNA is not incorporated into the genome, not replicated during the proliferation, and the maximum insert size is up to 30 kB. The
disadvantage of the adenovirus vectors is that, they trigger inflammatory reactions and toxicity. The recombinant adenoviruses can be used for gene therapy in different diseases, including cystic fibrosis treatment. To produce a recombinant, vectors used two or five serotypes.

During the manufacturing process the resistance against adenovirus infection may be a problem, which may reduce the efficiency of treatment.

During the first generation of the adenovirus vectors removes from the viral genome the E1 gene segment, which responsible for the replication of the virus. This was beneficial in many ways. Primarily the E1 genes are replaced by the therapeutic genes, and consequently the virus replication is inhibited. Finally, the method is not perfect, because antigen-dependent immunological responses develops, which ultimately reduced the therapeutic efficiency. This has necessitated the cultivation of second and the third-generation vectors. In these vectors, all but the E1, E2 and E3 genes were detected. These genes also play a role in viral replication. However, immunosuppressed patients still suffer problems with the application of these genes. One solution is packing the cells, but this is not possible for adenoviruses. The final solution is a helper-dependent vector system, in which the helper virus located all the viral genes that are essential for virus replication, but lacks the packing signal, without which coating of the virions does not happen. The other vector included is just the inverted terminal repeat (ITR), packing detection signal, and the therapeutic gene.

The main advantage of adeno-associated (AAV) vectors is that they are able to infect non-proliferating and proliferating cells. They can integrate into the genome and the maximal size of insert 5 kB. Their risks in gene therapy are the same as the risks of the adenoviral vectors. The adeno-associated viruses is actually nothing more than human parvovirus, which require the presence of helper viruses (which are adenoviruses) for their reproduction. There are currently six known human serotype, which have different cell access strategies. The major advantage to its use in gene therapeutic treatments is that it is not associated with human disease. Their genome consists of two genes. The rep gene is responsible for replication, while the cap gene is encoded by structure proteins.

Viral genes are not found in the genome of AAV, therefore it is not toxic for the human body and does not trigger inflammatory reactions. Its disadvantage is that only small inserts can be incorporated into the genome, or to accommodate its frequency, neutralizing antibodies may be generated that can reduce the effectiveness of the therapy. Today AAV is the most successfully used to produce coagulation factor IX in hemophilia B patients. Moreover, it is in experimental phase for the treatment of cystic fibrosis and muscular dystrophy.
Today, the most commonly used vectors include the retroviral vectors. The retroviruses are enveloped, positive double-stranded RNA viruses. With their help a maximum 7-7, 5 kb insert can be taken into the cell.

The first gene retroviral vectors developed for therapeutic purpose was the murine leukemia virus (MLV). In relation to other carrier systems, the retroviral vectors can infect many different cell types. Eventually the production of helper viruses has been reduced with development of so-called packing cells, and the production of lentivirus vectors also allows for infection of resting cells. Because the retroviruses are pathogens, it is important that the recombinant viruses which are used in medicinal treatment must be safe to use. Retroviral vectors are able to create so-called packing cells with the help of specific cell lines. In these cell lines, the viral vectors and the viral gene sequences expressed genes are segregated, as well as the progeny cells that virus genes do not include. The retroviruses can only infect proliferating cells. Therefore, previously in clinical practice, the cells with the help of \textit{in vitro} stimulation should stimulate reproduction, and then with \textit{in vitro} transduction enter the retroviral vector into the cell; finally the cells can be returned to the patient. This is called exogenous gene therapy. For the first time a patient suffering from multiple immunodeficiencies was injected by retroviral vector adenosine deaminase gene. Today, recombinant retroviral vectors are used in a growing mass. In this way the glucocerebrosidase gene was injected in the hepatocyte of patients suffering from hypercholesterolemia or Gaucher. In the fight against cancer retroviral vectors also play a role. Namely, it is also within the exogenous gene therapy by autologous tumor cell vaccines immune modulator (IL-2, IFN-) molecules that are placed in the tumor cells, or into the lymphocytes which infiltrates of them. In addition, they can be used to allocate multidrug-resistance genes into autologous bone marrow. Besides the cases mentioned, retroviral vectors are used in vivo brain tumors, melanoma, breast, prostate, and lung cancer treatment as well.

The above-mentioned viral vectors also have a number of other viral vectors in service of gene therapy. While retroviral vectors can infect only proliferating cells, the lentivirus can infect non-proliferating or slowly proliferating cells also. Herpes viral vectors may be used with good results in animal models of the nervous system, cancer diseases or cerebrospinal damage and in the treatment of nerve pain. In the future, clinical testing of these vectors is planned.
References

1. 52/2005. (XI. 18.) Ministry of Health regulation to be human applied drugs placing on the market, 2005.
LIPOSOMES AND NANOTECHNOLOGY

Therapies based on traditional drug forms (tablet, injection, solution etc.) do not provide satisfactory effects on patients’ recovery in many cases. Sometimes serious side effects occur due to the lack of targeting the drug molecule. To improve the efficacy of these therapies, researchers have endeavored to develop novel approaches in biomedicine. The revolution in nanotechnology resulted in the appearance of nano-sized drug carriers. The aim of this chapter is to give a brief overview on these recently invented new therapeutic agents.

1. Liposomes

The development of liposomes for drug delivery began in the early 1960’s and it took two decades to create the first pharmaceutical products with liposome carriers. Despite the considerable hurdles scientists had to face, the liposome field has realized great progress in the past few years. Nowadays, after solving many challenges of issues in biopharmacy, safety and technology, commercial preparations are available in various areas of therapy. However in this chapter we focus on biopharmacy, since basic knowledge about liposomes’ structure is fundamental to understand how they act in the body. Detailed data on manufacturing and other technical aspects are to be found elsewhere.

Liposomes are microscopic vesicles composed of phospholipid bilayers enclosing an aqueous core. Their size ranges from 10 nm to several micrometers in diameter. The different types of liposomes can be sorted according to their size, structural parameters, composition and applications. Structural parameters are shown in Table 14 and are illustrated in Figure 23. Several types of phospholipids can be used for liposome formulation. Phosphatidylcholine or sphingomyelin can be purified from natural origins (eg. egg or soy), but there are numerous synthetic phospholipid derivatives available for preparations as well. The formulation technology to develope new generations of liposomes were invented. In the following section these generations of liposomes will be discussed.
Table 14. The most common structural forms of liposomes and their parameters

<table>
<thead>
<tr>
<th>Type of liposomes</th>
<th>Abbreviation</th>
<th>Number of bilayers</th>
<th>Entrapped non-concentric vesicles</th>
<th>Usual size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small unilamellar vesicles</td>
<td>SUV</td>
<td>ONE</td>
<td>NO</td>
<td>10-100 nm</td>
</tr>
<tr>
<td>Large unilamellar vesicles</td>
<td>LUV</td>
<td>ONE</td>
<td>NO</td>
<td>&gt;100 nm</td>
</tr>
<tr>
<td>Multilamellar vesicles</td>
<td>MLV</td>
<td>Several concentric</td>
<td>NO</td>
<td>100 nm – 1000nm</td>
</tr>
<tr>
<td>Multivesicular vesicles</td>
<td>MVV</td>
<td>Several non-concentric</td>
<td>YES, several</td>
<td>&gt;1000 nm</td>
</tr>
<tr>
<td>Giant unilamellar vesicles</td>
<td>GUV</td>
<td>ONE</td>
<td>NO</td>
<td>10 µm - 100 µm</td>
</tr>
</tbody>
</table>

Figure 23. Schematic representation of the most common forms of liposomes and the phospholipid bilayer
1.1. Conventional liposome

One of the major characteristics of the first generation of liposomes is that they are typically composed of solely phospholipids and cholesterol. Their physicochemical properties, including size, surface charge and lipid composition can vary in wide ranges. Despite this variability, the in vivo behavior of conventional liposomes is rather consistent. The biggest problem with the first generation liposomes was their relatively short blood circulation time, due to rapid accumulation in the phagocytic cells of the reticuloendothelial system (RES). This phenomenon decreased many researchers’ enthusiasm for liposomes. In addition, the stability of conventional liposomes in biological fluids is very low (Figure 24). They are administered mainly intravenously, but after a short time they release the encapsulated molecules into the plasma, due to interactions with plasma proteins. Absorbing opsonins onto the surface of liposomes mediates their endocytosis by the RES.

**Figure 24.** Illustration of the fate of intravenously administered first generation liposomes. Panel “A” shows the opsonisation and the uptake of liposomes by phagocytic cells is shown. In panel “B”, the exchange of liposomal lipids with lipoproteins (mostly HDL), another destabilizing phenomenon, is schematically pictured.
On the other hand, recognizing the high level of uptake in the spleen and in the liver (the organs with the highest reticuloendothelial system activity) raised the issue of a possible liposomal therapeutic opportunity for RES. Hence, antimicrobial agents can be successfully delivered to infected macrophages (*Table 15.*). Delivery of immunomodulators to macrophages could be another application of conventional liposomes. This would increase their ability to kill neoplastic cells and therefore prevent tumor formation. Antigen delivery is the principle of liposomal-based vaccination. Trials on these vaccines are mainly still in different clinical stages, but a liposomal Hepatitis-A vaccine manufactured by a Swiss company is now available on the market (*Table 15.*).

### 1.2. Long-circulating Liposomes

The next milestone in liposomal drug delivery research was the introduction of the long-circulating liposomes. Some success has been achieved by the incorporation of polyvinylpyrrolidone polyacrylamide lipids or glucoronic acid lipids into liposomes or coating them with glycolipids, polysaccharides and proteins of red blood cells. At present, the most fruitful way to produce long-circulating liposomes is to attach polyethylene-glycol (PEG) covalently to the surface of the vesicles. These PEG-coated liposomes are also-called PEG-ylated, stealth or sterically stabilized liposomes, and this is the second generation of liposomes as well. The PEG polymers sterically prevent the interaction between serum proteins and the outer surface of the liposome due to their hydrophobicity and flexibility. By these mechanisms the average half-life of liposomes in humans has reached the 48 hours rate (*Figure 25*), which has restored much of the original promise of liposome research. Preparations available are listed in *Table 15."

### 1.3. Immunoliposomes

After solving the problem of the fast elimination of liposomes from the circulation, targeting vesicles like “magic bullets” have become the main purpose in the liposome field. The most promising way to reach this goal is to attach specific ligands or antibodies to the surface of the liposomes, which bind to receptors upregulated on the target cells. These systems have been investigated to deliver various types of drug molecules, but the primary focus was placed on the delivery of anticancer agents. Although, numerous studies investigating immunoliposomes targeted with antigens, including anti-HER2, anti-EGF, anti-CD19 and many others, have resulted in successful inhibition of tumor proliferation; products have not been approved for human therapy yet.
Figure 25. Typical blood circulation profiles of PEGylated liposomes and conventional liposomes when encapsulating the same drug molecules.

1.4. Other Types of Liposomes

Contemporary studies on liposomes investigate new technical solutions for developing more effective drug carriers. Here we briefly refer to some of the liposomes potentially to be used in the future. This newest generation of liposomes is frequently referred to as “intelligent” liposomes.

Fusogenic liposomes or virosomes

A fusogenic liposome or a virosome is a liposome coupled with a fusogenic viral envelope noncovalently. The presence of viral proteins results in the fusion of the virosome and the cell membrane. If the virosome could be targeted to the proper type of cells, the delivered drug would get into the cytoplasm directly. However, drug delivery by virosomes is not a new idea (studies initiated in 1975), and we still have only a few applications of them in the human pharmaceutical therapy. Their benefits are exploited mostly in vaccination, and will be discussed in section 1.5.
**Stimuli-Responsive Liposomes**

Stimuli-responsive liposomes, also called “sensitive” liposomes, are the most recently developed drug carrier vesicles. The release of the encapsulated drug molecules are triggered by the alteration of different environmental factors, such as pH, temperature, or ultrasonic waves. The benefit of using pH-sensitive liposomes is the possibility of avoiding lisosomal degradation. This innovation is aiming to solve a fundamental problem in the area of protecting delivered drug molecules. Many attempts on targeted delivery of therapeutic agents have failed, because after vesicles were internalized into the cell by endocytosis, the liposome degraded in the endosome, and the free label was lysed by lisosomal enzymes. The pH-sensitive molecules are prepared from phosphatidyl ethanolamine and acidic phospholipids. Below pH 6.5, the bilayer of the liposome undergoes protonation and becomes fusogenic. For this reason, after fusion of the liposome and the lisosome membrane, the liposomal content reaches the cytosol. Researchers managed to use these liposomes for nucleic acids delivery.

Temperature-sensitive liposomes are composed of phospholipids with a phase transition temperature of approximately 40°C. After administration, liposomes accumulate in different parts of the body, and even if the targeting was not completely successful, a local release of the liposomal content can be achieved by heating the treated sites. Nevertheless, these delivery systems have been effectively used *in vitro* and in experimental animal models; introducing them in the human therapy has not yet been realized.

**2. Liposomes as vaccines**

For vaccination usage liposomes can be administered intramuscularly, exploiting the slow release of the encapsulated antigens and their passive accumulation in the regional lymph nodes. Cationic surfactant based systems show potential not only in gene delivery (the purpose they were developed), but also in initiating an enhanced and diverse immune response. As it was mentioned previously, a formalin inactivated Hepatitis A virus particle containing liposomal vaccine is already licensed for clinical use. A virosome-based trivalent influenza vaccine has proven to be highly immunogenic and well tolerated in patients, and is available now on the market. This vaccine delivery system is comprised of spherical unilamellar vesicles with a diameter of about 150 nm. Other vaccines, against tetanus, diphtheria, *E. coli* infections are in clinical trials.
3. Liposomes in the human therapy

Table 15. Liposomal pharmaceutical preparations available or in clinical trials (CTs). AMD: age-related macular degeneration; PM: pathologic myopia; OHS: ocular histoplasmosis syndrome.

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Active ingredient</th>
<th>Type of formulation</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocet</td>
<td>Doxorubicin</td>
<td>Non-PEGylated liposome</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>Doxyl (USA), Caelyx (EU)</td>
<td>Doxorubicin</td>
<td>PEGylated liposome</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>Lipo-Dox</td>
<td>Doxorubicin</td>
<td>PEGylated liposome</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>Daunoxome</td>
<td>Daunorubicin</td>
<td>Non-PEGylated liposome</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>DepoCyt</td>
<td>Cytarabine</td>
<td>Multivesicular liposomes (administered intrathecally)</td>
<td>Lymphomatous meningitis</td>
</tr>
<tr>
<td>Ambisome</td>
<td>Amphotericin B</td>
<td>Non-PEGylated liposome</td>
<td>Leishmaniasis, fungal infections</td>
</tr>
<tr>
<td>Visudyne</td>
<td>Verteporfin</td>
<td>Non-PEGylated liposome</td>
<td>AMD, PM, OHS</td>
</tr>
<tr>
<td>DepoDur</td>
<td>Morphine sulfate</td>
<td>Multivesicular liposomes (administered epidurally)</td>
<td>Post-surgical pain</td>
</tr>
<tr>
<td>LipoPlatin (in CTs)</td>
<td>Cisplatin</td>
<td>PEGylated liposome</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>Onco-TCS (in CTs)</td>
<td>Vincristine</td>
<td>Non-PEGylated liposome</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>ThermoDox (in CTs)</td>
<td>Doxorubicin</td>
<td>Temperature-sensitive liposomes</td>
<td>Anticancer drug</td>
</tr>
<tr>
<td>Arikace (in CTs)</td>
<td>Amikacin</td>
<td>Non-PEGylated liposome (nebulized)</td>
<td>Lung infections</td>
</tr>
<tr>
<td>Epaxal</td>
<td>Inactivated Hepatitis A virus</td>
<td>virosome</td>
<td>Vaccination</td>
</tr>
<tr>
<td>Inflexal V</td>
<td>Influenza subunit</td>
<td>virosome</td>
<td>Vaccination</td>
</tr>
</tbody>
</table>

In Table 15 we have summarized the currently available liposomal pharmaceutical preparations and some formulas being investigated in clinical trials. As has been shown, using liposomes in cancer treatment is a major application of these delivery systems. Liposomes are expected to reduce cytotoxicity of anticancer agents in healthy tissues, because they accumulate in the tumor due to the increased permeability of vessels in neoplastic sites. Inasmuch as
liposomal antitumor preparations stay in circulation for extended periods of time and their content is released slowly, the frequency of administration is lowered. These benefits, and other believed potentials inspire researchers to keep on developing liposomal drug formulation.

4. Other nanoparticles in biomedicine

Nanoscience is definitely one of the most dominant disciplines in the 21st century. Nanotechnology has become part of our lives in numerous areas, including entertainment, information technology, nutrition and many others. In the few pages we have for this topic, we try to set out a very limited overview on nanoparticles being developed for medical applications, without attempting to be comprehensive.

4.1. Fullerenes

Fullerenes, the third allotrope of carbon, were discovered in 1985. Due to their unique physical and chemical properties they have been intensively studied over the past 25 years. For medical purposes, mainly the so-called Buckminsterfullerene or buckyballs are the focus. This is an icosahedral structure containing 60 carbon atoms. The potential utilization of these nanoparticles is believed to be drug delivery. Studies have been investigating paclitaxel-embedded buckysomes, which are to be designed similarly to Abraxane, the US Food and Drug Administration (FDA)-approved drug, which contains an albumin-bound form of paclitaxel with a mean particle size of approximately 130 nanometers. Functionalized fullerenes are believed to become functional MRI contrast agents; and C_{60} derivatives might constitute antioxidant compounds useful in biological systems. At present, these experiments are limited to in vitro and in vivo pre-clinical studies only, and have not reached the stage of clinical trials. Experts optimistically look forward to the future, however some papers have generated intense discussions on the safety and toxicity aspects of these nanomaterials.
4.2. Quantum Dot

Quantum dots are luminescent semiconductor nanocrystals. These particles are novel candidates in the field of molecular diagnostics and nanotherapeutics. These inorganic fluorescent nanocrystals typically comprise periodic groups of II–VI (e.g. CdSe and CdTe) or III–V (e.g. InP and InAs) semiconductor materials. The first application of quantum dots in biology was in highly sensitive cellular imaging in vitro, due to their brightness and stability. Later researchers recognized that, the high surface-to-volume ratio of quantum dots enables the construction of a "smart" multifunctional nanoplatform, where the quantum dots acts not only as an imaging agent but also a nanoscaffold catering to therapeutic and diagnostic (theranostic) modalities (Figure 26.).

As evident, quantum dot-based theranostics also need some further time to reach the clinical level.

**Figure 26.** Schematic illustration of a multimodal quantum dot.

Upon interacting with the target cell, the cell-penetrating ligand can be exposed, supporting the multifunctional quantum dot to enter the cell. Stimuli-sensitive antennae may be triggered by environmental stimuli (pH, temperature, or ultrasound), allowing subsequent intracellular release of the drug from the drug-loaded vesicle. The quantum dot itself serves as an imaging agent and also the carrier of other agents.
4.3. Miscellaneous

- Nanoshells: consist of a dielectric core (mostly silica) covered by an ultrathin metal shell, which is typically composed of gold. These particles can be designed to absorb or scatter light and are easily conjugated to antibodies and other biomolecules. Their potential biomedical applications include targeted drug delivery, photothermal cancer therapy, and imaging contrast agents.

- Nanobubbles: micelles made of detergents and lipids. They accumulate in tumoral tissues because of the elevated permeability of the endothelium. The nanobubbles create considerable head and pressure in the surroundings of the cancer cells, due to the coalescence caused by external stimuli, for example ultrasound or laser impulse. These impacts result in cell death and tumor destruction.

- Nanotubes: cylindrical tubes of graphite or fullerene with diameters on the order of 1 nm and a length up to a few millimeters. These particles are being developed for targeted therapeutic and diagnostic purposes especially in tumors. A possible risk to balk the application of these materials in humans is the general damaging effects of long fibres in the pleural mesothelium. Toxicological experiments are being processed.

- Some of the nanoparticles we have not mentioned yet are: nanopores, paramagnetic nanoparticles, nanosomes, dendrimers, and so on.
References

OPHTHALMOLOGICAL DRUG DELIVERY SYSTEMS

Introduction

The eye is a unique organ, both anatomically and physiologically, and contains several structures with independent physiological functions. For example, the cornea and the lens are the only tissues in the body besides cartilage that have no blood supply. The eye has the most differentiated tissue, the layer with the richest blood supply of the human and it has a special optical system, too. These systems allow for sight, which is one of our most important perceptions. Several diseases can cause serious disorders of this organ which can lead to blindness. The complexity of the eye provides unique challenges to drug delivery systems.

The Ebers-papyrus from around 1550 B.C. mentions saliva and urine as effective ophthalmological drugs.

Galenus was engaged in the structure of the eye and treating of its disorders. His theories and works helped in the development of treating ophthalmological diseases for a long time.

Much, like Hieronymus, famous ophthalmologists in e 16th century Europe, built their ophthalmological therapy on Arabic bases.

The world’s first universities of ophthalmology were founded in Budapest and Vienna in 1801.

The 20th century caused a significant breakthrough in the development of ophthalmological therapy. In the middle of the century, factory-made sterile ophthalmological solutions, suspensions, creams and ointments appeared.

The number of ophthalmological products and the length of their application period have been continually increasing since then.

1. Anatomical and physiological bases

The human eye is an organ which gives us the sense of sight, allowing us to observe and learn more about the surrounding world than we do with any of other four senses. Our organ of sight consists of three parts (Fig. 27): 1. the eye ball, 2. the optic nerve (an intraocular portion, an intraorbital portion and an intracranial portion), 3. additional organs: extraocular muscles, protective structures (eyelids, conjunctiva), lacrimal system (lacrimal gland, superior and inferior lacrimal punctum, lacrimal canaliculus, lacrimal sac, nasolacrimal duct) (Fig. 28) (17, 24).
The spherical, paired eyeball’s weight is 7.5 g; its diameter is 24 mm, and it is specialized for sense of sight. The bulb is located in the closed, bony orbital cavity.

We can differentiate three layers of the globe from outside to inside (Fig. 27.):

1. Fibrous layer which consists of the cornea and sclera.
2. The uveal tract which consists of three parts: iris, ciliary body and choroid.
3. The retina which has two parts: neuroretina (photoreceptive part) and retinal pigment epithelium.

The cornea is the anterior part of the eyeball. The cornea’s curvature is greater than the sclera’s curvature. It connects into the sclera like a watch-glass with a shallow sulcus (the limbus of the cornea) marking the junction of the two structures. The cornea is a transparent layer. The cornea is the most important refractive medium in the eye, it has 42 diopters. The
The normal average diameter of the adult cornea is between 11 and 12 mm. The thickness of the cornea is about 0.52 mm in the central part and 0.67 in the margin. It consists of five parts (Fig. 29.) from outside to inside: 1. epithelium (the surface of the cornea is formed by stratified non-keratinized squamous cells), 2. Bowman’s layer (this layer cannot regenerate, it has an important role in processing corneal scars), 3. stroma (it consists of many regular lamellae of collagen fibrils which can provide the transparency of the cornea), 4. Descemet’s membrane (the basement membrane of the endothelial cells), 5. endothelium (one layer does not regenerate).

The cornea is an avascular tissue, but it contains a lot of sensory nerves, which end in the epithelium layer. Therefore, injuries of the cornea expose sensory nerve endings and cause intense pain with reflective tearing and involuntary eye closing. The cornea has an important role in focusing the entered light.

![Cornea Diagram](image)

**Figure 29.** The cornea

The sclera and the cornea form the rigid outer covering of the eye. The sclera is the fibrous, whitish-opaque part of the eye, and consists of nearly acellular connective tissue with higher water content than the cornea. The sclera contains blood vessels and nerves.

The iris is the anterior part of the uveal tract. The color of the iris varies in the individual according to the melanin content of the pigment cells. The iris contains pupillae muscles. These muscles regulate the contraction and dilation of the pupil, so that the pupil can function as the aperture of the optical system of the eye.

The ciliary body extends from the root of the iris to the ora serrata, where it joins the choroid. The ciliary muscle is responsible for accommodation. Numerous ciliary processes extend into the posterior chamber of the eye. The suspensory ligaments, as known as zonules, extend
from the ciliary body and they are located between the ciliary processes and the lens capsule. A double-layered epithelium covering the ciliary body produces the aqueous humor.

The **choroid** is highly vascularized tissue which contains a vessel layer with large blood vessels and a capillary layer. The blood flow through the choroid is the highest in the entire body. It supplies a part of the retina.

The **retina** is the innermost of three layers of the globe. It consists of two parts: a photoreceptive part (consisting of the first nine of the 10 layers), and a nonreceptive part. The retina has two types of photoreceptors, the rods and cones. There are about 120 million rods, and they allow twilight and night vision. They are about 500 times more photosensitive than the cones. There are about 7 million cones in the macula which are responsible for daytime vision, resolution and color perception.

The **macula lutea** is a flattened oval area in the center of the retina. The avascular fovea centralis is located in the center of the macula lutea. This is the point at which visual perception is the sharpest. The fovea centralis contains only cones (no rods). Light stimuli in this region can directly activate the sensory cells. The intraocular portion of the optic nerve is visible on ophthalmoscopic examination as the optic disc. All the retinal nerve fibers merge into the optic nerve here. The complete absence of photoreceptors at this site creates a gap in the visual fields known as the blind spot.

**Inside of the globe we can also find the followings:**

1. The **anterior chamber** is bordered by the endothelium layer of the cornea, the trabecular meshwork, the anterior part of the iris, and the lens in the area of the pupil (here it gets connected with the posterior chamber). The anterior chamber is filled with clear aqueous humor.

2. The **posterior chamber** is bordered by the posterior surface of the iris, the ciliary body, the anterior surface of the lens and the zonules. The aqueous humor that is produced gets here first.

3. The trabecular meshwork is bordered by the corneo-scleral border and the iris root. It is loose sponge-like avascular tissue. It is responsible for draining out of the aqueous humor. The aqueous humor can flow out from the anterior chamber through two ways:
a. the trabecular meshwork, which receives about 85% of the outflow, and b. an uveoscleral vascular system which receives about 15% of the outflow.

The aqueous humor is formed by the ciliary processes and secreted into the posterior chamber. Then the aqueous humor passes through the pupil into the anterior chamber and flows out through the trabecular meshwork. About 1-2% of the aqueous humor is replaced each minute. It is responsible for the intraocular pressure and supplying the cornea with oxygen and nutrition.

If the outflow of the aqueous humor is obstructed, the intraocular pressure is elevated. The normal intraocular pressure is between 12 and 21 mmHg. The elevated intraocular pressure can be a sign of glaucoma. The term glaucoma covers several diseases, with different etiologies, that share the common finding of optic neuropathy with characteristic pathologic findings in the optic nerve head and a specific pattern of visual field defects. The disease is often associated with increased intraocular pressure. The final stage of glaucoma is blindness. Primary glaucoma refers to glaucoma that is not caused by other ocular disorders. Secondary glaucoma may occur as the result of another ocular disorder or as an undesired side effect of medication or other treatment. Simplex glaucoma is the more common type of the primary glaucoma. Primary open angle glaucoma begins in middle-aged and elderly patients with minimal symptoms that progressively worsen. The angle of the anterior chamber characteristically remains open throughout the clinical course of the disorder. Primary open angle glaucoma often does not show typical symptoms for years. The intraocular pressure is usually between 24-30 mmHg. In the case of primary angle closure glaucoma there is an acute episodic increase in intraocular pressure to several times the normal value due to sudden blockage of drainage. A typical glaucoma attack occurs unilaterally due to widening of the pupil either in dark surroundings or under emotional stress. During an acute glaucoma attack the intraocular pressure often is above 50 mmHg.

4. The lens is the other important refractive media (besides the cornea) of the eye and focuses incident rays of light on the retina. The refractive power of the lens is about 20 diopters. It is a biconvex, transparent structure. The lens lies in the posterior chamber of the eye between the posterior chamber and the vitreous body. Radially arranged zonule fibers that insert into the lens around its equator connect the lens to the ciliary
body. These fibers hold the lens in position and transfer the tensile force of the ciliary muscle.

Contraction of the ring-shaped ciliary muscle decreases the tension in the zonule fibers. The lens can approach a spherical shape. This change in the curvature of the lens increases the refractive power; the focus of the eye shifts to the near field and close objects appear on sharp contour. When the ciliary muscle relaxes, the tension on the lens increases and the lens flattens. The resulting decrease in refractive power shifts the focus of the eye into the distance and distant objects appear on sharp contours.

The lens is nourished by diffusion from the aqueous humor. The epithelium of the lens helps to maintain the ion equilibrium and permit transportation of nutrients, minerals and water into the lens. The transportation permits active transport of sodium, potassium, calcium and amino acids from the aqueous humor into the lens. Maintaining this homeostasis is essential for the transparency of the lens and is related to the water balance. The water content of the lens is normally stable and in equilibrium with the surrounding aqueous humor. The water content of the lens decreases with age, whereas the content of insoluble lens protein increases. The lens becomes harder, less elastic, and less transparent. A decrease in the transparency of the lens with age (known as cataracts) is unavoidable.

Refraction is defined as the ratio of the refractive power of the refractive media (cornea and lens) to the axial length of the globe.

In case of emmetropia (normal sight) the ratio of axial length of the eye to the refractive power of the refractive media is balanced. Parallel light rays enter into the eye and meet at a focal point on the retina. Ametropia is a mismatch between the axial length of the eye and the refractive power of the lens and cornea. The most common disorders are myopia (nearsightedness), hyperopia (farsightedness) and astigmatism.

Myopia is a discrepancy between the refractive power and axial length of the eye such that parallel incident light rays converge at a focal point anterior to the retina; the refractive power of the eye is elevated. If we put minus lens (concave lens) in front of the eye, the refractive power becomes weaker and the light rays converge on the retina. In hyperopia, there is a discrepancy between the refractive power and axial length of the eye such that parallel incident light rays converge at a focal point posterior of the retina. If we put plus lens (convex
lens) in front of the eye, the refractive power become stronger and the parallel lights converge on the retina.

Astigmatism means lack of focal point. This disorder is characterized by a curvature anomaly of the refractive media such that the parallel incident light rays do not converge at a point but are drawn apart to form a line. This disorder can be corrected with cylinder lenses.

Presbyopia is physiologic loss of accommodation in advancing age. The eye’s refractive power must alter to allow visualization of both near and distant objects with sharp contours. This accommodation is made possible by the elasticity of the lens. It begins when the range of accommodation falls below three diopters. Presbyopia can be compensated with converging lenses. We can correct the refractive failures with glasses or contact lenses. The contact lenses can be soft or hard lenses. Later the imprinted contact lenses can play an important role such as ocular drug delivery systems.

5. The *vitreous body* stabilizes the globe. The gelatinous vitreous body consists of 98% water and 2% collagen and hyaluronic acid. It fills the vitreous chamber which accounts for two-thirds of the total volume of the eye. The hyaluronic acid molecules fill the three-dimensional collagen fiber network and provide mechanical stability. The vitreous body contains neither blood vessels nor nerves.

*The tear film* that moistens the conjunctiva and cornea is composed of three layers:

a.) the outer oily layer (approximately 0.1 µm thick) is a product of the meibomian glands and the sebaceous glands and sweat glands of the margin of the eyelid. The primary function of this layer is to stabilize the tear film. It has hydrophobic properties therefore it prevents rapid evaporation like a layer of wax.

b.) The middle water layer (approximately 8 µm thick) is produced by the lacrimal gland and the accessory lacrimal glands. Its task is to clean the surface of the eye and to provide mobility of the palpebral conjunctiva over the cornea and a smooth corneal surface for high quality optical images.

c.) The inner mucin layer (approximately 0.8 µm thick) is secreted by the goblet cells of the conjunctiva and the lacrimal gland. It is hydrophilic with respect to the microvilli of the corneal epithelium, which also helps to stabilize the tear film. This layer prevents the watery layer from forming beads on the cornea and provides the watery layer moistens the entire surface of the cornea and conjunctiva. Lysozyme, beta-lysin, lactoferrin, and gamma-globulin (IgA) are tear-specific proteins that give the tear fluid antimicrobial characteristics.
The windshield wiper motion of the eyelids moves the tear fluid medially across the eye toward the medial canthus. The superior and inferior puncta lacrimales collect the tears, which then drain through the superior and inferior lacrimal canaliculi into the lacrimal sac. From there they pass through the nasolacrimal duct into the inferior concha (Fig. 28.).

Tear production is continuously reduced with aging. Keratoconjunctivitis sicca as a result of dry eyes is one of the most common eye problems between the ages of 40 and 50. As a result of hormonal changes in menopause, women are more frequently affected than men. Depending on the severity of findings (burning, reddened eyes, lacrimation), artificial tear solutions in varying viscosities are prescribed.

It seems from the above mentioned that the diseases of the eye, especially the intraocular disorders, are difficult targets for drug delivery systems. However, all diseases of the whole eye need to be made available and treatable via the different drug delivery systems.

2. Ocular drug delivery systems

The main object of clinical therapeutics is to provide and maintain an adequate concentration of drugs at the site of action. Furthermore, the release of drug can be constant, or cyclic or triggered by the environment or a chemical signal, and the drug delivering polymer can be broken down naturally by the body when it is no longer necessary.

The anatomy, physiology, and biochemistry of the eye render this organ highly impervious to foreign substances. A significant challenge to the researcher is to circumvent the protective barriers of the eye without causing permanent tissue damage. In addition, drug binding to tear proteins and to conjunctival mucin also inactivates a portion of the administered drug (25). Other factors that can limit the usefulness of topical drug application include the rapid and extensive precorneal loss that occurs as a result of drainage and tear fluid turnover (27). The most commonly applied drugs are the ocular solutions and ointments. Before applying the drugs the patients has to look up while the lower eyelid is pulled downward close the anterior margin. After instillation, the eye needs to be closed for about one minute so that the the drug can be most effective.

A large portion of the drug is wasted due to administration of an excess volume and as other large portions enters the nasolacrimal duct toward the nasal cavity (Fig. 28.). Following the removal of the excess solution from the front of the eye, a second mechanism of clearance prevails. The eye has an efficient system for tear turnover (1 µl/min). The two clearance mechanisms result in a biphasic profile for an instilled solution.
with a rapid initial clearance phase due to removal of excess fluid followed by a slower second phase due to tear turnover (9, 11).

It has been estimated that typically less than 5% of a topically applied drug permeates the cornea and reaches intraocular tissues. A significant portion of the instilled dose is absorbed systemically by the conjunctiva and significant systemic absorption also occurs when the solution enters the nasolacrimal duct and is absorbed by the nasal and nasopharyngeal mucosa (11). Two features, which render the cornea an effective barrier to drug absorption, are its small surface area and its relative impermeability. In contrast, the area of conjunctiva is significantly bigger and it is also between 2 and 30 times more permeable to drugs than the cornea (26). Therefore, following topical administration to the pre-ocular area, conjunctival drug absorption is an important loss factor that competes with corneal absorption (18). The drugs which enter systemic circulation by above mentioned ways can cause several side effects.

The main route for intraocular absorption is across the cornea. In addition the sclera has also been shown to have a high permeability, for example for a series of beta-blocking drugs.

Schematically, the cornea is a sandwich comprising a hydrophilic layer, the stroma, between two lipophilic layers, the epithelium, and the endothelium. The hydrophilic-lipophilic nature of the cornea clearly indicates that to be well absorbed, active ingredients have to exhibit to some extent both lipophilic and hydrophilic properties. Drugs acting on tear secretion, physicochemical status of the tear film, and blinking can modify transcorneal drug permeation. Indeed, a major issue is the ratio of precorneal disappearance/transcorneal penetration (25).

Four approaches may be used to deliver drugs to the eye: topical, systemic, intraocular and periocular (including subconjunctival, sub-Tenon’s, parabulbar, retrobulbar) (11).

We can talk about the eye penetration of systemically administered drugs, mostly anti-infectious and anti-inflammatory drugs. There are blood-eye barriers which are the most important. Aqueous humor is produced by the ciliary epithelium in the ciliary processes. It is frequently named as ultrafiltration, since the ciliary epithelium prevents the passage of large molecules, plasma proteins, and some antibiotics. Some molecules can be secreted in aqueous humor during its formation. Inflammation associated with injury, infection, or an ocular disease, e.g., uveitis, disrupts the blood-aqueous humor barrier and drugs enter the aqueous humor and reach the tissues of the anterior segment. There is a blood-retina barrier and there is one between blood and vitreous humor, complicated by the high
viscosity of the vitreous humor, which prevents diffusion of the drugs in the posterior part of the eye. Delivery of drugs to the posterior pole and to the retina is very difficult. Topical administration is usually preferred over systemic administration so as to avoid systemic toxicity, for rapid onset of action, and for decreasing the required dose. This applied method is the most convenient for the patient. However, bioavailability of the topical administered drugs is as low as 10%. We suppose that a correlation exists between the concentration of a drug at its intended site of action and the resulting pharmacological effect.

*Three main factors have to be considered when drug delivery to the intraocular tissue is attempted:*

a.) how to cross the blood-eye barrier (systemic to ocular) or cornea (external to ocular) to reach the site of action,

b.) how to localize the pharmacodynamic action at the eye and minimize drug action on other tissues,

c.) how to prolong the duration of drug action so the frequency of drug administration can be reduced (1).

**Formulation approaches to improve ocular bioavailability:**

Various approaches can be divided into two groups. The first is based on use of the drug delivery systems, which provide the controlled and continuous delivery of ophthalmic drugs. The second involves, minimizing precorneal drug loss.

*Some examples for the first case:*

1. To provide sustained and controlled drug delivery.
2. To increase ocular bioavailability of the drug by increasing corneal contact time.
3. To provide targeting within the ocular globes so as to prevent loss to other ocular sites.
4. To circumvent the protective barriers like drainage, lacrimation and diversion of exogenous chemicals into the systemic circulation by conjunctiva.
5. To provide comfort and compliance to the patient and improve the therapeutic performance of the drug over conventional systems.
So, the two main approaches attempted are improvement in bioavailability and controlled release drug delivery.

Recently there are many ophthalmic drug delivery systems available. These are classified as conventional, and newer drug delivery systems. Topically applied ocular drugs have to reach the inner parts of the eye, and transcorneal penetration is believed to be the major route for drug absorption. Corneal absorption is a much slower process than elimination. The sum of $K$ loss (first order elimination rate) and $K$ absorption (first order absorption rate) constants control the fraction of the applied dose absorbed into the eye. So the ocular bioavailability can be increased by decreasing $K$ loss or by increasing $K$ absorption. The former can be achieved by modifying the ocular dosage forms by formulating ocular dosage forms containing lipophilic prodrugs or by adding penetration enhancers. Therefore to optimize topical ocular drug delivery system, prolonged contact time with the corneal surface and better penetration through the cornea is necessary.

3. Enhancement in bioavailability of ocular drugs

3.1. Improvement of viscosity with viscous solutions and gels

The bioavailability of ocular drugs increases with prolonging precorneal residence time. Viscous solutions and hydrogels, based on the addition of hydrocolloids to simpler aqueous solutions, are the most common formulations. There is no clear-cut frontier between very viscous solutions and gels in terms of biopharmaceutical results. The most common polymers used in viscous solutions are cellulose derivatives, carbomers, polysaccharides, polyalcohol, polyacrylic acid and, recently, hyaluronic acid (25). Sodium carboxy methyl cellulose is one of the most important mucoadhesion polymers. Hyaluronic acid offers a biocompatible and biodegradable matrix for fabrication of ocular sustained release dosage form. Recently, hydrophilic polymers continue to be used in the production of ophthalmic drugs, which contribute to patient comfort and they are responsible for bioadhesion rather than viscosity enhancement. It offers several advantages like localizing a dosage form within a particular region, increasing drug bioavailability, promoting contact with surface for longer time, and reducing dosage frequency. Several synthetic and natural polymers are used for this purpose like sodium hyaluronate, chondroitin sulphate (natural polymers) and various polyacrylate, carbopol (synthetic polymers) (25).
Gel formation is an extreme case of viscosity enhancement through the use of viscosity enhancers, which allows the dosing frequency to be decreased to once a day. This has encouraged researchers to work on formulations that would be solutions while in the drug vials but would gel in the conjunctival cul-the-sac. Three main mechanisms have been explored to induce the sol-gel transition in the conjunctival pouch, namely a change in pH, a change in temperature, or a change in ionic environment (25). If the trigger is a change in pH, the low viscosity polymeric dispersion in water undergoes spontaneous coagulation and gelation after instillation in the conjunctival cul-de-sac. In the method of formation of gels from the solution is triggered by the temperature change. Sustained drug delivery can be achieved by the use of a polymer that changes from solution to gel at the temperature of the eye. But the disadvantage of this is characterized by high polymer concentration (25% Poloxamers). If the trigger is the change in ionic strength, there is an example called Gelrite. Gelrite is a polysaccharide, which forms a clear gel in the presence of mono or divalent cations. The concentration of sodium in human tears is 2.6 g/l, which is particularly suitable to cause gelation of the material when topically installed into the conjunctival sac (8, 22).

The high viscosity of the gel results in blurring of vision and malted eyelids which substantially reduce patient acceptability.

Penetration enhancers increase corneal uptake by modifying the integrity of the corneal epithelium. There are chelating agents, preservatives and surfactants. But their usefulness is diminished due to local toxicity associated with enhancers.

Prodrugs enhance corneal drug permeability through modification of the hydrophilic or lipophilic nature of the drug. The methods includes modification of chemical structure of the drug molecule, therefore making it selective, site specific, and a safe ocular drug delivery system (2, 23).

Cyclodextrins act as carriers by keeping the hydrophobic drug molecules in solution and delivering them to the surface of the biological membrane, where the relatively lipophilic membrane has a much lower affinity for the hydrophilic cyclodextrine molecules, therefore, they remain in the aqueous vehicle system. Chitosan is a bioadhesive vehicle suitable for ophthalmic formulation since it exhibits general biological properties such as biodegradability, non-toxicity and biocompatibility.
3.2. *Oil in water emulsions*

Phospholipids were used as the emulsifiers. Oil in water emulsion is useful for delivery of water insoluble drugs, which is solubilized in the internal oil phase.

3.3. *Colloidal particles*

The potential use of polymeric colloidal particles as ophthalmic drug delivery systems started in the late 1970’s. They could not enter commercial development because of various issues, like local toxicity, non-biodegradable polymer, and large scale sterilization. Dispersed systems based on liposomes, nanoparticles or nanocapsules have been extensively studied for potential ophthalmic use (12, 19). The retention of these particles in the conjunctival sac is a key consideration. This retention must be effective in providing an extended source of active drug and to allow the drug to leak out from the dispersed phase before the instilled formulation is drained away from the precorneal area.

3.4. *Liposomes*

The use of liposomes as a topically administered ocular drug delivery system began in the early stage of research in ophthalmic drug delivery. The results were favorable for lipophilic drugs and not for hydrophilic drugs. It was concluded that liposomes must be suitable for ocular drug delivery, provided, they had an affinity for and were able to bind to ocular surfaces, and release contents at optimal rates. Positively charged liposomes have a greater affinity to corneal epithelium which is thinly coated with negatively charged mucin. So they can increase both precorneal drug retention and drug bioavailability.

3.5. *Nanoparticles*

Nanoparticles provide sustained release and prolonged therapeutic activity when retained in the cul-de-sac after topical administration. They are solid, colloidal particles consisting of macromolecular substances that vary in size from 10 nm to 1000 nm. The drug of interest is dissolved, entrapped, adsorbed, attached or encapsulated into the nanoparticle matrix. Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a polymer membrane, whereas nanospheres are a matrix system in which the drug is physically and uniformly dispersed. The utility of nanoparticles as an ocular drug delivery system may depend on a.) optimizing lipophilic-hydrophilic properties of the polymer-drug system, b.)
optimizing rates of biodegradation in the precorneal pocket, and c.) increasing retention
efficiency in the precorneal pocket. It is desirable to formulate the particles with bioadhesive
materials in order to enhance the retention time of the particles in the conjunctival cul-de-sac.
Without bioadhesion, nanoparticles could be eliminated as quickly as aqueous solutions from
the precorneal site. Bioadhesive systems can be either polymeric solutions or particulate
systems (13, 14) Chitozan coated nanocapsules improve the bioavailability.
Polymer nanoparticles are devoid of any irritant effect of cornea, iris or conjunctiva and
therefore appear to be a suitable inert carrier for ophthalmic drug delivery (25).
Nanoparticles represent promising drug carriers for ophthalmic applications. Smaller particles
are better tolerated by patients than larger particles; therefore, nanoparticles may represent
very comfortable prolonged action ophthalmic delivery systems. The major developmental
issues in the case of nanoparticles include formulation stability, particle size uniformity,
control of drug release rate, and large-scale manufacture of sterile preparations (20).
Nanosystems having surface-segregated chitosan or polyethylene-glycol have been found to
be relatively stable and also efficient at overcoming mucosal barriers (3).
Nanoparticles made of non-biodegradable polymers are neither digested by enzymes nor
degraded in vivo through a chemical pathway (16). However, the biodegradable properties are
required in most cases (4). Most commonly used polymers are poly-alkyl-cyanoacrylates,
poly S-caprolactone and poly lactic-co-glycolic acid, which undergo hydrolysis in tears.
Nanoparticles as an ophthalmic drug delivery have been demonstrated for both hydrophilic
and hydrophobic drugs.
Erodible, biodegradable systems have an inherent advantage over other systems in that the
self-eroding process of the hydrolysable polymer obviates the need for their removal or
retrieval after the drug is delivered. A few examples of reported polymers for successful
preparation of nanoparticle are described as follows: a.) Polymethylmethacrylate (PMMA)
does not degrade either biologically or enzymatically, which makes them less attractive for
ophthalmic use. b.) Cellulose acetate phthalate is an emulsification of polymer in organic
solvent followed by solvent evaporation. This suspension, upon coming in contact with the
lacrimal fluid at pH7.2-7.4, gels in situ, thus averting rapid washout of the instilled solution
from the eye. The disadvantage of these preparations is vision blurring. c.) PACA (polyacryl-
cyanoacrylat) particles possess properties of biodegradation and bioadhesion. They are able to
adhere to the corneal and conjunctival surfaces, which represent their mucoadhesion property.
This polymer has the ability to entangle in the mucin matrix and form a noncovalent or ionic
binding with the mucin layer of the conjunctiva. PACA nanoparticles and nanocapsules have
been shown to improve and prolong the corneal penetration of hydrophilic and lipophilic drugs. Despite these positive results, the potential of the PACA nanoparticles is limited because they cause disruption to the corneal epithelium cell membrane. d.) PECL (poly-caprolactone) nanoparticles yielded the highest pharmacological effect. This was believed to be due to the agglomeration of these nanoparticles in the conjunctival sac. PECL nanocapsules also showed good performance in increasing the ocular availability of drugs such as metipranolol and betaxolol while suppressing their systemic absorption. More specifically these nanocapsules have been shown to increase ocular penetration of lipophilic drugs (metipranolol, betaxolol, amphotericin-B). The PECL did not cause any damage in the corneal epithelium cell membrane (6). e.) The bioavailability of nanoparticles coated with poly-l-lysine and chitosan (both have positive charge) were compared to that of noncoated nanoparticles. It was suggested that the specific nature of chitosan was responsible for bioavailability improvement rather than the charge. Chitosan-coated nanocapsules were more efficient at enhancing the intraocular penetration of some specific drugs (7, 10). f.) Eudragit®Retard polymer nanoparticles suspensions have been investigated as a carrier system for the ophthalmic release of nonsteroidal anti-inflammatory drugs (e.g. ibuprofen). This suspension is prepared from inert polymer resins. When loaded with drugs, these resins are proposed as delivery system to prolong the release and improve ocular availability of the drug. g.) Acyclovir-loaded PECA nanospheres prepared by emulsion polymerization technique showed increased drug levels in the aqueous humor compared to the free drug suspension in the rabbits.

3.6. Inserts

The first solid insert was described in 1948 in British Pharmacopoeia. It was an atropine containing gelatin wafer. Soluble inserts consists of all monolytic polymeric devices that at the end of their release, the device dissolve or erode. Soluble ophthalmic drug inserts are a soluble copolymer of acrylarnide, N-vinyl pyrrolidone and ethyl acrylate. It is a sterile thin film or wafer of oval shape. The system softens in 10-15 sec after introduction into the upper conjunctival sac, and gradually dissolves within 1 hour, while releasing the drug. These systems have the drawback blurred vision while the polymer is dissolving. A water soluble bioadhesive component in its formulation has been developed to decrease the risk of expulsion and provide prolonged residence in the eye, when combined with a controlled drug release. They are bioadhesive ophthalmic drug inserts. Due to difficulty with-self-insertion
and foreign body sensation, only a few insert products are listed, and pharmaceutical manufacturers are not actively developing inserts for commercialization.

We know two inserts which are more common products as drug delivery systems. There are Ocusert and Lacrisert. Ocusert is an insoluble delicate sandwich technology that is filled with sufficient pilocarpine for 7 days’ use, whereas Lacrisert is a soluble minirod of hydroxypropil cellulose non-medicated and dissolving within 24 h to treat dry-eye syndrome (21). The disadvantage of non-biodegradable systems is that they do not eliminate by naturally ways so they have to be removed from the eye after use. The biodegradable systems can adapt to physiologic conditions and they have less irritant effects (5).

Ophthalmic inserts (ocuserts) have been reported using alginate salts, modified collagen, and silicone elastomer based matrix that allows for the controlled release of an active ingredient over a period of at least 2 weeks. Other inserts are more like implants to be placed in the eye tissues by surgery.

3.7. Implantable systems

These systems are less popular as they require minor surgery. For example, there is an ocular implant for delivering gancyclovir for the treatment of cytomegalovirus. This delivers drug directly to the retina for over 5 months.

3.8. Minidisc

Minidisc is a controlled release monolithic matrix type device consisting of a contoured disc with a convex front and a concave back surface. They can be made hydrophilic and hydrophobic to permit extended release of both water soluble and water insoluble drugs. This system provides an effective therapy for viral retinitis. In this case a 3 mm diameter disc (Vitrasert) is placed into the vitreous body from which the gancyclovir can release continuously and for a prolonged length of time.

3.9. Soft contact lenses

The rationale for corneal contact devices has not been fully explored in therapy. It is generally accepted that soft contact lenses can act as a reservoir for drugs, providing improved release of the therapeutic agent. Imprinted soft contact lenses are promising drug devices able to provide greater and more sustained drug concentrations in tear fluid with lower doses than conventional eye drops. The most widely used material is poly-2-hydroxymethylmethacrylate.
Its copolymers are used both to correct vision and deliver drugs. Controlled release can be obtained by binding the active ingredient via biodegradable covalent linkages.

3.10. **Collagen shields**

Collagen shields were developed to promote wound healing and perhaps more importantly to deliver a variety of medications to the cornea and other ocular tissues. The shields are rehydrated in aqueous solutions of the drug whereby the drug is absorbed by the protein matrix and is released once the shield dissolves in the eye. Their size renders them impractical for a new drug delivery system. Suspensions of collagen micro particulates may be better accepted (15).

They are manufactured from porcine sclera tissue, which bears a collagen composition similar to that of human cornea. They are hydrated before being placed on the eye. Shields are not fully transparent and therefore reduce visual acuity. But they are appropriate delivery systems for both hydrophilic and hydrophobic drugs with poor penetration properties.

Among these drug delivery systems, only a few products have been commercialized. An ideal system should have effective drug concentration at the target tissue for an extended period of time with minimum systemic effect. Patient acceptance is very important for the design of any comfortable ophthalmic drug delivery system. Major improvements are required in each system like improvement in sustained drug release, large scale manufacturing, and stability. Combination of drug delivery systems could open a new directive for improving the therapeutic response of a non-efficacious system.
References


MEDICATIONS IN PREGNANCY AND LACTATION

1. Medication therapy in pregnancy

The use of medication during pregnancy is one of the least-developed areas of clinical pharmacology and drug research, however almost every gravid woman is exposed to some type of medication during pregnancy.

At the beginning of the 20th century the society had a misbelief that the fetus in utero was safe and could not be harmed by anything. Since the Contergan (thalidomide) disaster, treatment with drugs during pregnancy has been a reason for concern. Potential teratogenic risks of treatment, and potential risks for the mother of no treatment, have to be evaluated each time drug therapy is considered during pregnancy (1, 2).

Teratogens

A teratogenic, fetotoxic or embryotoxic agent is one that has the possibility to do harm to a developing embryo or fetus as a result of prenatal exposure. The most dramatic of these effects are the alteration in growth, the functional abnormalities, and structural malformation trait of teratogens. The baseline prevalence of congenital malformations is estimated to be 2-3% of all birth, and only 10% may be causally associated with prescription drug use (3). Established teratogens include chemicals (e.g., alcohol, mercury), viruses (e.g., rubeola, cytomegalovirus), environmental factors (e.g., irradiation, hyperthermia), and therapeutic drugs (e.g., isotretionin, inhibitors of the renin-angiotensin system, and warfarin) (1). Most of the drugs reach the fetus by the maternal bloodstream.

Therefore embryonic and fetal exposure depends on quite a few critical factors, such as:
- gestational age,
- route of administration,
- absorption of the drug,
- the dose of the drug,
- serum levels of the mother, and
- the maternal and placental clearance (1).
An agent is accepted as a human teratogen if it meets several criteria, such as:

- confirmed exposure at critical times during human development,
- constant dysmorphic findings recognized in well-conducted epidemiologic studies,
- specific defects or syndromes associated consequently with specific teratogens,
- rare anatomic defects related to environmental exposure, and
- proven teratogenicity in experimental animal models.

Physicians routinely use, almost solely, the FDA classification to take a position on whether to initiate, continue, discontinue, or replace a medication (*Table 16*).

**Table 16**: Drug Classification System by the U.S. Food and Drug Administration (1)

<table>
<thead>
<tr>
<th>FDA Category</th>
<th>Pregnancy Category Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>According to well-controlled studies no risks to humans have been reported.</td>
</tr>
<tr>
<td>B</td>
<td>Any risks in humans unlikely to occur. In animal studies, no evidence of harm to the fetus has been noticed, but there are not enough adequate and well-controlled studies in pregnant women.</td>
</tr>
<tr>
<td>C</td>
<td>Risks cannot be excluded in humans. Adverse effects have occurred in animal studies but there are no adequate studies in humans. or There are no appropriate data in animal studies or in humans.</td>
</tr>
<tr>
<td>D</td>
<td>There is clear evidence of human fetal risk, but the benefits from use in pregnant women may outweigh the risks (e.g. in a life-threatening situation).</td>
</tr>
<tr>
<td>X</td>
<td>Drugs contraindicated in women who are or may be pregnant.</td>
</tr>
</tbody>
</table>

A teratogen can cause birth defect if it acts during critical periods of embryonic or fetal development; therefore, teratogenic medications can either induce embryopathy or fetopathy. Human gestation is divided into three periods from the perspective of teratogenesis. These are the preimplantation (fertilization to implantation), the embryonic (second through ninth week), and the fetal (ninth week to term) periods. The preimplantation period is conventionally viewed as a gestational window characterized by an „all or nothing” phenomenon. This means that, injury to a huge number of cells during the early embryo development will inevitably cause the loss of the embryo. If only a small number of cells are damaged, the phenomenon of compensation can defend the embryo and ensure survival without malformation.

During organogenesis (2 to 8 weeks of gestation), when each system has a specifically vulnerable period, a teratogenic agent can cause severe malformations (e.g., the heart is mostly
affected if the teratogen acts between 6.5 weeks to 8 weeks post-conception). However, the fetus can also be disturbed by alterations in structure and function of the organs that have developed in a normal way during embryogenesis (1).

**Placental transport**

The placenta needs a high rate of metabolism to be capable to multiple transport and biosynthesis activities. Thus, sometimes its oxygen and glucose consumption is faster than in the fetus.

The most important function of the placenta is the transport of oxygen and nutrients to the fetus and the reverse transfer of CO₂, urea, and other catabolites back to the mother. In principle, those compounds that are indispensable to the minute-by-minute homeostasis of the fetus (e.g., oxygen, water, sodium, CO₂ are transported very rapidly by simple diffusion). Substances that are essential for the synthesis of new tissues (e.g., enzyme cofactors such as vitamins, amino acids) are transported by an active process. Compounds that are at the upper limits of permissible molecular size (such as some maternal hormones, which may modify fetal growth) may diffuse very slowly, whereas proteins (such as IgG immunoglobulins) possibly reach the fetus by pinocytosis.

This transfer takes place by at least 5 mechanisms:

- simple diffusion,
- facilitated diffusion,
- active transport,
- pinocytosis, and
- leakage (4).

**Placental transport of drugs**

The placental passage to the embryo (or fetus) is necessary for a medicine to exercise its teratogenic effect.

In turn, placental transfer depends intensely on:

- the metabolism of the mother,
- gestational age,
- protein binding and storage,
- charge,
- liposolubility of the drug, and
- molecular size.

The placental membranes are often mentioned as a „barrier“ to fetal transfer, but there are only few compounds (e.g., drugs) that cannot cross the membranes at all. The molecular weight of a substance is an important regulatory factor of its transplacental passage. Compounds with a very large molecular size or charge like heparin and insulin can hardly get across the placenta. This lack of transfer is almost exceptional among medications. Most of the drugs, with mass below 500 Da, are transferred from the maternal to the fetal circulation by simple diffusion, the rate of which is regulated by the concentration gradients of the medicines (1, 4).

Diffusion gradients are influenced by several serum factors, including the degree of drug-protein binding. Serum albumin concentration is significantly lower during gestation. Thus, those drugs that bind almost solely to plasma albumin (e.g., salicylates, warfarin), will have relatively higher unbound concentrations and, therefore, an effectively higher placental gradient (By contrast, carbon monoxide can attach itself so strongly to the increased total hemoglobin that there will be little left in the plasma for transport.)

Chemicals and drugs that are highly soluble in lipids are transferred much more easily across the placental barrier than are water-soluble molecules. Ionized drug molecules are exceedingly water soluble and are consequently poorly transmitted across the placenta. Ionization of chemicals depends partially on their pH-pK relationships, so there are multiple factors that determine this „simple diffusion“ of drugs across the placenta (4).

Recent studies have exemplified the importance of placental drug transport proteins, such as P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRPs) in reducing fetal exposure to drugs and toxins. These transport proteins that are members of the ATP-binding cassette (ABC), drug efflux proteins, are presented on both the microvillus brush border and basal membranes of the syncytiotrophoblast cells. They have recently been identified as playing an important part in facilitated and active transport. Over the last decade, the role of Pgp and BCRP in mediating drug transfer across the placenta and in the normal development and physiological function of the placenta has been investigated. In the genes encoding Pgp and BCRP, several single nucleotide polymorphisms (SNPs) have been described and are expected to have causality with altered protein expression, transporter activity and function in the placenta (Fig. 30.). The impact of these genotypes on fetal exposure and altered placental physiology or
development is also demonstrated. An understanding of this genotype-phenotype relationship will allow for prediction of susceptible or auspicious genotypes in order to personalize medication choices to minimize fetal exposure to teratogens, or to maximize the efficacy of the pharmacological therapy to the fetus (5).
physiologic changes induced by pregnancy is necessary to understand coincidental disease processes that can threaten women during the gestation period and the puerperium. Nutritional requirements, including those for vitamins and minerals, are increased during pregnancy, and several maternal changes occur to meet this demand. Most of the mother’s food intake is greater due to the increased appetite, but some women have a decreased appetite or experience nausea and vomiting. These symptoms may have an association with the relative levels of human chorionic gonadotropin (hCG), estrogen and progesterone. Several changes may be seen in the oral cavity during pregnancy. Pregnant women are predisposed to gingivitis and gingival hyperplasia due to the increased capillary permeability caused by the elevated circulating estrogen. The severity of these hormone-mediated inflammatory oral changes can be prevented or reduced with good oral hygiene. Swallowing difficulty associated with nausea may induce increased salivation. Changes in composition of saliva can also be noticed; these include a decrease in sodium and pH, and an increase in potassium, protein, and estrogen levels. (The risk potential of preterm labor is suggested to be determined with a screening test of estrogen levels, because salivary estrogen levels are higher in the women destined to have preterm babies compared to women having normal term deliveries.) Recent evidence suggests that the reduced gastrointestinal motility during pregnancy is due to elevated estrogen concentrations. In this way constipation may occur, because the transit time of food through the gastrointestinal tract can be slowed so significantly that more water than normal is reabsorbed. Production of the hormone gastrin increases materially. As a result of an increased intragastric pressure, decreased stomach pH, decreased esophageal peristalsis, slower gastric emptying time, and dilatation or relaxation of the cardiac sphincter, reflux may occur. Gastric reflux is more prevalent in later pregnancy due to elevation of the stomach by the enlarged uterus. These conditions may stimulate hiatal hernia and can also lead to heartburn. During pregnancy, gallbladder function is also changed because of hypotonia of the smooth muscle wall. Emptying time is slowed and emptying is sometimes incomplete, thus bile stasis may lead to gallstone formation. Functional alterations appear in the liver during normal pregnancy. A decrease in plasma albumin and a slight decrease in plasma globulins normally occur during gestation, while serum alkaline phosphatase activity can be doubled. The alterations in renal function that occur in gestation are probably related to increased maternal and placental hormones, including adrenocorticotropic hormone (ACTH),
antidiuretic hormone (ADH), aldosterone, cortisol, human chorionic somatomammotropin (hCS), and thyroid hormone. The glomerular filtration rate (GFR) increases during gestation by about 50%, while the renal plasma flow (RPF) rate increases by as much as 25-50% (due to the generalized increase in blood volume). Other changes such as increased frequency of urination, biochemical changes in the urine and blood, urinary stasis, and urinary tract infections can be noticed during this period.

Probably the most shocking physiologic alteration that pregnant women live through is the above mentioned increase in blood volume. The average increase in volume at term is 45-50%. In addition, women have to reckon with other cardiovascular changes such as increase in cardiac output, a decrease in blood pressure, and the potential occurrence of the supine hypotensive syndrome.

The increase in red blood cell mass is about 33%; therefore, the need for iron for the production of hemoglobin naturally increases. If iron is not promptly available, the fetus uses iron from maternal stores.

The total blood leukocyte count can increase significantly during normal pregnancy. Some studies have reported an apparent increase in the production of platelets and in the levels of several essential coagulation factors, too.

Dyspnea, hyperventilation, alterations in the oxygen intake and reserve, and an increase in both the tidal volume and minute ventilation rate are the main changes in the respiratory system that occur during pregnancy to accommodate the increasing size of the developing fetus and the maternal-fetal oxygen requirements (4, 6, 7, 8).
THE MOST COMMON DISEASES AND THEIR THERAPY DURING PREGNANCY

1. Gastrointestinal disorders

Nausea and vomiting of pregnancy (NVP)
Nausea and vomiting are common gastrointestinal complaints during the first half of pregnancy. These symptoms typically commence between the first and second missed menstrual period and persist until about 14 to 16 weeks. In most cases pregnancy-induced nausea and vomiting occurs in the morning, but they may remain throughout the day. The complete genesis of these disorders is not clear, but probably high levels of serum hCG and thyroxine may stand on the background. In some women vomiting becomes so severe that weight loss, dehydration, electrolyte and acid-base disturbances, and starvation ketosis may occur.

As an essential part of the treatment, proper eating habits should be recommended. Having small meals (that are low in fats and rich in carbohydrates) at more frequent intervals but stopping short of satiation is of value. Using vitamin B₆ as a first-line pharmacotherapy, which is safe and effective, should be ordered. Dopamine antagonists, such as promethazine (PIPOLPHEN) or metoclopramide (CERUCAL) are allowed for use during pregnancy, and have a high efficacy and a low risk of malformations occurring. Dimenhydrinate (DAEDALON) proved to be safe during early pregnancy, but because of its potential to stimulate uterine contractions it should be avoided in the third trimester.

Constipation

Constipation is common during pregnancy. The reasons can be the prolonged transit time, the compression of the lower bowel by the uterus, or the muscular relaxation of the colon which is accompanied by increased absorption of water and sodium. Some medicines, like the iron derivative (MALTOFER), that are ordinarily used during gestation, may cause such symptoms.

Constipation can be prevented with sufficient quantities of fluid, high-fiber diet and reasonable amounts of daily exercise. If it is necessary, osmotic laxatives (such as lactulose: DUPHALAC) or Suppositorium Glycerini can be ordered, because they are effective and are safe during pregnancy. Stimulant laxatives such as senna (TISASEN) and bisacodyl
(STADALAX, LAXBENE) are considered low risk for short term use, but long-term use is not suggested because hyponatremia, hypokalemia, dehydration may occur. Castor oil should be avoided because it can cause uterine contraction and even rupture.

**Diarrhea**

In most cases diarrhea is caused by several viruses, bacteriums, parasites, but food allergy, taking some kind of medicines (such as antibiotics), irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) can also cause diarrhea. Beside antibiotic therapy the treatment for symptom relief includes activated carbon (CARBO ACTIVATUS). Loperamide (IMODIUM, LOPEDİUM) and diphenoxylate (+atropine: REASEC) are deemed low risk, but can be used with discretion.

**GERD**

Heartburn, also called pyrosis, is reported in 30-50% of pregnancies. The retrosternal burning sensation is caused by esophagitis from gastroesophageal reflux related to the slower emptying time and dilatation or relaxation of the cardiac sphincter. Increased stomach volume and decreased stomach pH are also related to heartburn.

When lifestyle and dietary modifications (and raising the head of the bed) are insufficient, medical therapy should be considered. In animal studies, antacids that contain magnesium, aluminum, or calcium compounds (ANACID, ANTAGEL) proved not to have teratogenic effects. Since sucralfate (VENTER) is a non-absorbable drug and exerts a local rather than systemic effect, it is considered low risk during pregnancy. If severe symptoms persist, histamine-2-receptor blockers (H2RA) are chosen from which ranitidine (ZANTAC, ULCERAN) is deemed to be safe. Omeprazole (OMEP, LOSEC) is a drug of choice for reflux esophagitis in pregnancy and also appears not to be dangerous for the fetus. For other indications proton pump inhibitors (PPI) should be second choice drugs in gestation.

**Hemorrhoids**

Varicosities of the rectal veins occur frequently during pregnancy. Their development or aggravation in gestation is related to constipation and the increased pressure in rectal veins below the level of the enlarged uterus. Topically applied anesthetics (lidocaine: DOXIPROCT OM), warm soaks, and stool-softening agents can effectively relieve pain and swelling (4, 6, 7, 9).
2. Hematological disorders

**Thromboembolism**

Venous thromboembolism (VTE) is one of the leading causes of maternal mortality. Changes in the coagulation system, such as an increase in several procoagulant factors, reduction in endogenous anticoagulant activity, and suppression of fibrinolysis are main parts of the early physiological adaptations of pregnancy. Thus, pregnant women are at risk of thrombosis, which begins in the first trimester and continues until at least 6 weeks post-partum.

Since coumarin anticoagulants (warfarin: MARFARIN) can cause significant adverse teratogenic and fetal effects, their using should be avoided during pregnancy. Heparin – especially low-molecular-weight heparin (LMWH), such as dalteparin (FRAGMIN) - would be the good choice instead, because they are large enough not to cross the placenta and are not associated with congenital malformations. Urokinase (RHEOTROMB) can also be used safely during gestation.

**Varicosities**

Generally, these enlarged veins result from congenital predisposition and are exaggerated by pregnancy because of the increasing femoral venous pressure. As the first step, conservative therapy is used; this involves periodic rest with elevation of the legs, elastic stockings, or both. Calcium dobesilate and oxerutin (VENORUTON) can be applied topically while diosmin and hesperidin (DETRALEX) is suggested as oral medication (6, 10).

**Anemias**

Anemia is one of the most common diseases in obstetrics. The Centers for Disease Control and Prevention (1990) defined anemia as hemoglobin concentration less than 11g/dL in the first and third trimesters and less than 10,5g/dL in the second trimester (6). The slight, physiological fall in hemoglobin levels during pregnancy is caused by a comparatively greater expansion of plasma volume compared with the increase in red cell volume.

**Iron-deficiency anemia**

The main cause of anemia in gestation is iron-deficiency. In a normal singleton pregnancy, the need for iron averages close to 800 mg & 300 mg for the fetus and placenta respectively, and 500 mg would be ideal for maternal hemoglobin mass expansion. In most cases the iron stores of women cannot meet the demands, which results in anemia. However, hemoglobin production in the fetus is not impaired, because the placenta obtains iron even from the
severely anemic mother. With simple iron compounds (ferrous sulfate, ferrous fumarate: TARDYFERON, AKTIFERRIN, FEROglobin-B12), that provide about 200 mg daily of elemental iron, the restitution of iron stores can be accomplished.

**Folic Acid Deficiency**

In most cases, megaloblastic anemia beginning during pregnancy can be explained with folic acid deficiency. In addition, the role of folate deficiency in the genesis of neural-tube defects has been proven.

Folic acid requirement in non-pregnant women is 50 to 100μg/day, and increases to 400 μg/day during gestation. Pregnancy-induced megaloblastic anemia -that is usually coupled with iron-deficiency anemia- should be treated with folic acid (HUMA-FOLACID), nutritious diet, and iron (+folic acid: NEO FERRO FOLGAMMA) (6, 11).

**3. Hypertensive disorders**

Hypertension is a very common disorder that occurs in 5-7% of all pregnancies. Moreover, due to the estimates of the World Health Organization, over 100,000 women die from preeclampsia each year.

The most comprehensible, useful and widely accepted classification system of hypertension in pregnancy is based on the system elaborated by Davey and MacGillivray.

- Gestational hypertension
  - Gestational hypertension (without proteinuria)
  - Gestational proteinuria (without hypertension)
  - Preeclampsia (proteinuria and hypertension exists)

- Preexisting hypertension and/or renal disease
  - Chronic hypertension (without proteinuria)
  - Chronic renal disease (proteinuria and/or hypertension exists)
  - Chronic hypertension with superimposed preeclampsia

- Unclassified hypertension and proteinuria (12)

Pregnancies with severe hypertension are at increased risk of intrauterine growth retardation, placental abruption and preterm delivery. In these cases medication therapy is essential.
Methyldopa (DOPEGYT) is traditionally the most commonly used antihypertensive drug, whose long-term safety for mother and fetus (and in the long-term follow-up of the infants) has been adequately assessed. This compound is a central α-adrenergic agonist that inhibits vasoconstricting impulses from the medulla oblongata. It reduces total peripheral resistance without causing physiologically remarkable changes in heart rate or cardiac output. The most frequently reported side effects are sedation – that can be unbearable- and postural hypotension.

Calcium channel blockers cause direct arteriolar vasodilation by selective inhibition of slow inward calcium channels in vascular smooth muscle. The best-studied calcium antagonists during pregnancy are nifedipine (CORDAFLEX) and verapamil (ISOPTIN, CHINOPAMIL R). For the treatment of hypertension or cardiac arrhythmias (in the second and third trimester) they are the preferred first-line drugs in the group of calcium channel blockers. In recent years, nifedipine (CORDAFLEX) has gained popularity in the treatment of chronic hypertension in pregnancy. Use of this agent is considered safe, but cumulative evidence is not extensive enough for unequivocal statements. The principal side effect is headache, which can be very severe at the beginning of the treatment.

Dihydralazine (DEPRESSAN) is an arteriolar vasodilator that causes a secondary baroreceptor-mediated sympathetic response, increasing heart rate and cardiac output. It is usually used with a diuretic, methyldopa, or a beta blocker to minimize the undesired side effects, however, use of multiple agents is not recommended during pregnancy. In acute hypertensive crisis, it is used intravenously.

Beta blockers (such as propranolol: HUMA-PRONOL and metoprolol: BETALOC, EGILOK), which have been in long-term use, are widespread in the treatment of hypertension in pregnancy. Nevertheless, their use requires thoughtful risk-benefit analysis, because they have been associated with several fetal and neonatal complications. Angiotensin-converting enzyme (ACE) inhibitors (such as captopril: ACEOMEL, TENSIOMIN; ramipril: TRITACE, MERAMYL, RAMACE, perindopril: COVEREX, PRENESSA, ARMIX) are assessed to be fetotoxic, because of producing fetal hypocalvaria and renal defects. Angiotensin-II receptor antagonists (such as losartan: PORTIRON, TERVALON; valsartan: DIOVAN; irbesartan: APROVEL; telmisartan: MICARDIS, PRITOR) are contraindicated throughout pregnancy. Their use is only acceptable when all other treatment regiments have been ineffective. A detailed ultrasound diagnosis is advisable if exposure has occurred in the first trimester. Diuretics may reduce uteroplacental perfusion, and therefore, should also be avoided in pregnancy (7, 12, 13).
4. Diabetes mellitus

Pregnancy is a carbohydrate-intolerant state, which is poorly developed among pregnant women. Significant metabolic changes are necessary to provide proper energy delivery to the growing fetus. In all pregnancies, the rising circulating concentration of cortisol and human placental lactogen (HPL) lower glucose levels, promote fat deposition, and stimulate appetite while insulin resistance increases as the pregnancy advances. Because of this resistance the need for insulin grows gradually, especially in the second half of pregnancy. If insulin secretion is reduced due to several reasons, the rising insulin resistance may leads to hyperglycemia and GDM.

In the classification, diabetes (even type 1 or 2) existing before pregnancy (named pregestational diabetes) have to be distinguished from gestation diabetes mellitus (GDM). Patients with GDM had any degree of glucose intolerance at the beginning of pregnancy; it comes to light during gestation. In most cases glucose regulation will return to normal after delivery. To ensure normal levels of glycosylated hemoglobin before and during pregnancy, careful preconceptual analysis and counseling is needed. To accomplish this well-organized, high-quality care for patients with GDM multidisciplinary teamwork (including diabetologist, obstetrician, diabetes specialist nurse, dietitian, midwife and neonatologist) is required. The patient must take part actively (frequent home glucose monitoring, thoughtful control of diet, and stabilization of exercise) in the care of herself and her fetus to achieve and maintain near normal blood glucose.

Due to the increased risk of neural tube defects in a diabetic pregnancy, a higher dose of folic acid (5 mg HUMA-FOLACID) supplementation is necessary at the beginning of gestation. All patients should be taught self-monitoring of blood glucose because of the continuously changing glucose tolerance, and should receive dietary advice as well. Moderate physical activity is recommended to be included in every-day life. Patients with GDM are seen monthly until 32 weeks gestation, then twice a month until the 36th week and then weekly till term. Most practitioners initiate insulin therapy in women with gestational diabetes if dietary therapy fails to achieve the target glucose values (<8mmol/l: 1h post-prandially and <7 mmol/l: 2h post-prandially). Bolus-insulin given with meals (ACTRAPID, HUMULIN R) and basal-insulin given at bedtimes (INSULATARD, HUMULIN N) is more effective than twice-daily insulin regimens. The use of insulin by continuous infusion pump (CSII) should be considered if control is still not adequate. Oral hypoglycemic agents should be avoided for safety reasons (4, 14, 15).
5. Seizure disorders

Approximately three to five per 1000 pregnancies are complicated with epilepsy (17). It is one of the most serious and common neurological disorders encountered in pregnant women. Epilepsy can cause different alterations in fetal development and can affect the course of gestation, labor, and delivery. Generalized tonic-clonic seizures during pregnancy can cause inter alia maternal and fetal hypoxia and acidosis, fetal intracranial hemorrhage, miscarriage, and stillbirth. Seizures of different types can cause trauma, which can lead to ruptured fetal membranes or abruption of the placenta. Some studies demonstrated that children, whose mother had seizures during pregnancy, had increased risk for cognitive dysfunction, and for experiencing seizures in their own life.

Several anticonvulsant medications have incontestable teratogenic effects (such as microcephaly, cognitive dysfunction, intrauterine growth retardation, congenital heart disease, cleft lip/palate, neural tube defects, and urogenital defects). On the other hand, pregnancy can aggravate epilepsy by modifying the metabolism of these anticonvulsants. Thus, the risks associated with drug exposure to the fetus and newborn and the risks incurred by seizures need to be well balanced.

Because of the importance of drug exposure during the first gestational week, appropriate counseling is essential for women with epilepsy who plan to become pregnant. If the patient is asymptomatic for a few years, the need for continued antiepileptic drugs (AED) should be reconsidered by the physician. The withdrawal of AEDs should be tried long before conception. For those with active epilepsy, treatment with AEDs should employ monotherapy at the lowest effective dose – by continuous measuring of the drugs plasma concentration- to control the occurrence of seizures.

As pregnancy progresses, it can alter the pharmacokinetics of AEDs at all levels, (absorption, distribution, metabolism and elimination), resulting in declining plasma concentrations of the anticonvulsants. The most important mechanisms are the increased renal elimination and the increased metabolic clearance due to enzyme induction. In addition, albumin concentrations fall significantly during the first half of gestation. The total plasma concentrations of highly protein bound AEDs – such as phenytoin (DIPHEDAN), valproate (CONVULEX, DEPAKINE), carbamazepine (NEUROTOP, TEGRETOL, STAZEPINE) and phenobarbital (SEVENAL) - may decline due to decreased binding to plasma proteins. But since the unbound and pharmacologically active concentration of the drug remains relatively unchanged, it would be better to monitor the free concentrations instead of the total
concentrations of these drugs so as not to increase dose unnecessarily. Carbamazepine or phenytoin monotherapy may be used in pregnancy, but for women treated with valproic acid it should be changed for another anticonvulsant with less teratogenic potential. Different studies reported that lamotrigine (GEROLAMIC, LAMICTAL, LAMOLEP) and oxcarbazepine (TRILEPTAL) are the two AEDs with the most noticeable gestational-related pharmacokinetic changes. Increase in seizures among patients using lamotrigine has frequently been associated with a drop in the drug’s plasma concentrations during pregnancy. For an exact assessment, more clinical results would be needed about gestational use of new AEDs such as vigabatrin (SABRIL), gabapentin (GORDIUS, NEURONTIN), pregabalin (LYRICA), felbamate (TALOXA), topiramate (ETOPRO), and levetiracetam (KEPPRA) (6, 17, 18).

6. Antibacterial medication

Antibiotics are among the most commonly prescribed medications during pregnancy because treatment of infections is critical to the health of a mother and her fetus. Infection in early pregnancy represents one of the most important reasons for abortion, while contagions in the second and third trimester are the principal cause for premature membrane rupture, premature delivery and the resultant complications in the newborn child. In the event of a serious infectious disease of the mother, there is no contraindication for antibiotic treatment during pregnancy, because the ailment may cause much more harm—even on the fetus—than the drug itself.

Penicillins (such as amoxicillin: AMOXICILLIN-B, AKTIL, AUGMENTIN, ampicillin: SEMICILLIN, UNASYN) constitute the oldest group of effective antibiotics and are considered to be first-line antibiotics for use during pregnancy. They are capable of passing through placental barrier and reaching the fetus, but penicillins have practically no toxicity in humans at therapeutic doses. As side-effects associated with penicillin are quite rare, in fact, maternal allergy to penicillin represents almost the only potential therapeutic problem with this group of antibiotics.

Cephalosporins (such as ceftriaxone: LENDACIN, cefixime: SUPRAX, ceftibuten: CEDAX) are another first-line antibiotics used in gestation. They can cross the placenta and can reach
bactericidal concentrations in the amniotic fluids. In principle, more established cephalosporins should be given priority. In mothers treated with second- and third-generation cephalosporins, immune hemolytic reactions have been observed and in some cases, oversensitivity reactions (such as skin rash and anaphylactic shock) have also appeared. Hemolysis, bone marrow disorders, increase in transaminase levels and thrombophlebitis at the injection site may also occur. Nevertheless, based on the available data, cephalosporins at therapeutic doses—that should be well-controlled because of their increased clearance during pregnancy—are considered not to be teratogenic.

Macrolides (such as erythromycin: MEROMYCIN, ERYTHROTROP; spiramycin: ROVAMYCINE; roxithromycin: RULID; clarithromycin: KLACID, FROMILID; azithromycin: SUMAMED) may be used in gestation in case of penicillin allergy or if mandated by the bacterial spectrum. Very low levels of macrolides are able to reach the fetus due to their relatively large size. Erythromycin estolate (ERYTHROTROP) should not be administered in the second half of pregnancy because of its hepatotoxicity. Clarithromycin should be used with caution because of teratogenic effects, and cardiovascular defects have occurred in animals. In the early stages of pregnancy, spiramycin is the drug of choice for the treatment of toxoplasmosis.

Lincomamines (such as clindamycin: DALACIN) may have no teratogenic effects, but they should only be used in the event that penicillins, cephalosporins and macrolides are ineffective due to potential maternal side effects (diarrhea, pseudomembranous colitis).

Aminoglycosides (such as tobramycin: BRAMITOB, gentamicin: GENTAMICIN, amikacin: LIKACIN) have potential ototoxic and nephrotoxic effects, therefore they should generally not be used during the first 4 months of pregnancy.

Use of sulfonamides (sulfamethoxazole: SUMETROLIM) should be avoided before birth because it can cause kernicterus due to the displacement of bilirubin in the newborn.

Tetracyclines (doxycycline: DOXITIDIN, lymecycline: TETRALYSAL, oxytetracycline: TETRACYCLINE WOLFF, tigecycline: TYGACIL) can get across the placental barrier and can accumulate in developing long tubular bones, teeth and eye lines. As a result, they can cause tooth discoloration and growth inhibition of the long tubular bones as well as cataracta.
Several reports have mentioned a link between the use of tetracyclines and lethal liver damage in the mothers. Administration of this group of antibiotics is contraindicated after the 16\textsuperscript{th} week of pregnancy.

Quinolones (such as ofloxacin: TARIVID, ciprofloxacin: CIPROBAY, norfloxacin: NOLICIN, levofloxacin: TAVANIC, moxifloxacin: AVELOX) causes severe cartilage damage in animals, but in humans, irreversible joint cartilage defects have not been detected among prepartially exposed children (7, 19, 20, 21).

7. Pain relief and lower fewer

Pregnant women should not suffer unnecessarily from pain, because it can result in depression and anxiety, which can have an adverse effect on her pregnancy. Common analgesics such as paracetamol, aspirin, and non-steroidal anti-inflammatory drugs (NSAIDs) are relatively safe, if they are used appropriately.

Paracetamol (PANADOL, PARAMAX RAPID) is one of the most commonly used analgesic and antipyretic drugs in pregnancy. It can cross the placenta in its unconjugated form, but in case of short-term use in therapeutic doses, it does not seem to increase the risk of birth defects or other adverse pregnancy outcomes. Some studies found that acetaminophen may damage the fetal liver, but it is clinically not proven.

Aspirin (ASPIRIN, KALMOPYRIN) is usually used to treat mild pain and fever, but some obstetricians also prescribe low-dose aspirin to reduce the risk of adverse outcomes in pregnant women with antiphospholipid syndrome and recurrent miscarriages. Use of aspirin should be avoided in the first few weeks of gestation, because it may obstruct the implantation as a result of effects on the prostaglandin pathway. In the third trimester, taking this drug is contraindicated as it can cause premature closure of the fetal ductus arteriosus and persistent pulmonary hypertension. Due to the inhibition of thromboxane synthesis, serious fetal and maternal hemorrhage may occur during childbirth.

NSAIDs – including ibuprofen (ADVIL ULTRA, ALGOFLEX), naproxen (ALEVE, APRANAX), and indomethacin (INDOMETACINUM) – are inhibitors of cyclo-oxygenase,
that is a potent dilator of the ductus arteriosus and pulmonary resistance vessels in the fetus and newborn. Therefore, NSAIDs used in the first and third trimester can cause similar adverse effects in pregnancy like aspirin (problems with implantation, premature closure of the fetal ductus arteriosus, pulmonary hypertension). High doses of NSAIDs after 30 weeks gestation may also reduce perfusion of the fetal kidneys and decrease fetal urine output, so their use should be avoided in this period (7, 22, 23).
MEDICATION THERAPY IN LACTATION

Approximately 96% of women are physiologically able to breastfeed under normal conditions, and most of them initiate breastfeeding after the birth of their infant. Women on medication often choose formula feeding or not taking their drug therapy because they fear of exposing their child to the medication through the breast milk. Although most medications are considered compatible with nursing, it would be useful to make a case by case risk assessment before the mother initiates breastfeeding or drug therapy (24).

1. General principles of lactation and medication during breastfeeding

Benefits of breastfeeding
Breastfeeding is the optimal form of infant feeding for the first months of neonates’ life, because human milk provides species- and age-specific nutrients for the infant. Moreover, breast milk contains immunological factors, antibacterial properties, and factors that act as biological signals for promoting cellular growth and differentiation. Due to investigations, breastfed infants have a lower risk of developing necrotizing enterocolitis, gastrointestinal ailments, otitis media, sudden infant death syndrome (SIDS), insulin dependent diabetes mellitus, lower respiratory tract infections, respiratory syncytial virus infection, and allergies. One of the most significant benefits of nursing is the higher cognitive function of breastfed children. The benefits increase with the duration and exclusivity of breastfeeding. Women also have advantage from nursing their babies. Breastfeeding increases maternal levels of oxytocin, that can decrease postpartum bleeding and quicken uterine involution. Reduced risk of ovarian and pre-menopausal breast cancer is also a considerable benefit of breastfeeding. Certainly, it is the best way to increase maternal-infant bonding, and maternal sense of fulfillment and self-worth (24).

Endocrinology of Lactation
Complex humoral and neuronal mechanisms are involved in lactation. Estrogen, progesterone, and placental lactogen, as well as prolactin, insulin, and cortisol appear to have a role in stimulating the growth and development of the milk-secreting apparatus of the mammary gland (Fig. 31.). After delivery, the levels of progesterone and estrogen decrease abruptly and significantly, which cease the inhibitory influence of progesterone on prolactins lactation
stimulating effects, and the production of α-lactalbumin (in which prolactin also play a particular role). The increased α-lactalbumin stimulates lactose synthase and increase milk lactose. Oxytocin, which is secreted by neurohypophysis in a pulsatile fashion, stimulates milk expression from a lactating breast by causing contraction of myoepithelial cells in the alveoli and small milk ducts. Milk ejection is provoked by suckling (which stimulates neurohypophysis to liberate oxytocin) and can be inhibited by fright or stress (4, 6).

![Diagram of mammary gland and neurohypophysis](image)

**Figure 31.** Emptying of the mammary gland

*Drug transport into human milk*

The transport of compounds into breast milk is determined by several factors such as ionization, molecular weight, plasma protein binding, lipophilicity of the drug, and its pharmacokinetics in the mother. Generally, cationic drugs with low molecular weight, high lipophilicity and low plasma protein binding can be easily excreted into milk. This phenomenon is explained with the biochemical characteristics of milk including lower pH and higher lipid contents compared to plasma (24).

The transfer of drugs across the mammary alveolar cells is thought to be carried out in several ways. The most common route is transcellular diffusion for small molecules. Compounds of a larger size might get into the milk through intercellular diffusion, thereby avoiding the
Another process of transport is ionophore diffusion that might facilitate the transfer of charged ions. Other substances, in turn, may be bound to carrier proteins, such as organic cation and anion transporters, nucleoside transporters, and multidrug resistance associated proteins.

Breast milk is slightly acidic (average pH 7.1) as compared to plasma (average pH 7.4), so the drugs acid/base characteristics are very important because of the phenomenon of ion trapping. To explain this, take for example penicillins and NSAIDs as acidic drugs that will favor ionization in the relatively alkaline plasma and, thus, the transport into milk will be decreased. In this case, plasma is considered to be the ‘ion trap’ of acidic drugs. The reverse is true for basic drugs, such as β-blockers, so they accumulate in milk (25, 26).

**Figure 32.** Alveolar cell from lactating mammary gland

*N: nucleus, GJ: gap junction, SV: secretory vesicle, BM: basement membrane, ME: cross section through process of myoepithelial cell, RER: rough endoplasmic reticulum, MFG: milk fat globule*
Drug elimination systems in the infant

Besides the parameters of the drug and the dose in the mother’s serum, and the amount in the milk, the characteristics of the infant are crucially important. The infant’s ability to absorb, metabolize, and excrete the drug are depend on its age and maturity.

Due to the immaturity of drug elimination systems, clearance values are lower in neonates and young infants; consequently, they are exposed to higher amount of drugs.

Renal excretion of drugs is determined by glomerular filtration, and net tubular secretion. Glomerular filtration rate (GFR) of full-term neonates at birth is very low, and adult values are achieved by 3-5 months of age. The maturation of tubular function appears to be much slower than GFR’s.

Big differences can occur among the developmental profile of the drug metabolizing enzyme families. Take for example CYP3A7 enzyme that mediates the metabolism of endogenous steroids and xenobiotics. This enzyme is expressed mainly in fetal liver, but after birth, CYP3A4 replaces it, reaching 40% of the adult level after 1 month. CYP1A2 needs about 3 postnatal months to emerge, on the other hand, CYP2D6 and 2E1 expressions in liver increases within hours of birth (7, 24).

Determination of drug exposure in the infants

The maternal plasma concentration of the drugs significantly determines how much drug is available for getting into the milk. Diffusion occurs along a diffusion gradient, thus, high maternal serum levels of the medicine will produce high concentrations in the milk. Milk-to-plasma/serum concentration ratio (MP or MS ratio), which means the ratio between the drug’s existence in milk and in maternal plasma/serum, can also be influenced by the mother’s metabolizing ability (‘poor/slow’ or ‘normal’) or other maternal pharmacokinetics and compositional changes of milk. The drug exposure level of the breastfed infant can be expressed by the following equation:

$$\text{Exposure index} (\%) = \frac{100 \times MP \times A}{CL_i}$$

A= average milk intake (150ml/kg/day=0,1ml/kg/min)

MP= milk to plasma ratio

CL_i= the rate of drug clearance by the infant

This index becomes very high at a low infant drug clearance, due to the hyperbolic correlation, so it is essential to be aware of the infant’s pharmacokinetics.
The clinical dose of medication that the infant receives can be estimated with another equation:

\[(D_{\text{inf}}) = \text{drug concentration in milk (mg/L)} \times 0.15 \text{L/kg/day}\]

The relative infant dose (RID), which provides an estimate of the weight-normalized dose relative to the mother’s dose, is more meaningful for the clinician. It can be calculated with the formula below:

\[\text{RID} = \frac{\text{Dose}_{\text{infant}} \left( \frac{\text{mg}}{\text{kg} \cdot \text{day}} \right)}{\text{Dose}_{\text{mother}} \left( \frac{\text{mg}}{\text{kg} \cdot \text{day}} \right)}\]

Dose\_infant = dose in infant/day
Dose\_mother = dose in mother/day (24, 27)

2. Drug application during lactation

2.1. Drugs acting on the gastrointestinal tract

**Antacids.** Calcium-, magnesium- and aluminum-compounds and their complexes (TISACID, ANACID, and MAALOX) are available to treat heartburn. Their recommended doses can be used safely during lactation because the systemic absorption of antacids is negligible. Sodium bicarbonate is absorbed systemically and could cause alkalosis, so it should be avoided. Sucralfate (VENTER) inhibits pepsin activity and can be used for ulcer prophylaxis. It is compatible during breast-feeding, because only very small amounts of the drug are absorbed systemically.

**Antisecretory agents.** The histamine H2 antagonists -such as famotidine (QUAMATAEL, MOTIDIN), nizatidine (NAXIDIN), and ranitidine (ZANTAC, ULCERAN)- and the proton pump inhibitors -esomeprazole (NEXIUM), lansoprazole (PROTONEXA, REFLUXON), omeprazole (LOSEC), pantoprazole (CONTROLOC, NOLPAZA), and rabeprazole (PARIET, GELBRA, ACILESOL)- are used for heartburn and GERD. The histamine antagonists can be given during breast-feeding without anxiety – especially those with low concentration in breast-milk (for example, nizatidine)-, but the prolonged use of PPIs’ should be avoided because of their carcinogenic and mutagenic properties.
**Laxatives.** If a change in dietary habits is not successful, bulking agents (MUCOFALK), saline and osmotic agents (natrium phosphate: FLEET-PHOSPHO-soda, lactulose: DUPHALAC, glycerin), stimulants/irritants (bisacodyl: DULCOLAX, STADALAX) can be used during breastfeeding. Senna preparations (TISASEN, BOLUS LAXANS) are excreted into breast milk and may cause diarrhea in a nursing infant, even so they are also compatible with breast-feeding.

**Antidiarrheal agents.** The use of diphenoxylate (combined with atropine to prevent abuse: (REASEC) has the potential for toxicity in a nursing infant, but may be taken infrequently in lactation. Loperamide (IMODIUM, LOPERAMID) is low risk during breastfeeding and can be used temporarily. Activated carbon (CARBO ACTIVATUS) can be given for symptom relief without reservation.

**Inflammatory bowel disease.** If the mother is taking inflammatory bowel disease agents, such as mesalamine (PENTASA, SALOFALK) and sulfasalazine (SALAZOPYRINEN), close observation of the nursing infant is required. Several cases of diarrhea were reported in the nursing infants that appeared to be related to these agents (7, 25).

**2.2. Anticoagulants**

Due to heparins large molecular weight, it does not cross into milk and can be used safely during lactation. This would also be expected for the low molecular-weight preparations (dalteparin: FRAGMIN, enoxaparin: CLEXANE, nadroparin: FRAXIPARINE). The most widely used oral anticoagulants (warfarin: MARFARIN, acenocoumarol: SYNCUMAR) have a very high protein binding (> 95%), and therefore are not detectable in milk or infant serum.

The use of acetylsalicylic acid – certainly in low dose (80-300mg per day) - to inhibit thrombocyte aggregation is widespread and is well-tolerated in nursing. Breastfeeding can be continued during fibrinolytic treatment (streptokinase: STREPTASE, alteplase: ACTILYSE, urokinase: RHEOTROMB, tenecteplase: METALYSE) because these agents cannot be detected in breast milk, and they are not absorbed in relevant amounts from the GI tract (7, 25).
2.3. Cardiovascular drugs and diuretics

- **α-** methyldopa (DOPEGYT) and dihydralazine (DEPRESSAN) are among the antihypertensives of first choice during breastfeeding. They are not cumulated in the infant, and the dose that can be detected in breast milk does not reach the effective level.

- ACE –inhibitors, that have been available for a long time, such as captopril (TENSIOMIN), enalapril (RENITEC), perindopril (COVEREX-AS) can be used in lactation if they are indicated. They exist in mother’s milk in a low concentration; even so attention should be paid to edema and a possible increase in the infant’s weight – as indicators for disturbed kidney function - for safety reasons.

- β-receptor blockers are excreted in milk in high amounts and there is also a risk for accumulation in the infant, with the exception of propranolol (HUMA-PRONOL) that can get into breast milk in very small amounts. Metoprolol (BETALOC) and timolol (FOTIL eye drops) are also used during nursing, but the infant’s condition should be controlled frequently. Hypotension, bradycardia, and transient tachypnea can occur as side-effects due to long-term use of β-blockers.

- Ca-antagonists with high plasma protein binding – such as nifedipine (CORDAFLEX, CORINFAR) and nitrendipine (BAYPRESS) – are excreted into milk in very limited amount, so they can be ordered during breast-feeding without reservation. The use of verapamil (CHINOPAMIL R, ISOPTIN) is also allowed in nursing, but diltiazem (DILRENE, DILZEM) should be avoided.

- Diuretics should not be used primarily for treating hypotonia during breastfeeding. Thiazides are excreted in breast milk and have been used to inhibit lactation, but moderately dosed treatment with hydrochlorothiazide (HYPOTHIAZID) can be undertaken. Loop diuretics are also distributed into human milk (in very small amounts) and decrease milk production, but with appropriate indication furosemide (FURON) may be given to a nursing mother. Chlortalidone (HYGROTON) should be avoided because of its accumulation. Potassium sparing diuretics, such as spironolactone (VEROSPIRON) and amiloride may be compatible with lactation (7, 28, 29).
2.4. Agents affecting metabolism

- As for establishing a physiological state, substitution with thyroid hormones (levothyroxine: EUTHYROX, LETROX) should be continued during breast-feeding. Propylthiouracil (PROPYCIL) should be chosen as thyrostatic in preference to thiamazole (METOTHYRIN), but the thyroid parameter of the infant should be monitored to be safe. Iodine supplementation should be avoided in lactation.
- Insulin can be used during breast-feeding without reservation. Oral antidiabetics, such as metformin (MERCKFORMIN) and glibenclamide (GILEMAL, GLUCOBENE) may also be taken – with observation of the infant for symptoms of hypoglycemia. There are insufficient data on the other oral antidiabetics.
- Prednisolone (RHEOSOLON, AUROBIN), prednisone (RECTODELT), methylprednisolone (MEDROL, METYPRED) are often used corticosteroids for systematic treatment during lactation. After the administration of high doses, there should be a 3-4-hour pause of breastfeeding, if it is possible to arrange. There is nothing to worry about routine inhalation of corticoids for asthma (7, 25).

2.5. Antiepileptics

- Monotherapy with phenytoin (DIPHELAN, EPANUTIN) or valproate (CONVULEX) is considered to be safe during nursing because very small amount get into the breast-fed infant.
- Use of carbamazepine (NEUROTOP, TEGRETOL) requires the observation of the baby for symptoms, such as weak suck, vomiting and tiredness.
- Antiepileptic therapy with barbiturates (phenobarbital: SEVENAL, primidone: SERTAN), clonazepam (RIVOTRIL) and ethosuximide (PETNIDAN) should be avoided in lactation. They have a long half-life and they can also easily get into human milk, so accumulation in the infants may occur. If the use of these drugs is necessary, monitoring of the baby is essential.
- There has been little experience with the use of new antiepileptics (such as vigabatrin: SABRIL, gabapentin: GORDIUS, NEURONTIN, levetiracetam: KEPPRA), so the breast-fed infant should be well controlled and if any symptoms turn up, the serum concentration of the drug in the child should be measured.
- Combination anticonvulsive therapy is not compatible with breastfeeding (7, 16, 18).
2.6. Antibiotics

When nursing mothers are being treated with antibiotics, only minimal concentrations -less than 1% of the weight-related therapeutic dosage- are reached in the infant’s plasma.

The most commonly discussed risk factors of antibiotic using in lactation are the following:

- Alterations of the intestinal flora with the possibility of diarrhea.
- Effects may occur on bacteriological studies which might be necessary in case of the infant’s illness.
- Bacterial resistance can develop.
- Sensitization.

These side effects have not been proven yet. The most probable possibility might be the loosening of the stool consistency.

- Penicillins and cephalosporins have been found to produce only trace levels in human milk, so they can be used in lactation without anxiety. Though, substances that have been available for a long time are preferable.
- Erythromycin (MEROMYCIN) and roxithromycin (RULID) are also first-line antibiotics during breastfeeding, while azithromycin (SUMAMED), clarithromycin (KLACID) and spiramycin (ROVAMYCINE) are second-choice medications.
- Tetracyclines, such as doxycycline (DOXITIDIN), reach low levels in milk. Although short-term therapy is considered to be acceptable, long-term use should be avoided in lactation. It is a well-known fact that these drugs given during pregnancy or directly to children (under the age of 8) cause dental staining. This effect has not yet been proven on breast-fed infants with maternal use of tetracyclines.
- Quinolones has been reported to cause arthropathy in developing animals, but this has not been observed in human children. Pseudomembranous colitis has been noticed in a breast-fed infant whose mother administered ciprofloxacin (CIFRAN, CIPROBAY, and CIPRUM); in contrast, this drug has been used extensively in other patients without reported problems. However, quinolones
should only be used during breastfeeding when a complicated infection really requires them.

- Metronidazole has shown mutagenic and carcinogenic effects in laboratory, the existence of these risks in humans has not been supported, yet. Topical and vaginal application of the drug is of no concern because of the limited absorption via these routes of administration, but after large oral doses, breastfeeding should be interrupted for a few hours (7, 19, 27).

2.7. Analgesics and pain relief

- Aspirin is generally not recommended for pain relief during breastfeeding mainly because of the substantial serum salicylate levels that have been demonstrated in breastfed neonates. As a result of this, there may be significant adverse effects in infants, such as metabolic acidosis, bleeding, altered pulmonary circulation, and Reye’s syndrome. Nursing mothers should use aspirin cautiously, and large doses should be avoided.

- Use of paracetamol is considered to be safe during lactation because only 6% of the maternal dose gets into the infant. Far greater amounts of the drug are given to the children themselves if it is necessary, so there is no reason for concern.

- NSAIDs – such as naproxen (ALEVE, APRANAX), ibuprofen (ADVIL, ALGOFLEX, NUROFEN), piroxicam (HOTEMIN), and diclofenac (CATAFLAM, VOLTAREN) - do not reach high concentrations in breast milk, even so, they should be administered with caution by nursing mothers. Indomethacin (INDOMETACINUM) circulates enterohepatically and should be avoided. Most NSAIDs have the ability to displace bilirubin, so they can increase the risk of kernicterus and are naturally contraindicated in the jaundiced neonate.

- Morphine does not appear to be hazardous to most breastfed infants – so long as the maternal doses are low to moderate - owing to its rather limited levels in breast milk and its poor oral bioavailability. Codeine (e.g. in TALVOSILEN FORTE) is one of the most commonly used opioid analgesics among nursing mothers, despite neonatal sedation and apnea has occurred in some cases (7, 25, 27).
2.8. Psychotherapeutic drugs

Nowadays, approximately 10% to 15% of postpartum women suffer from depression, and almost 80% experience postpartum blues. During the 3 months after delivery, the majority of mothers go through sleep deprivation, pain and stress, which can be difficult to differentiate from clinical depression. Because of these symptoms, the vulnerability of women for psychiatric illness during the mentioned period raises the possibility that psychotropic drugs will be necessary. It is not an easy task to balance the benefits of breast-feeding, and the effects of untreated mental illness on the mother, and the adverse impact of her ailment on infant attachment and cognitive and behavioral development. However, in cases with certain diagnosis of clinical depression, withholding treatment would be too risky.

Sedatives and Hypnotics

- Benzodiazepines (such as diazepam: SEDUXEN, medazepam: RUDOTEL, midazolam: DORMICUM) do not reach excessive levels in human milk, but rarely, sedation, lethargy and poor suckling has occurred in a few breastfed infants. If the use of a sedative is necessary, the shorter half-life analogs – such as midazolam: DORMICUM – are preferred, but not for long intervals.
- Phenothiazine analogs – such as chlorpromazine (HIBERNAL) and promethazine (PIPOLPHEN) - should be avoided during breastfeeding, because they may increase sleep apnea and the risk of sudden infant death syndrome (SIDS).

Antidepressants

- Tricyclic antidepressants – such as amitriptyline (TEPERIN), imipramine (MELIPRAMINE), clomipramine (ANAFRANIL) - appear in human milk in very small amounts, so their use during breastfeeding is considered quite safe. Due to the anticholinergic symptoms - such as blurred vision, xerostomia and sedation- of these antidepressants, the patients’ compliance in connection with their use is not satisfactory.
- Selective serotonin reuptake inhibitors (SSRIs) are widely used in all segments of the population, also among pregnant women.
  - Sertraline (ASENTRA, STIMULOTON, ZOLOFT), fluvoxamine (FEVARIN), and paroxetine (REXETIN, PAROXAT, APODEPI) are the drugs of choice among SSRIs for nursing mothers because of their low transfer into human milk.
milk, and the even lower uptake by the infant. No untoward effects have been 
mentioned yet in connection with the maternal administration of these drugs in 
lactation.

- Owing to the long half-life and active metabolite of fluoxetine (PROZAC, 
  FLOXET), it reaches relatively high concentrations in breastmilk, and also 
significant plasma levels have been reported in infants. Some severe symptoms 
such as colic, sedation, seizure, or coma have occurred, so special attention 
should be paid to the suckling infant.

- Relevant amounts of citalopram (SEROPRAM, SEROTOR, and DALSAN) 
  and escitalopram (CIPRALEX) can appear in human milk. Infants, whose 
  mothers administer these drugs, should be closely monitored for somnolence.

  • Use of other atypical antidepressants such as mirtazapine (REMERON, MIZAPIN) 
    and bupropion (WELLBUTRIN) seems not to be dangerous in most breastfeeding 
    mothers. Nevertheless, because of the sedative effects of mirtazapine, it should be 
    used cautiously in premature infants and those subject to apnea.

**Antipsychotics**

Despite the poor transfer of phenotiazines such as chlorpromazine (HIBERNAL) into human 
milk, they should be avoided in nursing mothers because of their association with neonatal 
apnea and sedation.

  • It is suggested to choose the newer atypical antipsychotics for treating breastfeeding 
    mothers. Clozapine (LEPONEX), olanzapine (ZYPREXA), quetiapine (SEROQUEL, 
    KETILEPT), and risperidone (RISPERDAL) are considered to be acceptable in 
lactation (27, 30, 31).

2.9. **Drugs of abuse**

  • Despite the large volume of research proving the risks associated with tobacco use, 
    many pregnant and breastfeeding women smoke cigarette. Increased infantile colic, 
    increased susceptibility to respiratory infections, and decreased respiratory rates and 
    oxygen saturation following suckling characterize the infants of smoking mothers. 
    Nicotine is presented in human milk in high concentrations and as a weak base it may 
    accumulate in the slightly acidic milk through ion trapping. The infants’ exposure
index depends on the number of cigarettes smoked by the mother daily. Harm minimization would be the best advice to the mothers that can be solved with limiting smoking as much as possible, prolonging the time between the last cigarette smoked and breastfeeding, switching to nicotine patches, smoking outside the house and avoiding smoky environments.

- Breastfed infants with a significant maternal daily intake of caffeine may potentially accumulate toxic concentrations of the drug owing to the newborn’s surprisingly slow elimination. In these cases irritability, vomiting and insomnia can be noticed in the nursing infants. Restricting caffeine consumption (to less than 300 mg/day) during breastfeeding is necessary due to the drug’s prolonged half-life in newborns.

- Alcohol passes easily into breast milk at concentrations similar to those that exist in the mother’s bloodstream. Only a fraction of the maternal ingested alcohol gets into the infant, but we have to take into account its low detoxification capacity. Alcohol induces several adverse effects on suckling infants. After moderate levels of ethanol exposure on the newborn, impaired motor development, changes in sleep patterns, decrease in milk intake, weight gain, and risk of alcohol-induced hypoglycemia have been reported. For causing mild sedation, acute neuronal dysfunction or death, larger amount of alcohol would be necessary. Harm minimization strategies should be applied by the nursing mothers that include: feeding infant before alcohol ingestion, avoiding suckling for 2-3 hours after drinking alcohol. Chronic or heavy consumers of alcohol should not breastfeed (7, 32).
References


