

export (GS-X) pump or by the ABC-transporter (MRP2) (Borst et al. 2000).

Metallothionein (MT)

MT, a group of low-molecular weight (6–7 kDa) cysteine-rich intracellular proteins (Cherian et al. 2003), binds and forms complexes (Fig. 1) with a number of trace metals including zinc, copper, cadmium, mercury, platinum and silver, and also protects cells against heavy metal toxicity, suggesting that it may also have a functional role in drug resistance. It is generally considered that anti-cancer drugs that contain metal ions (such as, cisplatin) in their structures would be more sensitive to the intracellular expression of MT. In several studies, it was found that the elevated level of MT and MT mRNA regulates the resistance of human SCLC, ovarian, testicular and colon tumors as well as fibroblasts, to anticancer drugs including to cisplatin, melphalan, bleomycin and cytarabine (Chin et al. 1993; Dziegiel et al. 2003; Kasahara et al. 1991; Kondo et al. 1995). In addition, MT also functions as a potential negative regulator of apoptosis (Shimoda et al. 2003) and due to such an effect, several cancer cells like lung cancer, hepatoma and hepatocellular carcinoma are resistant to etoposide.

DNA topoisomerase II (Topo II)

Topoisomerase II (Topo II) has been identified as the site of action of several clinically used chemotherapeutic drugs, including doxorubicin, actinomycin D, mitoxantrone, etoposide, teniposide etc. (Liu 1989; Smith et al. 1993). These drugs, commonly called Topo II poisons, cause enzyme-mediated DNA damage (Tewey et al. 1984) followed by the induction of cell apoptosis, thus representing a promising and effective strategy for cancer chemotherapy. Cancer cells can defend themselves by altering the expression of Topo II with which the drug functions. The level of expression of Topo II in the cell nuclei is associated with the function of the drug (Fig. 1) as well as the sensitivity of the respective cells. Cells with reduced levels of Topo II were found to be resistant to Topo II poisons (such as DOX) as compared to cells overexpressing Topo II (Beck et al. 1993; Zhang et al. 2012), suggesting that the MDR of cancer cells is mediated through a reduction in enzyme-mediated DNA damage.

Catalytic enzymes

Thymidylate synthase (TS) catalyzes the methylation of fluorodeoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), an important process for DNA biosynthesis (Danenberg 1977; Jonston et al. 1995)

and this enzyme is the target of several chemotherapeutic drugs (Fig. 1), such as 5-fluorouracil (5-FU), methotrexate etc. It was reported that the prognosis of cancer patients with adenocarcinoma, colorectal cancer and non-SCLC (NSCLC) is significantly related to the expression of TS and its mRNA levels (Lenz et al. 1995; Shintani et al. 2003; Yamachika et al. 1998). On the other hand, the expression of dihydropyrimidine dehydrogenase (DPD) in solid tumors, a key enzyme responsible for the catabolism of 5-FU, was found to reduce the efficacy of 5-FU (Fischel et al. 1995). Therefore, the activity of these enzymes in tumors, including gastric cancer, colorectal cancer, breast cancer, and NSCLC is considered to be associated with chemosensitivity to 5-FU (Beck et al. 1994; Salonga et al. 2000).

Others

Including the factors discussed above, some other factors also play a role in the development of MDR by cancer cells. In MDR cancer cells, increased levels of detoxification enzymes such as cytochrome p450 rapidly metabolize and inactivate the internalized drugs (Gottesman et al. 2002). In addition, cancer cells can defend themselves against drug induced apoptosis through the upregulation of anti-apoptotic proteins such as survivin and Bcl-2 family members (Fig. 1) (Kanwar et al. 2011; Zhang et al. 2012;), where survivin stimulates drug resistance by directly suppressing apoptotic proteins such as caspase and procaspase signaling mechanisms, consequently resulting in the upregulation of the expression of MDR proteins such as P-gp, MRP-1 and MRP-2. In a variety of tumors, the deletion of tumor suppressor genes (p53) has been reported to cause drug resistance (Luqmani 2005). The drugs that enhance DNA damage leads to p53-mediated cell death, and the loss of p53 function thereby allows the cells to continue to replicate with damaged DNA, and for which the cancer cells become resistance to DNA-damaging drugs.

Strategies for overcoming cancer MDR using DDS

The resistance of cancer cells, lead by several precursor genes, to cytotoxic drugs is one of the major causes of the failure of cancer chemotherapy. Therefore, it becomes necessary to explore strategies for circumventing the MDR of cancers as well as to make the treatment effective by utilizing the available drugs. Strategies for circumventing MDR by using a DDS are presented in Fig. 2 and are discussed here because they have the potential for serving as an innovative and promising alternative to conventional small-molecule chemotherapeutics, by encapsulating and conjugating drug molecules within a nanocarrier.

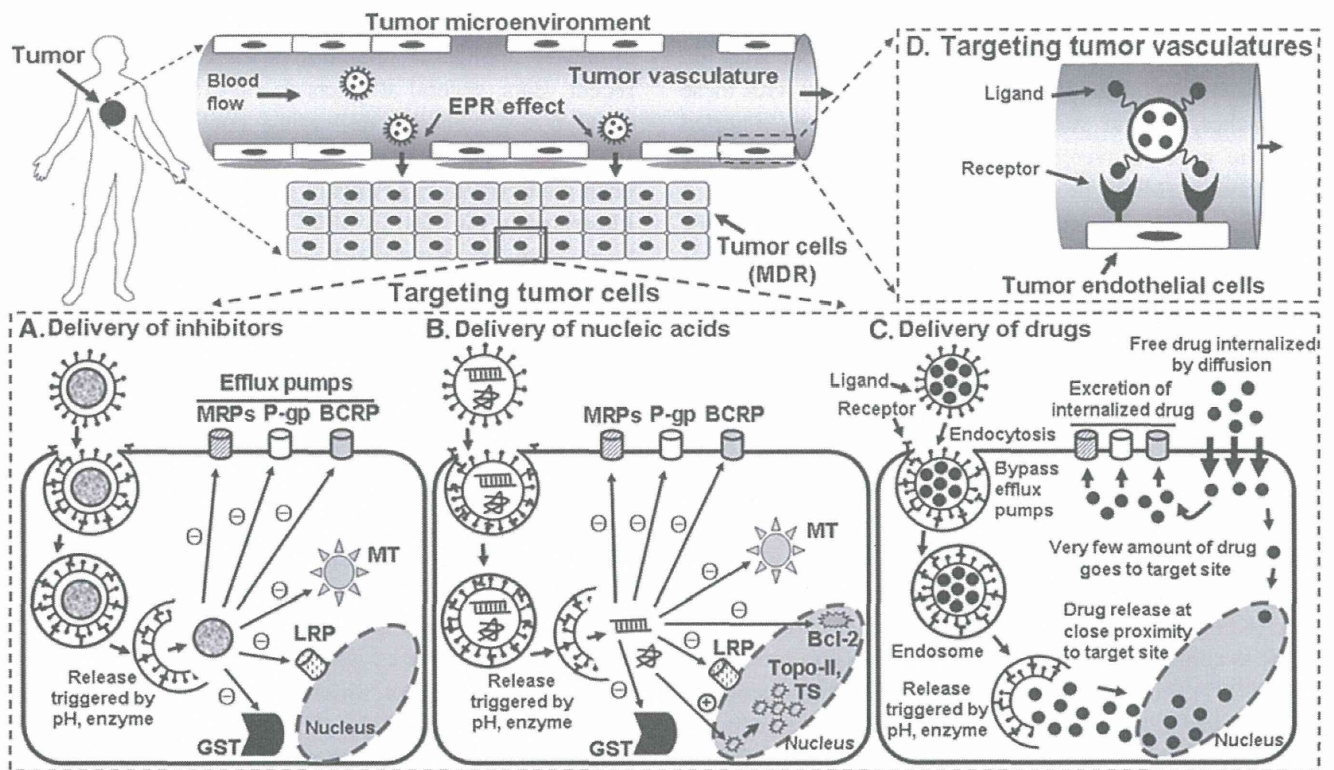


Fig. 2 Schematic representation of the application of a DDS for reversing cancer MDR. The expression of proteins or enzymes, responsible for MDR in cancer cells, can be controlled by delivering either a specific inhibitor or nucleic acids followed by the delivery of cytotoxic drug (either concurrently or separately) via a nanoparticle. The free drug, internalized by diffusion, can easily be detected by the

ABC-transporters and excreted out before going to the depth of the cells. Nanoparticles loaded with free drug can be endocytosed, thus permitting them to bypass the ABC-transporters and deliver their payload to the target organelle where the drug exerts its action. These delivery approaches would reverse the MDR of cancer cells by making them chemosensitive

Delivery of inhibitors of the MDR proteins

The activity of cytotoxic drugs that are internalized by cells depends on their concentration and availability in the cell cytosol and in nuclei. However, several proteins, such as P-gp, MRP, BCRP, LRP, play a role in transporting the internalized drug molecules to the outer environment of the cell (Fig. 1). In addition to these, GST detoxifies the internalized drug molecules through its enzymatic activity (Fig. 1). Therefore, to make drug molecules available as well as for them to function effectively in the cellular compartment, inhibiting the expression of the responsible proteins or enzymes is the prime concern. Several inhibitors of these proteins or enzymes have been identified (Choi 2005; Wang et al. 2003) and could be utilized to down-regulate their expressions. To inhibit and antagonize the function of P-gp, several potent and selective inhibitors, including verapamil, diltiazem, tariquidar, quinidine, cyclosporin A, astemizole, itraconazole, doxorubicin-gallium-transferrin conjugate etc. can be used (Choi 2005; Wang et al. 2003). Erythromycin, itraconazole, difloxacin, ofloxacin, rifampicin, indomethacin, NSAIDs, doxorubicin-gallium-transferrin conjugates etc. were found to

inhibit the activity of MRP (Choi 2005). Genistein, estrone, fumitremorgin C etc. were reported to be representative inhibitors of BCRP (Choi 2005). A variety of GST inhibitors, including ethacrynic acid, 6-mercaptohexanol derivative (NBDHEX), nitazoxanide, Haloenol lactone, Aloe-emodin, Benastatin A etc. (Laborde 2010) were shown to modulate drug resistance by sensitizing tumor cells to anticancer drugs.

While the inhibitors are efficient enough to antagonize the function of MDR proteins, they have no specificity towards the target sites and can block the functions of the target proteins in normal organs, which leads to the development of adverse effects. Cardiotoxicity, nephrotoxicity, and neurotoxicity, for example, are among the common side effects associated with these inhibitors (Rezzani 2004). To avoid such undesirable circumstances in normal tissues, it is necessary to specifically deliver the inhibitors to the tumor sites by encapsulating them in nanoparticles (Fig. 2A), which can further be modified with specific ligands to render them to be more specific to tumor cells. The delivered inhibitor can function in specific tumor cells to down-regulate the expression of the responsible MDR proteins (Fig. 2A). Following down-

regulation; the cytotoxic drugs can be delivered separately or con-currently with the inhibitors to the tumor sites via the administration of the nanoparticles. In line with these approaches, several studies have recently been reported with the goal of overcoming MDR through the specific inhibition of MDR proteins. Nanoparticles containing a combination of cytotoxic drugs and efflux pump inhibitors, such as cyclosporine, verapamil, and tariquidar, have shown promise in terms of reversing MDR in cancer cells (Patil et al. 2009; Soma et al. 2000; Wu et al. 2007). Nanoparticles co-encapsulated with cyclosporin A and DOX result in about two folds higher efficacy in DOX-resistant leukemia cells as compared to free cyclosporin A or only DOX-loaded particles (Soma et al. 2000). In another study, it was depicted that transferrin-conjugated liposomes containing both verapamil and DOX were expedited cellular internalization, resulting in a higher accumulation of DOX in a DOX-resistant leukemia cell line (K562), and thereby demonstrated the reversal of MDR as compared to the use of unmodified liposomes (Wu et al. 2007). In addition, biotin-conjugated poly lactic-co-glycolic acid (PLGA) nanoparticles loaded with paclitaxel and tariquidar, also showed improved therapeutic efficacy in breast cancer, as compared to the non-targeted formulations (Patil et al. 2009). Despite the immense role of MRP and BCRP on mediating MDR in cancer cells, the application of nanoparticles loaded with inhibitors aimed at suppressing the function of these proteins have not been extensively studied. As a potent GST inhibitor, ethacrynic acid was reported to efficiently potentiate the cytotoxic effects of chlorambucil and melphalan in human colon cancer cell lines (Clapper et al. 1990). Thus the use of nanoparticles loaded with this inhibitor and a cytotoxic drug would be a promising tool for overcoming the resistance of cancer cells where GST plays a pivotal role. Based on the above information, it is evident that the targeted delivery of chemosensitizers (inhibitors) and chemotherapeutics via the utilization of nanoparticles promises to be a safer and effective approach to the treatment of cancers that are resistant to chemotherapy.

Delivery of nucleic acids aimed to target MDR proteins

Gene delivery, aimed to controlling the activity of a specific gene via RNA interference (RNAi), has become a powerful tool in cancer therapeutics. RNAi is a post-transcriptional gene silencing mechanism that is mediated by small interfering RNAs (siRNAs) of 21–25 nucleotides (nt) (Filipowicz et al. 2005). The double-stranded RNA molecules are incorporated into the RNA-induced silencing complex (RISC), where they induce the degradation of target mRNAs in a sequence-specific manner (Filipowicz et al. 2005). In solid tumors, membrane transporter families

play a pivotal role in the distribution and excretion of clinically applicable chemotherapeutic drugs (Fig. 1). In recent years, several attempts to control the expression of ABC transporters by delivering nucleic acids (siRNA, miRNA etc.) loaded with nanoparticles to tumors have been made (Patil and Panyam 2009; Wang et al. 2010), as illustrated in Fig. 2B. In MDR cancer therapy, siRNA has been used to down-regulate MDR-related proteins by silencing MDR-1 (Liu et al. 2009), MRP1 and Bcl-2 (Saad et al. 2008). Nanoparticles loaded with P-gp siRNA and DOX were applied to the delivery of encapsulated contents into tumors (Meng et al. 2010) where the delivered siRNA silences the expression of P-gp which consequently increases the intracellular concentration of DOX. Following the same purpose, specific ligand modified liposomes containing P-gp siRNA or DOX were also used to treat drug resistant tumors (Jiang et al. 2010). Nanoparticles loaded with MDR-siRNAs showed enhanced gene transfection (Nakamura et al. 2011), which can be attributed to its systemic stability as well as target specificity, as compared to free siRNAs which are unstable in serum and show poor cellular uptake (Gao et al. 2009). In addition, ligand modified nanoparticles also capable to addressing the off targeting issue in siRNA delivery (Di Paolo et al. 2011), which is a prerequisite for down regulating the specific genes present in the MDR tumor tissues.

It was also reported that the co-delivery of paclitaxel and Bcl-2-targeted siRNA from cationic amphiphilic copolymeric self-assembled nanoparticles exhibited superior activity in a breast cancer cell line (MDA-MB-231) via the down regulation of Bcl-2 expression, as compared to the individual agents (Wang et al. 2006). In another study, it was reported that paclitaxel and P-gp siRNA loaded in PLGA-polyethyleneimine (PEI) copolymeric nanoparticles resulted in significantly higher paclitaxel retention in MDR cancer cells and a better activity in vivo (Patil et al. 2010), which showed minimal response to paclitaxel without silencing the P-gp. Liposomes loaded with DOX as well as MRP-1 and Bcl-2 siRNAs caused the induction of cell apoptosis as well as the reversal of MDR in lung cancer (H69AR, human SCLC) (Saad et al. 2008). Recently, a dual-sequential treatment strategy was applied in which siRNA and drug loaded bispecific antibodies (BsAb) modified targeted micelles were delivered to knockdown the expression of MDR1 (MacDiarmid et al. 2009), where the tumors were treated with siRNA loaded minicells followed by the administration of minicells loaded with shRNA. The subsequent administration of drug (5-FU and irinotecan) loaded targeted minicells showed a better pharmacological effect against drug resistant tumors. Furthermore, simultaneous administration of anti-MDR1 shRNA encoding vectors as well as DOX inhibited tumor growth by reversing MDR (Walther et al. 2010). Survivin

is a negative regulator of apoptosis and its expression is elevated in MDR cancer cells (Fig. 1). Therefore, inhibiting the expression of survivin would likely be effective in enhancing apoptosis in cancer cells (Kanwar et al. 2011). Considering this issue, polyamidoamine (PAMAM) dendrimer modified magnetic nanoparticles were used to deliver antisense oligodeoxynucleotides (asODN) with the objective of suppressing the survivin mRNA and protein levels in breast cancers (MCF-7 and MDA-MB-435) and in liver cancer (HepG2) cells. The above preparations showed the resensitization of MDR cancer cells to the drug molecules where they were initially resistant. Therefore, prior to delivering the cytotoxic drug to MDR cancer cells, it is immensely important to control the level of expressions of the respective genes (Fig. 2B) by delivering the nucleic acids via the nanoparticles.

Delivery of nanoparticles to modulate the uptake route of drugs

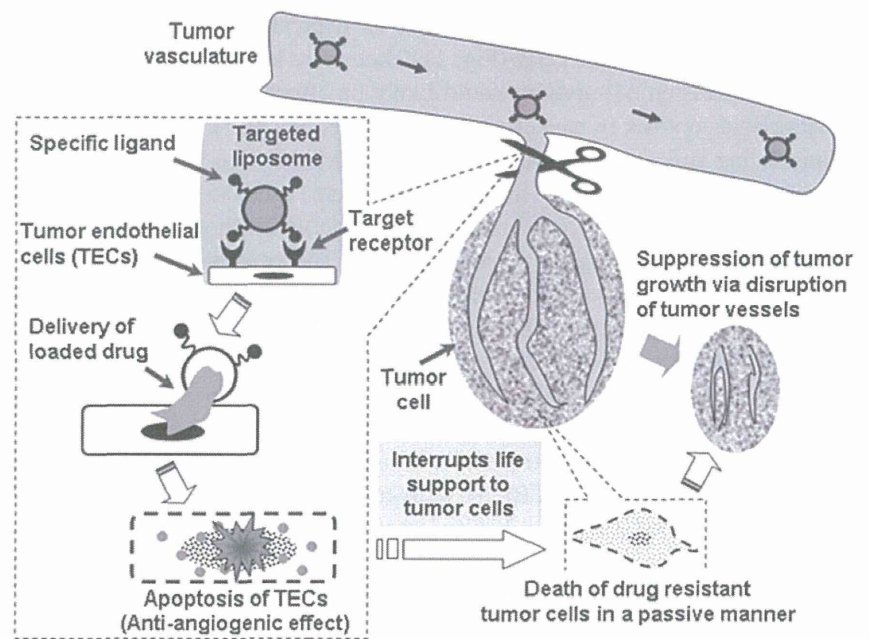
Drugs that are encapsulated in nanoparticles have different pharmacokinetic properties compared to the free drugs. Free drugs are generally internalized by diffusion across the cellular membrane and the drug efflux pumps present on the cell membrane can sense free drug molecules as they cross the membrane (Fig. 2C), and prevent them from entering the cell cytoplasm or making them vulnerable to capture by ABC transporters, with their subsequent ejection. To overcome the problems associated with the efflux of free drugs as well as to increase their efficacy, nanocarriers would be the effective tool where the drugs can be loaded or encapsulated and can deliver the payload to the cellular internal organelles (Fig. 2C). Nanocarriers are internalized into cells via a non-specific endocytosis pathway and cross the cell membrane in an 'invisible' form, thereby preventing the drugs from being recognized by efflux pumps (Huwlyer et al. 2002, Rejman et al. 2004). This type of endocytosis process is called "stealth endocytosis" (Fig. 2C), and results in a higher intracellular accumulation of the drug (Davis et al. 2008). The particles are internalized in endosomes that release drugs near the peri-nuclear region (or deep inside the cytoplasm) away from membrane ABC transporters (Shen et al. 2008). Following these steps, nanocarriers are able to bypass ABC transporters (Fig. 2) (Kunjachan et al. 2012) and the cytotoxic drugs are shielded from cytoplasmic detoxification enzymes such as MT and methionine synthase (Murakami et al. 2011). It was reported that taxol-containing liposomes exhibited antitumor effects in a taxol-resistant Colon-26 tumor model (Sharma et al. 1993). In addition, polymer-drug conjugate comprised of a paclitaxel-carboxymethyl dextran exhibited the in vivo antitumor activity against paclitaxel-resistant Colon-26

carcinoma cells (Sugahara et al. 2007). Therefore, by modulating the uptake route as well as by targeting sub-cellular compartments, DDSs utilizing nanocarriers would be the effective tool for reversing MDR in cancer cells.

Targeted anti-angiogenic therapy

For the growth and progression of a tumor, the tumor cells need glucose, minerals, and oxygen which are initially supplied by nearby blood vessels; but as the tumor grows, the cells in the interior of the tumor become farther away from the blood supply. To continue growing, tumor must have new blood vessels. Without the formation of new blood vessels, a tumor can not grow larger than about $1\text{--}2\text{ mm}^3$ (Bamias and Dimopoulos 2003). With the help of several key promoters secreted from the tumor cells, the new blood vessels are formed within the tumor microenvironment from pre-existing blood vessels, a process called tumor angiogenesis (Folkman 1995). To control the growth of tumors, several attempts have been made to inhibit tumor angiogenesis, a process that involves down-regulating key promoters as well as by delivering cytotoxic drugs to the tumor endothelial cells (TECs) present in the tumor blood vessels (Fig. 2D), a process that is referred to as anti-angiogenic therapy (Folkman 2007; Jain 2005). Tumor vasculatures are generally leaky, with endothelial cell gaps of $\sim 100\text{--}600\text{ nm}$ (Hashizume et al. 2000), although the length of cell gaps depend on the tumor type, malignancy, and the stage of the disease (Hashizume et al. 2000; Hobbs et al. 1998; Siwak et al. 2002). Nanoparticles with diameters of $\sim 100\text{ nm}$ (dnm) are used to target the tumor tissues which accumulate in tumor cells through the leaky tumor vasculature via the enhanced permeability and retention (EPR) effect (Maeda et al. 2000), a universal phenomenon in solid tumors (with some exceptions in the case of hypovascular tumors, such as prostate cancer or pancreatic cancer). Nanoparticles with dnm smaller than 10 cross the basement membranes in the glomeruli of kidneys and are rapidly cleared, which leads to a shorter blood half-life (Choi et al. 2010). Therefore, a particle size of $10\text{--}100\text{ dnm}$ would be suitable for in vivo tumor targeting based on the EPR effect (Gupta and Gupta 2005). Doxil, a typical successful example of a DOX loaded small size PEG-LP ($\sim 100\text{ dnm}$), functions against tumor cells via the EPR effect, and is used clinically in the treatment of breast cancer, ovarian cancer, AIDS related Kaposi's sarcoma etc. (Haley and Frenkel 2008; Yuan et al. 1994). However, it shows very poor or even no therapeutic efficacy against the cancers that are resistant to DOX. This circumstance is also true for other types of chemotherapeutic drugs once the cancer cells become resistant to them. Tumors that are resistant to chemotherapy would be difficult to treat by delivering drugs to the tumor cells. Therefore, an alternate approach for treating drug-resistant cancers would be highly

Fig. 3 Schematic illustration of the application of targeted anti-angiogenic therapy in the treatment of drug-resistant cancer. Ligand modified, drug-loaded nanoparticles deliver the drug and kill the TECs followed by the disruption of tumor blood vessels which supports the survival of tumor cells. The discontinuation of life supports leads the suppression of growth and progression of drug resistant tumor cells



desirable. TECs present in the tumor vasculatures provide life support to MDR tumor cells. Hence, targeting TECs would be an alternate and effective approach to the treatment of such types of notorious tumor cells (Figs. 2D, 3), where the drug is specifically delivered to the TECs via targeted nanoparticles, not to the MDR tumor cells.

Several specific markers, including integrin $\alpha v \beta 3$, aminopeptidase N (CD13), vascular endothelial growth factor (VEGF) receptors (VEGF-R2, neuropilin-1), tumor endothelial markers (TEMs) etc. are expressed by TECs (Ruoslahti 2002) on their surfaces. Therefore, to target the TECs, ligand or antibody modified nanoparticles having ~ 100 nm have been used to deliver a therapeutic moiety to TECs (Murphy et al. 2008; Pastorino et al. 2003). Antibody against the TEM8 marker exhibits an impaired growth of human tumor xenografts including melanoma, breast, colon, and lung cancer by

the selective inhibition of pathological angiogenesis (Chaudhary et al. 2012). In another study, it was observed that cationic liposomes loaded with oxaliplatin provides ionic interactions with the surface molecules of TECs, resulting in a remarkable anti-angiogenic activity in mice bearing melanoma (B16BL6) tumors (Abu-Lila et al. 2009). Recently, an effective anti-angiogenic therapy has been developed in which K237 peptide-conjugated nanoparticles loaded with paclitaxel (K237-PTX-NP) were used to deliver the drug to the TECs for the treatment of P-gp overexpressing and paclitaxel resistant human colorectal adenocarcinoma (HCT-15) (Bai et al. 2013). These targeting approaches can be applied as an anti-angiogenic therapy (Fig. 3) for the treatment of MDR cancers.

In leaky tumor vasculatures, the length of the gaps in TECs varies, depending on the type of tumor. Therefore,

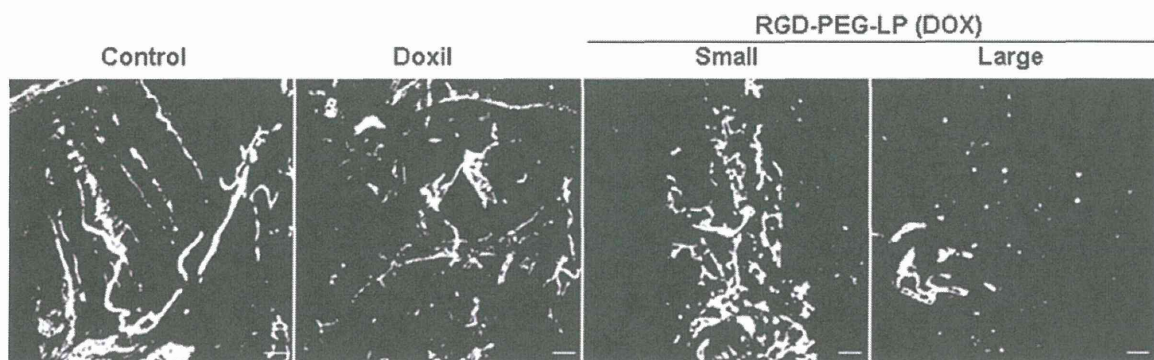


Fig. 4 Anti-angiogenic effect of DOX loaded PEG-LPs in DOX resistant human RCC tumor tissues. At a tumor volume of 150 mm^3 on the back of BALB/c male nude mice, 3 successive doses of 1.5 mg DOX/kg body weight were injected by tail vein. At 24 h post-injection, tumors were collected and observed under a microscope.

Tumor blood vessels (white) were stained with FITC-isolectin B4; scale bars $50 \mu\text{m}$. Large size RGD-PEG-LP (DOX) preferentially targets and delivers DOX to TECs followed by significant disruption of the tumor vasculatures as compared to others

the endothelial cell gap is an important issue to consider in designing nanoparticles for targeting TECs in a specific tumor type. For the treatment of DOX resistant renal cell carcinoma (RCC) via the targeting of TECs, we recently developed DOX loaded ligand modified size controlled PEG-LPs having ~ 300 nm (Kibria et al. 2013; Takara et al. 2012). The large size particles (~ 300 nm) showed a minimization of the EPR effect and preferentially targeted and delivered DOX to TECs, where the small size particles (~ 100 nm) largely act directly on DOX-resistant tumor cells via the EPR effect. The DOX, delivered by large size particles, functions to kill the TECs, leading to the disruption of the tumor vasculature (Fig. 4), and discontinues life support to the tumor cells, ultimately causing the death (apoptosis) or inhibition of the growth of the RCC tumor cells in a blood supply-dependent manner (Fig. 3). Therefore, the targeted anti-angiogenic therapy using drug-loaded nanoparticles also has the promise of reversing the utilization of chemotherapeutic drugs for the treatment of chemotherapy resistant cancers.

Future perspectives

Due to self defense mechanisms, cancer cells show resistance to chemotherapeutic drugs for which the drug molecules eventually become ineffective, finally resulting in the failure of cancer treatment and thereby patient mortality. For reversing tumor cell resistance, it is immensely important to identify the key factors responsible for MDR in a specific tumor type. Based on a successful identification, it would be easier to design and apply DDS techniques to control the functions of the responsible factors, followed by the delivery of the chemotherapeutic drugs to which the cancer cells are resistant. Such a rationale design and application of DDS would permit cancer MDR to be overcome by making the cells chemosensitive as well as by reverting back the activity of drug molecules in MDR tumors.

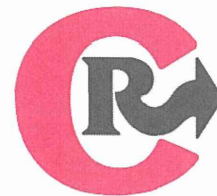
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RNAi-mediated gene knockdown and anti-angiogenic therapy of RCCs using a cyclic RGD-modified liposomal-siRNA system



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ABSTRACT

Angiogenesis is one of crucial processes associated with tumor growth and development, and consequently a prime target for cancer therapy. Although tumor endothelial cells (TECs) play a key role in pathological angiogenesis, investigating phenotypical changes in neovessels when a gene expression in TEC is suppressed is a difficult task. Small interfering RNA (siRNA) represents a potential agent due to its ability to silence a gene of interest. We previously developed a system for *in vivo* siRNA delivery to cancer cells that involves a liposomal-delivery system, a MEND that contains a unique pH-sensitive cationic lipid, YSK05 (YSK-MEND). In the present study, we report on the development of a system that permits the delivery of siRNA to TECs by combining the YSK-MEND and a ligand that is specific to TECs. Cyclo(Arg-Gly-Asp-D-Phe-Lys) (cRGD) is a well-known ligand to $\alpha_v\beta_3$ integrin, which is selectively expressed at high levels in TECs. We incorporated cRGD into the YSK-MEND (RGD-MEND) to achieve an efficient gene silencing in TECs. Quantitative RT-PCR and the 5' rapid amplification of cDNA ends PCR indicated that the intravenous injection of RGD-MEND at a dose of 4.0 mg/kg induced a significant RNAi-mediated gene reduction in TEC but not in endothelial cells of other organs. Finally, we evaluated the therapeutic potency of the RGD-MEND encapsulating siRNA against vascular endothelial growth factor receptor 2. A substantial delay in tumor growth was observed after three sequential RGD-MEND injections on alternate days. In conclusion, the RGD-MEND represents a new approach for the characterization of TECs and for us in anti-angiogenic therapy.

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1. Introduction

Angiogenesis is a major cause in cancer progression and metastasis [1,2]. Folkman et al. first proposed the theory that, to be supplied with oxygen and other nutrients, tumors with sizes over 1–2 mm³ inevitably required angiogenesis, and that, if tumor vasculature development could be inhibited, tumor tissue would shrink, as the result of a lack of oxygen and other nutrients [3]. Since this publication, anti-angiogenic therapy has evolved as an innovative treatment for various cancers. A mono-clonal antibody against vascular endothelial growth factor (VEGF), which is referred to as Avastin, is currently used in the treatment of various types of cancer [4,5].

Small interfering RNA (siRNA) was predicted to be a potentially useful drug for this purpose, due to the ability to inhibit the expression of any genes of interest in a sequence-specific manner [6]. However, its instability in the blood and the low permeability of the plasma membrane require drug delivery systems that target specific cells in order to achieve an effective therapy by siRNA [6,7]. We previously developed

a liposomal siRNA system, a multi functional nano-device (MEND) [8,9]. In the past report, the use of a MEND composed of a pH-sensitive cationic lipid, YSK05 (YSK-MEND) caused a significant gene reduction in tumor tissue when intratumorally and intravenously injected into tumor-bearing mice [10,11]. A number of pH sensitive siRNA carriers, such as liposomes [12,13], polyplexes [14] and micelles [15], have been evaluated for use in tumor targeting. pH-sensitive carriers are generally thought to be more suitable for tumor targeting than conventional cationic carriers because of their highly specific fusogenicity in acidic endosomes [16]. YSK05 consists of two linoleyl fatty acid chains and a tertiary amino group, which are responsible for pH-responsive fusogenicity in endosomes. In this study, we incorporated a ligand that is specific to tumor endothelial cells (TECs) into YSK-MEND to achieve anti-angiogenic therapy using siRNA.

Cyclo (Arg-Gly-Asp-D-Phe-Lys) (cRGD) peptide is a well-validated ligand for $\alpha_v\beta_3$ integrin, which is highly and selectively expressed on the cell surface of TECs and some types of cancer cells themselves [17]. cRGD is a known antagonist of $\alpha_v\beta_3$ integrin, and the injection of free cRGD suppresses tumor progression in many cancers such as glioblastomas and lung cancer [18]. This is because $\alpha_v\beta_3$ integrin plays a key role in angiogenesis in tumor tissue [19]. Moreover, the cRGD peptide can be

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used for a variety of purposes, including cancer imaging and therapy by conjugating cRGD with imaging probes, anti-cancer agents or drug carriers [20]. Concerning the *in vivo* delivery of nucleic acids using cRGD, several reports have appeared in which tumor growth was inhibited by the systemic injection of anti-tumor and/or anti-angiogenic oligonucleotides encapsulated in micelles [21] and lipoplexes [22–24]. However, in almost all of those reports it was not clear whether siRNA was delivered to cancer cells and TECs, and no direct evidence showing that a gene reduction in TECs was mediated by RNA interference. In this study, we verified gene silencing by siRNA in TECs using quantitative RT-PCR (qRT-PCR) and rapid amplification of the 5' cDNA ends (5' RACE-PCR), which was the only method available for confirming RNAi-induced silencing [7].

We chose renal cell carcinomas (RCCs) as a therapeutic model cancer by inhibiting angiogenesis, since it is well known that RCCs effectively respond to anti-angiogenic therapy [25]. Since RCCs are known to respond poorly to conventional anti-cancer drugs, interleukin-2 and interferon- α injections are currently the standard treatment for patients with progressive RCCs [26]. In recent years, however, novel agents targeting angiogenesis pathways have been developed as the result in advances in our understanding of tumor biology. Actually, Afinitor and Toricel (mTOR inhibitors) and Sutent (a multi kinase inhibitor) are currently being applied for metastatic RCCs in addition to Avastin.

Although anti-angiogenic treatment has had significant therapeutic effects for cancer progress and metastasis, it has been reported that some patients are refractory or acquire resistance to VEGF inhibition [27]. Several mechanisms are thought to be involved in the resistance anti-angiogenic treatment by VEGF blockade. Compensation by other pro-angiogenic mechanisms, such as basic fibroblast growth factors (bFGF), platelet-derived growth factor (PDGF) and angiopoietins, appears to be a dominant factor in the development of acquired resistance to VEGF inhibition. Moreover, recent reports suggest that the recruitment of other cells, such as pericytes and bone marrow-derived myeloid cells, to tumor vessels is implicated in the resistance to anti-angiogenic therapy [28,29]. A methodology that will permit the complete control any gene that is expressed in TECs is needed for further elucidating the mechanism of anti-angiogenic therapy resistance, and hence developing a better therapy that targets tumor angiogenesis. In the study, we report that the RGD-MEND represents an efficient siRNA delivery system for cancer treatment through anti-angiogenic therapy.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycerophosphocholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycerophosphoethanolamine (POPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol₂₀₀₀ (PEG-DMG), 1,2-distearoyl-sn-glycerol, methoxypolyethylene glycol₂₀₀₀ (PEG-DSG) and N-hydroxysuccinimide-polyethylene glycol₂₀₀₀-1,2-distearoyl-sn-glycerophosphoethanolamine (NHS-PEG-DSPE) were purchased NOF (Tokyo, Japan). Cholesterol (chol), RPMI-1640 medium and DMEM were obtained from SIGMA Aldrich (St. Louis, MO). Egg phosphatidyl choline (EPC) and 1,2-distearoyl-sn-glycelo, methoxy polyethylene glycol (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). siRNAs were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). [³H]-cholesteryl hexadecyl ether (CHE) was purchased from PerkinElmer Life Science (Tokyo, Japan). DiI and DiD were purchased from Invitrogen (Carlsbad, CA).

2.2. Synthesis of cRGD conjugates

We synthesized cRGD-conjugated PEG (RGD-PEG) as previously reported [30]. In brief, cRGD peptide was incubated with NHS-PEG-DSPE in 20 mM phosphate buffered saline (pH 7.4, PBS) at 37 °C for 12 h. The mixture was then subject to dialysis using Spectra Por 6 (MWCO

1000Da, Spectrum) to remove un-conjugated RGD. The molecular weight of the conjugate was determined by MALDI TOF-MS.

2.3. MEND preparation

YSK-MENDs were prepared as previously reported [10,11]. Briefly, 1500 nmol of YSK05, 750 nmol of POPE, 750 nmol chol and 150 nmol PEG-DMG were dissolved in 400 μ L of 90% (v/v) aqueous tertiary butanol (t-BuOH). When the fluorescence was incorporated into the YSK-MENDs, 0.5 mol% (of the total lipid) DiD was added to the tubes and the organic solvent was removed by evaporation before the lipid solution was mixed. Two hundred microliters of siRNA solution (concentration 0.8 mg/mL in 2 mM filter-sterilized citrate buffer (pH4.5)) was gradually added to the shaking lipid solution, and homogeneous particles of liposomal siRNA were spontaneously formed by drastically diluting the siRNA-lipid mixture to 2 mL with 20 mM citrate buffer. The t-BuOH was then removed by ultrafiltration. For RGD-modification, a RGD-PEG solution was incubated with a YSK-MEND solution at 60 °C for 30 min at various molar ratios (RGD-PEG/total lipid of YSK-MEND). The YSK-MENDs were characterized by a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worcestershire, UK). The encapsulation efficiency and recovery ratio were calculated using RiboGreen (Invitrogen) as previously described [10]. siRNA encapsulation efficiency rate of all MENDs used in this study was over 90%. The sequences of the used siRNAs are shown in Supplemental Table S1.

2.4. Cell culture

OS-RC-2 cells and HEK293T cells were cultured in RPMI-1640 and DMEM, respectively. These media were supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μ g/mL). TECs, which were previously isolated by Ohga et al. [31], and HUVEC were cultured in EBM-2 medium supplemented with 2% FBS (v/v) and bullet kits (Lonza, Walkersville, MD). All cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere.

2.5. Evaluation of antigen expression

For evaluating the expression of $\alpha_v\beta_3$ integrin, 1.0×10^6 trypsinized cells were suspended in 1 mL of FACS buffer (0.5% bovine serum albumin and 0.1% sodium azide in 20 mM PBS), and the suspension was centrifuged at 4 °C for 4 min at 500 \times g. The cells were incubated in 100-fold diluted anti human $\alpha_v\beta_3$ integrin rat IgG (R&D systems, Minneapolis, MN) for 30 min on ice. The antibody solution was then removed by centrifugation and the cells were washed twice with 500 μ L of FACS buffer. Two hundred-fold diluted Alexa633-labeled anti rat IgG goat F(ab') (Invitrogen) was added to the cells. The cells were washed twice with 500 μ L of FACS buffer, and re-suspended in 1 mL of FACS buffer. The cell suspension was analyzed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

2.6. Animal study

Male, 4-week-old ICR mice and BALB/cAJcl-nu/nu were purchased from Japan SLC (Shizuoka, Japan) and CLEA (Tokyo, Japan), respectively. For preparing OS-RC-2-bearing mice, 1.0×10^6 OS-RC-2 cells in 75 μ L of sterilized PBS were inoculated into anesthetized BALB/cAJcl-nu/nu mice on the right flank. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals.