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COMMENTARY

Quantitative Aspects of Intracellularly-Targeted Drug Delivery

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INTRODUCTION

Many pharmacological agents act intracellularly, need to be endocytosed, and reach the site of action in specific organelle to exert their action. The cell's interior is highly compartmentalized, and complexity of the cellular endocytosis and trafficking pathways (1,2) leads to suboptimal magnitude and duration of pharmacological effects at the organelle of interest, as well as to non-specific effects due to exposure of additional organelles to the drug. Thus, attaining efficient and selective pharmacological effects for intracellularly acting drugs requires development of specialized drug delivery systems (DDS) that should be targeted to specific organelle and deliver the drug in a controlled fashion (3,4).

For this purpose, particle or vesicle (liposome)-based DDS can be used, and intracellular targeting can be achieved by decorating the drug or the DDS with organelle-specific targeting moieties. This approach relies on recognition of these moieties by the endogenous intracellular trafficking mechanisms and preferential delivery of the drug or the DDS into specific organelle (3,4). Feasibility of targeted delivery of drugs and model compounds into individual organelles has been assessed in several studies (summarized in Table I). However, the quantitative aspects of this targeting, in terms of targeting efficiency and kinetics of drug delivery to the specific intracellular organelles, are not yet clear and should be intensively studied in order to pave the way to the clinical application of the intracellularly targeted DDSs. This commentary analyzes the existing approaches for intracellular drug targeting, their efficiency and limitations. For an introduction to intracellular drug delivery, the reader is referred to several excellent recent reviews (3,5). Delivery of DNA and RNA molecules is not in the scope of this commentary and will be mentioned only briefly.

TARGETING EFFICIENCY

Efficient intracellular targeting of a drug should lead to preferential accumulation of the drug in the target organelle, and not in the other intracellular organelles, following endocytosis. Assessment of drug amounts that have reached the individual organelles, i.e. the drug intracellular distribution, can be done using fluorescence-based (confocal microscopy) (6) and biochemical (7,8) approaches. However, both these approaches are limited to semi-quantitative analysis of drug content in the selected set of organelles and do not allow detailed analysis of the drug's intracellular distribution and mass balance calculations. Therefore, studies on intracellular drug targeting usually report relative drug accumulation in the target organelle, but not in other organelles. In some studies, intracellular drug concentrations are not quantified, and the extent of drug accumulation in the target organelle is deduced from the bioactivity of the organelle-targeted *vs.*

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Table I Examples of Intracellularly-Targeted Drug Delivery Approaches

Study	Drug and formulation	Target organelle	Experimental system	Outcomes	Quantitative formulation/delivery parameters
Tachibana *et al.* (10)	NLS-FITC-albumin in pH-sensitive liposomes	Nucleus	*In vitro* uptake and intracellular localization in rat peritoneal macrophages	Penetration of NLS-FITC-albumin but not of FITC-albumin into the nucleus	4–5 NLS residues per FITC-albumin molecule. ~1% of the endocytosed FITC-albumin-NLS molecules were estimated to reach the nucleus
Misra & Sahoo (17)	Doxorubicin-loaded PLGA nanoparticles decorated with nuclear localization signal	Nucleus	*In vitro* uptake, intracellular localization, and cytotoxicity in MCF-7 human breast cancer cells	Enhanced cellular uptake, nuclear accumulation and cytotoxicity of the targeted nanoparticles	443 targeting residues per nanoparticle
De la Fuente & Berry (26)	Gold nanoparticles decorated with Tat peptide	Nucleus	*In vitro* uptake by primary human fibroblasts and intracellular localization by electron microscopy	Penetration of the nanoparticles into the nucleus	–
Kang *et al.* (27)	Gold nanoparticles decorated with RGD peptide and nuclear localization signal	Nucleus	*In vitro* uptake, intracellular localization, and cytotoxicity in human malignant (HSC-3 oral squamous cell carcinoma) and nonmalignant cells	Nuclear accumulation and enhanced cytotoxicity of the targeted nanoparticles	–
Hoshino *et al.* (16)	Quantum dots (QD) conjugated with nuclear or mitochondrial localization signal	Nucleus or mitochondrion	*In vitro* uptake and intracellular localization in Vero monkey kidney epithelial cells	Enhanced accumulation of the QD in the target organelle	48 and 62 targeting residues per QD, respectively; rapid nuclear accumulation of the QD (within 15 min).
Boddapati *et al.* (28)	Liposomes encapsulating ceramide and decorated with mitochondriotropic triphenylphosphonium cations	Mitochondrion	*In vitro* uptake and cytotoxicity assays (in 4T1 mouse mammary carcinoma and human COLO205 cells), *in vivo* tumor growth inhibition (4T1 cells growth in mice)	Enhanced delivery of liposomes and ceramide to mitochondria, enhanced cell death *in vitro* and *in vivo*	Increase by 20% in metabolic ceramide toxicity in mitochondria-targeted *vs.* nontargeted liposomes
Yamada *et al.* (29)	Octaarginine-modified liposomes loaded with GFP	Mitochondrion	Membrane fusion with isolated rat mitochondria, *in vitro* uptake and intracellular localization in HeLa human cervical carcinoma cells	Enhanced cellular uptake and accumulation in the mitochondria	~1% encapsulation efficiency of GFP in the liposomes, ~4–5-fold increase in mitochondrial accumulation in octaarginine-modified *vs.* octaarginine-lacking liposomes
Hayashi *et al.* (14)	Fusogenic liposomes loaded with ER signal peptide-antigenic peptide conjugate	Endoplasmic reticulum	*In vitro* antigen presentation assay, *in vivo* CTL induction and tumor protection assays	Enhanced and prolonged CTL activation, enhanced protective immunity against antigenic peptide-presenting tumors	Prolonged cross-presentation of the antigen (more than 140 h).
Matsuo *et al.* (15)	Poly(γ-glutamic acid) nanoparticles loaded with ER signal peptide-antigenic peptide conjugate	Endoplasmic reticulum	*In vivo* CTL induction and ELISPOT assays	Enhanced CTL activation	–

untargeted formulations. In both cases, efficiency of drug targeting to a specific organelle (i.e., drug/DDS accumulation in the target organelle in comparison to the other organelles) cannot be readily estimated.

Preferential drug delivery to target organelles was claimed in several studies (see Table I). Targeting moieties that were used for this purpose included 1) peptide sequences that are recognized by the cytosolic transport systems of the host cell, such as nuclear localization signal (NLS), mitochondrial localization signal, endoplasmic reticulum (ER) signal peptide or ER-retrieval sequence, etc., and 2) peptide or non-peptide molecules that preferentially interact with the membrane of the target organelle, e.g. mitochondriotropic arginine-rich peptides or positively charged compounds.

Endosomal 'Escape' of the Drug/DDS

Both peptide and non-peptide targeting moieties are active within the cytosol and will not be able to target the drug or the DDS to the organelle of interest if they are located inside one of the organelles, such as endosome or lysosome. Thus, the *first limiting step* that affects the intracellular targeting efficiency is the ability of the drug or the DDS to reach the cell's cytosol. If the targeting signal is conjugated directly to the drug, the resulting drug-targeting moiety conjugate is usually small and can efficiently permeate the intracellular membranes and reach the cytosol following its release from the DDS. The situation is different for the targeting-moieties-conjugated particle or vesicle-based DDSs. 'Escape' of these DDSs from the endosomes to the cytosol following endocytosis is the major obstacle for their efficient intracellular targeting. Only a small fraction of the DDS-containing endosomes spontaneously degrades and releases its contents to the cytosol, while the majority of the endocytosed material is degraded in the endosomal-lysosomal compartments and is not able to reach the cytosol and subsequently traffic to the target organelles. Destabilization of the endosomal membrane using fusogenic liposomes or cell-penetrating peptides can substantially enhance the endosomal escape of the DDS (4,9). It was estimated that as much as 12% of the endocytosed material was able to reach the cytosol when administered in pH-sensitive liposomes (10). Endosomal membrane destabilization, though, can be toxic to the cells and may be unsuitable for intracellular delivery of some therapeutic agents. PLGA nanoparticles are able to escape from the endo-lysosomes without opening them. The mechanism of this escape involves direct interaction of the nanoparticles with the endo-lysosomal membrane due to selective reversal of the surface charge of PLGA (from anionic to cationic) under acidic conditions (11,12).

Cytosolic Rates of Mobility, Degradation, and Uptake by the Target Organelle

Once the drug or the DDS have reached the cytosol, they can traffic to and accumulate in the organelle of interest. The *second limiting step* that affects the intracellular targeting efficiency is derived from the interplay of the processes and factors that act on the drug or the DDS in the cytosol, i.e., stability and mobility of the targeted drug/DDS, efficiency of recognition of the targeting residues by their targets, rate of uptake by the target organelle, etc. Our understanding of the mechanisms and efficiencies of the forces that act on the drug/DDS in the cytosol is very limited. It is known that small molecules, such as peptides and small soluble proteins, are highly mobile in the cytosol (D=2–3 $\mu m^2/s$) and are able to diffuse through the entire cell in 5–7 s (13). This time is short enough and may result in efficient uptake by the target organelle even for peptide drugs that are rapidly degraded by cytosolic proteasomes and proteolytic enzymes ($t_{1/2} \approx 7$ s) (13). Thus, conjugation of even a single targeting residue to a peptide-based drug may be sufficient for its preferential accumulation in the target organelle following delivery to the cytosol. For instance, conjugation of the ER signal peptide to the antigenic peptide (1:1 drug:targeting residue ratio) was reported to enhance its accumulation in the endoplasmic reticulum of the target cells following its release from the nanoparticle or liposome-based DDS (14,15).

Cytosolic mobility of the particle and vesicle-based DDS is much lower, and recognition of the targeting residues-decorated DDS by the cytosolic transport systems can contribute to their targeted delivery to the organelle of interest. The number of targeting residues required for this targeted delivery is not known. For a small protein (e.g. albumin, ~67 kDa), decoration with 4–5 targeting residues may be sufficient for preferential trafficking to the target organelle (10). On the other hand, bigger DDSs, such as nanoparticles, may require decoration by dozens or hundreds of targeting residues for preferential trafficking to the target organelle. For example, enhanced nuclear or mitochondrial accumulation of quantum dots (average diameter of 20–40 nm) was obtained following their conjugation with 48 and 62 targeting residues, respectively (16). Enhanced nuclear accumulation and toxicity of doxorubicin-loaded PLGA nanoparticles (average diameter of 230 nm), however, required conjugation of ~443 nuclear localization signals per particle (17). Although the targeting efficiency was not quantified in these studies, it looks plausible that nanoparticle and vesicle-based DDSs that are densely decorated by the targeting residues could be relocated by the cytosolic transport systems to the organelle of interest. This relocation should proceed rapidly, since peptide-based targeting sequences that were used in the

above-mentioned studies (16,17) are rapidly degraded in the cytosol and lose their efficiency. The limited amount of the targeting residues that can be chemically conjugated to a single DDS during its preparation, combined with their rapid cytosolic degradation, apparently limit the efficiency of DDS intracellular targeting. Decoration of the DDS with more metabolically stable non-peptide targeting residues, therefore, is expected to substantially enhance targeting efficiency.

It appears that identification of the targeting residues by cytosolic transport systems may help to bring the intracellularly targeted DDS in close proximity to the target organelle and may contribute to the DDS uptake into the target organelle, although the mechanism of this uptake is not clear. DDS should not necessarily permeate into the target organelle, and drug release from the DDS in close proximity to the organelle of interest may also result in preferential drug accumulation in this organelle.

KINETICS OF INTRACELLULARLY TARGETED DELIVERY

In addition to increasing the *efficiency* of drug delivery to the target organelle, intracellularly targeted DDS may allow control over *kinetics* of drug release and the time-course of the resulting pharmacological activity. Specifically, nanoparticle-based DDSs can be designed to release the drug in a prolonged fashion (up to several days or weeks) at higher or lower rates by changing their composition and preparation protocol. Vesicle-based DDSs are also suitable for prolonged drug delivery to the target organelle, but allow less tight control over kinetics of drug release. It is not clear whether kinetics of intracellular delivery affect the efficiency of cytotoxic or pro-apoptotic drugs. However, it is anticipated that controlled kinetics of drug release and of pharmacological activity can be crucial for the therapeutic success of certain treatments, such as correction of 'metabolic' cellular defects in mitochondrial diseases, lysosomal storage diseases, ER protein-misfolding, etc.

Efficiency of the anti-cancer vaccination with antigenic peptides is also highly dependent on the kinetics of their delivery to the antigen-presenting cells (APCs). For efficient vaccination, antigenic peptides should be delivered to the APC and reach the intracellular organelles where the antigen cross-presentation process takes place (predominantly the ER and endosomal compartments). Prolonged cross-presentation of the antigen by the APCs is critical for efficient vaccination and activation of cytotoxic T lymphocytes (CTLs) directed against the tumor cells (18,19). For instance, prolonged delivery of an ER-targeted antigenic peptide using liposomal DDSs resulted in prolonged cross-presentation of the antigen by the APCs (more than 140 h), more efficient activation of the CTLs, and enhanced *in vivo* anti-tumor effects (14). The authors suggested that this prolonged cross-presentation resulted from efficient targeting of the antigen into the ER and its long-term retention in the ER compartment, which has much less proteolytic activity as compared to the cytosol (14). The same group reported enhanced *in vitro* CTL activation using nanoparticle-based DDS loaded with ER-targeted antigenic peptide (15). It should be noted, though, that very prolonged delivery of the antigenic peptide may induce tolerance to this antigenic peptide on behalf of the host's immune system and diminish the anti-cancer response following vaccination. To avoid tolerance development, DDSs for anti-cancer vaccination should be designed to gradually release the antigenic peptide over the desired time period (several days) and may incorporate additional immunostimulatory cargo (e.g., activators of the innate immune system) (20).

SUMMARY

Intracellularly targeted drug delivery is a promising new approach for enhancing and controlling the drug pharmacological activities. It appears that conjugation of specific targeting residues can affect the intracellular fate of the drug/DDS and result in its preferential accumulation within an organelle of interest. In most cases, cytosolic permeation of the drug/DDS is a pre-requisite for its targeting to the organelle of interest. After arrival to the cytosol, the targeting efficiency of the drug/DDS to the target organelle is apparently affected by the relative kinetics of three major processes, namely drug/DDS cytosolic mobility, degradation rate of the drug/DDS in the cytosol, and rate of drug/DDS uptake by the target organelle. The choice of drug, targeting residues, and formulation type will determine which of these processes will be rate-limiting for the overall targeting efficiency.

Currently applied intracellular-targeting delivery approaches have limited efficiency. To develop clinically applicable DDSs, studies that quantitatively assess the mechanisms, barriers, and efficiency of intracellular drug delivery are needed. Mathematical modeling approaches will be of great importance to determine the barriers and limiting factors in intracellular drug delivery. For this purpose, mathematical models of the intracellular drug trafficking mechanisms (i.e., intracellular pharmacokinetics) and the relationship between the drug concentrations in the individual organelles and the pharmacological effects (i.e., intracellular pharmacokinetic-pharmacodynamic models) should be developed. Several such models have been proposed, e.g., for the description of receptor-mediated endocytosis and trafficking (21) and for lysosomal delivery of

pharmacological agents (22). Mechanisms of intracellular gene delivery were studied more extensively and were quantified more thoroughly, as compared to small-molecule pharmacological agents, apparently due to somewhat easier quantification of DNA molecules. Subsequently, several mathematical models have been suggested to describe the mechanisms of DNA intracellular traffic and efficiency of nuclear delivery and of the resulting gene expression (23,24).

It is expected that intracellularly targeted drug delivery approaches will be the focus of intensive research during the next decade and that efficient and controlled delivery of the drug to specific organelles will be attained using specialized DDSs. For successful clinical application of these DDSs, two levels of targeting are required: targeted uptake by the cells of the specific organ/tissue at the first stage, followed by intracellular targeting within these cells. Most probably, future DDSs will be composed of multiple layers with individual layers responsible for efficient targeting at the whole body and on the cellular levels (25).

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