CHAPTER 1

An Introduction to Subcellular Nanomedicine: Current Trends and Future Developments

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Drug therapy is based largely on the paradigm that an ideal drug will selectively exert a desired pharmacological activity free from negative side effects to modulate either the symptoms or the underlying biochemical cause of a disease to provide a benefit to the patient. In order to have such selective action, the drug molecule should ideally interact with only the disease-associated biochemical pathway but have no activity with respect to any normal biochemical pathway. This principle was famously explored by Paul Ehrlich in his search for agents with selective toxicity toward bacteria. Ehrlich's work is widely accepted to have given rise to the concept of the ideal drug molecule as a "magic bullet," a term that he used for the first time in his Harben Lectures [1]. Finding such selective molecules is relatively easy when there are significant differences between the disease-causing process and normal human biochemical pathways, as in the case of infectious diseases. Not surprisingly, in the decades since Ehrlich's work infectious diseases have become much easier to treat but it does bear consideration that the lack of activity in non-disease cells is dose dependent and not absolute. Most drugs that are considered to be selectively toxic to invading pathogens are in fact toxic to human cells as well but just at higher doses. However, given that the new challenges in drug therapy lie in the treatment of diseases associated with malfunctions

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of normal human biochemical pathways in certain tissues, the concept of the magic bullet perhaps needs to be redefined or at least clarified. In the infectious disease example it is essential to understand that the so called magic bullet did not necessarily have to home in on the disease agent but could in fact accumulate to the same level in both host and pathogen cells. Just as long as the agent was toxic only to the pathogen it was considered by many to be a magic bullet. In fact, Ehrlich's Nobel Prize Lecture from December 11, 1908 speaks of "outlining the principles of selective toxicity" rather than selective accumulation. Therefore one could argue that for a molecule to be a magic bullet, it does not have to selectively accumulate at its intended site of action but just that it should not exert its action anywhere but at that intended site of action. This is different from what is now referred to as drug targeting.

The term "targeting" is most often meant to imply that the molecule is in some way able to selectively accumulate at an intended site of action and that the selective accumulation is associated with its selective action as a magic bullet. Unfortunately (perhaps due to the widespread use of the noun target to describe a potential molecular site of action), there is often the misconception that a drug that is believed to act at a molecular target (noun) is by default also able to target (verb) or "home in" on that target (noun). It would therefore be more appropriate to define a true magic bullet as a drug molecule that is specific in its activity for a molecular target but that is also able to selectively accumulate at this molecular target and exert a selective therapeutic action by virtue of both its specific activity and its selective accumulation. This distinction is important when we consider the daunting challenge of developing magic bullets for diseases like cancer and neurodegenerative diseases like Alzheimer's, as well as hormone imbalance diseases like diabetes that are becoming more widespread. Unless unique molecular targets found exclusively (or at sufficiently higher levels) in the diseased state and not in normal state are discovered, magic bullets by the traditional definition or the compromise of dose-dependent activity at the site of action may not be feasible. Strategies to effectively control the disposition of drug molecules thus represent an important tool for successful therapy.

At a very basic level, selective accumulation is influenced by bioavailability and subsequent biodistribution. In the context of drug molecules, biodistribution is primarily related to physicochemical properties. Many potent drug candidates exhibit low bioavailability due to their limited water solubility. On the other hand, water-soluble compounds display a very limited ability to cross biological membranes, which essentially can exclude them from the cell interior. To overcome the limitations that a compound's physicochemical nature can impose on its potential pharmaceutical application, the process of large-scale screening of chemical libraries has been extended beyond just identifying desired bioactivity. Screening approaches routinely incorporate selection for desirable physicochemical properties that might confer high bioavailability as well. On the down side, this approach leads to many potent molecules being

excluded from further development because they aren't true magic bullets; that is, while they may have a potent pharmacological action at a desired molecular target they aren't able to find their way exclusively to that target. There is most certainly a growing list of such molecules that are in essence potential drugs if only a delivery strategy can be devised to get them to their molecular target in the human body. As the search for the perfect magic bullet continues there is a significant effort to improve the action of currently available molecules by using targeted delivery approaches. It is not surprising that the field of drug targeting has grown significantly in the effort to develop better therapy. Based on the experience over the decades since Ehrlich first introduced the magic bullet concept, it seems more reasonable to separate the functions of pharmacological action and selective accumulation into properties desired in a drug molecule and in a delivery system, respectively, rather than the traditional expectation that the drug molecule alone possess both properties.

Generally, drug disposition may be modulated via three broad approaches First, the drug molecule might be modified subtly to change its physicochemical properties without adversely affecting its inherent pharmacological action. This is essentially the intent of medicinal chemistry approaches and the concept of structure–activity relationship (SAR) studies that have now become standard practice and often aren't even considered a means of achieving targeting. The second approach might be considered to be an extension of the first but is different in that it involves using chemistry to conjugate ligands that are often larger than simple organic functional groups to change the biodistribution of a molecule. Again this approach works as long as the conjugation does not adversely affect the desired pharmacological activity of the molecule. Conjugation using selectively cleavable linkers is an extension of this strategy. The third strategy involves the use of some sort of delivery system or a carrier system and does not involve chemical modifications to the pharmacologically active molecule. Pharmaceutical nanocarriers fall into this category and several such technologies are being developed that are fast becoming applicable to a variety of pharmacologically active molecules.

Pharmaceutical nanocarriers offer what might be viewed as a nonchemical approach to modify the disposition of drug molecules. All chemistry can be performed on the components of the nanocarrier system that can then be loaded with the drug to afford targeted delivery [2–5]. Most pharmaceutical nanocarriers can be modified for some level of targeting to specific tissues if not specific cell types. Long circulating liposomes and nanoparticles are able to passively target areas of leaky vasculature by virtue of the EPR effect and can additionally be modified with antibodies or other targeting ligands to afford cell specific recognition [6–10].

However, despite such advances, the improvement in drug action is not always dramatic. This is likely because many drugs act at molecular targets inside the cells and these molecular targets are often in well-organized subcellular structures inside the mammalian cell.

The interior of a cell is very different from an aqueous buffer solution, in which small drug molecules can freely diffuse and randomly interact with potential cosolutes. In addition to the presence of the cytoskeletal network and various dispersed organelles, the cytoplasm contains a large amount of dissolved macromolecules. The concentration of dissolved macromolecules in the nucleoplasm and cytoplasm of living cells has been determined to be between 50 and 400 g/L [11, 12]. Subsequently, transport or diffusion events in such a crowded solution cannot be expected to be the same as those in buffer solutions. Generally, intracellular diffusion has been characterized as hindered diffusion, reflecting among other factors the high level of molecular crowding [13, 14]. Additionally, the fluid-phase viscosity of the cytoplasm and binding to intracellular components are believed to influence the diffusion of solutes inside a cell [15, 16]. While efforts aimed at thoroughly understanding cellular material properties such as cytoplasmic viscosity are currently underway [17], it is generally accepted that the physicochemical properties of the drug also play a major role in determining the subcellular fate of the drug molecule. Consequently, the ability to predict the influence of various properties of the drug molecule on the likely site of accumulation within the cell could prove to be a powerful tool in drug design to either select molecules with a desired subcellular accumulation or identify molecules that would benefit from subcellular targeting strategies.

There is apparent fractal symmetry between the case of drug delivery to a cell and drug delivery to a molecular target inside a subcellular compartment. The cell could be viewed as being a small, slightly simpler but nonetheless highly organized "body" with "organs" (organelles) and "cells" (defined structures and molecular arrangements) within these organs. It should therefore stand to reason that controlling drug disposition within the cell might also be necessary for optimal drug action [18–27]. Consequently, the next logical step in the development of targeted nanocarriers would be to extend our control over nanocarrier distribution to the subcellular level as well.

At least two major schemes can be imagined to be useful in the design of nanocarriers with the potential for subcellular targeting: the first based on the inherent predisposition of the nanocarrier for a particular compartment and the second based on attaching subcellular targeting ligands to the surface of nanocarriers to redirect their accumulation to the desired compartment. Essential to the latter of these approaches is the use of a subcellular targeting ligand. Such ligands could be, as in the case of leader sequences, derived from normal cellular trafficking processes or, as in the case of triphenyl phosphonium, based on observations of a predisposition of an organic compound for subcellular compartments. The availability of a wide range of subcellular stains is proof enough that there are several molecules with an inherent ability to accumulate in a particular subcellular compartment.

Based on the intracellular distribution of a large variety of fluorophores, a quantitative structure–activity relationship (QSAR) model for predicting cellular uptake and intracellular distribution of low molecular weight compounds

has been proposed [28]. This QSAR approach was recently applied to identify potential common chemical features of molecules that are known to selectively accumulate at or inside mammalian mitochondria within living cells [29]. The QSAR approach has additionally proved useful for the modeling of cationic transfection lipids [30] and could therefore be applicable to predicting the subcellular disposition of a potential therapeutic molecule and even to design molecules with a desired subcellular affinity for the development of subcellular targeting approaches.

Approaches to nanocarrier-mediated subcellular delivery are based on the principle that the subcellular destination of a drug is the same as that of the nanocarrier. Nanocarriers by virtue of their particulate nature are believed to be subject to endocytic cell entry mechanisms and subsequent endolysosomal processing. As such, nanocarriers could be considered to be ideally suited for delivering bioactive molecules to the endolysosomal system. Indeed, directing nanomedicine complexes to the endolysosomal system has increasingly gained attention, as pathological conditions associated with endosomes and lysosomes could potentially benefit from therapies targeting these pathways [31–34]. Although endocytosis is a common mechanism that almost all cells possess for the internalization of macromolecules, a wide array of such vesicular internalization mechanisms exist [31]. For example, nanoscale drug carrier systems taken up by clathrin-dependent receptor-mediated endocytosis (RME) are most likely to undergo lysosomal degradation, while clathrin-independent RME may lead to endosomal accumulation [31]. Consequently, the type of targeting moiety displayed by the nanocarrier system will determine whether the carrier delivers its cargo to either endosomes or lysosomes.

Well-characterized endocytic targeting moieties potentially useful for nanocarrier-mediated drug delivery are folic acid, low-density lipoprotein, cholera toxin B, mannose-6-phosphate, transferrin, riboflavin, the tripeptide RGD, ICAM-1 antibody, and nicotinic acid, as recently reviewed by Bareford and Swaan [31]. The cellular internalization mechanisms utilized by these ligands involve clathrin-dependent RME, caveolin-assisted endocytosis, lipid raft associated endocytosis, and cell adhesion molecule (CAM) directed cellular uptake [31].

In addition to several approaches to exploiting the inherent tendency of nanoparticles to accumulate in the endolysosomal compartment for possible therapeutic purposes, there is a growing body of work that suggests the feasibility of modifying nanocarriers to redirect delivery of their cargo to other subcellular compartments as well. Liposomes modified with mitochondriotropic ligands have been shown to improve the efficacy of an anticancer drug both in vitro and in vivo [35]. AuNPs have already been targeted to the nucleus using the adenoviral nuclear localization signal (NLS) and integrin binding domain [36]. Such an approach has been reported to be useful in the development of probes for cell tracking by surface-enhanced Raman scattering [37]. Modification with a leader sequence peptide has also been applied to creating delivery systems for mitochondria. A mitochondrial leader peptide

(MLP), derived from the nucleocytosol expressed but mitochondria localized ornithine transcarbamylase, has been reported to render polyethylene imine (PEI) mitochondriotropic and represents a potential approach for mitochondrial DNA delivery [38].

It is interesting to note that the examples discussed above share a common assumption. Nanocarriers are assumed to have a predisposition for the endolysosomal pathway by virtue of their nanometer size and, without a subcellular targeting ligand, all nanocarriers would remain in the endolysosomal compartment. However, it is interesting to also consider the disposition of a nanocarrier made exclusively of a molecule with a predisposition for a subcellular compartment. A good example is the mitochondriotropic amphiphile dequalinium chloride. A serendipitous discovery while screening mitochondriotropic drugs potentially able to interfere with the mitochondrial DNA metabolism in *Plasmodium falciparum* [39] revealed this self-associating tendency of dequalinium chloride and its ability to form vesicles. At the time of their discovery, these unusual vesicles were termed DQAsomes (pronounced dequasomes), that is, dequalinium (DQA) based liposome-like vesicles [40]. Based on the fact that these carriers were composed exclusively of mitochondriotropic molecules and that they were able to bind and protect DNA, DQAsomes were explored as potential mitochondria-specific DNA delivery vehicles for direct mitochondrial gene therapy [41–44]. DQAsomes have also been explored as a mitochondria-targeted nanocarrier system for small drug molecules, in particular, for anticancer drugs known to trigger apoptosis via direct action on mitochondria [45, 46]. It would therefore appear that, for now, a basic proof of concept for an alternative strategy toward the design of subcellular targeting nanocarriers seems to have been established. It is also obvious that in order to design similar carriers for other subcellular compartments it would be necessary to first find self-assembling molecules with an affinity for the intended subcellular compartment. To this end, recent work on the subcellular distribution of micelle-forming agents offers some interesting insights [47–51].

Based on the examples discussed so far, it would seem that there is indeed hope that nanocarrier systems could be designed to achieve true molecular level targeting inside cells. However, to say that these systems will be available soon is perhaps premature given what little we know about the subcellular dynamics associated with nanoparticle trafficking. There are in our opinion several unanswered questions. For example, do all nanocarriers remain intact upon cell entry and subsequent disposition? Are there differences in the disposition of vesicles in comparison to particles? What is the true influence of size on the intracellular disposition of various nanocarriers? Most important, however, is the question of the mechanism by which the nanocarrier is able to achieve selective uptake and delivery into the subcellular compartment. All the strategies described so far report observations of altered or improved subcellular accumulation that appears to result in improved activity, but how exactly this happens is still unclear. Do the nanocarriers remain intact upon

internalization and then get trafficked as intact structures? If so, how is the therapeutic cargo released to the correct subcellular compartment? Alternatively, it could be imagined that once taken up into the early endosomal vesicle, the nanocarrier components undergo a redistribution to become part of the endosomal vesicle. There is some evidence to suggest that in fact cells actively traffic nanocarriers in cell membrane-derived vesicles [52]. Assuming the targeting ligand was able to redistribute to the surface of the endosomal vesicle, it might be possible then that the vesicle would have an altered subcellular fate that could involve transport to and association with a target compartment other than the lysosome. While this may seem to be far-fetched speculation, there has already been some work along similar lines toward the development of nanocarrier systems for delivery of molecules to the nucleus and even the mitochondria. A strategy that involved stepwise membrane fusion was devised based on the premise that, to efficiently deliver DNA to the nucleus, a delivery system must penetrate through the plasma membrane, nuclear envelope, prior to DNA release in the nucleus. Using a multilayered nanoparticle called a Tetralamellar Multifunctional Envelope-type Nano Device (T-MEND) and consisting of a DNA-polycation condensed core coated with two nuclear membrane-fusogenic inner envelopes and two endosome-fusogenic outer envelopes, which are shed in stepwise fashion, transgene expression in nondividing cells was reported to be dramatically increased [53]. A similar approach in designing a mitochondria-specific delivery sytem has been reported as well. Liposomal carriers called MITO-Porters, which carry octaarginine surface modifications to stimulate their entry into cells as intact vesicles (via macropinocytosis) were prepared with lipid compositions that were identified in various experiments to promote both fusion with the mitochondrial membrane and the release of liposomal cargo to the intramitochondrial compartment in living cells. Using GFP protein as a model cargo, it was shown that MITO-Porter liposomes are able to selectively deliver their cargo to mitochondria [54, 55].

It is also interesting to note that changes in nanoparticle architecture result in changes in subcellular disposition [56]. Fluorescein isothiocyanate labeled layered double hydroxide (LDH) nanoparticles were prepared from Mg_2Al under conditions that yielded either hexagonal sheets (50–150 nm wide and 10–20 nm thick) or nanorods (30–60 nm wide and 100–200 nm long). A comparison of the subcellular distribution of these two types of preparations revealed that the nanorods trafficked to the nucleus but the hexagonal sheets remained in the cytoplasm [56]. Not surprisingly, an active microtubule mediated transport process is hypothesized to be responsible for the observed rapid nuclear accumulation of the nanorods [56].

As discussed so far, various nanocarrier platforms have already undergone preliminary investigation for their ability to control the subcellular disposition of drugs and a potential improvement in therapy through the use of nanocarriers for subcellular targeting of bioactive molecules. A common paradigm that currently seems to apply to most of these approaches is the use of

subcellular targeting ligands to control subcellular distribution. Given the relative ease of modifying nanocarriers with various surface functionalities and the fact that such approaches are already in use to achieve targeting at the cellular and organ levels, the ligand-based approach does seem to be a logical extension of current technology. It helps that new tools are concurrently being investigated to understand some of the physicochemical aspects of how small molecules [28, 29] as well as proteins [57] are able to selectively accumulate in certain subcellular compartments to afford the rational design of a wide repertoire of subcellular targeting ligands. However, one must be cautious in the knowledge that the approaches described so far are based only on current understanding of subcellular trafficking. Current knowledge of subcellular trafficking processes is based largely on studies with solid nanoparticles and on quantum dots [58–64]. Whether these observations can be extended to vesicular carriers like liposomes and micelles remains in question and in our opinion is due in large part to current limitations in imaging technology. We are however hopeful that technological advances in real-time fluorescence confocal imaging of live cells [65–68], as well as the emergence of new imaging techniques like total internal reflection microscopy [69] and label-free approaches like Raman microscopy [70–72], will allow some of the questions raised to be more satisfactorily answered. This book is our attempt to bring the best of current knowledge together to provide a comprehensive resource for anyone interested in this emerging area of drug delivery. The subsequent chapters discuss in full detail the current state of the art in the various approaches to nanocarrier-mediated bioactives to cell organelles as well as emerging research methods for the identification of subcellular targeting ligands and the study of subcellular transport processes.

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