

Introduction to Cellular Drug Delivery

Present Realities and Future Prospects

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1. Introduction

Drugs play a central role in modern medicine (1). The total global pharmaceutical market in 2002 was more than \$400 billion, and the annual growth rate of the global market has been more than 10%. The large market size allows big pharmaceutical companies to invest heavily in research and development (R&D). In 2002 alone, the combined R&D budget of Pfizer, Pharmacia, Merck, Eli Lilly, and Bristol-Myers Squibb was more than \$16 billion. Despite an ever-increasing R&D budget, development of new drugs has been slow, and the number of new drugs introduced has become smaller than the number of drugs that went off patent protection. The real cost of developing a new drug is not well established, but it is generally agreed to be about \$800 million. One of the reasons for such a high cost is that many drug candidates are abandoned owing to their unfavorable physicochemical and biochemical properties. It is also becoming more and more difficult to develop new drugs due to the increasing difficulty of reaching their intended targets. One of the ways of making drug development more efficient is to utilize drug-delivery technologies from the early stages onward. The development of new drug-delivery technologies also makes the existing drug more useful.

Drug-delivery technologies contribute significantly in various stages of drug development. For a drug to exert its bioactivity, it has to enter the target cells. This seemingly simple process requires a series of steps that are often difficult to overcome. The steps of drug delivery to the target cells are described in Fig. 1. A drug first has to dissolve in aqueous solution first to have any chance of being bioactive (Step A in Fig. 1). Many newly developed drug candidates are poorly water soluble so a large number of potentially useful drugs are abandoned. Poorly soluble drugs can be dissolved in aqueous solution via polymeric micelles, polymeric nano/micro-particles, or polymeric prodrugs. New protein drugs can increase their bioactivity by protecting themselves in blood using various drug delivery technologies, e.g., PEGylation and microencapsulation. Once a drug is in solution, it has to be delivered to the target cells (Step B in Fig. 1). Commonly, a drug is delivered throughout the body, which causes unwanted side effects. Targeting minimizes such side effects. Targeting may be the only way for some treatments. For example, whether gene therapy will be successful or not depends entirely on whether genes can be delivered to the target site and effectively enter the

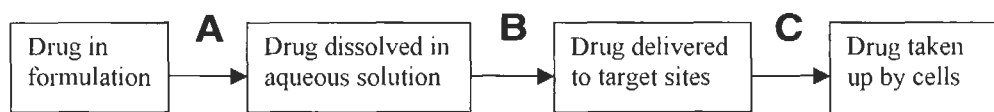


Fig. 1. Drug molecules in different stages.

target cells for expression (Step C in Fig. 1). Entering into the target cell is half of the process. The genes also must escape from endosomes to make themselves available for expression. For most drugs, the ultimate bioactivity is appreciated after they have entered the cells, and thus, cellular uptake can be considered one of the most important steps in drug delivery. In this regard, cellular drug delivery is a very timely topic to consider.

2. Current Technologies in Cellular Drug Delivery

There is a saying in China that you win every battle if you know both your enemy and yourself. Knowing one's enemy is critical in winning the war. In the war against disease, we have to know the enemy, such as viruses and bacteria. At the same time, we have to know ourselves, that is, the cells in our body. This book is about understanding ourselves in preparing for the war against diseases. We have to understand how our body functions and then apply that knowledge in the design of new strategies for efficient drug delivery.

Cellular Drug Delivery: Principles and Practice provides information on both drug uptake into cells and methods of improving cellular uptake. As listed in Table 1, the book discusses the cellular processes, drug delivery to cells, and targeting and formulation. The first three chapters provide background on how cells uptake nutrients and drugs, and other basic information on cell biology. We have a good understanding of transport processes through cell membranes that can be exploited in cellular delivery of various drugs (*see* Chapters 1 and 2). Understanding the biological processes of cell-mediated responses (*see* Chapter 3) should provide strategies for promoting cell-mediated immunity against viral infection, such as influenza virus and, it is hoped, the virus causing severe acute respiratory syndrome (SARS). Two chapters are dedicated to nonviral DNA-delivery systems (*see* Chapters 4 and 5). Although viral vectors are most effective in gene delivery, the inherent danger of using live viruses has been proven to be problematic. There is no doubt that development of nonviral vectors will be essential in routine clinical applications of gene therapy in the future. One of the approaches for efficient cellular delivery may be utilizing naturally occurring peptides that are known to enhance entry into cell membranes (*see* Chapter 6). Transporters not only absorb drugs and nutrients into cells, but also they can efflux absorbed drugs, and thus understanding various transporters in cell membranes is important in optimizing drug therapy (*see* Chapters 7 and 8). Because the majority of drugs are delivered by oral administration, understanding the roles of various transporters in intestinal drug absorption is critical in screening various drugs (*see* Chapter 9).

The two chapters on targeting using colloidal carriers and protein nanocomplexes should be useful for the delivery of various types of drugs (*see* Chapters 10 and 11). The last six chapters discuss formulation-related problems, which often, unfortunately, are neglected, for without proper formulation, no drugs would be clinically useful. Bulk manufacturing of polynucleotides in their stable forms in effective delivery sys-

Table 1
Organization of the Book

Topic	Chapter
Cell biology	1. Transport processes through cell membranes
	2. Cell growth and cell cycle
	3. Cell-mediated immunity
Cellular delivery	4. Nonviral DNA delivery
	5. Cellular delivery of nucleic acid
	6. Membrane permeation promoting peptides
	7. Transporters
	8. MDR modulators (inhibitors)
Targeting	9. Intestinal cell permeability
	10. Colloidal carriers
	11. Protein complexes
Formulation	12. Preservation of DNA integrity
	13. Cationic microparticles
	14. Transdermal delivery
	15. Immunoglobulin formulation
	16. Antibody formulation for cancer treatment
	17. Pharmaceutical profiling

tems is essential in widespread use of nucleotide-based therapeutics (*see* Chapters 12 and 13). One of the first applications in controlled drug-delivery technologies was the development of transdermal products, and one of the first therapies of antisense oligodeoxynucleotides may be by transdermal delivery (*see* Chapter 14). Immunoglobulins are considered to be good vectors to deliver T-cell epitopes to antigen-presenting cells (APCs), and targeted delivery of immunoglobulin-engineered microparticle systems have a great potential (*see* Chapter 15). Antibody-mediated targeted delivery of anticancer agents also holds great promise (*see* Chapter 16). Chapter 17 on the automated pharmaceutical profiling assays is one of the most interesting chapters of the book. Rapid screening of physicochemical and biochemical properties of drug candidates that affect cellular drug delivery provides new opportunities in pharmaceutical research beyond in vivo animal experiments.

3. Opportunities in Cellular Delivery

The concept of controlled drug delivery is more than four decades old, and the drug-delivery area is still evolving. Through the years, numerous novel drug-delivery systems have been developed and a large number of formulations are helping patients. Although the advances made to date are impressive, a number of breakthroughs are necessary for solving various problems facing us today. The main progress to be made is in the precision of targeting. Until a few decades ago, destroying a target in the enemy territory during a war required delivering hundreds of thousands of bombs that literally destroyed the whole town. Nowadays, laser-guided smart bombs or bombs with global positioning capability brought a new concept of one bomb per one target. The ultimate success in drug delivery is expected to bring about a concept of one drug

molecule per one target. In fact, all that is required for successful gene therapy is to deliver a gene into a cell as long as the delivered gene is properly expressed. This means that the precision targeting has to be accompanied by effective cellular delivery.

For delivery of macromolecular drugs, such as anticancer drug molecules grafted to polymer chains, protein drugs, and genes, absorption into cells by endocytosis may be the most effective way. Endocytosis occurs when macromolecular drugs or drug carriers interact with the receptors on cell membranes, and receptor-mediated cellular uptake has been exploited quite extensively. In addition to receptor-mediated endocytosis, nonspecific electrostatic interaction with cell membranes is also known to cause endocytosis. One of the possible mechanisms of enhanced cellular absorption by cell penetration peptides (2,3) and trans-activating transcriptional activator (TAT) (*see* Chapter 6) is nonspecific electrostatic interactions between positive charges in side chains of those peptides with cell membranes. Effective endocytosis based on nonspecific electrostatic interaction is important for designing the cellular delivery systems that also have the additional function of escaping from endosomes. Drug-delivery vehicles can be designed with high flexibility for effective endocytosis with a minimum number of specific ligand molecules that bind to receptors on cell membranes. Enhanced cellular delivery may also exploit the unusual ability of neutral block copolymers, such as poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymers, to get inside cells. They are known also to decrease significantly the P-glycoprotein efflux activity in P-glycoprotein overexpressing membranes, probably through incorporating themselves into cell membranes, leading to increased fluidization (4). The cumulated data clearly demonstrated that those block copolymers are taken up inside cells to spread throughout the cytoplasm. Recent studies using fluorescent micelles showed that micelles made from polycaprolactone and poly(ethylene oxide) enter into cells by endocytosis to distribute themselves within several cytoplasmic compartments, but not in the nucleus (5). There is a great potential in improving cellular uptake using various polymeric micelles and in order to control the distribution of the absorbed drug in the cytoplasm.

Opportunity also exists in designing the delivery systems that have the ability to escape from endosomes once they enter cells by endocytosis. One of the main disadvantages of nonviral vectors as compared with viral vectors is their inability to deliver drugs intact from endosomes into the cytoplasm. A number of approaches have been attempted for providing endosome-disruptive properties. Endosome-disruptive hemagglutinin peptides from influenza virus and membrane-active amphipathic peptides have been used to release the delivered agents from endosomes to the cytoplasm (6,7). Although these approaches are promising, they suffer from the shortage of such peptides. One recently developed approach is to utilize synthetic polymers that can disrupt the endosomal membranes at pH around 6.0. Polymeric histidine has shown its ability to disrupt endosomes when pH is lowered to deliver drugs into cytosol (8–10). Combining polycationic nature for endocytosis and polyhistidine for endosomal escape may be a practical combination for enhanced cellular delivery of a variety of drugs ranging from low molecular-weight anticancer drugs to proteins and genes.

Understanding cell biology, membrane interfacial phenomena, protein chemistry, and polymer chemistry are all essential in developing effective cellular delivery systems. Being an expert on any of these areas provides a great potential to open new avenues of cellular drug delivery. If the history of the pharmaceutical industry is any guide, one can easily imagine the ever-increasing values of such research areas and the

high rewards accompanying them. Opportunities in the future basically are infinite and only our imagination is a limit. *Cellular Drug Delivery: Principles and Practice* will certainly serve as an excellent resource in expanding our imagination in developing novel drug-delivery systems for the most efficient methods of cellular delivery.

References

1. Drews J. In Quest of Tomorrow's Medicines. New York, NY, Springer-Verlag, 1999.
2. Langel Ü. *Cell-Penetrating Peptides. Processes and Applications*. Boca Raton, FL, CRC Press, 2002.
3. Nielsen PE. *Peptide Nucleic Acids. Methods and Protocols*. Totowa, NJ, Humana Press, 2002.
4. Batrakova EV, Li S, Alakhov VY, et al. Optimal structure requirements for Pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J Pharmacol Exp Ther* 2003;304:845–854.
5. Savic R, Luo L, Eisenberg A, Maysinger D. Micellar nanocontainers distribute to defined cytoplasmic organelles. *Science* 2003;300:615–618.
6. Wagner E, Curiel D, Cotten M. Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Adv Drug Del Rev* 1994;14:113–135.
7. Plank C, Zauner W, Wagner E. Application of membrane-active peptides for drug and gene delivery across cellular membranes. *Adv Drug Del Rev* 1998;34:21–35.
8. Midoux P, Monsigny M. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconj Chem* 1999;10:406–411.
9. Putnam D, Gentry CA, Pack DW, Langer R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc Natl Acad Sci USA* 2001;98:1200–1205.
10. Benis JM, Choi JS, Mahato RI, et al. pH-sensitive cationic polymer gene delivery vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer. *Bioconj Chem* 2000;11:637–645.

I

FUNDAMENTALS OF CELL BIOLOGY AND CELLULAR DRUG DELIVERY

1

Cellular Structure, Function, and Membrane Transport

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Numerous scientific disciplines have contributed significantly to the study of cellular drug delivery. From activity and toxicity screening of potential active ingredients to elucidating the biological mechanisms of site-specific interactions, the science of cellular drug delivery is a complex, integral part of the treatment and prevention of life-threatening illnesses. Every year, new information is made available from many different research areas allowing for continuous improvement through technological advancements. In order to continue along this route, a multidisciplinary background is necessary to understand the process of drug delivery at the cellular level. The purpose of this chapter is to present basic principles of cellular biology and their importance in cellular drug delivery. Subheading 1 deals with the fundamentals of cellular structure and intracellular compartments. Membrane structure, transport inside the cell, passive transport through the membranes, and other membrane properties are discussed in Subheading 2, as well as how these membranes serve as a major barrier to the drug-delivery process. Finally, Subheading 3 describes receptor proteins, how they function, where they are located on a cellular level, how they act as transporters to deliver drugs to the cell, and their role in multidrug resistance.

The effectiveness of a drug therapy is dictated by several factors, including the rate and extent of the drug molecule's penetration into, and permeation through, the body tissues and cells to reach a site of action. Many barriers prevent the drug from reaching the site of action. They can be grouped according to size, including the organism itself, on a 1–2 m scale, an organ representing the 1–100 mm scale, tissue in the 1–100 μm size, and the extracellular and intracellular space of only 1–100 nm (1). Understanding the processes governing a single cell and its makeup is the fundamental objective of this chapter.

1. Internal Organization and General Function

Although there are many different types of cells, every cell has some common components necessary to carry out life processes. In general, the chemical make-up of cells and their internal compartments or organelles are similar (2). From a drug-delivery point of view, the cell or a group of cells is in some way the “site of action,” whether the therapy is to destroy cancer cells or produce antibodies through vaccination. There are several traits that an ideal drug carrier should possess in order to deliver the drug to this site of action. First, carriers should target the area or group of cells specific to their

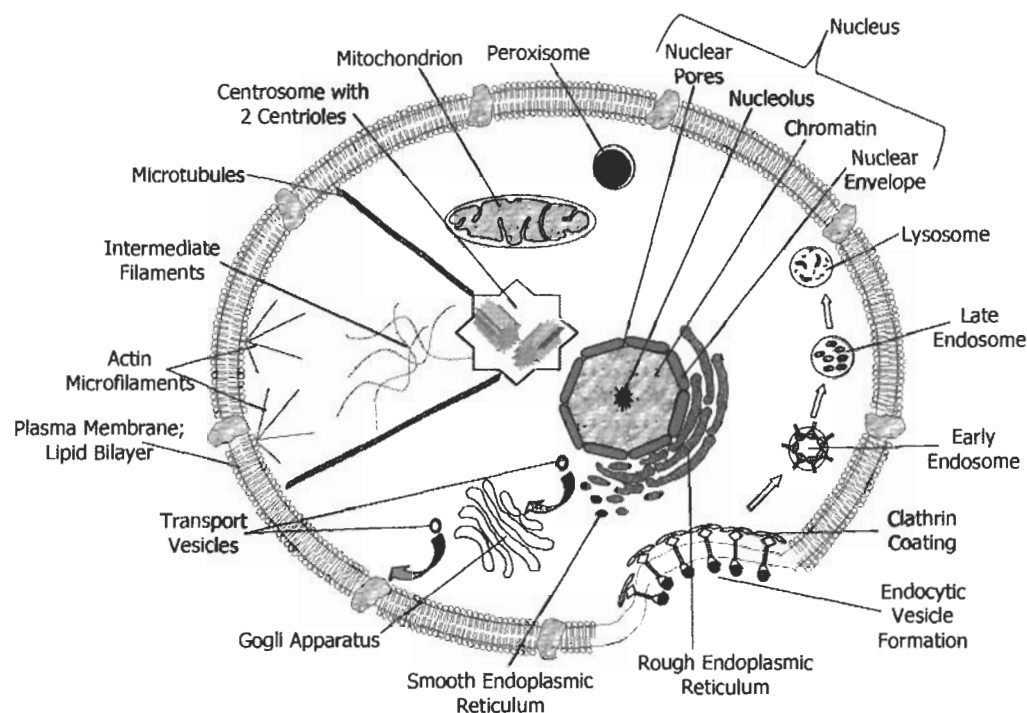


Fig. 1. Schematic of a typical eukaryotic cell, including key internal compartments and endocytic vesicle formation and transformation to lysosomes.

action while avoiding interactions with nontarget cells. They should release therapeutically relevant amounts of bioactive substances at that site for a required duration and frequency. They should also be readily excretable or degradable without causing undesirable biochemical or immune reactions in the body (3). There are a number of cellular compartments common to most eukaryotic cells that a drug carrier may encounter in pursuing its therapeutic goal. A schematic of a typical cell and its compartments is shown in Fig. 1, and Table 1 summarizes how each cellular compartment can act as a drug-delivery barrier.

1.1. The Plasma Membrane

The plasma membrane forms the boundary around the cell and allows it to maintain chemical coordination. The membrane itself is formed from a series of phospholipids that are noncovalently linked together by hydrophobic interactions to form the bilayer around the cell. Imbedded within the membrane are proteins that function to transport molecules from one side to the other. Only specific types of molecules can be recognized, internalized, or transported by these proteins making passage into the cell difficult for noncompatible molecules. There are other means of transporting molecules into the cell, including fluid-phase pinocytosis, phagocytosis, receptor-mediated endocytosis, membrane fusion, passive diffusion, and channel or pore transport, which will be discussed in Subheading 2.1.2.

Table 1
Internal Cellular Organelles Can Act as Barriers to Drug Delivery

Compartment	Drug-delivery barriers
Plasma membrane	Membrane transport protein specificity
	Lipid bilayer penetration
Cytoplasm	Slow macromolecular transport
	Diffusion rate limited
Nucleus	Double lipid bilayer penetration
	Pore complex size and specificity
	Nucleus membrane receptor protein specificity
Endoplasmic reticulum	Metabolic enzymes
Golgi apparatus	Metabolic enzymes
Lysosomes	Presence of hydrolytic enzymes
	Low pH microenvironment
Mitochondria	Double lipid bilayer penetration
Peroxisomes	Presence of catalases
	Presence of membrane-bound oxidative enzymes
Cytoskeleton	Microtubules restrict internal traffic

1.2. The Cytoplasm

After drug molecules cross the plasma membrane, they may encounter the cytoplasm, depending on the mechanism of uptake. The cytoplasm consists of the cytosol and the organelles or compartments of the cytoplasm. These compartments include the nucleus, mitochondria, peroxisomes, endoplasmic reticulum (ER), golgi apparatus, lysosomes, endosomes, and cytoskeletal elements. The cytosol makes up about 54% of the total cell volume and provides the semi-liquid media where proteins are synthesized and degraded and where most intermediate metabolism occurs. The main transport mechanism through the cytosol is diffusion. Macromolecules such as enzymes or high molecular-weight drug molecules diffuse slowly in the cytosol owing to constant interactions with various cellular organelles. On the other hand, smaller molecules diffuse relatively efficiently (4,5).

1.3. The Nucleus

The nucleus is located in the cytoplasm and is bound by the nuclear envelope consisting of a double lipid bilayer membrane. These two bilayers are about 20–40 nm apart and form a significant barrier limiting molecular penetration into the nucleus. The main passage into the nucleus is through pores of about 60–100 nm in diameter formed in the bilayers. This passage is regulated by pore complexes composed of about 50 different proteins called nucleoporins, which are arranged in an octagonal shape. There are approx 3000–4000 pore complexes in a nuclear envelope of the typical mammalian cell transporting many function-essential substances. Within the pore complexes are open aqueous channels that permit small molecules of less than 5000 Daltons to

diffuse readily while sufficiently preventing passage of macromolecules of 60,000 Daltons or greater. Macromolecules rely on specific receptor proteins to carry them into or out of the nucleus (2,5,6). If a macromolecule is unrecognized by nuclear receptor proteins, it is prevented from entering the nucleus, making it difficult to use macromolecular targeting carriers. To overcome this impasse, researchers have developed macromolecular carriers, which can be used to target drugs specifically to the nucleus. They are made of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, containing targeting sites for cell specificity, and degradable drug units. As the carrier enters the cell—probably through endocytosis—and is carried to the lysosomal compartment, the drug unit is cleaved from the polymer backbone by lysosomal enzymes. The free drug molecule then enters the cytoplasm and rapidly diffuses into the nucleus to carry out its effect (7). Other research groups have utilized modular recombinant transporters (MRTs) to target cellular nuclei and deliver photosensitizer agents for anti-cancer therapy. These MRT constructs are made up of several components including α -melanocyte-stimulating hormone, Simian virus (SV40) large T-antigen nuclear localization signal and a translocation domain of the diphtheria toxin, allowing selective drug targeting to melanocyte nuclei (8).

The nucleus is one of the most important components of the cell because it contains the cell's genetic information encoded through deoxyribonucleic acid (DNA). Inside the nucleus, DNA is packaged into chromosomes by associating with proteins called histones to form chromatin. There are a variety of functional enzymes, strands of messenger ribonucleic acid (mRNA) and transfer ribonucleic acid (tRNA), and ribosomal components within the nucleus that are constantly constructed and transported into the cytosol. The ribosomal components (ribosomal RNA or rRNA) are synthesized and assembled in the subunit of the nucleus called the nucleolus, which has a spherical structure composed of 5–10% RNA, protein, and DNA (5,6). Continuous exchange of components across the nuclear membrane is necessary for proper cellular function and can occur through specific transporters in the nuclear pore complex called importins and exportins (9). This nucleocytoplasmic transport pathway could be exploited as a potential mechanism to deliver drugs to the nucleus. Many diseases may be more effectively treated with direct delivery of genetic material into the nucleus to treat or kill target cells. Potential delivery systems, both viral and nonviral vectors, to deliver genes to the nucleus are reviewed in Chapters 5 and 6.

1.4. The Endoplasmic Reticulum

Interspersed throughout the intracellular space and continuous with the outer nuclear membrane is the endoplasmic reticulum (ER). It accounts for more than half of the total membrane systems in the cell. It is a network of flattened sacs with bound ribosomes (the rough ER) and branching tubules (the smooth ER), each with different structures and functions. Inside the ER is the lumen or ER cisternal space, which is separated from the cytosol by the ER membrane. The rough ER is responsible for the synthesis and modification of membrane proteins and phospholipids as well as the synthesis of secretory proteins such as insulin. One of the main functions of the smooth ER is to bud off transport vesicles containing the newly synthesized proteins and lipids. In specialized cells, such as hepatocytes, the smooth ER is the site of drug and lipid metabolism and a site for the storage of calcium ions. The enzymes in the smooth ER are broad acting, such that encountering more than one drug of the same type will cause proliferation of the smooth ER, increasing metabolism and potentially leading to drug tolerance (2,5).

1.5. The Golgi Apparatus

After being released from the smooth ER, many transport vesicles travel to the Golgi apparatus. The vesicle fuses with the Golgi membrane, releasing the contents into the Golgi apparatus. Here the proteins and lipids are modified for specific functions and stored before being repackaged into other vesicles bound for a specific part of the cell or the cell surface. The Golgi apparatus is also the manufacturing site for certain macromolecules, such as glycosaminoglycans and some carbohydrates. It is similar in structure to the rough ER, containing a series of flattened sacs or cisternae stacked upon each other. It is a polar membrane with *cis* and *trans* sides, and thus can receive and export transport vesicles, respectively, at opposite membranes.

1.6. Lysosomes

Lysosomes are membrane-bound organelles whose specific function is digestion or chemical breakdown of various substances. They function to recycle some macromolecules produced in the cell by breaking them down into the constituent monomers. Lysosomes contain about 40 different hydrolytic enzymes and all of them are acid hydrolases. The internal pH of the lysosome is maintained at about 5.0 providing a more effective hydrolyzing environment. The lack of proper lysosomal enzymes can result in cellular damage owing to accumulation of undigested toxic substances. For example, Pompe's disease is a disorder owing to indigestion of glycogen in the liver, and Tay-Sach's disease sufferers are missing a lipid-digesting enzyme in the brain, causing impairment (2,5). Many of these kinds of diseases are treatable with the delivery of specific enzymes into the lysosomal compartment or genetic material into the cell, allowing production of the necessary lysosomal enzymes.

Delivery of drug molecules to cells is often a challenge because most endocytosed drug molecules eventually suffer degradation in the lysosomal compartment. Some researchers have developed a method to circumvent lysosomal degradation using pH-responsive polymers capable of lysing the endosome and releasing the endocytosed drug molecules into the cytoplasm. These polymers are based on α -alkyl acrylic acids such as poly(2-ethylacrylic acid), which, upon exposure to pH 6.3 or below, undergo a conformational change that disrupts the lipid bilayer of the endosome. The main mechanism of action is the polymer permeabilizes phosphatidylcholine membranes, disrupting the lamellar structure of the bilayer and causing lysis (10). This type of technology can be useful in delivery of biomolecules to the cytoplasm; however, little is known of the potential toxic effects of releasing the endosomal components into the cell's cytoplasm.

1.7. The Mitochondria and Peroxisomes

The mitochondria within cells function to extract energy from sugars and fats to synthesize ATP. They are contained by a double lipid bilayer with an internal membrane that has a large surface area owing to complex infoldings called cristae. In the heart of the mitochondria enclosed by the inner membrane is the mitochondrial matrix, where many of the proteins and enzymes reside. The mitochondria contain their own DNA, mitochondrial DNA, which is different from nuclear DNA, and their own ribosomes, which synthesize the proteins and enzymes necessary for specific functions. In a typical mammalian cell, there may be thousands of these 1–10 μm long mitochondria making up about 20–22% of the total cytoplasmic volume (2,5,6).

Peroxisomes are also metabolic centers. They are bound by only a single lipid bilayer and produce hydrogen peroxide as a by-product from reactions of breaking down vari-

ous substrates. They grow by incorporating cytosol-produced lipids and proteins into their membrane and split into two when they reach a certain size. Contained within the membrane of the peroxisomes are oxidative enzymes that act to break down fatty acids, sugars, and macromolecules for use in other cellular processes. Another important function of the peroxisomes, especially in liver and kidney cells, is the detoxification of substances that enter the blood, such as alcohol.

1.8. The Cytoskeleton

The cytoskeleton provides the structural support necessary for the cell to maintain its shape. It consists of a network of fibers that extend throughout the cytoplasm. There are three main types of fibers of the cytoskeleton: the large microtubules, the intermediate filaments, and the actin filaments or microfilaments. Each serves a slightly different purpose within the cell.

The microtubules are small tube-like structures measuring 25 nm in diameter and 200 nm to 25 μ m in length. They are made from the protein tubulin and dictate the positions of various organelles while directing intracellular transport. The intermediate filaments have an intermediate diameter of 8–12 nm. They are constructed of a diverse family of proteins called keratins and may serve as the framework for the entire cytoskeleton. Some intermediate filaments line the inside of the nuclear envelope, providing a strong support and barrier to protect DNA. Microfilaments are not hollow like microtubules. Instead they are solid rods composed of the globular protein actin. Actin filaments are flexible structures of 5–9 nm in diameter and tend to organize themselves in tight linear bundles. Their main purpose is to provide structural support so that the cell holds its shape.

2. Passive Transport Into and Through the Cell

The complexity of cells and their compartments pose tremendous challenges to drug-delivery scientists. Nearly every component in and around the cell can be viewed as a barrier to drug delivery. Targeting specific pathways, receptor proteins, or various cellular transport mechanisms can help scientists overcome those barriers. In this section, the transport mechanisms occurring into and throughout the cell will be elucidated and several examples found in the literature will be given. Mostly passive transport will be talked about here as active transport and the proteins responsible for active transport are reserved for Subheading 3.

2.1. Transport Across the Lipid Bilayer

The lipid bilayer or plasma membrane surrounding cells is composed of amphipathic molecules arranged in a dual-layer configuration that is about 5 nm thick. Fatty lipid molecules make up 50% by weight of the membrane, with the remainder being embedded proteins that serve to actively transport molecules across the membrane, catalyze membrane associated reactions, and provide many other functions. These lipid components act as formidable barriers to aqueous soluble substances. If a drug molecule approaches the cell surface attempting to gain entry, how does it cross the lipid bilayer and obtain access to the internal aqueous cytoplasm? There are several possible mechanisms described below.

2.1.1. Passive Diffusion

One of the main routes for both hydrophilic and hydrophobic drug molecules to enter the cytoplasm is through passive diffusion. The driving force for passive diffu-

sion is a concentration gradient. The molecules move from an area of high concentration to an area of low concentration by random thermal motion. The factors that can affect the diffusion coefficient include solubility and molecular weight. Usually small (<300 Daltons) molecules that are uncharged species and have a moderate oil/water partition coefficient, such that they are somewhat hydrophobic, can diffuse readily. If the partition coefficient is too high, the drug exhibits high lipophilicity and is unable to reach the membrane because it cannot dissolve in the aqueous extracellular compartment. On the other hand, if the drug has a very low partition coefficient and hence is very hydrophilic, it cannot diffuse through the lipophilic membrane to reach the interior of the cell. As molecular weight increases, the rate of diffusion decreases. Larger macromolecules greater than 300 Daltons, such as many oligonucleotides and sucrose, are essentially excluded from the lipid bilayer with a diffusion half-life of 4–10 d (11). Nevertheless, large macromolecules may gain access into the cell by other means.

2.1.2. Endocytic Processes

Many large macromolecules and even small particles can be taken up by the cell through a process called cytososis, or endocytosis. The lipid bilayer forms a cavity or indentation owing to an external stimulus, and through normal fluid motion and rearrangement of the membrane, an envelope, or endocytic vesicle forms engulfing the substance, including any surrounding extracellular fluid, in the cavity. Most cells undergo endocytosis on a regular basis and several different types of endocytosis exist based on the specificity and the size of the material engulfed (11–13). Several types of coating proteins are involved in the process to coat newly formed vesicles. For the internal compartments of the cell, there is a similar mechanism for vesicle formation with a major difference in the protein used to coat the cytosolic side of the membrane. One type of coating protein that has been identified in several types of endocytoses, including pinocytosis, trancytosis, and potocytosis, is caveolae. This protein is unique because it uses part of the lipid bilayer that is very high in concentration of cholesterol to form vesicles. Caveolae have been involved in the internalization of Simian virus (SV40) and cholera toxin, and it is known that caveolae play a role in membrane fusion and intracellular trafficking of transport vesicles. Owing to the high numbers of caveolae in pulmonary capillary epithelial cells, some researchers have investigated macromolecular delivery to the lungs, specifically targeting caveolae membrane domains (14,15).

Pinocytosis is a process in which both small and large molecules dissolved in the extracellular fluid flow into the formed cavity and are pinched off as vesicles within the cell. Because pinocytosis deals with fluids, it is also referred to as fluid-phase pinocytosis, and the vesicle internalized via fluid-phase pinocytosis is called an endosome. Much of the time, the dissolved drug molecule interacts with membrane protein receptors and basically is “caught” in the cavity through specific (receptor mediated pinocytosis) or nonspecific (adsorptive pinocytosis) binding to the receptor when an endosome is formed (13). One fate of the endocytosed material is that its vesicle fuses with an organelle of the cell called an early endosome, which has an acidic environment. Some of the material, especially membrane components and receptor proteins, is then selectively recycled back to the membrane or exocytosed, while the rest becomes a late endosome containing hydrolases and an even more acidic environment with a pH of approx 5.0–6.0. The late endosomes move toward the interior of the cell and become lysosomes containing more digestive enzymes that act to degrade the contents previously endocytosed.

A less destructive endocytic process is transcytosis. Often, the endocytosed material is specifically diverted from the lysosomal route and sent to the plasma membrane, thereby being recycled through exocytosis, or more importantly in drug therapy, transferred to the other side of the cell. Proteins and peptides such as immunoglobulin G (IgG), nerve growth factor (NGF), and epidermal growth factor (EGF) are known to undergo pinocytosis and transcytosis through intestinal mucosa cells to reach the blood vessels on the other side. In some cases, as with ricin, Shiga toxin, pertussis toxin, and cholera toxin, the endocytosed protein toxin is transported to the Golgi apparatus initially, instead of undergoing the normal transitions from endosomes to lysosomes. The toxins then, undergo retrograde transport to the ER (16). Elucidating these types of mechanisms provides insights not only into intracellular transport mechanisms, but also on potential treatments for these toxins.

Another type of endocytosis involves the uptake of large substances such as drug particles, cells, bacteria, or viruses. This is called phagocytosis and occurs in a series of steps similar to pinocytosis. Drug particles or other large substances adhere to the cell membrane. The binding usually occurs at a specific site that activates phagocytic mechanisms within the cell, similar to the process of when an antibody segment bound to a virus particle binds specifically to the Fc receptor on the surface of macrophages initiating uptake (2). The entire particle along with some extracellular fluid is ingested or engulfed by the membrane, forming a detached vesicle or phagosome within the cell. The phagosome then fuses with a lysosome, and the particle and other phagocytosed material is digested while the bilayer materials are recycled. Phagocytosis only occurs in specialized cells called professional phagocytes that exist in specific areas of the body, mainly the reticulo-endothelial system (RES). This includes the liver, bone marrow, and spleen macrophages and circulating blood monocytes. The phagocytes are tremendously important in regulating the body's immune system because they destroy foreign substances and remove particulate antigens. Their importance in drug therapy is limited to those drugs that are delivered in particulate carriers to the cell such as liposomes, erythrocytes, or microspheres. The therapy can be targeted with those drug molecules whose site of action is some region of the RES (13).

Receptor-mediated endocytosis is the most versatile type, involving a high degree of recognition and specificity. Agonists binding to surface receptors activate the endocytic pathway causing invagination of the bilayer and vesicle formation. The receptors themselves are usually recycled back to the cell surface and the agonist either participates in metabolism or is taken back to the cell surface. The size of material that can be endocytosed via receptor-mediated endocytosis ranges from smaller than 100 Daltons to in excess of 1 million Daltons, such as a virus particle. Many drug-delivery efforts focus on targeting various receptors on the cell surface so that particular biochemical pathways can be exploited. Agonists are typically developed to extend the duration of action via altered metabolic pathways in the lysosomal compartment, or to reduce side effects by specific binding to certain receptors on certain cells.

One interesting drug-delivery application developed to exploit receptor-mediated endocytosis is to engineer specific receptors on the cell surface so that targeting can be more specific. This approach, investigated at the University of California, Berkley, involves feeding the cells with chemically modified sugars that can be metabolized and incorporated as the terminal sugar on membrane glycoproteins. The chemical modification adds an electrophilic group that dangles off the cell membrane. When nucleophilic-bound receptor molecules are presented to the cell surface, they bind

instantly to the electrophile to provide the cell with newly inserted receptors. Specifically designed drug molecules or virus particles can bind to these receptors to initiate receptor-mediated endocytosis for targeted drug delivery (17,18).

2.1.3. Membrane Fusion

Membrane fusion occurs mostly intracellularly. However, similarities exist between membrane fusion occurring from extracellular space into the plasma membrane and from transport vesicles into the membranes of other internal organelles. Virus particles typically undergo membrane fusion to gain entry into host cells. The mechanism of action is not well-known, but there are several hypotheses. Two of the most commonly accepted ones will be discussed briefly below.

One set of hypotheses are called “proximity models” where a cell and another bilayer surrounded entity come so close together that hydrophilic molecules are displaced and the two lipid bilayers fuse into one membrane spilling the contents of the entity into the cellular cytoplasm (19). There are proteins that apparently catalyze all membrane fusions by anchoring the two membranes together to initiate the process. Inside the cell, these proteins are called soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) (20) proteins and they function to fuse transport vesicles with a target organelle.

A second group of hypotheses are called “fusion pore models,” where the space between the two membranes is bridged by a protein or lipid pore complex (19,21). Upon external activation, the pore complex undergoes a conformational change and an aqueous channel is formed linking the internal compartments of the two entities. The lipid layers then migrate inward opening up the channel until fusion is complete. This second hypothesis seems to explain many exocytic pathways that have been experimentally observed, and some authors suggest that this is the most likely theory (19,21).

2.1.4. Pore Transport

The passive transport of solutes, small molecules, or ions across the lipid bilayer can occur through aqueous pores or ion channels. Membrane-transport proteins in the lipid bilayer form aqueous pores connecting the extracellular space with the cytosol. Owing to the narrow channel in these pores, only small molecules and ions can effectively diffuse through, thereby protecting the cell from larger, potentially harmful substances. Some bacterial toxins manage to destroy cells by enlarging these pores to allow the entry of large harmful molecules (4). Aqueous pores may also be formed between two adjacent cells joining their cytoplasms. This type of pore is called a gap junction and is typically much wider, allowing larger molecule exchange between the cells. In either case, this route of cell entry is very fast, much more so than active transport through carrier proteins. The ion channels, which are specifically designed to transport inorganic ions across the lipid bilayer, can allow up to 100 million ions to pass per second.

One important difference between aqueous pores and ion channels is that aqueous pores remain open, but ion channels are gated and open and close in response to specific stimuli. There are several types of stimuli that can cause an ion gate to open. Voltage-gated channels open when a voltage change across the membrane occurs. Mechanical-gated channels open when a mechanical stress is applied or the membrane is stretched, and ligand-gated channels open when a specific ligand or receptor on the cell surface is bound. If the ligand is an extracellular mediator such as a neurotransmitter, it is a transmitter-gated channel. If the ligand is an intracellular mediator such as an

ion or nucleotide, it is an ion-gated channel or nucleotide-gated channel, respectively. The main importance to cellular drug delivery is that there are many means to open an ion channel and these paths may be exploited to deliver drug molecules into cells. Under normal circumstances, however, ion channels allow only tiny ions to pass and may not be a feasible route to deliver any but the smallest drug. Similarly, aqueous pores may only be useful for delivering small-molecule drugs into the cell unless a novel drug carrier can act to enlarge the pores or channels, as some bacteria and neurotoxins do, permitting entry of larger substances.

2.2. Intracellular Transport

Many of the barriers encountered at the plasma membrane also occur within the cell because lipid membranes surround most of the intracellular compartments. An overall schematic of general intracellular transport is shown in Fig. 2.

As described earlier, movement of many substances through the cytosol occurs via diffusion. The rate of diffusion is inversely proportional to the size of the molecule. In addition, throughout this semi-liquid media, even the diffusion of small molecules is slow compared with that in water. Small drug molecules entering the cytosol are often metabolized and then taken up into the ER, mitochondria, or peroxisomes through transmembrane transport. Other substances, such as macromolecules or macromolecular assemblies, are transported into the nucleus through gated channels or nuclear receptors, which only allow specific molecules to pass through. In most cases, large macromolecules in the cell have sorting signals that make them selective to certain organelles. If taken up into the mitochondria or peroxisomes, the substances are handled through the metabolic pathways to generate energy. If taken up into the ER, they become part of the biosynthetic secretory pathway in which they enter the Golgi complex, and become modified or metabolized and packaged. Eventually they are sent off as secretory vesicles bound for the cell surface or are diverted to lysosomes.

3. Receptor Proteins and Active Transport

Much research has been done to determine receptor protein structure and function and how these receptors initiate uptake mechanisms within cells. The research efforts continue so that every minute detail can be known as to how cells work. This knowledge can help us better understand specifically what causes diseases and what drugs or types of therapy can be used to cure or treat them by allowing us to target specific receptors on specific cells. In this section, receptor proteins and their function will be discussed, with some specific examples given as to how proteins interact with drugs during cellular drug delivery.

3.1. Receptor Proteins

Proteins are composed of a specific sequence of amino acids. They are of great importance as enzymes in the cell, as receptors on the cell surface, and as biological motors and switches within the cell. But what makes some proteins function as receptors specific only to certain molecules and how do these certain molecules “fit” into the receptor so well? The answer lies in the numerous noncovalent interactions that play a major role in protein folding and receptor binding.

The long polypeptide chain of a protein, anywhere from 50 to 2000 amino acids long, is able to adopt many different conformations, limited mainly by steric interactions from the amino acid side chains. The sequential order of amino acids, where the

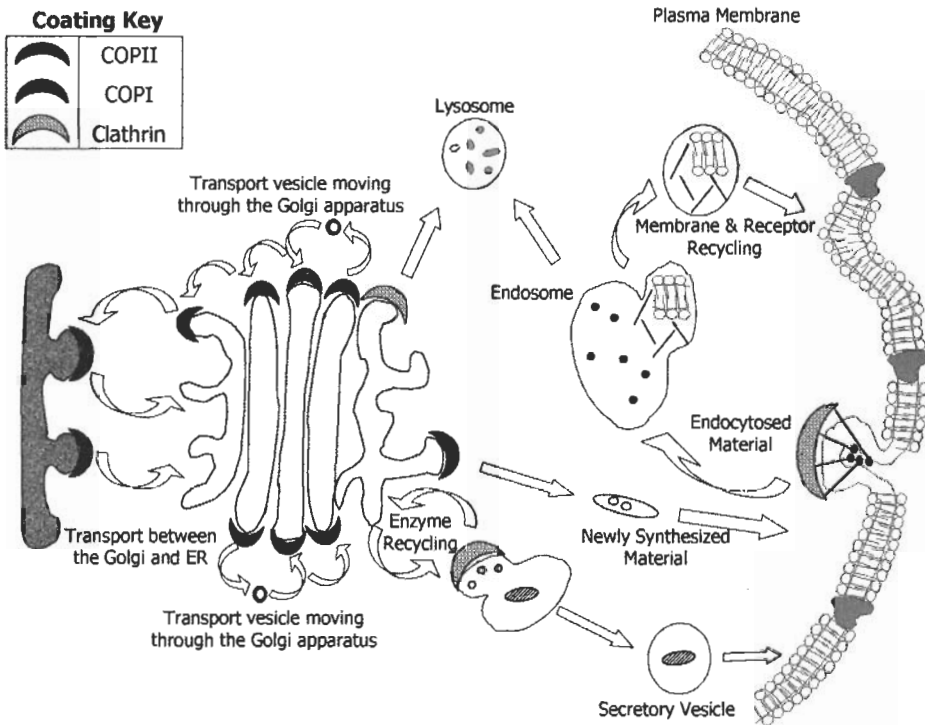


Fig. 2. General overview of the intracellular transport pathways occurring in most eukaryotic cells, including recycling of receptor proteins and membranes.

polar vs nonpolar groups are situated along the backbone, determines the folded shape of the protein as corresponding hydrogen bonds, ionic bonds, and van der Waals attractions hold specific sections together. The final conformation is usually the one in which the free energy of the system is at a minimum.

In order for a biological function to be carried out or activated, binding of a protein ligand or a drug to a protein receptor must occur. There are several different ways a ligand can bind to a particular protein receptor and most deal with only protein ligand or protein drug interactions at receptors. In the first case, the surface of one entity interacts with an extended string-like portion of another in a surface-string interaction. The second interaction, sometimes called a protein-protein interaction, occurs when two α -helices interact to form a coiled-coil structure. This type of interaction is often found in some gene-regulatory proteins and usually requires each entity to be a protein or large macromolecule with a coil structure. The third type of interaction is the most common one, generally stronger and more specific. It is the surface-surface interaction and it occurs when two protein surfaces are precisely matched up, such as the interaction between antibody and antigen. In most cases, the interaction is essential for the protein to function.

The binding of ligands or substrates to a receptor protein can initiate a protein conformational change. This plays a major role in cell signaling and enzyme regulation. For example, many antagonists or inhibitors bind to one site and prevent binding of other substances by causing the protein to shift conformations hiding other active sites.

This property of having more than one, differently shaped surface binding site is known as allostery and occurs in most receptor proteins. One site normally acts as a regulatory site for feedback mechanisms in the cell, whereas the other is the active site. When one site is bound, the protein “switches” on or off depending on which site is activated, and a new conformation with different surface contours is adopted. These types of changes not only occur to regulate pathways in the cell or signal changes, but also to move subcellular units around in the cell or move the cell itself around, as seen in motor proteins. Allosteric proteins can also use the energy from adenosine 5'-triphosphate (ATP) hydrolysis to accomplish active transport across cellular membranes. This mechanism of transport in addition to several examples will be discussed next.

3.2. Active Transport

There are several different kinds of active transport including ion transport through ion channels, electron pumps, and carrier-mediated transport through transporter proteins. In order for each of them to work, binding of one or more substrates must occur. The process involves specificity at the binding site and the potential for saturation of the binding sites owing to a high concentration of ligand. The transporters are capable of not only moving substances down a concentration or electrochemical gradient as in passive transport, but they can also pump substances against this gradient actively, which often occurs to get them inside the cell.

In general, carrier proteins bind drug molecules to bring them inside the cell without altering their chemistry. The process involves a reversible conformational change of the bilayer-embedded carrier proteins in order to initially bind to the ligand outside the cell and subsequently release it inside the cell. In most cases of active transport, energy is required to pump the ligand against the concentration gradient. There are three mechanisms in which cells achieve active transport: coupled carriers, ATP-driven pumps, and light-driven pumps.

3.2.1. Coupled Carriers

There are three kinds of carrier-mediated transporters. Those only transporting one type of substance from one side of the plasma membrane to the other are called uniporters. The other two types are symporters and antiporters, which transport either two types of substances at the same time from one side to the other or two types of substances in opposite directions across the membrane, respectively. In the latter case, the coupled carriers often use the electrochemical gradient driving force of one of the substrates to transport the other. In many cells, Na^+ is the co-substrate providing the driving force into the cell. When Na^+ comes into the cell, an ATP-driven pump embedded in the plasma membrane is activated to transport it out of the cell, thus maintaining a large electrochemical gradient for the influx of a particular substance. In other cases, H^+ ions are the co-substrate as found in the uptake of enalapril, an angiotensin-converting enzyme (ACE) inhibitor, by small intestinal brush border membrane vesicles of rabbits (22). More information on transporters, their superfamilies, and prodrug approaches to cellular drug delivery utilizing membrane transporters is discussed in Chapter 7.

3.2.2. ATP-Driven Pumps and Light-Driven Pumps

Light-driven pumps are mainly found in bacterial cells and will not be discussed here in great detail. They use the energy from light to actively transport ligands against the electrochemical gradient.

ATP-driven pumps have an important role in cell regulation. The mechanisms that pump H^+ ions into lysosomes and other organelles where a low pH is required act similarly to the motor proteins. One of the best understood pumps of this sort is the Ca^{2+} pump in muscle cells, which uses the energy of ATP hydrolysis to change conformations. This allows it to bind ions, bring them inside the cell, and release them while returning to the lower energy conformation. Another critical ATP pump in cell regulation is the Na^+-K^+ pump, or Na^+-K^+ ATPase, as it is sometimes called. It is crucial not only to pH regulation, but also in the regulation of cell volume, propagation of nerve impulses, and transport of essential nutrients.

3.3 Proteins Involved in Multi-Drug Resistance

Finding a specific drug with high potency, delivered to the patient with a suitable and efficient formulation or drug carrier are not the only challenges facing drug-delivery scientists. Often, therapy is hindered by multi-drug resistance (MDR), and thus the understanding of the mechanism as well as how to overcome it becomes a tremendous task. There are many different resistance mechanisms that hinder the effective treatment of infectious and malignant diseases, but the resistance mechanism involving the ATP-binding cassette (ABC) superfamily of transporter proteins has gained the most attention over recent years owing to its role in some of the most common, widespread diseases such as malaria, leishmaniasis, and cancers (23). In many cases, drug influx into the cell is not enough to overcome drug efflux out of the cell, resulting in the lack of drug accumulation inside the cell and thus unsuccessful therapy. Several transporters responsible for drug efflux are members of the ABC superfamily. These proteins act as ATP-driven pumps and use the energy from ATP hydrolysis to actively transport substances across the plasma membrane. They include P-glycoproteins (P-gp), multi-drug resistance-associated proteins (MRP), and lung resistance-related proteins (LRP) (24–26). Many MDR cell lines have been found to exhibit excess numbers of these transport proteins, thus causing a lack of drug accumulation within the cell owing to extensive efflux. Additionally, the intracellular pH has been linked to MDR, as found in drug-resistant mouse renal proximal tubule cells. Alkalinization of the endosomes and lysosomes by lysosomotropic agents have been found to stimulate the efflux of vinblastine, an anticancer drug (26).

Cancer is one of the most well-studied illnesses in which MDR is a major problem. The reason many cancer chemotherapy regimens fail is multifactorial. Extracellular factors including bioavailability of the drug, drug metabolism, cell-cycle stage, and proliferation status, as well as intracellular factors of drug influx and efflux involving these ATP-driven efflux pump proteins, DNA replication, and cellular repair mechanisms, have all been identified as contributing significantly to MDR (24). One method to address MDR is to block efflux of drugs from the cell to increase retention. Several different types of drugs have been studied as effective efflux blockers and chemosensitizing agents. They include verapamil, phenothiazine calmodulin inhibitors, cyclosporins, and tamoxifene, and have generally been shown to be effective in overcoming resistance (24). The ABC superfamily and MDR is discussed in more detail in Chapters 8 and 9, and cancer and its treatment modalities are reviewed in Chapters 11 and 12.

4. Conclusion

The complexity of even a single cell presenting formidable barriers to drug delivery both intracellularly and extracellularly can be overwhelming. By studying the mecha-

nisms employed by viruses, bacteria, and toxins in exerting their effect on cells, the drug-delivery scientist can gain important insights on how to exploit these mechanisms to deliver drugs effectively to target cells or target organelles, overcome barriers associated with uptake either passive (endocytosis) or active (transport proteins), or conquer MDR. A multidisciplinary background is necessary to understand the science of cellular drug delivery so that continuous improvements can be made in the quality of therapeutics.

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References

1. Saltzman WM. Drug Delivery: Engineering Principles for Drug Therapy. New York, NY, Oxford University Press, 2001.
2. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell, 4th ed. New York, NY, Garland Science, 2002.
3. Tomlinson E. Pathophysiology and the temporal and spatial aspects of drug delivery. In: Site-Specific Drug Delivery. Tomlinson E, Davis SS, eds. Chichester, UK, John Wiley & Sons, 1986, pp. 1–26.
4. Voet D, Voet JG. Biochemistry, 2nd ed. New York, NY, John Wiley & Sons, 1995.
5. Campbell NA, Reese JB, Mitchell LG. Biology 5th ed. Menlo Park, CA, Benjamin/Cummings, 1999.
6. Kuchel PW, Ralston GB. Schaum's Outline of Theory and Problems of Biochemistry. New York, NY, McGraw-Hill, 1988.
7. Jensen KD, Nori A, Tijerina M, et al. Cytoplasmic delivery and nuclear targeting of synthetic macromolecules. *J Control Rel* 2003;87(1–3):89–105.
8. Rosenkranz AA, Lunin VG, Sergienko OV, et al. Targeted intracellular site-specific drug delivery: Photosensitizer targeting to melanoma cell nuclei. *Russ J Gen* 2003;39(2):198–206.
9. Dargemont C. Nuclear export of proteins: molecular mechanisms and functions. *M S-Med Sci* 2002;18(12):1237–1244.
10. Lackey CA, Murthy N, Press OW, et al. Hemolytic activity of pH-responsive polymer-streptavidin bioconjugates. *Bioconj Chem* 1999;10(3):401–405.
11. Washington N, Washington C, Wilson CG. Physiological Pharmacutics: Barriers to Drug Absorption, 2nd ed. New York, NY, Taylor and Francis, 2001.
12. Hopkins CR. Site-specific drug delivery-cellular opportunities and challenges. In: Tomlinson E, Davis SS, eds. Site-Specific Drug Delivery. Chichester, UK, John Wiley & Sons, 1986, pp. 27–48.
13. Hillery AM. Drug delivery: the basic concepts. In: Hillery AM, Lloyd AW, Swarbrick J, eds. Drug Delivery and Targeting: For Pharmacists and Pharmaceutical Scientists. New York, NY, Taylor and Francis, 2001, pp. 1–48.
14. Gumbleton M, Hollins AJ, Omid Y, et al. Targeting caveolae for vesicular drug transport. *J Control Rel* 2003;87(1–3):139–151.
15. McIntosh DP, Tan XY, Oh P, et al. Targeting endothelium and its dynamic caveolae for tissue-specific transcytosis in vivo: a pathway to overcome cell barriers to drug and gene delivery. *Proc Natl Acad Sci USA* 2002;99:1196–2001.
16. Sandvig K, van Deurs B. Membrane traffic exploited by protein toxins. *Ann Rev Cell Dev Bio* 2002;18:1–24.
17. Bertozzi C, Bednarski M. C-glycosyl compounds bind to receptors on the surface of escherichia-coli and can target proteins to the organism. *Carb Res* 1992;223:243–253.
18. Handel TM, Bertozzi C, Hubbell JA. Biopolymer engineering and design: beyond the genome—Editorial overview. *Curr Opin Chem Biol* 2001;5(6):675–676.
19. Lindau M, Almers W. Structure and function of fusion pores in exocytosis and ectoplasmic membrane-fusion. *Curr Opin Cell Biol* 1995;7(4):509–517.

20. Tsui MMK, Tai WCS, Banfield DK. Selective formation of Sed5p-containing SNARE complexes is mediated by combinatorial binding interactions. *Mol Biol Cell* 2001;12(2):521–538.
21. Lentz BR, Malinin V, Haque ME, Evans K. Protein machines and lipid assemblies: current views of cell membrane fusion. *Curr Opin Struct Biol* 2000;10:607–615.
22. Kitagawa S, Takeda J, Sato S. Uptake of enalapril by rabbit small intestinal brush-border membrane vesicles. *Biol Pharm Bull* 1999;22(7):762–764.
23. Cole SPC, Deeley RG. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. *BioEssays* 1998;20(11):931–940.
24. Krishan A, Fitz CM, Andritsch I. Drug retention, efflux, and resistance in tumor cells. *Cytometry* 1997;29(4):279–285.
25. Sharma R, Awasthi YC, Yang Y, et al. Energy dependent transport of xenobiotics and its relevance to multidrug resistance. *Curr Cancer Drug Targets* 2003;3(2):89–107.
26. Ouar Z, Lacave R, Bens M, et al. Mechanisms of altered sequestration and efflux of chemotherapeutic drugs by multidrug-resistant cells. *Cell Biol Toxicol* 1999;15(2):91–100.