A Strategy to Bypass Natural Size Restrictions of Receptor-Mediated Endocytosis of Drug Carriers

M. Ansar1#, D. Serrano2#, I. Papademetriou3, T. Bhowmick1 and S. Muro1,3*

1Institute for Bioscience & Biotechnology Research, University of Maryland, College Park, MD 20742, USA; 2Biological Sciences Graduate Program, University of Maryland, College Park, MD 20742, USA; 3Fischell Department of Bioengineering, University of Maryland, College Park, MD 20742, USA.

#These authors contributed equally; *muro@umd.edu

ABSTRACT SUMMARY

Most endocytic receptors used for intracellular drug delivery mediate uptake via clathrin or caveolar pathways associated with \( \leq 200 \)-nm vesicles, greatly restricting design of drug carriers. Endocytosis mediated by intercellular adhesion molecule 1 (ICAM-1) is an independent pathway that allows uptake of nano- and micro-carriers in cells and in vivo. This is due to recruitment of cellular sphingomyelinases, which leads to ceramide generation at carrier binding sites. Ceramide then enables engulfment of carriers within a wide size-range. Here we adapted this paradigm to enhance uptake of drug carriers targeted to receptors different from ICAM-1 and associated with size-restricted pathways (e.g. the clathrin route). We coated sphingomyelinase on the surface of those carriers. This allowed ceramide generation and enhanced endocytosis independently from the receptor being targeted by the carrier. Hence, it is possible to maintain targeting toward a selected receptor while bypassing natural size-restrictions of its associated endocytic route. This strategy holds considerable promise to enhance flexibility of design of drug carriers for intracellular delivery.

INTRODUCTION

Targeting drug carriers to endocytic receptors is a common strategy to facilitate intracellular drug delivery.1 Receptor associates with particular pathways, whose biological features rule the efficacy of transport.1 Most receptors relate to transport via either clathrin, caveolae, phagocytosis, or macropinocytosis.2 Macropinocytosis and phagocytosis are mostly present in immune cells, precluding delivery to tissues.2 Clathrin pits and caveoli appear in most cells in the body and, hence, are preferable.2 However, caveoli and clathrin pits have sizes \( \sim 50-100\)-nm and \( \leq 200\)-nm, respectively, which greatly limits design of drug delivery strategies.1,2

Although still largely underappreciated, a few receptors operate independently from these pathways.3 An example is cell adhesion molecule (CAM)-mediated endocytosis induced by binding to intercellular adhesion molecule 1 (ICAM-1).4 ICAM-1 is a transmembrane protein overexpressed on many cells under pathological states, whose natural ligand is a micron-sized multivalent “object”: white blood cells.5 Due to its biological function, endocytosis of drug carriers via ICAM-1 is efficient within a wide range of carrier sizes (from 200-nm to several \( \mu \)m), as shown both in cell culture and in vivo.6 This is due to the fact that binding to ICAM-1 induces recruitment of cellular sphingomyelinase enzymes, leading to ceramide generation at carrier binding sites.4 Ceramide enrichment has been shown to provide signals for cytoskeletal reorganization, formation of large engulfment structures and micron-size invaginations, leading to uptake.4

Our goal here was to investigate if receptor targeting can be de-coupled from the subsequent endocytic signaling by designing carriers capable of ceramide generation even when targeting receptors different from ICAM-1, to enhance endocytosis regardless of carrier size.

EXPERIMENTAL METHODS

We used nano- and micro-particles (200-nm, 1-\( \mu \)-m, \( \sim 5\)-\( \mu \)-m) made of polystyrene to avoid confounding effects of polymer degradation and focus on targeting and transport.4 Carriers were coated with targeting antibodies via surface absorption.4 We targeted carriers to the size-restricted clathrin route using antibodies to the mannose-6-phosphate receptor (M6PR) or the transferrin receptor (TfR), and compared them to carriers targeting size-permissive ICAM-1. Non-specific IgG carriers served as a negative control. Carriers bearing both targeting antibodies and neutral sphingomyelinase (NSM), to induce ceramide generation and enhance endocytosis, were compared to carriers containing only targeting antibodies. Binding to endothelial and epithelial cells, endocytosis, intracellular transport, transport mechanism, cell viability, and in vivo biodistribution
in mice after i.v. injection were tested using fluorescence or radioisotopes.4

RESULTS AND DISCUSSION

We compared anti-ICAM carriers, associated to size-permissive CAM endocytosis, to carriers targeted to M6PR7 or TfR8 associated to the size-restrictive clathrin route, and IgG carriers served as non-specific controls. Within each carrier size (200-nm, 1-μm or ~5-μm), all carriers had a similar final size, polydispersity, and antibody valency.

Since clathrin pits (not the ICAM-1 route) are restricted to sub-micrometer sizes, we first compared 1-μm carriers. Anti-M6PR carriers bound to cells less efficiently than anti-ICAM carriers (19-fold below; 30-min) yet specifically vs. control IgG carriers (10-fold over). Despite specific binding and due to size restriction of clathrin pits, anti-M6PR carriers were poorly endocytosed: only 22% of carriers vs. 70% of carriers for anti-ICAM counterparts (30 min). These differences were accentuated even more over time.

With the goal of mimicking the ICAM-1 pathway, which operates via natural cell production of ceramide,4 we coated anti-M6PR carriers with NSM enzyme.9 NSM can hydrolyze sphingomyelin (a cell membrane phospholipid) into ceramide.9 Hence, we may be able to induce endocytosis similar to the ICAM-1 pathway even if we are targeting other receptor (M6PR). As hypothesized, endocytosis of anti-M6PR/NSM carriers was significantly enhanced (~3-fold increase), resulting in ~65% the level of uptake of anti-ICAM carriers. This was achieved without affecting carrier binding, which remained similar for anti-M6PR carriers and anti-M6PR/NSM carriers, indicating that the enzyme impacts endocytosis without contributing to binding. Indeed, binding was validated to be solely provided by the targeting anti-M6PR antibody. Neither the intracellular trafficking of internalized anti-M6PR carriers, the degradation of their degradable components (the coat), or the cellular viability were affected by the presence of NSM on the carrier coat.

Next, carriers of different sizes (200-nm, 1-μm, ~5-μm) and containing different NSM doses (20% vs. 80% of the carrier surface) were compared. Again, while binding to their receptor (M6PR) was not affected by NSM, endocytosis was enhanced by NSM and this was dependant on the carrier size and enzyme dose: the larger the carrier size, the greater the improvement provided by NSM and the greater the dependence on the enzyme dose.

Although anti-M6PR carriers bound to M6PR not to ICAM-1, NSM presence on the carrier surface rendered ceramide enrichment at sites of the cell membrane where carriers were bound. This also induced actin stress fibers formation, similar to those observed for anti-ICAM carriers.4 Using an inhibitor panel, we determined that NSM shifted the uptake pathway of anti-M6PR from clathrin-dependent to CAM-like endocytosis. Hence, by coating NSM on a carrier surface one can, for the first time, de-couple binding to a receptor from the subsequent signaling leading to uptake: binding and endocytosis can now operate independently, bypassing size restrictions associated with natural pathways. This was validated using carriers targeted to other receptors (transferrin receptor associated with size-restrictive clathrin pits) and other cells types (endothelial and epithelial, primary and established, healthy and cancer-like), highlighting the broad applicability of this approach.

Finally, presence of NSM on the coat of carriers targeted to the M6PR or TfR resulted in enhanced specific uptake by target organs after i.v. injection in mice, validating the impact of this strategy.

CONCLUSION

Functionalization of carriers with transducers of CAM endocytosis (sphingomyelinase) permitted us to highjack key signal transduction routes of said pathway (ceramide), independently from the receptors being targeted. This lead to enhanced uptake of carriers even when targeting receptors of size-restrictive pathways, without affecting carrier binding, intracellular trafficking, or cell viability. This is the first time that properties of an endocytic route are induced in cells independently from the receptor being bound and its natural pathway. This may greatly impact drug delivery by enhancing intracellular transport of drug carriers regardless of their size and target receptor, allowing wider design options with regard to carrier geometry.

REFERENCES

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