Local co-administration of gene-silencing RNA and drugs in cancer therapy: State-of-the art and therapeutic potential

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Abstract: Gene-silencing miRNA and siRNA are emerging as attractive therapeutics with potential to suppress any genes, which could be especially useful in combination cancer therapy to overcome multidrug resistant (MDR) cancer. Nanomedicine aims to advance cancer treatment through functional nanocarriers that delivers one or more therapeutics to cancer tissue and cells with minimal off-target effects and suitable release kinetics and dosages. Although much effort has gone into developing circulating nanocarriers with targeting functionality for systemic administration, another alternative and straightforward approach is to utilize formulations to be administered directly to the site of action, such as pulmonary and intratumoral delivery. The combination of gene-silencing RNA with drugs in nanocarriers for localized delivery is emerging with promising results.

In this review, the current progress and strategies for local co-administration of RNA and drug for synergistic effects and future potential in cancer treatment are presented and discussed. Key advances in RNA-drug anticancer synergy and localized delivery systems were combined with a review of the available literature on local co-administration of RNA and drug for cancer treatment. It is concluded that advanced delivery systems for local administration of gene-silencing RNA and drug hold potential in treatment of cancer, depending on indication. In particular, there are promising developments using pulmonary delivery and intratumoral delivery in murine models, but further research should be conducted on other local administration strategies, designs that achieve effective intracellular delivery and maximize synergy and feasibility for clinical use.

Keywords: Drug delivery system; Combinatory treatment; Tumor; Synergy; RNA; Therapeutic substance

Introduction

Cancer is the collective name given to a range of diseases in which cells of the body start to divide uncontrollably and invade surrounding tissues. There are numerous cancer types, differing in the location in the body, the cell type they originated from and genotypic characteristics. The underlying genetics behind formation of cancer cells and their behavior is highly complex. Some cancer genes are rather specific for certain cancer types, while others are commonly found in a wide range of cancer types [1]. It has also been shown that due to branched evolution the genetic profile commonly differs between primary and metastatic tumors [2, 3] and even within individual tumors [3]. Further complexity is added by that epigenetics also plays an important role in cancer development and progression [4, 5], metastasis [6, 7] and response to drug treatment [8].

Given the large number of different cancers, different cell behaviors due to genetic and epigenetic profiles and circumstances associated with location and stage of cancer, it is no surprise that different scenarios require different treatments. Common currently used cancer treatments are surgery, chemotherapy, radiation therapy, targeted therapy (acting mainly on cancer cells through selectivity for factors other than rapid cell division) and immunotherapy [9]. Although there has been much advancement in methodologies and available treatments, chemotherapy remains a main treatment, sometimes used alone, but often used before or after surgery, or in conjunction with radiation or other therapeutic substances [10]. Unfortunately, chemotherapy associated with the use of very toxic drugs at high concentration distributed through whole body is causing a range of side effects from action also on non-cancerous cells. Common ones are nausea and vomiting, diarrhoea, mucositis and myelosuppression [11].

One approach to reduce off-target side effect is to maximize the localization of drug to tumors and cancer cells. Smart nanocarriers that selectively accumulate at tumors or are taken up preferentially by cancer cells have been extensively researched [12]. However, another intuitive means to reduce off-target effects and systemic toxicity is to administer drug locally at the tumor site [13]. Several strategies have been explored for localized cancer therapy including drug-releasing implants, intratumoral injection [13-16] and pulmonary administration (inhalation) in the case of lung cancer [17, 18]. Although not widely used, intratumoral injection of drugs is employed in the clinic, while for lung cancer there have been clinical studies on pulmonary administration, as evident from searches in the ClinicalTrials.gov database, but the authors are not aware of any formally approved drugs/drug delivery systems for this specific purpose.

In chemotherapy and targeted therapies (which may be considered a subclass of chemotherapy) a major limitation for effective treatment is that tumors often develop multidrug resistance (MDR) [8, 19, 20], with recent indications that this is due to outgrowth of resistant subpopulations that initially were present at low frequencies, but that were favored under the selective pressure of the treatment [20, 21]. There is now substantial evidence that subpopulations of so called cancer stem cells (CSC) contribute to this MDR-development [20]. Thus, a contemporary research topic is to combine substances that suppress MDR with cytotoxic agents, so that also the MDR subpopulations of cells in tumors are killed [22-24].

One therapeutic substance that currently is heavily investigated for the purpose of MDR suppression in combination therapy is gene-silencing RNA, such as small interfering RNA (siRNA) and microRNA (miRNA) [25-28]. Although those RNAs could in theory silence any gene, there are several challenges before clinical realization, for example stability of the RNA in the body, limited uptake by cells and off-target effects [24, 25, 27, 29]. Nanoparticles currently

receive much academic interest as carriers for co-delivery of siRNA and drug to overcome such issues, with promising results in model systems [23, 25, 26, 28, 30]. However, the translation of circulating nanoparticles with advanced designs from laboratory studies to clinical use has proven challenging. It seems that drug delivery systems focusing on maximization of the synergistic effect of co-delivered gene-silencing RNA and drug after local administration to tumors could have a more straight-forward translation to clinical use.

In this review we explore and discuss the potential and challenges for using local-administration drug delivery systems to maximize synergistic effect between gene-silencing RNA and anticancer drugs in cancer treatment. First a background is given on the use of nanoparticles to co-deliver gene-silencing RNA and drug to cells. Subsequently, a brief background is given on drug delivery systems for local administration. This is followed by a review and discussion of literature on local co-administration of RNA and drug in cancer treatment. Finally, an outlook is provided on expected future developments.

Designing nanocarrier-mediated co-delivery of gene-silencing RNA and drugs

There has been extensive research on nanocarrier-mediated delivery of drug [12] and gene-silencing RNA [27, 30] alone. However, co-delivery of gene-silencing RNA and drug to achieve enhanced efficacy against cancer cells is now emerging [31, 32]. In achieving synergy between drugs and RNA it is critical that they reach their target cells and molecules simultaneously, or at least within timeframes where their effects overlap and could act synergistically. This could be achieved by administering individual formulations of RNA and drug that reach their targets independently, but that are designed so that effects overlap. In theory, drug molecules could be free in solution or in nanocarriers, but RNA has exclusively

been loaded into nanocarriers in clinical trials [33], likely due to stability, cell penetration issues and extended release requirements. Another appealing approach is to co-formulate the RNA and the drug into nanocarriers so they simultaneously reach and enter individual cells. Fig. 1 presents a schematic drawing of intracellular delivery of gene-silencing RNA and anticancer drugs co-loaded in nanocarriers or simultaneously administered individually, with common target mechanisms for synergistic effect indicated.

There are several recent achievements that highlights the potential of nanoparticle-mediated co-delivery of RNA and drug in cancer treatment. For example, Xu et al. showed that self-assembled nanocarriers of biodegradable poly(lactide-coglycolide)-b-poly(ethyleneglycol) diblock copolymer and cationic lipid nanocarrier, co-delivering a prodrug of cisplatin and siRNA against the *REV1* and *REV3L* genes involved in the error-prone translesion DNA synthesis pathway (a postreplication DNA repair mechanism), achieved greatly enhanced cytotoxicity against Lymph Node Carcinoma of the Prostate (LNCaP) [34]. Salzano et al. investigated self-assembled nanocarriers of polyethyelene glycol (PEG)-phosphatidyl ethanolamine to co-delivery paclitaxel and anti-survivin siRNA via intravenous administration in a murine ovarian xenograft model. They reported much improved reduction of tumor volume and weight for co-delivered substances, although nanocarrier delivered anti-survivin siRNA alone also achieved substantially better effect than the paclitaxel-only formulations [35]. In another approach, doxorubicin was conjugated to PEI using pH sensitive crosslinkers and the conjugates were complexed with siRNA against BCL2 and survivin. For both the siRNAs the nanocarrier-mediated co-delivery with doxorubicin achieved little-to-no improvement in cytotoxicity against lung cancer cells *in vitro* compared to administrations with free doxorubicin, but improved efficacy was observed *in vivo* in murine lung cancer models after pulmonary administration [36, 37]. Saad et al. reported

that co-delivery of siRNA against *MDR1* and *BCL2* genes with doxorubicin in cationic liposomes achieved greatly improved cytotoxicity against MDR breast and ovarian cancer cells compared to administrations using free doxorubicin or co-delivery in nanocarriers with only one of the siRNAs [38]. Functionalization of the nanocarrier with ligands for surfaces receptors on target cancer cells can increase the effectiveness of co-delivering nanocarrier systems. For example, Cao et al prepared folic acid-functionalized poly(ε-caprolactone)-PEI nanocarriers for doxorubicin and siRNA against BCL2 and reported enhanced apoptotic effect *in vitro* against hepatic cancer cells for the functionalized nanocarriers [39].

The above highlight different approaches, and phenomena for nanocarrier-mediated delivery of siRNA and drug to achieve synergistic effect and suppression of MDR. Importantly, *in vitro* cytotoxicity is not directly transferable to *in vivo* since accumulation and residence time at site of action are important factors. The results that combined siRNA against multiple targets achieved improved efficacy is important since, in contrast to conventional drugs, different siRNA will have similar physicochemical properties and can thus generally be co-loaded into a nanocarrier. The discussed studies represent a selection of the literature and the interested reader is encouraged to find more information, with suggestions being [28, 31, 32, 40-47]. As mentioned, a major focus of nanomedicine and nanocarriers has been intelligent nanoparticles that travel the circulatory system and selectively affect target cells. However, there have been recent advancements in the use of nanomaterials for local administration of therapeutic agents directly to the site of action, thus circumventing the considerable challenges in preparing nanocarriers that are stable and maintain their intelligent targeting in the highly complex environment of the body. Below, a background is given on drug delivery systems for local

administration, followed by a review and discussion on their use in co-delivery of gene-silencing RNA and drugs in cancer treatment.

Drug delivery systems for local administration: basic concepts

Local administration of therapeutics is successfully trialed already and clinically applied in cancer treatment [48]. However, since the purpose of local administration often is to achieve therapeutic concentration at the target site, while reducing off-target effects by minimizing concentration elsewhere, it is beneficial to increase residence time of the drug at the administration site. This can be achieved through a carrier matrix that remains at the site of administration and releases therapeutic substances in a controlled manner. Examples of systems for local administration are injectable *in situ* gelling materials [49-51], beads [48], inhalable particles [36, 52, 53], nanocarriers that remain at the site of administrations [54-56], microneedles [57, 58], liquid crystalline phases [59], creams and ointments [60, 61] and metal drug releasing implants [62, 63].

To extend therapeutic duration from a single local administration, controlled release functionality is desired and to maintain the extended release and large payloads are needed. However, burst release of a fraction of the therapeutic substance(s) is commonly observed [64], which can lead to dangerously high concentrations at short times. Furthermore, release of hydrophilic therapeutic molecules is generally fast and may be completed in less than 24 h [65]. To minimize burst release nanocarriers and nanostructured depots have been used to encapsulate the therapeutic molecules so that extended release with desired release kinetics is achieved [65-68]. The extended therapeutic duration also puts demand on protection of sensitive therapeutic molecules that are prone to degradation in the body, such as RNA. Again, nanoparticles and

nanostructured materials can be used to protect the substances until released to their targets, as demonstrated for RNA [56, 59, 69, 70]. This protective effect can be achieved by denying enzymes, such as RNAase, access to the therapeutic molecules, by providing a suitable chemical environment, and by keeping the substances in a stable physicochemical state. A schematic of basic concepts of a local-administration delivery systems is shown in Fig. 2.

Local administration of siRNA and drug

Local administration of siRNA per definition circumvent the challenge of reaching the target site (although there may be biological barriers to overcome) and the focus can thus be on design of materials with suitable release profiles, excellent protection of siRNA and effective cell internalization and gene-silencing. This is a contemporary research topic with efforts both in localized delivery and design of receptor-targeting nanocarriers that enter cells and release the RNA in a designed fashion, with nanostructured/nanocomposite materials emerging [71].

Local co-administration of gene-silencing RNA and drug is recently emerging and already at this early stage there are indications of excellent potential in cancer treatment. The previously mentioned self-assembling nanocarriers of biodegradable poly(lactide-coglycolide)-b-poly(ethylene glycol) diblock copolymer and cationic lipid, carrying cisplatin prodrug and siRNA against REV1 and REV3L were investigated for antitumor effect after intratumoral administration in a LNCaP xenograft model. The authors reported that co-delivery of drug and siRNAs effectively suppressed tumor growth. Noteworthy, all mice that received the co-delivered siRNAs and drug survived over the 50 days of the experiment. In contrast, for control groups administered saline solution, free cisplatin prodrug, cisplatin prodrug in nanocarriers, and siRNA in nanocarriers in combination with free cisplatin prodrug, all mice died over the time of

the experiment [34]. The Oh's group also investigated nanocarrier-mediated intratumoral delivery. They used cationic liposomes and solid lipid nanoparticles to co-deliver siRNA against MCL1 and a MEK inhibitor [46] or paclitaxel [47]. Both formulations were evaluated in a murine KB xenograft model and achieved much improved tumor suppression compared to controls. Lee et al prepared small (< 10 nm) complexes of Pluronics F-68, siRNA against BCL2 and benzethonium chloride [72]. In a murine xenograft model of aggressive and resistant breast cancer they found that peritumoral administration of the complexes resulted in synergy between the siRNA and drug so that improved tumor inhibition was achieved. The effectiveness was likely due to that the small size allowed for penetration and distribution of the complexes in the tumor after the peritumoral administration.

An attractive local administration route for lung cancer is inhalation (pulmonary delivery), as it potentially provides a non-invasive route directly to the tumor [17, 73]. There are recent promising results on nanocarrier-mediated co-delivery of siRNA and anticancer drug to battle lung cancer. Xu et al. prepared conjugates between doxorubicin and polyethyleneimine (PEI) using a pH sensitive liker. They complexed the conjugates with siRNA against BCL2 or survivin and evaluated antitumor effect in a mice B16F10 xenograft lung cancer model [36, 37]. Co-delivery with either of the siRNA improved tumor suppression compared to controls. Interestingly, after 24 h the *in vitro* cytotoxicity against cancer cells was lower for the co-delivered formulation compared to free doxorubicin, while after 48 h the cytotoxicity was comparable. The delayed effect in cytotoxicity combined with the improved performance *in vivo* indicates that the controlled release of doxorubicin from the carriers limited cytotoxicity *in vitro*, but improved *in vivo* effect, likely due to sustained residence time in the lungs. The results highlight the discrepancy that may exist between *in vitro* and *in vivo* performance. Taratula et al.,

reported similarly promising results for pulmonary delivery of siRNA against BCL2 and MDR1 with paclitaxel in lipid nanocarriers modified with luteinizing hormone-releasing hormone for targeting of receptors overexpressed by lung cancer cells [74]. The co-delivered siRNA and drug reduced the tumor volume towards zero over the 24 days of the experiment. The paclitaxel-nanocarrier without siRNA also achieved some reduction of tumor volume, while for other controls tumor growth was observed. They further reported that the pulmonary administration mainly resulted in localization to lungs, while for intravenous administration the majority of the nanocarriers accumulated in the liver. Interestingly, they observed that for pulmonary delivery the targeting functionality corresponded with preferential localization to tumor tissue. In another study the authors prepared similarly targeted porous silica nanoparticles for co-delivery of the same siRNAs and drugs. Again they reported excellent localization to the lungs after pulmonary delivery and improved cytotoxicity *in vitro* of the co-delivered substances [75]. However, the tumor suppression remains to be tested *in vivo*.

In an elegant and advanced design, Conde et al. prepared near-infrared (NIR) photo-responsive hydrogel patches loaded with anti-*KRAS* siRNA-gold nanoparticles and avastin-gold nanorods for triple-therapy (siRNA, drug and heat) [76]. The patches were implanted peritumoral in a mice LoVo-6-Luc-1 colorectal xenograft model and excellent results were achieved. The triple-treatment achieved greatly reduced tumor volume and all animals survived the 145+ days of the experiment under the survival criteria. In contrast, for the various control treatments all animals died within 45 days under the survival criteria (see Fig. 3). In another study the authors prepared implantable hydrogels of polyamidoamine G5 dendrimers complexed with triple-helixes of two miRNAs, one suppressing tumor RNA and one replacing down-regulated miRNA [77]. The di-miRNA hydrogels greatly outperformed hydrogels loaded with different anticancer

drugs when implanted peritumoral in a triple-negative breast cancer mouse model. Although not a combination of gene-silencing RNA and drug, the study do highlight the potential of local administration of substances with different target mechanisms in treatment of cancer.

An overview of formulations for local administration of co-delivered siRNA and drug is given in Table 1.

Outlook and conclusions

Although the few available studies seem limited to intra/peritumoral and pulmonary administration, the excellent results suggest that localized co-delivery of gene-silencing RNA and drugs can improve cancer treatment through these routes. Related studies indicate that other local administration routes and more advanced formulations should also be possible. For example, topical delivery of siRNA alone has been investigated for skin cancer treatment with promising results [58] and topical co-administration of siRNA and drug has been shown to inhibit skin inflammation [78]. The recently reported tri-therapeutic nanostructured implantable hydrogel patch [76] presents an interesting concept with a macroscopic depot housing siRNA/drug-loaded nanocarriers that are released in a controlled manner. Nanoparticle/nanocarrier composites can be designed with the macroscopic matrix being insoluble and biodegradable [79], which could offer a means to further protect the loaded siRNA and drug from degradation until released in a controlled manner. It seems that advanced implantable devices for co-delivery could be useful in some indications, but the invasiveness of the procedure may be limiting. An attractive approach from a clinical perspective is injectable gels [51]. Micro- and nano-structured injectable gels have been shown to offer excellent control

over release of substances [49, 66, 67, 80, 81] and enhance miRNA efficacy [82]. It was recently reported that an *in situ*-forming, microcapsule-containing biodegradable gel loaded with chemokine and dual siRNA/DNA attracted dendritic cells and silenced Interleukin-10 gene expressions [80] and an injectable gel containing siRNA polyplexes achieved antitumor effect for four weeks from a single administration in a murine model [83]. It is expected that advanced injectable nanostructured gels could be developed for improved cancer treatments through topical or intra/peritumoral co-delivery of siRNA and anticancer drugs. In addition, the excellent results to date on local co-administration of gene-silencing RNA and anticancer drugs have been performed without much optimization of the delivery systems, generally comparing the performance of the delivery system with various controls, but not varying the properties of the delivery system itself. Thus, there is both room and need for fundamental studies that focus on critical design parameters of individual systems, the results from which will enable improved antitumor efficiency and design of local administration systems for co-delivery of siRNA and drug in various cancers.

To conclude, co-delivery of gene-silencing RNA and anticancer drugs through local administration is emerging and pre-clinical results are so far promising. Developments in advanced, often nanostructured, local delivery systems are expected, as are fundamental studies to determine critical design parameters. Although speculative, with advancement of the nanomedicine and nanomaterials fields co-delivery of RNA and drugs may become established in cancer treatment and it seems that local administration has great potential in selected indications.

Conflict of interests

Dr. Larsson and Prof. Losic report research activity in the field of biomedical materials and potential for grant applications relevant to the present work in the future. Prof. Liu and Ms. Huang report a grant from Ministry of Science and Technology of the Republic of China, during the conduct of the study.

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Figure legends

Fig. 1. Drawing of intracellular delivery of gene-silencing RNA and anticancer drug through co-delivery in nanocarriers or simultaneous individual delivery, with common target mechanisms for synergistic effect indicated. Typically, the gene-silencing RNA is used, through the RNA interference pathways, to degrade target mRNA from genes that counteract the effects of anticancer drugs. Examples of suppressed mechanisms include; DNA repair, drug efflux, anti-apoptosis and activities of cancer related kinases. Note that for individual delivery of anticancer drug, the drug could be loaded in a nanocarrier, but for clarity only the case with free drug is included in the drawing. (A color version of the figure is available in the online version of the article)

Fig. 2. Schematic showing basic functionalities of a delivery system for local administration. The drug loaded material (depot) contains a high concentration of therapeutic substance that over time is released through diffusion and/or degradation of the depot, resulting in a locally high and systemically low concentration of the therapeutic. It is important that the therapeutic substance is stable in the depot until it is released, this can be achieved by (i) providing a suitable chemical environment and (ii) preventing enzymes from reaching the therapeutic substance. Note that the size of the depot may range from individual nanoparticles to macroscopic samples and that degradation of the therapeutic substance is not always an issue. (A color version of the figure is available in the online version of the article)

Table 1. Formulations evaluated *in vivo* for co-delivery of siRNA and anticancer drugs.

Formulation	**siRNA target(s)**	**Drug**	**Administration route**	**Animal model**	**Results summary**	**Ref.**
Suspension of self-assembled nanocarriers of biodegradable poly(lactide-coglycolide)-b-poly(ethylene glycol) diblock copolymer and cationic lipid.	*REV1/REV3L*	Cisplatin prodrug	Intratumoral	Murine prostate xenograft	Chemosensitization of cancer cells to platinum treatment. Synergistic effects on repression of tumor growth.	[34]
Suspension of N',N''-dioleylglutamide-based cationic liposomes.	*MCL1*	MEK inhibitor PD0325901	Intratumoral	Murine KB xenograft	Co-delivery reduced expression of Mcl1 and pERK1/2 proteins and reduced tumor cell survival. Co-delivery also much improved inhibition of tumor growth.	[46]
Suspension of cationic 1,2-Dioleoyl-sn-glycero-3-ethylphosphocholine-based solid nanoparticles	*MCL1*	Paclitaxel	Intratumoral	Murine KB xenograft	Co-delivery achieved greatest effect *in vitro* and greatest inhibition of tumor growth.	[47]
Nano-sized hydrophobically associated multiple monocomplex of Pluronics F-68, benzethonium chloride and siRNA.	*BCL2*	Benzethonium chloride	Peritumoral	Murine MDA-MB-231 xenograft	Simple physical mixing resulted in complexes with size < 10 nm. Peritumoral administration achieved synergistically enhanced effect against aggressive and resistant triple-negative breast cancer model.	[72]
Suspension of Doxorubicin-cis-aconitic anhydride-PEI conjugates complexed with siRNA.	*BCL2*	Doxorubicin conjugate	Pulmonary	Murine B16F10 xenograft	Co-delivery achieved enhanced inhibition of tumor growth compared to controls.	[36]
Suspension of siRNA complexed to doxorubicin conjugated with PEI through hydrazine bond.	*BIRC5* (Survivin)	Doxorubicin conjugate	Pulmonary	Murine B16F10 xenograft	Co-delivery achieved improved antitumor efficacy with low level of side effects.	[37]
Nanostructured lipid nanocarriers dressed with PEG and modified synthetic analog of luteinizing hormone-releasing hormone as a targeting moiety.	*MRP1* and *BCL2*	Paclitaxel or doxorubicin	Pulmonary	Murine A549 xenograft	Effectively co-delivered anticancer drugs and siRNA. Enhanced anti-tumor effect and reduced accumulation in non-target organs compared to intravenous injection.	[74]

Conjugates of siRNA-gold nanoparticles and drug-gold nanorods embedded in dextran/dendrimer hydrogels patch	*KRAS*	Avastin	Peritumoral implantation	Murine LoVo-6-Luc-1 CRC xenograft	The triple-combination therapy (siRNA/drug/heat) led to tumor regression and survival of all test animals over the 145+ days of the investigation.	[76]

Figure(s)

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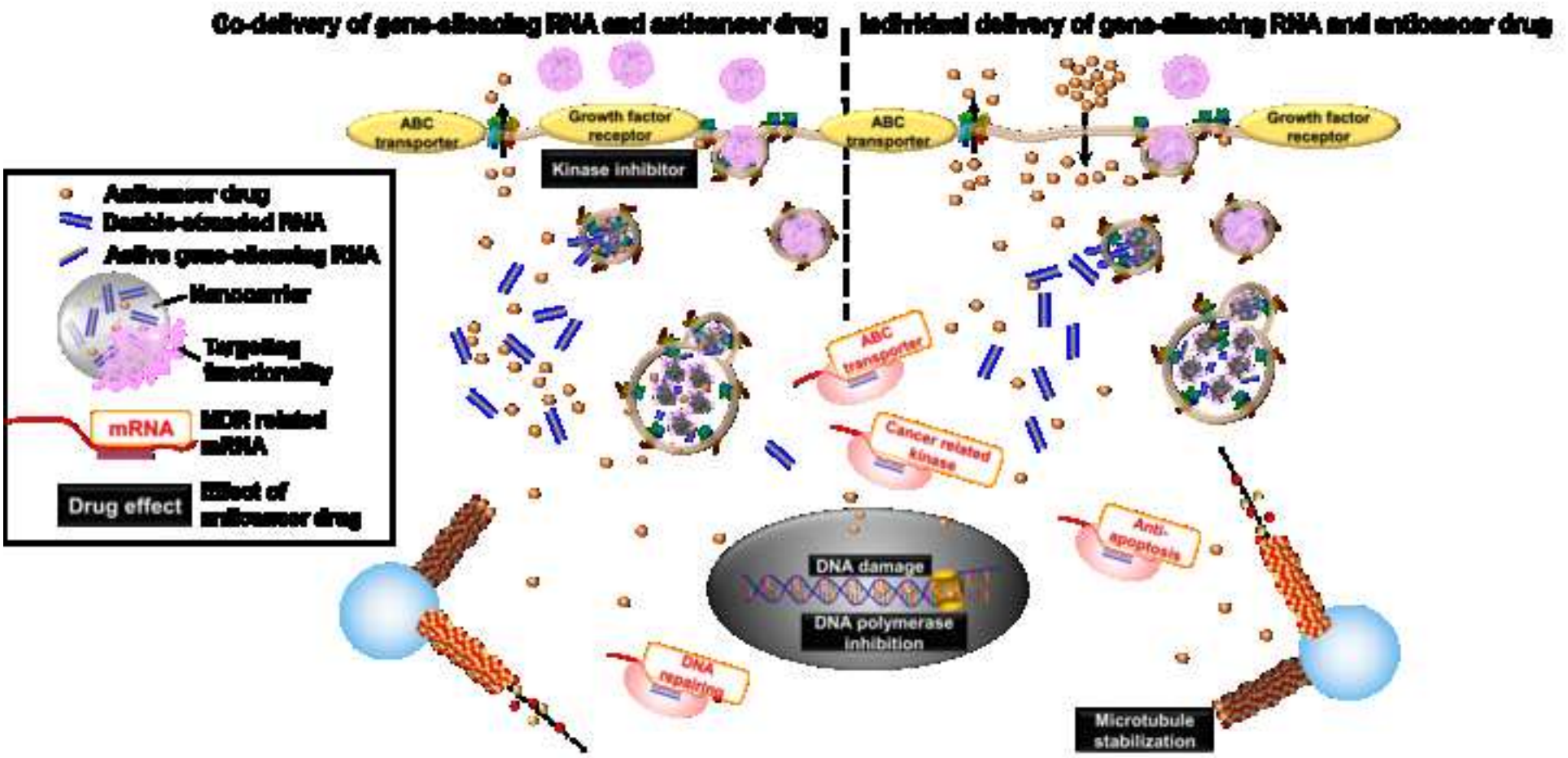

Fig. 2

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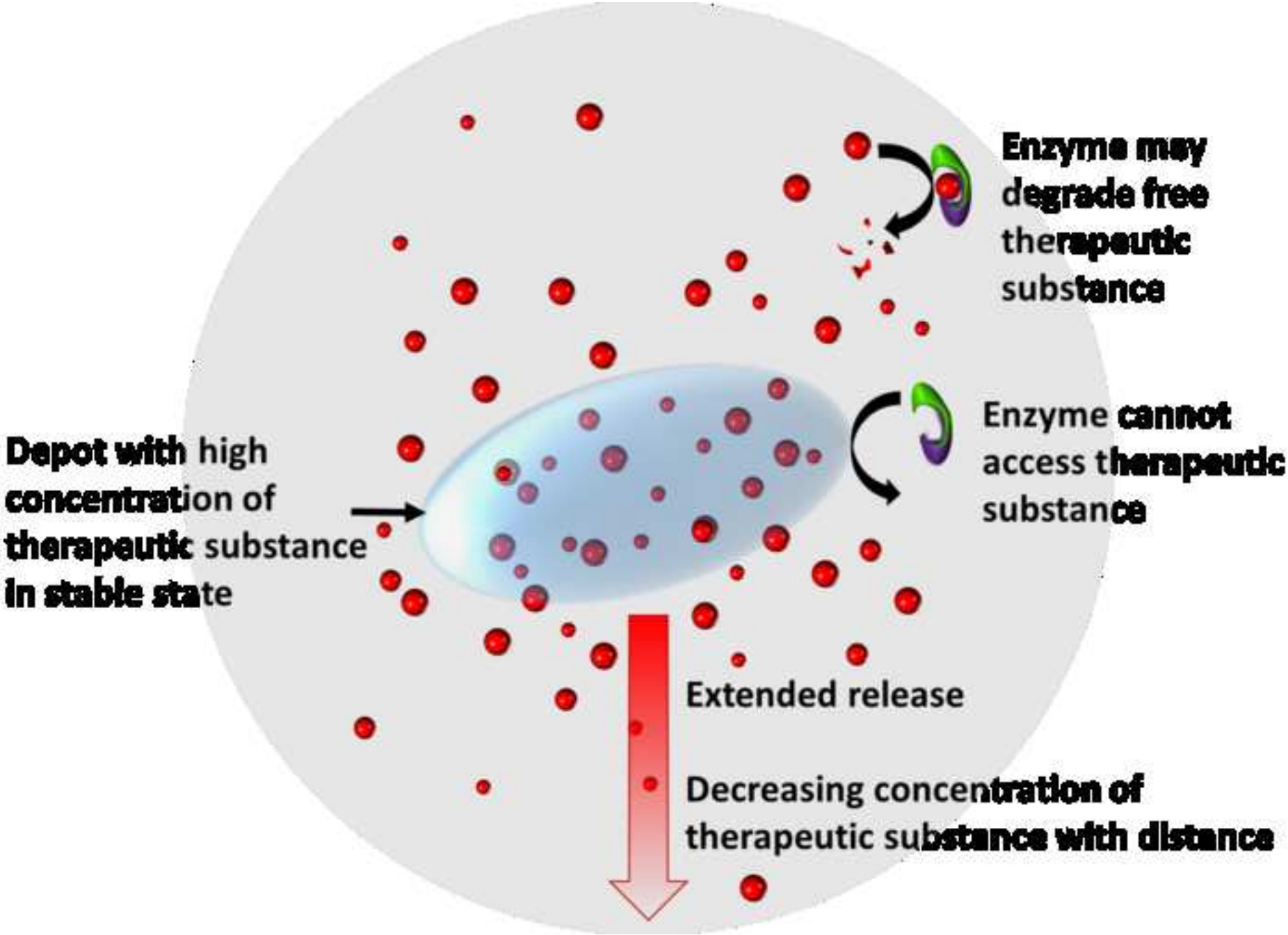

Note: Fig. 3 is excluded from the post print to meet the reproduction licensing agreement. The reader is referred to the original publication of the figure in REF no. 76: Conde J, Oliva N, Zhang Y, Artzi N. Local triple-combination therapy results in tumour regression and prevents recurrence in a colon cancer model. Nat Mater 2016;15:1128-38.