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Nanotechnology to target angiogenesis.

Foundation review: Antiangiogenic therapy using nanotechnological-based delivery system

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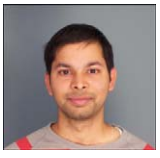
Of the many approaches for the treatment of cancer, angiogenesis and the additional promotion of apoptosis in cancer stem cells by using combinatorial therapy is usually the most recommended. There has been increased interest in the use of antiapoptotic and antiangiogenic biomolecules, such as antiangiogenic microRNA, small interfering RNA, inhibitor of apoptosis protein-binding peptides and Von Hippel-Lindau tumor suppressors, as well as targeting ligands, such as aptamers. Therefore, it is tempting to suggest that such molecules could be used for anticancer therapy. As we review here, such exploitation can be achieved by using nanotechnology and RNA-carrying cationic cell-penetrating peptides, for better protection from the enzymatic digestion and enhanced cellular internalization of these biomolecules.

Introduction

Carcinogenesis is a multistep process, resulting from a combination of environmental and genetic factors. It leads to a series of genetic and epigenetic changes that occur at various stages in the development and progression of the disease [1]. Cancer cells arise from a single transformed cell that has undergone genetic and epigenetic changes. Thus, the cancerous state of a cell is the result of several sequential events triggered by various factors, including genetic predispositions, transformation by viruses, radiation and/or certain chemicals. Many genetic and cytoplasmic events, including deactivation of tumor suppressor genes, such as those encoding protein 53 (p53) or retinoblastoma protein (Rb), trigger various events that lead to cancer [2].

Tumor invasion and metastasis is one of the major causes of treatment failure in patients with cancer. In addition, tumors have the capacity to generate new blood vessels by using pre-existing endothelium and/or endothelial precursor cells, by a process known as angiogenesis [3,4]. This, in turn, involves complex and diverse actions, such as extracellular matrix degradation, the proliferation and migration of endothelial cells and the morphological differentiation of endothelial cells to form capillary tubes. A range of factors, including various growth factors,

Jagat Kanwar, group leader of the Laboratory of Immunology and Molecular Biomedical Research, has an international reputation in investigating fundamental and applied molecular aspects of cancer and chronic inflammation. He is an immunologist, molecular biologist and cell biologist. He has extensive training and expertise in studying the molecular mechanisms of, and devising treatments for, diseases such as cancer, and chronic inflammatory diseases, such as asthma, atherosclerosis, inflammatory bowel disease (IBD), arthritis and multiple sclerosis in both *in vivo* and *in vitro* models. From 2002 to 2006, within Lactopharma, his main research project involved the identification of milk bioactive molecules and/or fractions for the treatment of cancer and used monotherapy (gene therapy, immunotherapy or antiangiogenic molecules) or combinational therapy with milk and natural plant bioactives; the results obtained have generated three patents and two provisional patents awaiting submission. He is currently working on nanotechnology-based peptide, siRNA and miRNA delivery for targeting survivin, HIF-1α and apoptotic cell signaling molecule expression in cancers and inflammations. For commercially funded grants, his research group carries out research in the areas of dairy/grain bioactives as immunomodulators, their role in bone and muscle development, osteoarthritis and wound healing. Presently, his 12 PhD students are working on various nanobiotechnological oral delivery systems for gene transfer technology and proteins in cancers. His publications have added to the body of knowledge in the fields of nanobiotechnology, cancer gene therapy, cell biology and immunology. Kanwar's research work has generated a total of eight patent/PCTs with two in preparation. Five of these patents have been licensed for commercialization to the biotech companies Antisoma, NeuronZ, Neuren Pharmaceuticals and Fonterra. He has been invited as a speaker to more than 30 conferences and has chaired sessions in immunology, nanotechnology, nanomedicine and biotechnology.



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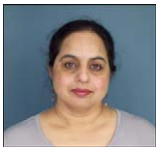

Rupinder Kanwar is a cell biologist and molecular immunologist who has extensive training and expertise in applying cutting-edge techniques of immunology, cell biology, molecular biology to the study of the pathophysiology and therapeutic targets of human cancer and chronic inflammatory diseases, including atherosclerosis, asthma, multiple sclerosis and osteoarthritis, in both *in vivo* and *in vitro* models. She has also delivered industry-oriented research. Her research at the University of Auckland, New Zealand during 2000–2005 on the efficacy of dairy bioactives, especially milk fat lipids, to combat chronic inflammation and cancer and the use of cell-permeable peptidomimetics in the control of chronic inflammation, generated four patents, with five live applications as a key inventor on devising novel peptides to treat inflammation and anti-inflammatory bioactives as neutraceuticals from bovine milk. To follow this, she currently has live patent applications in several countries, including Australia, the USA, Canada, China and Japan. Her expertise in chronic inflammatory has led to 25 papers, mostly in high-impact peer-reviewed publications. She also has national and international associates, including collaborators in India, China, and New Zealand and the USA.

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cytokines, lipid metabolites and cryptic fragments of homeostatic proteins, are involved in angiogenesis [5].

There are many anticancer, antiangiogenic drugs either approved or in phase II clinical trials (http://www.cancer.gov/CLINICALTRIALS). However, most of them are either chemical and/or fungal derivatives (mainly steroid-containing compounds) and these have many side effects when metabolized (i.e. after producing their secondary metabolites). Therefore, natural biodegradable compounds are the preferred alternative for decreasing patient compliance. In this regard, microRNAs (miRNAs) are ideal candidates to consider. These noncoding RNAs are reported to be involved in the temporal and spatial regulation of genes in different organisms, in addition to their tumor-suppressing and oncogenic properties. Certain classes of miRNAs (e.g. the let7 family) have been shown to have antitumor properties by their inhibition of rat sarcoma (RAS), a factor involved in cell proliferation. Therefore, it is tempting to use these tiny wonders for antitumor therapies. In this review, we describe the development, progression and metastatic stages of tumor development and the angiogenic switch and molecular regulators involved. In particular, we discuss the possible roles of miRNAs, aptamer biology and Von Hippel-Lindau (VHL) tumor suppressor as combinatorial therapy for the eradication of cancer and the development of drugs for clinical use [1–10].

Cancer as a disorder

Cancer is a lethal disorder caused by a group of mutated cells that are able to avoid programmed cell death (apoptosis), a natural process by which aged and/or virally infected cells die [6]. Of total deaths recorded, 13%, on average world wide, are as a result of cancer. As shown in Fig. 1, cancer progression occurs via different stages, beginning with initial aggressive growth, followed by the penetration of adjacent healthy tissues and a final metastatic state, after the formation of a pathological angiogenic vasculature. The mutations and/or modifications in key regulatory biomolecules contribute to the ability of cancer cells to avoid apoptosis. This is mainly as a result of mutations in various factors involved in cell cycle arrest, such as p53, Rb and some tyrosine kinases, which are regarded as cell cycle check points.

Apoptosis: a well planned suicide

Mammalian cells undergo apoptosis by two pathways: (i) an intrinsic pathway, which is mediated by Fas ligand and/or APO-1/CD95, and several other proteins, such as Fas-associated death domain (FADD) [11] and caspases 8–10 [12]; and (ii) an extrinsic pathway, which is mediated by B-cell lymphoma 2 (Bcl-2) family proteins [10,13]. These pathways are modulated by a defined set of genes, indicating that apoptosis is a process with genetic intervention. Bcl-2 family members regulate apoptosis in response to

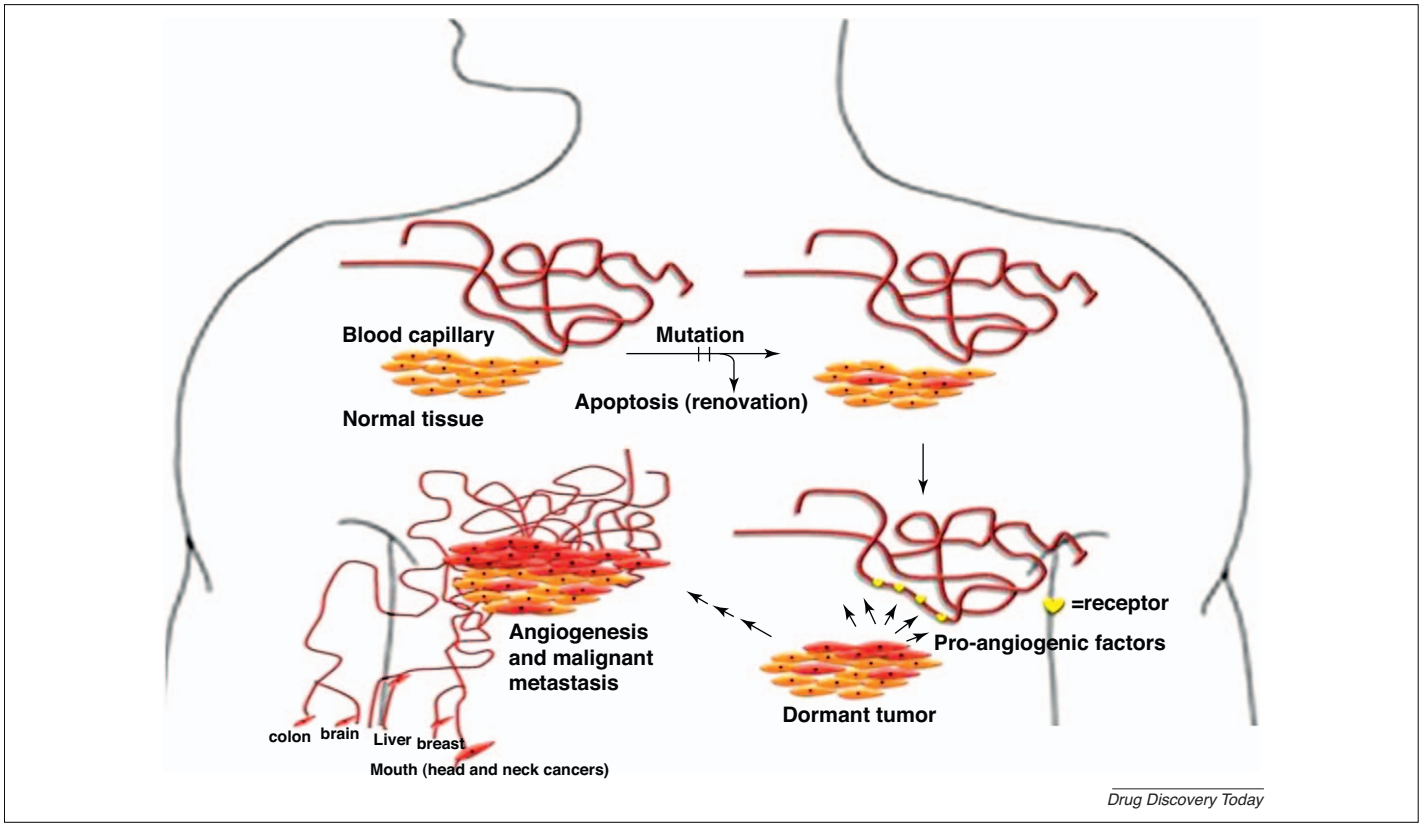


FIGURE 1

Stages in the development of malignant metastatic cancer. The development of a metastatic tumor is as complicated as the development of therapeutic treatments for cancer. Healthy cells, if they avoid apoptosis by mutation in one or other of various molecular events related to programmed cell death, form a group of cells, normally regarded as a dormant tumor. This tumor then produces various angiogenic factors and a balance between pro- and antiangiogenic factors decides the fate of tumor angiogenesis. Once blood vessel formation occurs, the tumor is regarded as malignant, and grows independently of the surrounding tissues by absorbing nutrients and oxygen required for growth. Mutated cancer cells from this tissue can travel to other healthy tissues via the established angiogenic network, before establishing themselves as solid tumors.

various death-inducing stimuli and, so far, more than 15 members of this family have been identified. Death antagonists and anti-apoptotic proteins include Bcl-2, Bcl-XL, myeloid leukemia cell differentiation protein 1 (Mcl-1), Bcl-W and A1, which provide protection, whereas death agonist members, including Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (Bad), Bcl-Xs, BH3 interacting domain death agonist (Bid), Bim, Bcl-2-interacting killer (Bik) and activator of apoptosis harakiri (Hrk), increase sensitivity to death-inducing signals. The death agonist:antagonist signal ratio determines the susceptibility of the cell to death stimuli [14] (Fig. 2).

In addition, members of the inhibitor of apoptosis protein (IAP) protein family, including c-IAP1, c-IAP2, X-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin, apollon, melanoma inhibitor of apoptosis protein (ML-IAP), livin and insulin-like peptide 2 (ILP-2) function as endogenous inhibitors of caspases. Among the IAP members, survivin and livin are highly expressed in cancer cells and transformed cells, but show little or no expression in normal differentiated tissues [15,16]. Colocalization of survivin antibodies and livin antibodies has been reported in sera of patients with breast cancer [17] and it has been demonstrated that combinational therapy using antisense survivin and B7-1 immunogene therapy eradicates EL4 thymic lymphoma tumors [18]. There are a few reports of the use of human and/or murine survivin antagonists as anticancer vaccines [19–21]. Heat shock protein 90 (HSP90) is a molecular chaperone believed to be involved in survivin regulation; interestingly, it has been shown that the HSP inhibitors geldanamycin and 17-allylamino-17-demethoxygeldanamycin (17- AAG) increase survivin expression [22], which suggests that the dual inhibition of Hsp90 and survivin is an effective anticancer therapy.

Angiogenesis and tumor progression

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels and/or endothelial progenitor cells (Fig. 3). It has implications in many physiological conditions, such as embryo development, ovulation, wound healing and some pathological conditions, such as arthritis, diabetic retinopathy and metastasis [23]. Angiogenesis can be classified into three major types, depending on the physiological processes in which it is involved: (i) *de novo* angiogenesis occurring in embryonic development and in female reproduction; (ii) degenerative angiogenesis in tissue repair; and (iii) pathological angiogenesis occurring in certain disorders, such as cancer and diabetic retinopathies. As a

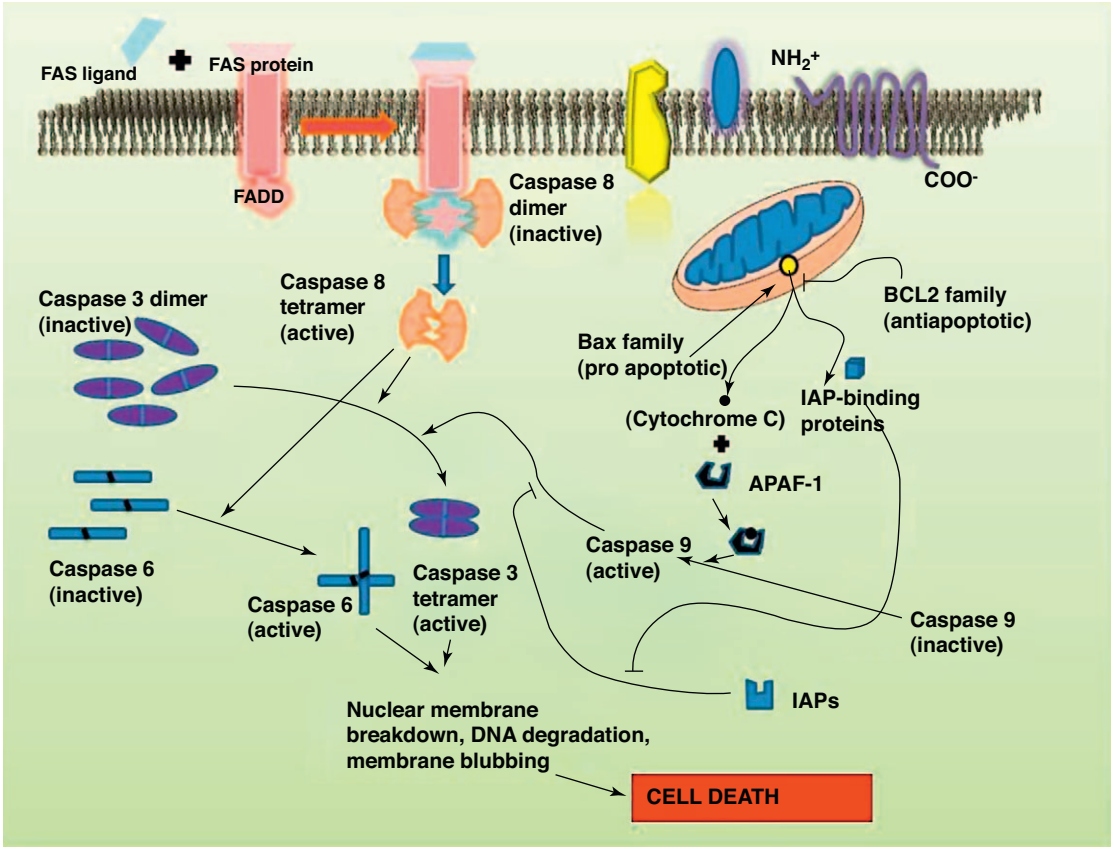


FIGURE 2

To help the organism with the overload of the cells, apoptosis can occur in ripened cells by one of the two mechanisms, as shown in the figure. The extrinsic pathway causes apoptosis by the activation of the FAS protein by specific ligands and subsequent activation of caspase 8, which in turn activates downstream caspases in a cascading manner. The intrinsic pathway occurs in response to the intracellular pathogens, and is triggered by cytochrome C, a mitochondrial protein. Various Bcl families of pro- and antiapoptotic proteins regulate this pathway. Both pathways share a common pathway at the point of caspase 3 activation and subsequent cell death. Members of the IAP family of proteins inhibit apoptosis by preventing caspase activation and/or other mechanisms.

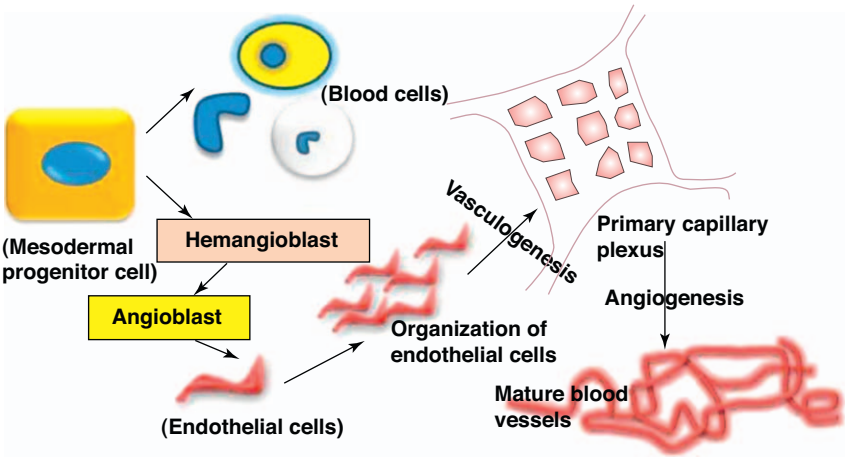


FIGURE 3

The process of angiogenesis is essential for embryogenesis; however, tumor cells misuse this process. Embryonic angiogenesis begins with formation of an angioblast from mesodermal precursor cells (yellow). Endothelial cells formed from these angioblasts subsequently form primary capillary plexus (PCP), by the process of vasculogenesis. Through the process of angiogenesis, these PCPs form mature blood vessels.

result, tumor vessels tend to break conventional rules of microcirculation, as they spread without organization and change vessel diameter, with some missing differentiation in arterioles, capillaries and venules.

Metastasis continues to be a major hurdle for the successful and complete treatment of malignant tumors. It has been observed that some human tumor lines do not form visible tumors when inoculated into immune-suppressed mice. Interestingly, when these cells are transfected with pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), dormancy was overcome by these microscopic tumors. On the basis of autopsy data, it has been estimated that more than one third of women between the age of 40 and 50 years carry *in situ* tumors in their breast tissue, but that only 1% had been diagnosed with breast cancer. A similar effect is seen in men with prostate cancer and in some individuals and patients with Down's syndrome with thyroid cancer [24]. These examples support the phenomenon of an 'angiogenic switch', which is used to denote the close relationship between angiogenesis and tumor progression [25]. It has been shown that hypoxia induces angiogenesis; the high proliferation of tumor cells creates hypoxic areas that are necessary for the induction of VEGF expression. In addition, the identification of transcription factor HIF-1 (hypoxia-inducing factor) as the upregulator of VEGF under low oxygen conditions provided further insights into the mechanisms of tumor angiogenesis [26]. Hypoxia has also been shown to stimulate other angiogenesis-supporting growth factors, such as platelet-derived growth factor (PGDF) and fibroblast growth factor (FGF) [27]. It has also been reported that development of micrometastasis into macrometastasis occurs as a result of the activation of an angiogenic switch. Embryogenic progenitor cells from bone marrow, rather than from pre-existing endothelium, were found to be the crucial regulators of this angiogenic switch. Immobilization of endothelial progenitor cells (EPCs) by blocking the expression of transcription factor id-1 leads to the inhibition of angiogenesis and further reduction in tumor progression [28]. The switch involves more than the simple upregulation of angiogenic activity and is thought to be the result of an inappropriate balance of positive and negative regulators.

Modulators of angiogenesis

Many factors are implicated in the process of angiogenesis, and are termed positive (angiogenic factors) or negative (angiostatic factors) regulators depending on their respective roles in stimulating or inhibiting angiogenesis. Integrins, VEGF, angiopoietins, FGF, transforming growth factor (TGF) and CXC chemokines are examples of angiogenic factors, whereas angiostatic factors include angiostatin, endostatin and thrombospondulin. Previous reports have shown that combinatorial therapy involving plasmids containing the gene encoding angiostatin combined with B7.1 immunogene therapy reduced solid EL4 lymphomas in syngenic C57BL/6 mice, which supports the exploration of angiostatin and/or other modulators as agents in combinatorial therapy [29]. In metastatic mice baring 4T1 breast tumors, it has also been shown that low-dose metronomic (LDM) chemotherapy downregulated the VEGF-2 receptor and upregulated antiangiogenic thrombospondin-1, which supports the application of LDM for advanced breast cancer treatment [30].

Usage of chemicals and/or fungal derivatives can be reduced and replaced with the biomacromolecules that have fewer side effects. Targeting the specific proteins expressed excessively in tumors and/or angiogenic blood vessels is the key point in developing an antitumor drug. Molecules used for this purpose can be distinguished by: (i) their capacity to discriminate between healthy and malignant forms of the proteins involved in signaling pathways; (ii) their ability to quantify the level of expression of the oncogenic forms, which applicability *in vitro* and *in vivo* and; (iii) their ability to block the activity of the oncogene product and, thus, able to be used as a therapeutic intervention. Interestingly, it has been observed that blockage of one particular growth factor results in cancerous cells adopting other pro-angiogenic factors [31]. Therefore, combinational therapy using different molecules could overcome this problem. Antiangiogenic and/or anticancer

peptides, DNA vaccines, oligomers [such as miRNAs and small interfering RNAs (siRNAs), which control the synthesis of gene products post-transcriptionally] and small oligomeric aptamers are promising agents in this regard. Here, we discuss miRNA, aptamers and other targeting agents that have attracted recent attention.

The role of miRNAs in cancer and angiogenesis

It has been speculated for some time that only 2% of the animal genome encodes functional protein-coding genes and that the remaining 98% is junk. However, recent advances have brought non-protein-coding RNAs into the spot light. miRNAs are a type of non-coding RNA involved in cell growth, regulation, differentiation and apoptosis (Fig. 4). Nearly 400 miRNAs have been identified in humans and the number is increasing. By using bioinformatics tools, it has been observed that one miRNA can recognize more than 200 messenger RNAs. Initially, miRNAs are transcribed as large precursors by RNA-polymerase II [32] and are then processed further in the nucleus by the RNAse Drosha [33,34], and subsequently by Dicer in the cytoplasm into 22 nucleotide double-strand RNA duplexes. The precursor molecules are exported to the cytoplasm from the nucleus by exportin5 in a Ran guanosine phosphate-dependent manner [35]. These duplexes, in turn, are incorporated into the RNA-induced silencing complex (RISC) complex. The RISC–miRNA complex then binds to the corresponding mRNA and represses its translation by blocking translation initiation or by inducing endonucleolytic cleavage of mRNA (Fig. 4).

Cloning of the first miRNA, lin4, was achieved by genetic analysis of the timing of development in *Caenorhabditis elegans* [36–38] and its prey, lin14 mRNA, was identified by Reinhart *et al.* while doing heterochronic analysis [39]. Recently, it has been found that carcinogenesis strongly associates with the inappropriate expression of miRNAs regulating gene expression at the translational level. On the basis of these observations alone, lin4 and let7 miRNAs are thought to be potential tumor suppressors and interest in them has increased following the finding that these molecules are conserved in mammals [40,41]. Subsequent reports show that let7 miRNA and other miRNAs belong to the same family act as tumor suppressors by targeting the 3′ untranslated region (UTR) of RAS mRNA, thereby affecting the RAS protein [42]. The first evidence for the link between miRNA and cancer came from work by Clain and co-workers, who showed that deletion in the region encoding miR-15a and miR-16-1 is the probable cause of overexpression of antiapoptotic protein Bcl-2 in many patients with chronic lymphoid leukemia (CLL), thus highlighting the tumor suppressor functions of miRNA [43]. There are also reports that an important group of miRNAs, the Let-7 family, regulate RAS and/or myelocytomatosis (MYC) oncogene expression at the translational level so that their expression is often downregulated in human lung tumors, owing to the growth repression functions

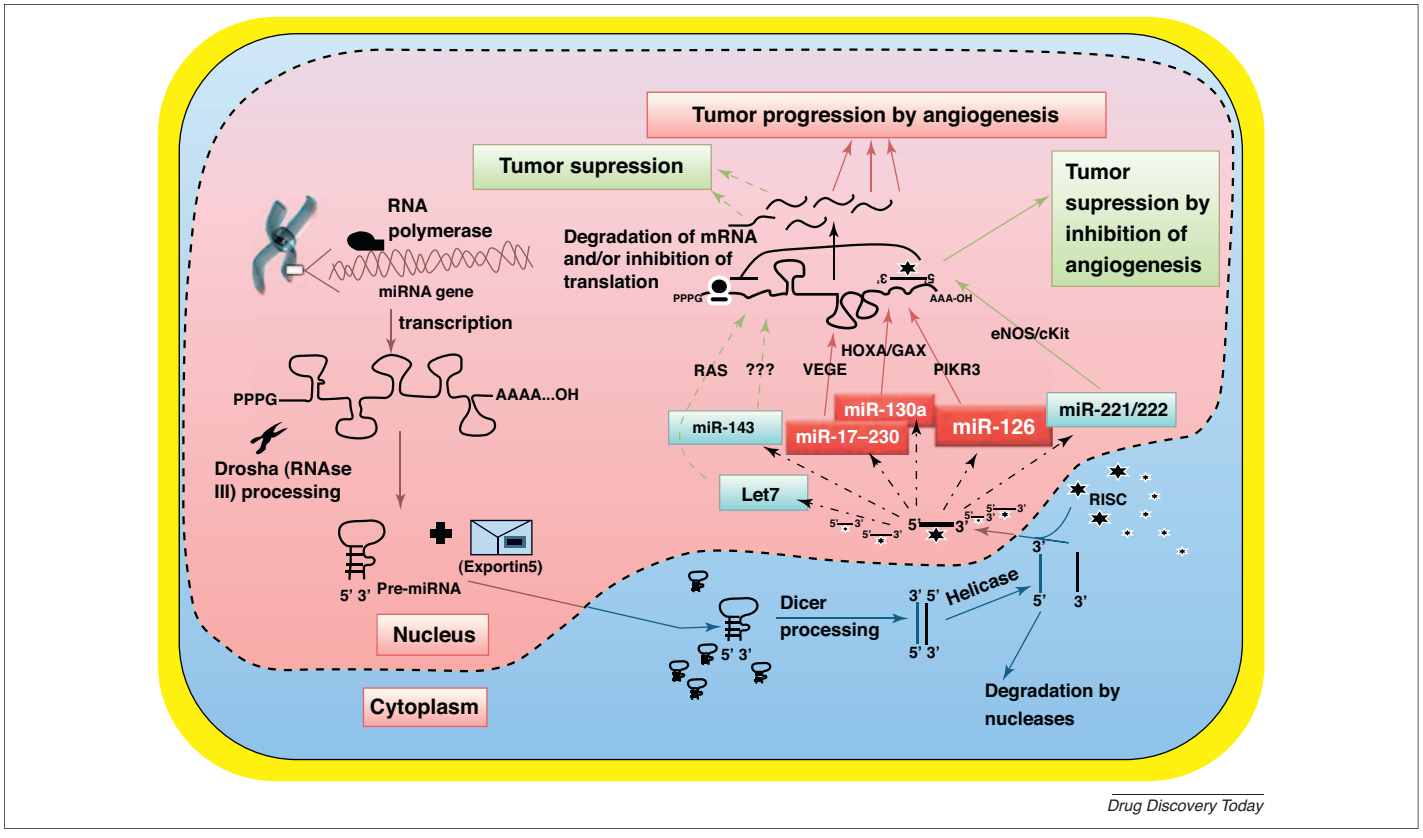


FIGURE 4

Biogenesis of miRNA, its cytoplasmic translocation and subsequent inhibition of translation. Exportin 5 (in blue), with the help of RAN Gap, helps in the export of pre-miRNAs (red), which are formed from its precursor gene by Drosha and/or Pasha processing. These pre-miRNAs, belonging to different classes of gene, are then degraded further by Dicer and helicase, before becoming mature miRNA. The RNA-induced silencing complex (RISC) can degrade the target mRNA by binding to the correct miRNA and by inhibiting translation at the 5′ end.

TABLE 1

miRNAs in cancer progression and angiogenesis

miRNA	Target mRNA	Outcome of interaction	Refs
miRNAs that are implicated in cell proliferation and apoptosis			
Let7	RAS	Downregulates RAS and leads to tumor suppression	[42,175,176]
miR15a, miR16-1	Bcl-2	Tumor suppression and downregulation observed in CLL and multiple myeloma	[45]
miR155	MYC	Upregulation of lymphoma cells	[48,49]
miR372/miR373	LATS-2	RAS-mediated cellular transformation	[57]
miRNAs that regulate angiogenesis			
miR130a	HOXA/GAX homeobox proteins	Migration, tube formation and proliferation of endothelial cells, leading to angiogenesis	[177]
miR17–miR230	HIF 1a	Promotion of VEGF-dependent angiogenesis	[57]
miR17-92	TGF, TSP1	Upregulated in MYC-induced tumors	[178]
miR221/222	eNOs/cKit	Inhibition of endothelial cell proliferation	[52]

of this family [44]. Recent evidence suggests that miR-143 and miR-145 are frequently downregulated in colorectal tumors [45]. Downregulation of these miRNAs also been noted as a common occurrence in breast carcinomas and breast tumor cell lines [46]. By contrast, miRNAs acting as oncogenes have also been identified recently, owing to speculations about their role in tumorigenesis [47,48]. miR-21 was demonstrated to be upregulated in glioblastoma [49], whereas miR-372 and miR-373 were found to block RAS-induced cellular senescence and potentiate RAS-mediated cellular transformation [50].

miRNAs have also been reported to be involved in the development and/or regulation of angiogenesis (Table 1). Hypoxia is a condition observed in cancer malformation and has implications in the formation of blood vessels. It has been shown that hypoxia upregulates VEGF [51,52], owing both to increased transcription mediated by HIF-1 and an increase in VEGF mRNA stability dependent on the 3′ region of mRNA [53]. Interestingly, a recent study [54] showed that a specific spectrum of miRNAs (including miR-23, -24, -26, -27, -104, -107, -181, -210 and -213) is induced in response to low oxygen concentrations. A similar study showed that miR-120 overexpression (induced in hypoxia) enhanced the formation of capillary-like structures and migration of endothelial cells [55]. Promotion of cell survival, tumor growth and angiogenesis by miR-378 was predicted by assaying for luciferase activity in constructs synthesized containing the 3′UTR of the possible downstream effecter molecules, SuFu and Fus-1, of miR-378 [56]. In another study, miRNA generation was impaired by silencing the molecules involved in its biogenesis (Dicer and Drosha) by siRNA; the study also found reduced angiogenesis in endothelial cells in parallel with the decreased spectrum of miRNAs [57]. miR-126 was found to regulate many aspects of endothelial cell biology [58]; for example, it regulates endothelial cells derived from mouse embryonic stem cells, by promoting VEGF signaling [59]. VHL disease is a familial cancer syndrome caused by an autosomal dominant trait, owing to mutations in the tumor suppressor gene, *VHL*. It has been observed that highly vascular tumors, especially glioma, renal cell carcinoma and pheochromocytomas, are the most common implications of this disease [60]. On the basis of this disease, it has been shown that application of VHL causes reduced tumor malignancies. Studies have reported the regression of solid tumors by using a combination therapy of VHL and antisense HIFα. It has been observed that VHL inhibits HIF by binding to 1α as well as 2α subunits [61]. Therefore, the use of VHL as an antitumor agent in combination therapy is promising. In this regard, a recent study demonstrated the downregulation of VHL and stabilization of HIF 1α by miR-92-1 in CLL B cells [62]. It would be interesting to see whether these miRNAs have any effect on regulating HIF-dependent VEGF and/or other pro-angiogenic factor genes and whether they are implicated further in the development of metastasis by supporting angiogenesis.

In summary, the identification of novel miRNAs with dual antitumor and antiangiogenic effects could lead to the discovery of potential anticancer drugs. In addition, the inhibition of miRNAs that are implicated in enhancing angiogenesis is another option. One can also use molecules such as antisense 2′-O-methyl oligoribonucleotides directed against a particular pro-angiogenic-miRNA. In relation to developing a novel and effective drug towards the prevention of cancer progression and metastasis, we discuss here biomolecules that could be used in addition to those discussed above for increased efficiency, effective targeting and/or for easy penetration of the drug into the systemic circulation.

Uses of aptamers and natural products in cancer and angiogenic therapy

Aptamers (Latin; *aptus*, to fit, *meros*, part or region) are composed of oligonucleic acid or peptide molecules that can bind to target molecules that are usually expressed on membrane surfaces. Aptamers are usually created following their selection from a large random sequence pool by using specific techniques, such as selective evaluation of ligands by exponential enrichment (SELEX), developed in the laboratory of Larry Gold at the University of Colorado [63]. Natural aptamers also exist in riboswitches. Aptamers are either DNA/RNA or protein aptamers depending on their chemical nature and can bind to a range of molecular targets, including nucleic acids, proteins, small molecules and even cells [64]. They can be used for basic research and as macromolecular drugs for clinical purposes. To be able to make these molecules cleave in the presence of their target, aptamers can be coupled with catalytic RNA (ribozymes). This gives these compound molecules additional research, industrial and clinical applications, including diagnostics, therapeutics, biosensors and tools for probing fundamental cellular processes [65]. Aptamers are more advantageous than antibodies in the sense that they are easy to synthesize in bulk, rather than using cell-based expression systems. During the phosphoramidite chemical synthesis of aptamers in the lab, fluorescent dyes or the chemical modification of functional groups can be achieved. These modifications can further help in *in vivo*

applications of the molecules bearing the aptamer or for better conjugation of the aptamer to other functional group moieties for the investigation of cellular processes [66].

Similar to other oligopeptides, their biggest limitation is their bioavailability upon oral administration. However, their biggest advantage is their low immunogenicity. Currently, the therapeutic application of these molecules is still in its infancy; however, one aptameric drug has been approved by the FDA. Pegaptanib is an RNA aptamer directed against VEGF and has been implicated in the treatment of all types of neovascular age-related ocular vascular diseases [67]. In one study using A5399 cells, the adenoviral system was used to deliver an RNA aptamer (AP50) against nuclear factor (NF)-κB, to overcome non-small cell lung cancer tumor resistance to doxorubicin [68]. However, using a viral system for drug delivery has its own side effects. More recently, PEGylated, angiopoietin-2-inhibiting RNA aptamers were shown to inhibit tumor angiogenesis and growth, by inhibiting Tie-2 phosphorylation [69].

Natural anticancer molecules

Several natural anticancer compounds, including curcumin, capsaicin and neem extracts, have been studied for their non-toxicity and specific effects on cancer cells. More recently, the antiangiogenic and anticancer effects of matrine, a traditional Chinese herb, have been shown on primary and metastatic breast cancer cell lines [70]. Another natural milk-based protein, lactoferrin, which is secretory protein, grabbed the attention of the scientific world. In addition to its main function (i.e. iron absorption in the intestine), lactoferrin also has a role in protection against infections, myelopoisis and autoimmunity. Previous reports have shown that 100% iron saturated bovine lactoferrin (FebLf) augments the antitumor cytotoxicity of certain chemotherapeutics in range of tumors [71,72] by increasing cytokines produced by Th1 family, which includes interleukin (IL)-18, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, as well as nitric oxide (NO), which have all been reported to sensitize tumors to chemotherapy. Increased cytokine production helps in the infiltration of cytotoxic T cells (CTL), CD4+, CD8+, natural killer (NK) and natural killer T (NKT) cells into the tumor tissue. Thus, using FebLf, along with a drug formulation, can enhance uptake by the systemic circulation and reduce tumor angiogenesis, blood flow and increase tumor apoptosis. FebLf has also been shown to inhibit tumor growth by enhancing antitumor immunity, via its ability to generate antitumor cytotoxic T-lymphocytes (CTLs) thereby enhancing leukocyte infiltration (CD4+, CD8+, IFN-γ, NK and dendritic cells) to tumors.

RNAi for cancer therapy

Double-stranded RNA (dsRNA) containing a sequence homologous to a specific gene causes sequence-specific gene silencing, which is termed 'RNA interference' (RNAi). RNAi was first discovered in *C. elegans*, an organism in which gene expression is downregulated by long dsRNA [73]. Remarkably, the basic molecular mechanism of RNAi is conserved in mammalian cells, and its applicability to mammalian systems was recently discovered using short dsRNAs (19–23 bp; siRNAs) and short hairpin RNAs (shRNAs) [74–76]. The mechanism of RNAi is crucial in protecting the host from various challenges, including viral and/or foreign gene replication [77]. RNAi-mediated gene silencing is now an essential strategy in analyzing gene functions owing to its high specificity

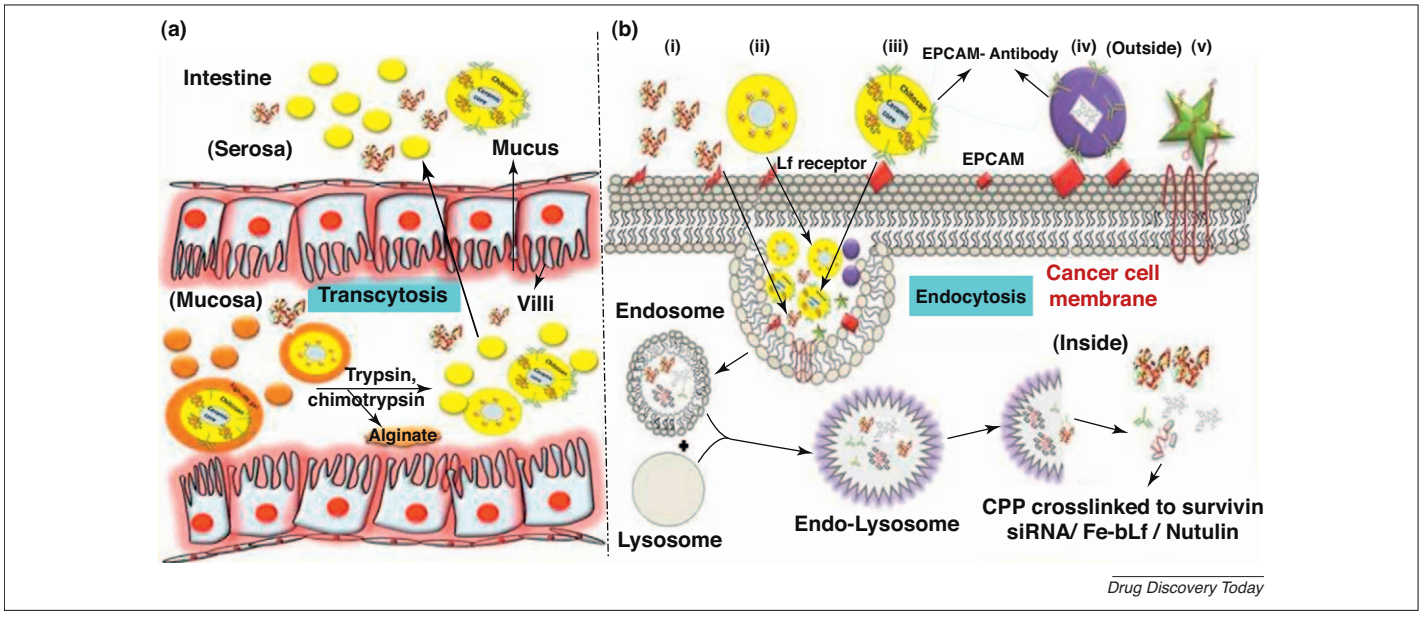


FIGURE 5

Diagrammatic representation of the transcytosis and absorption of orally delivered FebLf [72], ACSC–FebLf nanoparticles [162] and EpCAM-targeted CPP–siRNA–ACSC nanoparticles [171,173] **(a)**. **(b)** The internalization into cancer cells of: (i) FebLf; (ii) FebLf-loaded ACSC nanoparticles; (iii) CPP-linked survivin siRNA, oncogenic antisense miR-27a loaded on to ACSC nanoparticles; along with intratumorally delivered nutulin-loaded EpCAM nanoparticles (iv) [167] and aptamer-loaded nanoparticles (v) [191]. Here, ACSCs are shown as being protected from various gastric enzymes *in vitro* and *in vivo*, based on surface modification by chitosan (yellow) and alginate (orange). After transcytosis from the gut cells and subsequent endocytosis into the tumor tissue, anticancer biomacromolecules embedded in the nanocore silence key cell cycle regulators, thereby eliminating the tumor.

and efficiency. In addition, it offers one of the most attractive methods for gene therapy for many diseases, including viral infectious diseases and cancers [78,79]. Many types of disease have been demonstrated to be potential targets for RNAi-based therapy in laboratory experiments.

Gene delivery to target cells

The RNAi-mediated gene-silencing effect is limited in cells reached by RNAi effectors, which makes the delivery of RNAi effectors to target cells important in achieving successful RNAi-based therapeutic treatment. Many *in vivo* delivery methods of RNAi effectors have been developed, including those developed and optimized for the delivery of plasmid DNA (pDNA) for gene therapy for inhibiting angiogenesis and/or immunomodulation by co-stimulatory pDNA immunogene therapy [80–85]. Early studies on *in vivo* RNAi often used the expression of reporter genes that were expressed from vectors co-administered with RNAi effectors [86–88]. Similarly, suppression of tumor cell growth by efficient RNAi induction in tumor tissue by intratumoral injection has also been studied [89]. By contrast, only a few studies have reported the successful induction of gene silencing in target cells after systemic administration of shRNA-expressing pDNA. ShRNA-expressing plasmids, which are encapsulated in the interior of 85 nm PEGylated immunoliposomes (PILs), can suppress gene expression in tumor cells intracranially inoculated into the brain [90,91]. Among the non-viral carriers are cell-penetrating peptides (CPPs), which have the ability to enter cells by crossing the plasma membrane directly or through uptake by the endocytotic pathway [92]. We [93] and many other research groups have demonstrated the effective use of CPP [92,94–96]. Here, we summarize CPP-based RNA delivery strategies, and focus on recent improvements that enhance the release of siRNAs trapped in endosomes into the cytosol.

Aptamer–siRNA chimeras

Bioconjugated nanoparticles are important analytical tools with emerging biological and medical applications. Aptamer-conjugated siRNA chimeras are the recent addition to the growing list of anticancer agents. This promising technology offers the advantage of high-affinity binding onto the cell surface receptors based on the aptamer–ligand conjugates [97–99]. Prostate-specific membrane antigen (PSMA) has been exploited for treatment using PSMA-binding aptamers [100,101], RNAse-resistant 2′fluoro anti-PSMA aptamer tailored with polo-like kinase 1 (PLK-1) and Bcl-2 specific siRNAs. Intratumoral delivery, as well as the *in vitro* data, suggests a decrease in the transcript level of PLK-1 and Bcl-2 [102]. A non-covalent conjugation of glyceraldehyde phosphate dehydrogenase (GAPDH) siRNA with an anti-PSMA aptamer by using avido-biotin cross linkage with a reducible disulfide linker in between was used. siRNA-mediated inhibition of gene expression was reported after internalization of the chimeric siRNA within 30 min [103]. In another study, it was found that bivalent PSMA aptamer Ef2 siRNA chimeras resulted in increased and more-efficient PSMA cell death compared with monovalent chimeras [104]. A chimeric design was tailored by the addition of truncated a PSMA-specific A10PLK1 aptamer (39 nt instead of 71 nt) to a siRNA [105]. These chimeric molecules modified by additional nucleotides at the 3′ end, along with passenger and guide strand transactions in the siRNA domain of the chimera, favored dicer processing and thus increased silencing activity and specificity. *In vivo* silencing was prolonged by appending a PEG moiety, which even decreased the level of therapeutic dosage. More recently, combination of aptamer–siRNA chimeras with zoledronic acid [106] and tumor-targeted delivery of nonsense-mediated decay (NMD) factor-specific chimeric siRNAs [107] were reported. These results suggest the potential of aptamer–siRNA chimeras in cancer therapy.

In summary, aptamers that can bind to specific markers on tumor vasculature can be used along with certain tumor, HIF and/or angiogenic inhibitors to deliver them to the specified target. However, aptamers are little used in the treatment of tumor angiogenesis because of their low bioavailability, although they have the same action in retinal angiogenesis. One can imagine a wonder drug based on antitumor peptides and inhibitors of pro-angiogenic factors that could be delivered orally, along with ligands, such as aptamers, VHL and lactoferrin, incorporated in nanoparticles to protect them from challenges in the milieu interior [108].

CPP-mediated RNA delivery into mammalian cells

CPPs, also referred to as protein transduction domains (PTD), were first discovered when the HIV-1 Tat-protein, which transactivates transcription of the HIV-1 genome, was observed to cross the plasma membrane by itself [109,110]. The subsequently identified peptide fragment (49–59 amino acids) that confers cell permeability to the protein is called the Tat-peptide, and is one of the better characterized CPPs [111,112]. A variety of CPPs derived from natural protein sequences have since been discovered [113–115]. One example of a non-viral CPP (Penetratin™ from *Drosophila* antennapedia homeodomain protein) is a sequence of 16 amino acids [116]. Most of these CPPs contain a high density of basic amino acids (arginines and/or lysines), which are proposed to interact with the anionic surface of the plasma membrane and enhance internalization of the peptides. The CPPs are efficiently internalized by cells both *in vitro* and *in vivo*, irrespective of whether the cells are proliferating or non-proliferating [117–120]. In addition, CPPs provide cellular internalization not only to fused proteins, but also to non-covalently conjugated cargo molecules [121,122]. Tat-peptide, along with an oligoarginine (8–11mer) peptide [123,124] and penetratin [116], has been utilized for cargo delivery. Cytotoxicity with CPPs is either low or undetectable, within the CPP concentration range used for molecular delivery. CPPs for RNA delivery are used primarily in the following systems.

CPP covalently attached to RNA

If the cargo molecules are polypeptides or peptide analogs, such as peptide nucleic acids (PNAs), CPPs can be covalently linked by gene recombination or solid phase peptide synthesis. By contrast, if the cargos are non-peptide molecules, such as inorganic molecules or nucleic acids, chemical reactions must be used for covalent attachment of the CPPs to the cargo molecule. Covalent attachment of a CPP to a cargo molecule confers its permeability. Covalent attachment of CPPs to siRNAs has also been attempted (Table 2) [125–129]. There were concerns regarding the specificity of siRNA bound covalently to CPPs, which were addressed

TABLE 2

CPP-mediated RNA delivery into mammalian cells

Interaction	CPP	Target	Sequence	RNA concentration	Evaluation	RNAi efficiency (%)	Refs
Examples of siRNA delivery by covalent CPP–siRNA conjugates							
Covalent	Penetratin	SOD1 in neuron cell	RQIKIWFQNRRMKWKK	8 nM	mRNA	85	[126]
		Luciferase in CHO cells	CRQIKIWFQNRRMKWKK	25 nM	Luminescence	53	[127]
	Tat(48–60)	p38 in L929 cells	GRKKRRQRRRPPQ	10 mM	mRNA	40	[128]
	Tat(47–57)	EGFP in HeLa cells	YGRKKRRQRRR	200 nM	Fluorescence	70	[125]
	Transportan	Luciferase in CHO cells	CLIKKALAALAKLNIKLLYGASNLTWG	25 nM	Luminescence	63	[127]
	Tat–LK15	*Bcr-abl* gene in K562 cells	YIGSRKKRRQRRRGGG	3.5 mM	mRNA, protein and fluorescence	90	[179]
Examples of siRNA delivery by non-covalent CPP–siRNA conjugates							
Nonspecific	Polyarginine (R9)	EGFP in GC cells	RRRRRRRRR	200 nM	Fluorescence	43	[135]
	MPG	Luciferase in HeLa cells	GALFLGFLGAAGSTMGAWSQPKKKRKV	50 nM	Luminescence	78	[136]
		GAPDH in HS-68 cells	GALFLGFLGAAGSTMGAWSQPKKKRKV	100 nM	Western blot	60	[136]
	MPGΔNLS	Luciferase in HeLa cells	GALFLGFLGAAGSTMGAWSQPKSKRKV	50 nM	Luminescence	90	[136]
		GAPDH in HS-68 cells	GALFLGFLGAAGSTMGAWSQPKSKRKV	25 nM	Western blot	80	[136]
	MPGα	Luciferase in ECV304 cells	GALFLAFLAAALSLMGLWSQPKKKRKV	25 nM	Luminescence	75	[134]
	Penetratin	Luciferase in HeLa cell	RQIKIWFQNRRMKWKK	100 nM	Luminescence	0	[139]
	HA2-penetratin	Luciferase in HeLa cell	GLFGAIAGFIENGWEGMIDGRQIKI-WFQNRRMKWKK	100 nM	Luminescence	35	[139]
	Bovine PrP	Luciferase in HeLa cells	MVKSKIGSWILVLFVAMWSDVGLCKKRPKP	100 nM	Luminescence	48	[139]
	MPGΔNLS	Luciferase in HeLa cells	GALFLGFLGAAGSTMGAPKSKRKV	100 nM	Luminescence	55	[139]
	TP-10	Luciferase in HeLa cells	AGYLLGKINLKALAALAKKIL	100 nM	Luminescence	18	[139]
	EB1	Luciferase in HeLa cells	LIRLWSHLIHIWFQNRRLKWKKK	100 nM	Luminescence	55	[139]
	Fusogenic peptide	GFP in MDA-MB-435 cells	WEAKAKA LAKALAKHLAKALAKALKAC EA	0.72 mM	mRNA	74.9	[180]
Specific	Tat(47–57)-U1A-Alexa546	EGFP in CHO cells	YGRKKRRQRRR	200 nM	Fluorescence	65	[181]
	Polyarginine	HER-2 in COS-7 cells	R15	7 mM	Fluorescence and luminescence	50	[182]
	Stearoyl carrier peptides	FAM and VEGF in S-180 cells	CH2R4H2C, GH2R4H2G, CH2R4H2C	13 mM	Fluorescence and ELISA	80	[183]
	Arginine-grafted peptide	VEGF in PC3 cells	CBA-DAH-R	0.1 μM	Fluorescence	88.2	[184]

[130,131] in that various kinds of CPP–siRNA conjugates were prepared and purified by HPLC, and their RNAi effects measured. The results showed that moderate suppression of p38 (20–60%) is observed at a concentration of 10 μM CPP–siRNA, which is almost two orders of magnitude higher than previous reports [130,131]. Additionally, the site of attachment of the CPP to the siRNA affected the RNAi efficiency [128]. Thus, the CPP should be covalently attached to the appropriate site in the siRNA (the 3′-end of the sense strand) to generate an effective CPP–siRNA complex and the resultant CPP–siRNA should be purified to exclude the effects of free CPP [128].

Non-covalent CPP–RNA complexes

Some CPPs used as molecular delivery carriers interact with their molecular cargos through non-covalent interactions. Protein delivery by this mechanism was reported before the approach was used for RNA delivery. Pep-1 has been used for protein delivery, especially for intracellular antibody delivery, using a tryptophan-rich hydrophobic region [132,133]. Formation of CPP–cargo complexes by these nonspecific interactions is a simple and easy approach that provides cell permeability to macromolecules (Table 2). siRNAs have a negatively charged backbone; therefore, positively charged CPPs can bind to the siRNAs through nonspecific electrostatic interactions. In the presence of excess CPP, CPP–siRNA macrocomplexes are formed and provide cell permeability by covering the siRNA surface with positive charges from the CPP [131,134–140]. It has been demonstrated by simple administration of the complex to cell cultures that the *N*-methylpurine DNA glycosylase (MPG) peptide–siRNA complex efficiently suppressed gene expression [136,141].

Sequence-specific binding of CPP-fused RNA-binding protein to RNA

To deliver nonpeptide cargo molecules using CPPs, specific biomolecular interactions, such as biotin–streptavidin or antibody–protein A binding, have occasionally been used instead of covalent attachment [142–146]. The advantage of this strategy is the specific molecular delivery without covalent linkage. For siRNA delivery, this strategy can be employed by using a sequence-specific RNA-binding protein (RBP).

Advantages of delivering antiangiogenic and/or anticancer molecules with nanoparticles

Many delivery routes have been formulated for drug administration to be able to attack different diseases [108]. Several parenteral and non-parenteral administration routes for drug delivery have

been developed and devised previously for therapy [147]. However, complications, such as thrombophlebitis or tissue necrosis and poor patient compliance, have stimulated the investigation of non-parenteral routes [148]. Among such routes, oral administration is usually preferred because it is non-invasive, most acceptable and convenient for the patient and requires no skilled worker for drug injection. However, the oral bioavailability of these prodrugs or enzymes and peptides, as well as of protein drugs, is generally low, owing to the acidic conditions of the stomach, proteolytic activity of the gastrointestinal enzymes present in the intestinal tract, and poor permeability across the intestinal mucosa [149]. Various approaches have been proposed to overcome the biopharmaceutical limitations associated with these drugs, such as inhibition of enzymatic degradation [150], chemical modifications [151], an *in situ* gel system [152] and the formulation of polymer- or microsphere-based carrier systems [153]. Application of nanocarriers for drug delivery, especially RNA, pDNA, prodrugs and bioactive drugs, is an expanding area of research that has designed biomaterials with controlled rates of drug release [154]. Recent advances in chemotherapy involve the use of nanomaterials with a broad range of chemistry, including PEGylated, hydrophobically, glycol chitosan moieties and other poly (esters). These nanowonders are reported to protect the drug from the extremities of pH and enzymatic degradation. Drug delivery is one of the promising biomedical applications of nanotechnology. Several nano-based cancer drugs, for example Doxil® and Abraxane, are in clinical trials to assess their use in cancer chemotherapy. Ideally speaking, a suitable nanocarrier would have minimal side effects and greater hangover time in the blood. Nanocarriers made up of two or more different polymers can spontaneously assume various shapes in specific solvents and bring several advantages to the nanocore-like size, stability, drug loading and release efficiencies.

To develop an oral administrative, careful design of the core and exterior coat is required. Recent developments in bone marrow tissue engineering, dentistry, orthopedics and plastic surgery are making use of these kinds of composite on a macroscale; utilizing the same principle on a nanoscale will ensure more promising drug development [147]. Ceramic nanoparticles are being designed that have improved protein adsorption and which have various layers of biodegradable polymers coated on their surface both to protect them from lytic or gastrointestinal enzymes and to obtain sustained release kinetics of the loaded drug. Ceramics are solid compounds that are formed by a chemical reaction between a metal and a nonmetallic elemental solid or between a nonmetal and nonmetallic elemental solids [155–162]. Some of the ceramic material that are being used for nanomedicine include: (i) calcium phosphate ceramics. Various forms of calcium phosphate ceramics have been in use for the past three decades, including hydroxyapatite, beta-tricalcium phosphate, biphasic calcium phosphate amorphous calcium phosphate, carbonated appetite and calcium-deficient hydroxyapatite [162]; (ii) calcium phosphate cements. These consist of a powder phase of calcium and/or phosphate salts together with an aqueous phase that react at room or body temperature and form a calcium phosphate crystal that sets by entanglement of crystals; (iii) bioactive glass. This is produced in the same way as conventional glass, in which basic components are SiO_2, Na_2O, CaO and P_2O_5. It is commercially available as Bioglass®.

Characteristics of these ceramic materials include high mechanical strength, good body response and strong binding ability with proteins, which helps the sustained release of the drug. However, they are less biodegradable, which might be a drawback, although one that could be overcome by adding biopolymers, such as chitosan. This not only improves their biodegradability, but also increases their tolerance towards variations in pH and protects against enzyme action in the gut. Polymers widely used include: (i) polylactic acid (PLA) and/or polyglycolic acid (PGA) polymers, such as PLA, PLLA and PDLLA, poly (lactic-co-glycolic acid) (PLGA) and polycaprolactones; (ii) natural proteins, such as collagen, gelatin, fibrin and casein; and (iii) carbohydrates and their derivatives, such as chitin, chitosan, alginate, cellulose, starch, hyaluronan and amylopectins.

Uses of nanoparticles in cancer treatment

Blood vessels in tumor vasculature are found to be leaky and have pores in the range of 10s and 100s of nanometers [147]. This fact can be exploited for drug delivery to the tumor site, using nanocarriers with a diameter of 1–100 nm, a process called enhanced permeability retention (EPR) effect [163,164]. Currently, natural and synthetic polymers are used as drug delivery vectors, and some are detailed in Table 3. Other nanocarriers used with polymer conjugates include polymeric nanoparticles, lipid-based carriers, (such as liposomes and micelles), dendrimers, carbon nanotubules and inorganic nanoparticles. There has been growing interest in the development of biodegradable, polymeric nanoparticles for the diagnosis and treatment of cancer. As mentioned above, there is significant progressing being made in the development of anticancer biomolecules targeted with nanocarriers, especially polymeric nanoparticles and dendrimers. Recently, some formulations have been tested by using chemotherapeutic drugs, along with polymeric nanoparticles (Table 4). A formulation with two distinct layers of biodegradable polymer with an inner nucleus containing an antitumor agent (doxorubicin) and a PEGylated outer envelope with an antiangiogenic agent has been successfully tested [165]. In another study, multidrug resistance (MDR) in breast cancer cell lines was overcome by poly(ethylene oxide) (PEO)–poly-ε-caprolactone (PCL) nanoparticles that co-delivered paciltaxel and ceramide to the tumor site [166]. The anticancer drug, nutlin-3a, has been actively targeted using epithelial cell adhesion molecule (EpCAM) antibodies, loaded on to PLGA, and has been tested successfully [167]. Lodamin is the mPEG-PLA modification of TNP-470, using methylated PEG and PLA, which has fewer side effects compared with its counterpart, TNP and which was tested successfully in melanoma and Lewis lung carcinoma (LLC) cell lines [155]. It was reported to be a promising oral administrative for antiangiogenic therapy. In addition, there are some polymer conjugates in clinical use as cancer treatments, such as Zinostatin Stimalmer®, Oncaspar® and Neulasta® for cancer treatment [157].

Recently, Saraf *et al.* [160,161] developed nanoparticles that can be used for oral delivery of peptides. However, to the best of our knowledge, these alginate-coated nanocarriers (ACNC) have not yet been used for pDNA or RNAi delivery for gene targeting in any disease *in vitro* or *in vivo* in either human or animal systems. It is tempting to expand the utility of nanoparticles for the delivery of therapeutic enzymes, because particles larger than 20 μm are prone to be washed out and are therefore inefficient for mucosal

TABLE 3

Different types of nanocarrier

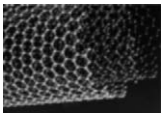
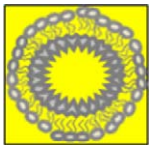
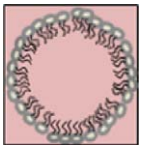
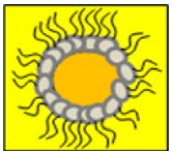
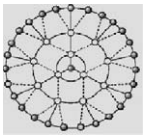
Name	Structure	Composition	Salient futures and uses	Refs
Carbon nanotubule		Composed of fullerenes (C60 compounds) and/or carbon-based hollow cage-like structures	Increased internal volume and ease of functional modification of internal and external surfaces. Single-walled nanotubes are used for drug and gene delivery. Double-walled nanoparticles have better implications for transfection	[158,159]
Liposome		Amphipathic lipids	SUVs composed of single lipid bilayer, where as LUVs consist of multiple layers. They alter the pharmacokinetic profile of loaded drug molecules	[160]
Solid lipid nanoparticle		Amphipathic lipids	Comprise a hydrophobic core (50–100 nm in diameter); they can be used as an effective adjuvant for vaccines and as non-viral transfection agents	[156]
Polymeric nanoparticle		Biodegradable and biocompatible polymers	Nanocapsules entrap the drug and nanospheres can be used for coating the drug on their surfaces. They enable greater control of the pharmacokinetic behavior of the loaded drug, leading to more appropriate steady levels of the drug	[161,162]
Polymeric micelles		Amphipathic lipids	They are formed in hydrophobic environments and are suitable for the delivery of water-insoluble drugs because of their core shell structure	[185]
Dendrimer		AB_n type monomers	They offer several advantages, such as nanometer size range, ease of modification and of preparation. They can be used as coating agents for the protection and delivery of drugs to various sites	[186]
Functionalized nanoparticle		Inorganic metals, such as Pt, Pd, Au and Ag	Fluorescent properties can be incorporated to be used as biosensors. They can also be used as markers for research in molecular biology, targeted drug delivery and biosensing	[187]

TABLE 4

Examples of polymeric nanodrug formulations tested against cancer angiogenesis

S/no. and name	Formulation	Outcome	Cell lines tested	Refs
Nanocell	Nucleus with doxorubicin and covered by a pegylated lipid envelope	Chemotherapeutic agent in the nucleus disrupts the solid tumor and the anti-angiogenic agent disrupts tumor vasculature	Melanoma and LLC	[165]
PEO–PCL nanoparticles	Tamoxifen and paciltaxel encapsulated with polyethylene oxide-modified poly(ε-caprolactone)	Combination therapy of tamoxifen and paciltaxel nanoparticles can overcome multidrug resistance	Breast cancer	[166]
Folate–SPIO–DOX micelles	Doxorubicin, Fe_2O_3 in a co-polymer of PEG and poly(ε-caprolactone) with folate as targeting ligand	Used for both diagnostic and therapeutic purposes, as Fe_2O_3 can be detected with MRI	Hepatic carcinoma	[188]
Lodamin	TNP-470 with an mPEG–PLA co-polymer	Tumor vasculature is reduced with fewer side-effects of TNP-470	Melanoma and LLC	[155]
PEC micelles	VEGF siRNA with polyethylene glycol and polyethyleneimine	Inhibition of VEGF-mediated angiogenesis	Prostrate carcinoma cell lines	[189]
PECM	SiRNA–PEG–fusogenic peptide (KALA)	VEGF gene silencing	Prostrate carcinoma cell lines	[190]

delivery [168] and for the maintenance of the native structures [169]. In this regard, innovative techniques have led to the use of ceramics in high-tech applications such as the delivery of chemicals and biologicals effectively *in vitro* and *in vivo* [170].

We recently used for the first time the covalently crosslinked CPP (R9 and Tat peptides) complexed with siRNA to survivin, an inhibitor of apoptosis that is overexpressed in tumors and inflammation (unpublished observations). We designed covalently crosslinked CPP (R9-siRNA to survivin and Tat peptide-siRNA to survivin peptides) complexed with siRNA to survivin for designing ACNC for effective loading and protection of active acid-labile and alkaline-labile large nanocarriers for oral administration. We tested these loaded targeted nanoparticles in human colon cancer cells (Caco-2 and HT-29) in transwell assays. As an attempt to increase the bioavailability, sustained release, mucoadhesiveness and conformational stability, we loaded covalently complexed CPP with survivin siRNA into ACNC nanocarriers [162]. Prolonged activity was obtained owing to the slow release of the protein or RNA from the ACNC and the intact structure without denaturation or dehydration during delivery and storage. The encouraging results obtained in this study suggest that ACNC should be included in future *in vivo* studies, especially in the delivery of RNA, DNA, proteins, peptides and drugs. These novel nanocarriers were also found to be promising in the protection of the spatial qualities needed for exhibiting improved therapeutic effects. However, further studies in terms of their pharmacokinetics and toxicology, as well as animal studies, are required for clinical utility of the formulation. More recently, we were able to load cell-permeable dominant-negative survivin R9 (DNSurR9) [85,171–174] and survivin and HSP-90 antagonists, 'shepherdin' on alginate gel-encapsulated, chitosan ceramic nanocore nanocarriers (ACNC-NPs) and could induce apoptosis and disintegrate the mitochondria of colon and breast cancer cell lines (but not normal control cells) more efficiently in *in vitro* cell-based assays. In this study, we loaded non-covalently crosslinked CPP (R9-siRNA to survivin and Tat peptide-siRNA to survivin peptides) complexed with siRNA to survivin and oncogenic antisense miRNA-27a (as-miR-27a) on ACNC-NPs and transferred them to human breast cancer MDA-MB-231 and MCF-7 cell lines by endocytosis (Fig. 5). Our results show that both R9 and Tat CPP peptides covalently complexed with as-miR-27a-loaded ACNC-NPs exhibit oncogenic activity. Suppression of miR-27a inhibits breast cancer cell growth and invasion. Simultaneous covalently complexed CPP (R9 and Tat-peptide) with siRNA to survivin and oncogenic as-miR-27a-loaded ACNC-NPs results in a faster and more efficient decrease in the expression of genes that are important for cell survival and angiogenesis faster than compared with monotherapy. In addition, these responses were accompanied by decreased expression of the genes encoding survivin and molecules involved in angiogenesis, including survivin isoforms, VEGF and VEGF receptor 1 (VEGFR1). We also demonstrated the downregulation of survivin expression in western blot in the covalently complexed CPP (R9 and Tat-peptide) with siRNA to survivin and oncogenic as-miR-27a-loaded ACNC-NP-treated cells. A TUNEL assay, caspase activity assay and changes in mitochondrial membrane potential showed that cell death was mainly through the intrinsic apoptosis pathway. Oral delivery of siRNA to survivin and oncogenic as-miR-27a-loaded ACNC-NPs induced apoptosis, necrosis and cytotoxicity in a xenograft breast cancer model. Oral administration of covalently complexed CPP (R9 and Tat-peptide) with siRNA to survivin and oncogenic as-miR-27a ACNC-NPs in combination regressed tumor growth faster and inhibited angiogenesis in the xenograft breast cancer mouse model as compared with monotherapy [171]. We also compared our results with doxorubicin- and taxol-loaded ACNC-NPs. Taken together, our results are encouraging for the development of combination nanotherapeutic strategies that combine gene silencing and drug delivery to provide more potent and targeted therapeutic, especially in late and metastatic cancers.

Outlook and conclusions

The ability to target functionally biorelevant molecular signals precisely and differentially in cancers will establish a new paradigm in cancer management; one that focuses on defining the uniqueness of each tumor and tumor-host processes and interactions following rational target prioritization using computational systems biology algorithms. This, then, would allow for the exploitation of the 'attack vulnerability' of the rewired cancer network by deconstructing essential hubs and linkages, multiply targeting and eliminating them. Anticancer protein, miRNA, siRNA and shRNA effectors are attractive opportunities. The ability to potentiate activity using a bifunctional design might further enhance the safety and efficacy of such effectors. Although shRNA seems ideal for cancer-related therapeutic development, new technologies, such as bifunctional RNAi, might provide an even greater opportunity for potency enhancement and heightening safety, thereby increasing the opportunities for multiple target therapy. This, of course, is contingent on the optimization of delivery and minimization of off-target effects, which will need to be established through early clinical testing. RNAi has rapidly become established as an experimental tool and is expected to be used as a therapeutic treatment for various diseases in addition to cancer. As well as the biomolecules mentioned above, shRNA-expressing pDNA is also a promising candidate for RNAi-based therapeutic treatment. As shRNA-expressing pDNA and biomolecular therapy have advantages and disadvantages, they should be chosen on a case-by-case basis.

There are still difficulties in the successful therapeutic application of RNAi. However, considering the pace of new findings and developments in the application of these factors, we believe that these problems will be solved and that RNAi and/or anticancer peptides will become a major therapeutic treatment in the near future. RNA delivery for RNAi-mediated gene silencing is a comparatively new area of CPP-mediated molecular delivery. Endosomal entrapment of RNAs delivered by CPPs is a problem that must be overcome for practical CPP-based cellular RNA delivery. A few groups have addressed this problem by using endosomolytic peptides and reagents, as well as photoinduced endosomal escape strategies. In addition, strategies targeting the CPP–cargo complex to a specific organ, to cancer, or to virus-infected cells, will be necessary for therapeutic applications. Recently, there has been a great advance towards therapeutic applications, in particular through the use of non-covalent strategies to form cargo–CPP complexes. The photoinduced RNAi strategy might also be useful as a targeting strategy for therapeutic applications. As RNAi is one of the most promising strategies for gene therapy, further advances in CPP-based RNA delivery with nanoparticles are expected in the near future.

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