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Inhibiting Molecular Intracellular Targets in Cancer

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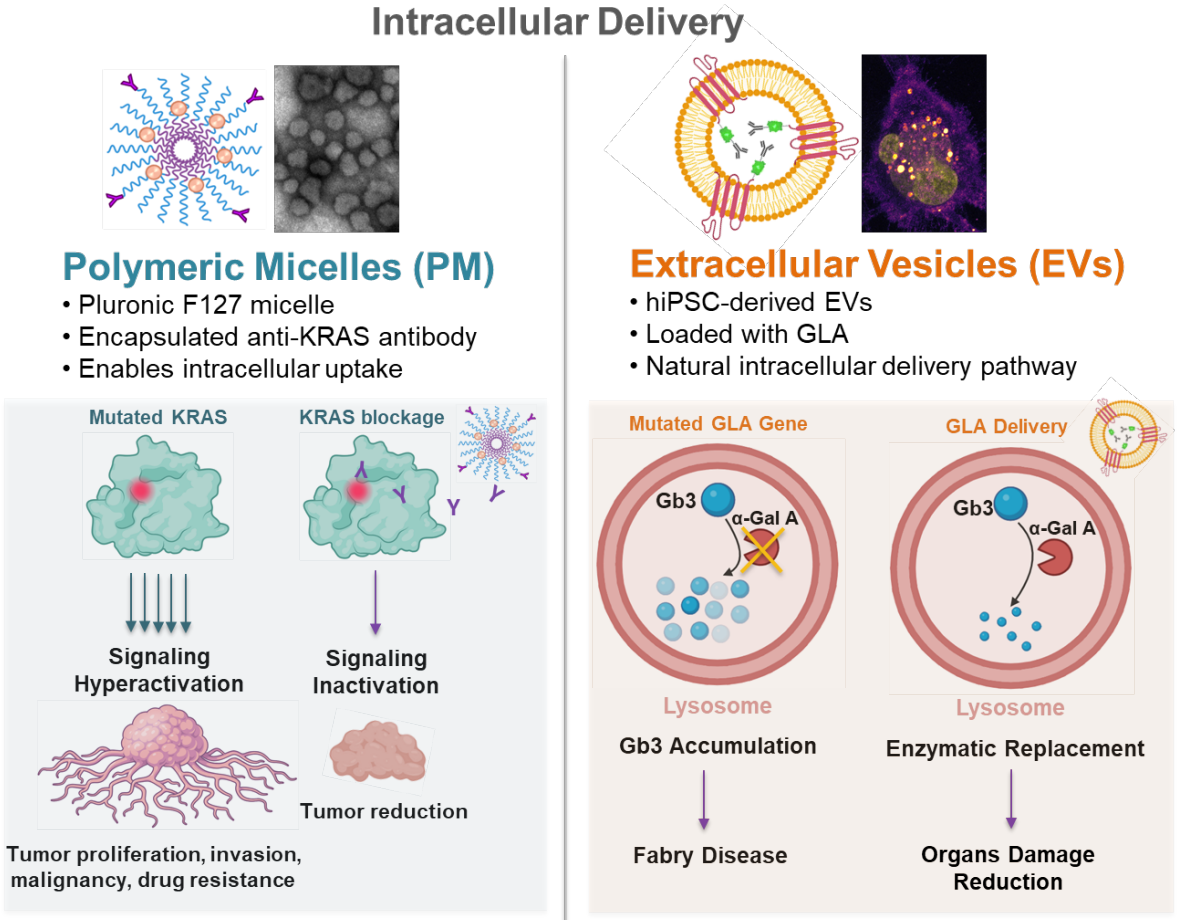
Graphical Abstract



Abstract

Advanced cancer is still considered an incurable disease because of its metastatic spread to distal organs and the progressive gain of resistance to given treatments. Even though more effective therapies have become available over the past years, long-term recurrence and undesirable side effects remain

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the main drawbacks of current clinical protocols. Because of this, there is a strong need to generate new effective anticancer treatments. Particularly, against specific molecular intracellular targets, most of which still remain elusive because many targets are unfortunately challenging to inhibit or undruggable. Therefore, the use of antibodies to target intracellular oncological proteins is regarded as a promising strategy, even though intracellular antibody uptake is, *per se,* suboptimal, as antibodies cannot efficiently enter cells. Here, we present a summary of our efforts to generate antibody delivery systems based on polymer micelles and extracellular vesicles, and to use them to deliver recombinant proteins across different disease models.

Keywords: Nanobodies; KRAS; Polymer micelles, Extracellular vesicles; Cancer; Intracellular targets, Nanomedicine.

Introduction

There are numerous antibodies in the market and under development against extracellular targets, while the number of available potential intracellular targets represents around 50% of the human proteome. In this context, the design of nanomedicine-based strategies for intracellular delivery of antibodies or nanobodies has become an attractive option.

An example is the use of extracellular vesicles and polymer micelles to deliver antibodies and nanobodies against KRAS (*Kirsten Rat Sarcoma viral oncogene homolog*), one of the most well-known proto-oncogenes in colorectal and pancreatic cancer models. KRAS is a well-known oncogenic kinase whose mutations are detected in PDAC (95% of cases) and also in many other cancer types (e.g., colorectal, lung, ovarian). Although it has long been considered an undruggable target, several inhibitors have been developed recently (e.g., Sotorasib^{R}, Adagrasib^{R}) that are effective against the KRASG12C mutation. Despite their promising clinical prospects, the early emergence of adaptive resistance mechanisms driven by secondary mutations (most often in KRAS) is clearly disappointing [1]. Moreover, other frequent KRAS mutations remain undruggable, even though some drugs are currently entering clinical trials. In addition, most tumors harboring KRAS mutations are 'addicted' to the hyperactivation of the KRAS pathway and therefore are often more sensitive to its inhibition than healthy cells. Because of this, developing new strategies that can inhibit KRAS independently of its wild-type or (most frequent) mutational status (KRASG12C and KRASG12D) should be considered to generate a wider range of usable anticancer therapies.

Discussion

We have developed KRAS antibodies (KRAS-Ab) encapsulated in Pluronic® F127-based polymeric micelles (PM-KRAS) [2]. The potential of PM for antibody encapsulation, as well as the conformational changes of the polymer and its intermolecular interactions with antibodies, was investigated for the first time through *in silico* modelling. *In vitro* studies demonstrated that PM encapsulation enabled intracellular delivery of KRAS-Ab across multiple pancreatic and colorectal cancer cell lines. Notably, PM-KRAS significantly impaired proliferation in KRAS-mutated HCT116 and MIAPaCa-2 cells, while showing minimal effects in KRAS–wild-type or KRAS-independent HCT-8 and PANC-1 cells, respectively.

Furthermore, PM-KRAS strongly suppressed cell colony formation under low-attachment conditions in KRAS-mutated cells. Remarkably, *in vivo*, intravenous administration of PM-KRAS markedly reduced tumor growth in HCT116 subcutaneous xenografts compared with vehicle controls. Mechanistic analysis revealed that the therapeutic effect of PM-KRAS involved substantial inhibition of ERK phosphorylation and downregulation of stemness-associated genes in both cell cultures and tumor samples. These findings provide clear evidence that PM-mediated delivery of KRAS-Ab can safely and effectively diminish tumorigenicity and stemness in KRAS-dependent cancers, opening new avenues for targeting intracellular oncogenic pathways.

We have also studied the potential use of extracellular vesicles (EVs) as delivery platforms of nanobodies against KRAS. In this context, several inhibitory sequences against KRAS have already been identified from phage display experiments showing good inhibitory activity against KRAS. Moreover, *in silico* AI-based approaches have been used to optimize protein-binding affinities and design and select the best-performing KRAS inhibitor nanobody, ensuring the highest sensitivity

for preclinical studies. EVs are naturally occurring lipid-bound nano- and micro-sized membrane vesicles that originate from the endosomal system or bud off from the plasma membrane. They are a heterogeneous group of membranous structures that contain cytosolic material enclosed by a lipid bilayer and are secreted by secretory cells. Moreover, EVs are secreted by *in vivo* and *in vitro* cells, and this process is conserved throughout evolution from bacteria to humans and plants [3]. The most common EVs secreted by the cells are exosomes, microvesicles (MVs), and apoptotic bodies, which differ in size, biogenesis, membrane composition, cargo, and function. MVs range in size from 50nm to 1mm in diameter, are directly shed from the cell membrane by budding, and their typical markers are phosphatidylserine, actinin-4, and mitofilin. Exosomes are the smallest vesicles with a size range of 50-100 nm. The nature and abundance of EV cargoes are cell-type-specific and are influenced by the physiological or pathological state of the donor cell. Because EVs accommodate functional proteins from their parent cells, no external factors are required to load bio-substances such as intrinsically folded proteins and nucleic acids [4]. In this respect, Stem cells, such as Mesenchymal Stem Cells (MSCs), have been widely used in cell therapy approaches in regenerative medicine due to their pluripotency and self-renewal properties [5]. Nonetheless, the principal therapeutic efficacy of MSCs arises from EVs that contain bioactive molecules [6]. MSCs can produce large numbers of EVs and are the most suitable candidates for tissue regeneration because of their ability to differentiate and proliferate. Unfortunately, as the number of MSCs in cell culture increases, cell activity and EV production decrease significantly, hampering their clinical use [7]. Because of this, our strategy aims to use human-induced pluripotent stem cells (hiP-SCs) instead. These are generated from somatic cells that are reprogrammed by ectopic expression of transcription factors such as OCT4, SOX2, KLF4, and MYC [8]. While iP-

SCs have comparable abilities to MSCs; their ability to produce large numbers of EVs over the long term makes them more attractive as a source for designing cell-engineered ATMPs (*Advanced Therapy Medicinal Products*). This is an important subject, as the selection of parental cells as the source of EVs is a critical factor in defining their characteristics and functionalities, which should be carefully aligned with their intended therapeutic application [4].

EVs are present in various body fluids and contain cytosolic proteins, proteins from the plasma membrane, from endosome membranes (more than 41860 different proteins), lipids, mRNAs, non-coding RNAs (ncRNAs), and DNA [9]. In fact, exosomal proteins play essential roles in various life processes, including.

(i) metabolic reprogramming and regulation,

(ii) modulation of receptor-ligand signaling pathways,

(iii) angiogenesis,

(iv) apoptosis and cell death mechanisms,

(v) cell survival and proliferation,

(vi) immune regulation, and

(vii) gene transcriptional regulation and translation [10].

Exosomes affect recipient cells via surface molecules or molecular cargo [11]. Cellular interactions involve exosomal interactions with the plasma membrane of recipient cells, or by exosomal cargo release following the uptake of exosomes [12]. The content of EVs enables cells to exchange genetic information and protein cargoes with cells from which they originate. EVs and exosomes, therefore, play an essential role in *cell-to-cell* communication through the exchange of proteins and other molecular cargo. In addition, their ability to cross biological barriers like the blood-brain barrier makes them attractive for delivering therapeutic proteins, potentially overcoming limitations of traditional drug delivery methods. Moreover, EVs can be reprogrammed through genetic engineering and other methods to improve their therapeutic potential because protein overexpression in the cell cytoplasm through transfection allows partial loading during EV biogenesis in a concentration-dependent manner [4]. Therefore, overexpression of proteins in mammalian cell systems (cell factories) provides for most proteins to be endogenously up-loaded into cellular EVs. In this context, we have recently demonstrated [13] that EVs isolated from cell supernatants from mammalian cells overexpressing alpha-galactosidase A (GLA) or N-

sulfoglucosamine sulfohydrolase (SGSH) enzymes, which are defective in Fabry and Sanfilippo A rare diseases, respectively, showed a high active content of GLA and SGSH proteins, restoring lysosomal functionality much more efficiently than the recombinant enzyme in clinical use. Remarkably, a single intravenous injection of EV-GLA reduced globotriaosylceramide substrate levels in clinically relevant tissues, demonstrating that EVs from cells overexpressing lysosomal enzymes act as natural protein delivery systems, thereby improving the activity and efficacy of the recombinant proteins. Accordingly, we have successfully produced anti-KRAS recombinant nanobodies expressed in EVs from cell factories, demonstrating their *in vitro* efficacy in targeting KRAS in PDAC and colorectal cancer models.

Moreover, EVs have several protein markers that are a structural part of their lipidic membrane, such as LAMP2b, ALIX, and TSG101, associated with multivesicular bodies (MVB), as well as CD63, CD81, and CD9 from the tetraspanin family. These are abundant in EVs and can also be used to enrich the content of specific proteins of interest by producing fused proteins [14,15]. Recently, in collaboration with Oregon Health and Science University, USA, we have demonstrated that EVs from Cancer Stem Cells (EV-CSC) of head and neck squamous cell carcinomas preferentially targeted MHC-II-macrophages and PD1+ T cells in the tumor microenvironment using genetic approaches to specifically label EV-CSC with lentiviral transfer plasmids coding for CD63-eGFP fusion protein to tag total EVs [16]. This indicates that the use of genetic technologies capable of tracking EVs without *in vitro* isolation is a valuable tool for unveiling the biology of native EV *in vivo*. We are currently using this strategy to validate the use of modified cells (ATMPs) as EV-producing factories for long-term, continuous *in vivo* cancer treatment protocols using endogenously produced anti-KRAS nanobodies loaded into EVs.

Conclusion and perspectives

Methods and procedures for designing and optimizing nanobodies, aptamers, and antibodies to inhibit protein targets are already available and widely used and are also under GMP. Moreover, there is a plethora of intracellular proteins that can be potentially used to develop new therapeutic strategies. Delivery systems such as polymer micelles and extracellular vesicles can be used to deliver intracellular proteins, including antibodies and nanobodies. This opens the door to effectively inhibiting intracellular undruggable molecular targets and providing new therapeutic opportunities against human diseases, including oncology applications.

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Conflicts of Interest: The author declares no conflict of interest. For a written statement, please contact the journal office.

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