Cell-penetrating properties of the transactivator of transcription and polyarginine (R9) peptides, their conjugative effect on nanoparticles and the prospect of conjugation with arsenic trioxide

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Cell-penetrating peptides (CPPs) are short chains of amino acids with the distinct ability to cross cell plasma membranes. They are usually between seven and 30 residues in length. The mechanism of action is still a highly debated subject among researchers; it seems that a commonality between all CPPs is the presence of positively charged residues within the amino acid chain. Polyarginine and the transactivator of transcription peptide are two widely used CPPs. One distinct application of these CPPs is the ability to further enhance the therapeutic properties of a range of different agents. One group of agents of particular importance are nanoparticles (NPs). Most NPs have no mechanism for cellular uptake. Hence, by conjugating CPPs to NPs, the amount of NPs taken up by cells can be increased, and therefore, the therapeutic benefits can be maximized. Some examples of this will be explored further in this review. In addition to CPPs, the concept of conjugation with the anticancer drug arsenic trioxide is reviewed and the prospect of transactivator of transcription-conjugated arsenic trioxide albumin microspheres is also discussed. Recent locked nucleic acid technology to stabilize nucleotides (RNA or DNA) aptamer complexes able to target cancer cells more specifically and selectively to kill tumour cells and spare normal body cells. NPs tagged with modified locked nucleic acid-aptamers have the potential to kill cancer cells more specifically and effectively while sparing normal cells. *Anti-Cancer Drugs* 23:471–482 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **23**:471–482

Keywords: aptamers, arsenic trioxide, cancer, cell-penetrating peptides, nanomedicine, nanoparticles, polyarginine peptides, transactivator of transcription peptides

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Received 2 September 2011 **Revised form accepted** 14 December 2011

Introduction

The cell membrane represents a cell's primary defence mechanism, demonstrating its ability to avoid the uptake of foreign and unwanted materials [1]. It is this mechanism that can make the development of suitable therapeutics quite challenging. One method of circumventing this challenge is the development of drug-delivery systems (DDSs). Using methods of conjugation, encapsulation and complexing, the delivery of agents to a cell can be made more efficient. In more recent times, proteins were discovered that contain protein transduction domains (PTDs), which have the ability to cross a plasma membrane, bypassing the cell's primary defence mechanism. The isolation of these PTDs has led to the development of cell-penetrating peptides (CPPs). These short chains of amino acids are the regions within proteins that allow for cell transduction. Many types of CPPs have been discovered such as transportan, penetratin and VP22. Others have been engineered with cell internalization in mind, such as MAP, KALA and proline-rich peptides [2]. A common factor among all CPPs is the presence of cationic residues within the amino acid chain. Two widely used CPPs are the transactivator of transcription (TAT) peptide, derived from the transcription-transactivating protein of the HIV-1 virus, and polyarginine, designed after key features of CPPs were identified and extensively studied. These two peptides have been used to increase the cellular uptake of therapeutic agents such as proteins, DNA, antibodies, imaging agents, nanoparticles, virus-like particles (VLPs) [3], radioisotopes [4], liposomes and micelles [5]. Conjugation and complexing of CPPs to these particles markedly increase their uptake by cells. CPPs have also been found to exhibit no immune response and to be relatively inert [6,7]. The internalization mechanism for CPPs is still a highly debated topic; evidence exists that supports many different mechanisms of uptake by cells, including endocytotic methods such as caveolae-mediated endocytosis, lipid raft macropinocytosis and clathrin-mediated endocytosis [6]. Evidence also exists to the contrary, indicating that uptake can be nonendocytotic and receptor mediated [8]. Internalization of some CPPs still occurs at 4°C [9]. Heparan sulfate proteoglycans (HSPG) are reported to play an important role in macropinocytosis, a role that will be

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32835065ed

further elaborated in a later section [10]. Mitchell *et al.* [11] also proved that internalization of polyarginine was energy dependent by treating cells with sodium azide, an agent known to deplete a cell's ATP and GTP levels. Cells treated with sodium azide did not take up any detectable levels of fluorphore-R7, and yet, untreated cells internalized the conjugated peptide [11]. When internalized through endocytotic means, certain CPPs allow for the disruption of endocytotic vesicles and the contents to be released into the cytoplasm [1,12]. Other types of CPPs require the presence of other factors to exit vesicles, such as lytic peptides, pH-sensitive polymers, swellable dendritic polymers or cationic polymers such as polyethylenimine (PEI) or poly(propylacrylic acid) [6,13].

As mentioned, the common feature in most CPPs is the presence of positively charged peptides contained within the amino acid chain. The TAT peptide contains six arginine residues and two lysine residues. It was predicted that these amino acids confer cell-penetration ability due to their positive charge. Mitchell *et al.* [11] performed experiments to determine cellular internalization of heptamers of arginine (R7), lysine (K7) and histidine (H7). It was found that polyarginine (R7) was much more effective at cell penetration than lysine or histidine, even though they are all cationic peptides. It was found that the guanidinium group contained within the arginine peptide must interact with the plasma membrane to allow internalization [11]. To test this theory, Mitchell *et al.* [11] used a chemical called citrulline, almost identical to arginine, except that the guanidinium group had been replaced with a urea group. It was found that polycitrulline had no cell-penetrating abilities, suggesting that guanidinium is the important group for internalization [11].

Transactivator of transcription peptide

With the advent of AIDS in the mid 1980s, much research was being performed on the HIV that was linked to the onset of AIDS. The TAT peptide was found to be an integral protein necessary for virus replication [10]. The TAT peptide exhibits antiviral silencing suppressor (RSS) activity, inhibiting the host's ability to interfere (RNAi) with viral replication [14]. The TAT peptide does this by inhibition of an RNAi enzyme, Dicer [15,16]. The TAT protein interacts specifically with human cyclinT1, which is a regulatory partner of cyclin-dependent kinase 9 in the positive transcription elongation factor complex [17]. This complex then interacts with the transactivation response element [17]. This final complex then activates Cdk9 kinase activities, which in turn cause the hyperphosphorylation of RNA polymerase II, leading to the formation of full-length HIV-1 viral RNA [17]. The TAT protein is controlled by two exons, the first exon encoding for regions 1–72 of the protein, enough to allow functionality [5]. While studying these proteins, Frankel *et al.* [18] discovered that the protein had the ability to cross the plasma membrane of many types of cells when incubated with the protein. In 1994, Fawell *et al.* [19] discovered that by conjugating portions of the TAT protein (1–72 and 37–72) to other functional proteins, one could allow internalization of the peptide and conjugate into cells. In 1999, Schwarze *et al.* [20] used the PTD from the TAT protein and conjugated it to a biologically active β-galactosidase. A solution of TAT–β-galactosidase was injected intraperitoneally into mice. The enzyme was successfully delivered to all organs of the body, including the brain. An important piece of information gathered from this experiment was that TAT-bound molecules have the ability to cross the blood–brain barrier. This makes it feasible to create therapeutic DDSs to target the brain and the central nervous system (CNS) [20]. Although the TAT peptide has the ability to cross the plasma membrane, it has little to do with the internalization of the HIV-1 virus into cells [10]. The ability of an HIV-1 virus to infect a cell is mediated by Gp120 and Gp41 proteins on a virus shell, a process that is independent of the TAT peptide and its function [10]. These proteins bind to cell surface proteins of CD4 antigens and chemokine receptors, and then fuse with the cell [10]. The TAT protein consists of three regions: an acidic terminal for transactivation, a cystine-rich RNA binding region and a nuclear localization sequence [2]. The region responsible for protein transduction is contained within the nuclear localization sequence [2]. Although variants exist, the main sequence of the proteins that have a PTD consists of the following:

$$\text{TAT}_{48-60}\ \text{GRKKRRQRRRPPQ}$$

Interestingly, the same peptide was formed with D-amino acids (as opposed to L-amino acids) and functionality was unaffected, supporting the theory that it is the presence of the guanidinium groups that determines functionality, not chirality [11]. Wender *et al.* [21] also showed that chirality was not important by synthesizing D-amino acids (D-TAT_{49-57}) and retro-inverso isomers (TAT_{57-49} and D-TAT_{57-49}). They found that the analogues of TAT were all more effective at crossing the plasma membrane, further supporting the idea that it is the presence of the guanidinium groups, not chirality, that enables internalization [21].

Considerable work has been carried out on the conjugation of TAT as a means of increasing the cellular uptake of therapeutic agents. Torchilin *et al.* designed DDSs consisting of a hydrophilic therapeutic agent encapsulated within a liposome. Conjugated close to the surface were TAT peptides, sterically shielded by pH-sensitive polyethylene glycol (PEG) chains and an antibody for cell targeting [9,13,22,23]. When an anticancer agent is encapsulated into the liposome and the liposome is introduced into an individual's system, the pH-sensitive PEG chains keep the TAT peptides shielded to ensure that they do not interact

with noncancerous cells. The PEG layer ensures a lengthy time in the system, giving the antibody enough time to actively target the tumour. The harsh conditions within a tumour include lack of nutrients and oxygen and low pH, caused by ischaemia. This lower pH inside an acidic environment in tumour, the pH-sensitive PEG chains release and allow tumour cells to interact with the TAT peptide [9]. This in turn allows the tumour cells to take up the liposomes and introduce an anticancer agent directly into the tumour cells.

The use of DNA/RNA in therapeutics has been greatly hindered by the lack of perfected DDSs for their safe delivery. The current method of nucleic acid delivery involves complexing with PEI [24]. PEI is a cationic polymer that is capable of compacting nucleic acids, but PEI can lead to liver necrosis and host death [25]. Alternative methods have been suggested with the use of TAT conjugated to different nanoparticles, such as VLP [3], solid lipid nanoparticles [25] and a conjugation of TAT–PEG–PEI [26]. The VLP discussed by Wei *et al.* in 2009 consists of an MS2 bacteriophage capsid containing antisense RNA and a TAT peptide conjugated to the capsid. The delivery of RNA for therapeutic reasons is hindered by the presence of RNases and is hence protected by the capsid. The bacteriophage capsid contains proteins that associate closely with RNA, allowing for stability, whereas the TAT peptide causes uptake of the whole system and internalization of antisense RNA into the cytoplasm [3]. Research performed by Rudolph *et al.* [25] resulted in the formation of solid lipid nanoparticles conjugated with dimeric TAT peptides (TAT_2) that associates with plasmid DNA. This was designed to improve on the harsh side effects of PEI-mediated gene delivery. The dimeric TAT system resulted in a much higher gene expression when compared with the PEI complex [25]. One of the more interesting designs would be to make use of the advantages of PEI, but attempt to eliminate or decrease the side effects. By conjugating the TAT peptide (for cell penetration) to a PEG spacer and to PEI (for DNA complexing), Kleemann *et al.* [26] have created a system that reduces the cytotoxicity of PEI and results in a transfection 600% higher than that achieved by PEI on its own.

The therapeutic use of synthetic nucleic oligomers, such as peptide nucleic acid and phosphorodiamidate morpholino oligomer, has become feasible with the use of CPPs [27]. These have antisense capabilities and, as they are uncharged, can be conjugated to CPPs. The introduction of peptide nucleic acid or phosphorodiamidate morpholino oligomer into a cell where binding to mRNA takes place results in the formation of a duplex and consequent cleaving by RNase H [27]. As previously mentioned [19,20], TAT can be conjugated to proteins without affecting functionality. TAT was conjugated to heat shock protein 70 (Hsp70), a protein with the ability to protect cells against heat shock, oxidative stress and apoptotic stimuli [7]. A neuroblastoma cell line called SH-SY5Y was treated with 6-OHDA, a drug that selectively targets these types of cells and causes oxidative stress [7]. TAT-Hsp70 was found to have a cytoprotective effect on these cells, reducing oxidative stress, whereas unbound Hsp70 showed little effect [7].

Jin *et al.* used a human catalase gene and fused it to the TAT peptide gene and the nonarginine (R9) gene. This was then translated through a bacterial vector, yielding TAT and nonarginine-fused human catalase [28]. The purpose of this was to create a conjugate that can eliminate reactive oxygen species intracellularly, instead of extracellularly. The fusion proteins were then introduced into HeLa mammalian cells and treated with H_2O_2. It was found that the cell viability was very high for the cells treated with the proteins. The secondary aim of this experiment was to determine whether the fusion proteins produced could penetrate animal skin. The protein was found to penetrate the epidermis as well as the dermis [28].

An interesting method to treat HIV/AIDS would be with a TAT-mediated therapeutic. Rao *et al.* [29] conjugated protease inhibitors to the TAT peptide in an attempt to target HIV-1 virus particles within the CNS. The CNS is a primary region for HIV-1 replication [29]. Prolonged presence of HIV-1 in the CNS leads to dementia, memory loss, HIV-mediated encephalopathy and cerebral complications [29]. Protease inhibitors block protease receptors, but proteases on their own show poor absorption, extensive protein binding and the 'first pass effect', where the liver breaks down the vast majority of a drug before serving its function [29]. By encapsulation in a poly(L-lactide) polymer capsule approximately 100 nm in size, permeability into the CNS was increased. There were also approximately 2000 TAT peptide molecules per nanoparticle [29]. The TAT peptide has also been used in the delivery of radioisotopes to breast cancer cells *in vivo* and *in vitro* [4]. A radioisotope of iodine, ^{123}I, which has a half-life of 13 h, is commonly used in imaging [4]. By conjugation to an antibody (mIgG), which actively targets cells, and the internalization factor of the TAT peptide, this system can accurately image tissues, while also minimizing toxicity [4].

Gold nanoparticles are being used more frequently because of their biological and chemical inertness. Berry *et al.* [30] designed gold nanoparticles with nuclear delivery in mind, and produced gold nanoparticles 5 and 30 nm in size. Delivery to the nucleus was achieved with 5 nm sized gold particles, but not the 30 nm sized gold particles [30]. This could be because delivery to the nuclear envelope was hindered by the nuclear pore size. In addition, Zhang *et al.* used block copolymer nanoparticles functionalized with the HIV TAT PTD to assess the shape of nanoparticles in relation to internalization

into cells by Zhang *et al.* [31] The results indicated that nanoparticles with a spherical shape had a much higher rate of entry than those with a cylindrical shape in Chinese hamster ovary cells. The level of nanoparticle entry into the cells was directly related to the amount of PTD conjugated to the particle as was the rate of escape from the cell [31]. This concept becomes important when targeted delivery is aimed at, as nanoparticles entering cells without specific targets would be required to escape without releasing their cargo, for example an anticancer compound in a normal cell.

Lei *et al.* [32] delivered TAT peptide-labelled quantum dots into mesenchymal stem cells. CdSe/ZnS quantum dots (3 nm) were coated with a PEG layer and conjugated to the TAT peptide. The conjugated quantum dots were then introduced into mesenchymal stem cells and they penetrated the plasma membrane easily. The introduction of the quantum dots did not affect the sensitive nature of stem cells, an achievement in itself. The quantum dot-labelled stem cells were further introduced into mice and the distribution of the stem cells was observed through fluorescence microscopy [32]. Fluorescence was observed in the liver, spleen and lungs but no fluorescence was observed in the brain, heart or kidney. Superparamagnetic iron oxide particles have many possible applications, such as cell and cell-content sorting, and as biosensors and imaging agents. Morgul *et al.* [33] conjugated the TAT peptide to nanosized superparamagnetic iron oxide to study what happens to human hepatocytes once transplanted. The labelled hepatocytes were detectable through MRI, although they were not introduced into humans; it was simply a proof of concept [33]. It can be surmised that the TAT peptide has great capabilities as a cell-penetrating protein, whether on its own or conjugated to a small or even a large particle. Although its applications seem endless, the fact that it was obtained from the HIV-1 virus may hinder its clinical usage despite the fact that there is no evidence for its involvement in the viral infection phase.

Polyarginine

With the discovery of TAT and its residues, it was theorized that a small chain of arginine may have cell-penetrating capabilities. Mitchell *et al.* [11] also performed experiments with different chain lengths of arginine with different chiralities (D or L) to determine ideal functionality. These small chains of arginine were conjugated to fluorphores and measured fluorescence was assumed to be a measure of uptake. Uptake in Jurkat cells was measured and it was found that the D-arginine conjugates were more effective in crossing the plasma membrane [11]. The resulting data are most likely because D-amino acids are more resistant to proteolysis when compared with naturally occurring L-amino acids. Polyarginines less than six residues long were found to be less effective at protein transduction. Transduction was found to be optimum with arginine chains between six and 15 residues, with penetration increasing as the chain length increased. Anything above a chain length of 15 or more, however, had less membrane-crossing ability, with increased cytotoxicity [11]. Wender *et al.* [21] also demonstrated that a polyarginine chain (R9) was 20-fold more efficient at crossing the plasma membrane than the TAT peptide, as measured by Michaelis–Menten kinetic analysis. A chain of D-arginine (r9) (as opposed to the L-arginine R9) was found to be 100-fold more efficient than the TAT peptide. Matsui *et al.* [34] created bacterial vectors that would produce polyarginine (R7, R9 and R11) and TAT, followed by enhanced green fluorescent protein. The proteins produced were then introduced into Cos-7 cells for 30 min. R11-enhanced green fluorescent protein was found to be most effective at cell membrane crossing, followed by R9, TAT and R7.

Implementation of R9 as a protein carrier has been used in studies using dominant-negative survivin. Survivin, a natural inhibitor of apoptosis protein, is often overexpressed in many cancer cells [35]. Cheung *et al.* demonstrated the capability of R9 to effectively carry a dominant-negative mutant form of survivin (DNsurR9-C84A) into prostate (DU145) and HeLa cells grown in 3D culture. Western blotting of cell lysates at time periods after exposure with the protein indicated that DNsurR9-C84A was able to penetrate the cell within 1–2 h [36]. Furthermore, dominant-negative survivin was able to perform its activity and block the action of overexpressed wild-type survivin [36].

In addition, mutant survivin bound to R9, SurR9-C84A, has been delivered to neuroblastoma cells in order to prevent apoptosis after exposure to oxidative stress by H_2O_2. Baratchi *et al.* [37] demonstrated that when cells were preexposed to SurR9-C84A, they were protected against the neurotoxicity that is normally induced by H_2O_2. It was also found that the delivery through R9 of this mutant survivin could protect against retinoic acid-induced neurotoxicity by increased cell proliferation and cell division by the binding of the mutant survivin to microtubules within the cell once it had gained entry through the R9 carrier [38]. Polyarginine, r9, has also been used to aid the internalization and activity of β7-12 integrin in TK-1 cells, reducing inflammatory activity [39]. Krissansen *et al.* delivered integrin β7-12 within a known CPP YDRREY fused to a 9-D-arginine polymer (r9) and observed rapid internalization and activation of anti-inflammatory responses, indicating the successful delivery of β7-12.

The use of DNA as a therapeutic has been hindered by the difficulties involved in the uptake of cells of negatively charged DNA particles. Antisense RNA and small interfering RNA (siRNA) have great potential to be the platform for the next generation of therapeutic agents. SiRNA has only recently been discovered and has sparked a huge amount of research [1]. Unaided, siRNA

has no mechanism for uptake into cells, making its use as a therapeutic difficult; however, through the implementation of CPPs and polyarginine, this may be overcome [40]. Meade *et al.* covalently conjugated an arginine chain of R8 to an siRNA molecule. Studies demonstrated that this would be sufficient to allow uptake into cells, and yet, after extensive purification, minimal transduction was observed [1]. Meade *et al.* theorized that due to the negatively charged backbone of siRNA ($\sim$40 residues), the small arginine chain was insufficient to increase cellular internalization. The reason why studies showed experimental evidence supporting this idea is that they did not have such an extensive purification step. This then led Meade *et al.* [1] to believe that nano-sized complexes between free R8 and siRNA would allow for an overall positive charge and therefore internalization. By combining the two at specific ratios, the complexes were able to cross into HeLa cells.

The delivery of fully functional proteins can also be achieved using polyarginine. An R9 peptide was fused to the well understood enzyme RNase A [41]. The polyarginine did not compromise enzyme functionality and it facilitated purification by adsorption onto glass slides. A genetic variant of RNase (G88R RNase A) is also found to be cytotoxic to several cancer cell types, by means of avoiding ribonuclease inhibitors. Conjugation of R9 to this peptide resulted in an increased uptake into cancer cells [41].

Mitsui *et al.* [42] used polyarginine fused to ovalbumin (OVA) in an attempt to cause dendritic cells to produce CD8 + -specific and CD4 + -specific antigens for tumour cell targeting. OVA was produced with TAT and R9 peptides fused to it, plus haemagglutinin (HA) to allow for monoclonal antibody staining for the detection of distribution in the cell. R9 was found to be the most efficient at membrane translocation, followed by the TAT peptide and free HA-OVA [42]. The dendritic cells were then introduced into mice models and it was found that with repeated injections of dendritic cells, tumour cell sizes were reduced. This strategy has the potential to provide a new way of reducing tumour size in patients using the immune system [42].

Polyarginine has also been conjugated to proteins with the intention of preconditioning cardiomyocytes to be more resistant to cell death during the effects of ischaemia [43]. By targeting a cell system called protein kinase C with analogues of proteins called RACKs (receptors for activated C-kinase), researchers were able to increase the resistance to the damage of isolated cardiomyocytes. The analogue used was named ψεRACK, but this peptide has no means of cell transduction. Four different CPPs were used (penetratin, TAT, R7 and r7) and linked to ψεRACK with cysteine disulfide bonds. This was because, to exert any effect, the ψεRACK needs to be free in the cell; disulfide bonds were reduced once in the cell. R7 conjugated to ψεRACK without disulfide bonds does not reduce cell damage [43]. R7 and r7 (L-arginine and D-arginine) were most effective at cell damage reduction.

Conjugation to polyarginine can also confer proteins with the ability to cross through skin. Cyclosporin A is an anti-inflammatory drug used in the treatment of psoriasis, severe atopic dermatitis and rheumatoid arthritis [44]. It is administered orally, as it is not absorbed when applied topically. Oral administration of cyclosporin A, however, is hindered by nephrotoxicity and internal drug interactions [44]. A heptamer of D-arginine was used with a pH-sensitive linker to cyclosporin A. To test that polyarginine conjugates were able to cross the stratum corneum layer of the skin, R7 was conjugated to biotin, which binds with a very high affinity to streptavidin. The conjugates were applied in a lotion to mice. After 2 h, skin biopsies were performed and stained with fluorescein-conjugated streptavidin. Fluorescence was detected in the epidermis and the dermis, indicating that the conjugate can successfully be applied topically. Biotin–R7–cyclosporin A was then synthesized and applied topically to a human skin graft on mice. The same method as above was used and fluorescence was again detected in the epidermis and the dermis [44].

Polyarginine has been used to create self-assembling micelles for the delivery of hydrophobic therapeutic agents [45]. A hydrophobically modified amino acid, poly(2-hydroxyethyl aspartamide), was conjugated to a long hydrophobic alkyl chain, which was then conjugated to octaarginine (R8). Once introduced into an aqueous solution, the conjugates formed micelles ranging in size from 8 to 40 nm [45]. These micelles were effectively internalized into HeLa cells through endocytosis.

Liposomes have also been conjugated with polyarginine to aid cellular uptake [46]. These liposomes can encapsulate many different cargos including DNA and therapeutic drugs. The conjugation of octaarginine to liposomes took place by a different method than previously mentioned. R8 was conjugated to a stearyl group and mixed with the lipids during liposome formation. The hydrophobic stearyl group interacts with the hydrophobic region of the lipids, stabilizing it and conferring the liposome with a positive charge, although it was not determined whether this method of conjugation leads to less stable liposomes [46]. R8-conjugated liposomes also require fewer peptides per liposome for successful internalization [46].

Mechanism

The mechanism that causes the internalization of CPPs is still highly debated among researchers. Some researchers claim that it is a nonreceptor, energy-dependent endocytotic mechanism, whereas others claim that the internalization mechanism is receptor mediated. Evidence also exists indicating that HSPG play a role in endocytotic

Table 1 Description of cell-penetrating peptides used in combination with nanoparticles and their potential use

Cargo	Cell-penetrating peptide	Aim of the study	References
β-Galactosidase, horseradish peroxidase, RNase A	TAT protein fragments	Test protein transduction ability of TAT protein fragments	[19]
β-Galactosidase	TAT peptide	Test distribution of protein conjugates throughout the body	[20]
PEGylated liposomes	TAT peptide	Development of a drug delivery system	[9,13,22,23]
MS2 bacteriophage-derived virus-like particles (VLP)	TAT peptide	Safe delivery of intact therapeutic RNA	[3]
Solid lipid nanoparticles	TAT peptide	Design a safer gene delivery system than PEI	[25]
TAT–PEG–PEI conjugates	TAT peptide	Delivery of DNA into cell	[26]
Steric block oligonucleotides (PNA and PMO)	TAT peptide	Deliver noncharged synthetic oligonucleotides for antisense applications	[27]
Heat shock protein 70 (Hsp70)	TAT peptide	Confer cytoprotective properties by reducing oxidative stress	[7]
Human catalase gene	TAT peptide and nonarginine (R9)	Eliminate reactive oxygen species intracellularly	[28]
Protease inhibitors	TAT peptide	Target replicating HIV-1 inside CNS	[29]
Iodine (^{123}I)	TAT peptide	Delivery of a radioisotope into cells *in vitro* and *in vivo* for imaging purposes	[4]
Gold nanoparticles (5 and 30 nm)	TAT peptide	Delivery of particles into the nucleus	[30]
Quantum dots	TAT peptide	To determine the distribution of mesenchymal stem cells in mice	[32]
Superparamagnetic iron oxide particles	TAT peptide	Label human hepatocytes for MRI detection	[33]
siRNA–octaarginine complexes	Octaarginine (R8)	Delivery of siRNA into cells successfully	[1]
RNase A	Nonarginine (R9)	Potential anticancer drug	[41]
Ovalbumin	Nonarginine (R9) and TAT peptide	Cause dendritic cells to produce specific antigens for tumour cell targeting	[42]
Receptors for activated C-kinase (RACK)	Heptaarginine (R7 and r7) TAT and penetratin	Preconditioning cardiomyocytes to be more resistant	[43]
Cyclosporine A	Heptaarginine (r7)	To develop an anti-inflammatory for topical application	[44]
Self-assembling micelles	Octaarginine (R8)	Delivery of hydrophobic therapeutics	[45]
Liposomes	Octaarginine (R8)	Development of a drug delivery system	[46]

CNS, central nervous system; PEG, polyethylene glycol; PEI, polyethylenimine; PMO, phosphorodiamidate morpholino oligomer; PNA, peptide nucleic acid; siRNA, small interfering RNA; TAT, transactivator of transcription.

uptake of these peptides. When cells have been treated with a chemical to degrade HSPG or an HSPG-deficient cell line is used, internalization of polyarginines and the TAT peptide is inhibited [2,10]. Internalization of peptides is also inhibited by a low temperature. At 4°C, a temperature at which endocytosis does not occur, internalization of some CPPs is inhibited [47]. Yet, at 4°C, internalization of polyarginine can still take place, suggesting alternative internalization mechanisms [10].

Nakase *et al.* [10] provide an explanation that takes into account many of the factors affecting internalization. The presence of cationic peptides at the cell membrane surface disrupts the membrane, leading to the formation of small vesicles. The HSPGs are thought to localize the positive charge of the cationic peptides, facilitating easier internalization through endocytosis [10]. Uptake through endocytosis is thought to take place by three different mechanisms: clathrin-coated uptake, caveolae-mediated endocytosis and macropinocytosis. Clathrin-coated uptake is the formation of concave structures on the plasma membrane. This results in the invagination of the cell membrane and the formation of small vesicles (120 nm) [10]. Caveolae-mediated endocytosis occurs much the same way, but with the formation of smaller vesicles [10]. Macropinocytosis involves an actin-induced disruption of the plasma membrane and the formation of large endosomic structures ($\geq 1\,\mu m$) [10]. Inhibitors of macropinocytosis also inhibit the internalization of polyarginine and the TAT peptide, supporting the theory of macropinocytosis-mediated internalization.

Much work has been carried out using CPPS in the design of new-age pharmaceuticals (Table 1). CPPs present an efficient way of crossing the plasma membrane, negating the need to design drugs with this functionality in mind. It is clear that more research needs to be carried out on methods through which CPPs can enter a cell. If this can be achieved, it would mark a milestone in the development of therapeutic agents using CPPs.

Arsenic trioxide

The use of arsenic trioxide (As_2O_3) as a therapeutic agent dates back to traditional Chinese remedies and has since been harnessed as a potent anticancer compound. In the mid 1990s, much research on As_2O_3 was performed on leukaemia, particularly on acute promyelocytic leukaemia (APL). In 1996, Chen *et al.* conducted experiments with As_2O_3 *in vitro* on a human leukaemia cell line. Results indicated that apoptosis was induced after As_2O_3 treatment at micromolar concentrations. Apoptotic induction was thought to be achieved through the downregulation of Bcl-2 proteins both at the mRNA and at the protein level [48]. In 1997, the same research group extended their research to a broader range of treatment concentrations from 0.5 to 2 μmol/l. They found a dose-dependent, dual effect of As_2O_3 in its ability to induce apoptosis at high concentrations and induce partial differentiation of NB4 leukaemia cells at lower concentrations *in vitro*. In addition, analysis of samples from patients treated with As_2O_3 for 2–3 weeks showed that degenerative cells increased where levels of promyelocytic leukaemia cells increased [49].

Table 2 Arsenic trioxide has been studied as a treatment for various solid tumour forms of cancer as well as extensively as a treatment for acute promyelocytic leukaemia

Cancer type	Model	Observation	References
Acute promyelocytic leukaemia	*In vivo*, *in vitro*	Apoptosis	[48,49]
Neuroblastoma	*In vitro*	Apoptosis by activation of caspase 3	[51]
Gastric cancer	*In vitro*, *in vivo* (mouse xenograft)	Apoptosis	[52,53]
Lung cancer	*In vitro*	Apoptosis by inhibition of Bcl-2 genes	[54]
Breast carcinoma	*In vitro*	Apoptosis, cell cytotoxicity	[55]
Prostate cancer	*In vitro*	Apoptosis	[56]
Liver cancer	*In vivo* (mice)	Apoptosis by inhibition of Bcl-2 genes	[57]
Head and neck cancer cells	*In vitro*	Cell cycle arrest	[58]
Cervical cancer	*In vivo, in vitro*	Reduces the adhesive, migration and invasive properties of cells	[59]

Clinical trials in 1997 concluded that As_2O_3 was a relatively safe drug for the treatment of APL, with limited side effects [50]. Among individuals with APL who were administered 10 mg of As_2O_3 per day, 90% went into complete remission and the remaining 10% went into remission with combinational treatment of both As_2O_3 and low doses of chemotherapeutic drugs. After proving a successful APL chemotherapeutic, considerable studies have been carried out on the effects of As_2O_3 as a treatment for solid tumours (Table 2). Studies of tumour cells in breast [55], lung [54], gastric [52,53] prostate [56] and cervical [59] cancer have demonstrated the ability of As_2O_3 to induce apoptosis through the activation of caspase 3 [51], downregulation of Bcl-3 genes [57] or cell cycle arrest [58]. As_2O_3 demonstrates severe toxicity on cancer cells; however, it also exhibits a strong effect on normal cells. The chemotherapeutic side effects can be severe and the necessary doses required to kill certain tumours may be lethal to the host. A possible solution to the problem was addressed by Krissansen *et al.* [39] by combinational treatment with an immunotherapeutic protein ligand from the B7 family, mouse B7H3, which induced antitumour immunity. In-vivo studies were performed on induced large hepatocellular carcinoma subcutaneous tumours in mice. Once tumours were developed, mice were pretreated with B7H3 before being administered As_2O_3 48 h later. Studies found increased levels of cytotoxicity and apoptosis to the point where complete eradication of tumours occurred [60]. Studies also found that neither treatment worked as a monotherapy, highlighting the need for As_2O_3 to be used in conjunction with another treatment or delivery method.

Arsenic trioxide and nanoparticles

Another significant prospect to overcome the heightened toxicity of As_2O_3 is through the concept of nanoencapsulation. Using this method, As_2O_3 can be safely encapsulated within a nanoparticle or a microparticle, travel through the body to the tumour site and specifically target only cancer cells. This would allow minimal exposure and damage to the rest of the host while consequently increasing the effectiveness and safety of potent chemotherapeutics such as As_2O_3. In 2005, Zhou *et al.* successfully synthesized As_2O_3–albumin microspheres for use *in vitro*. As_2O_3–albumin microspheres (As_2O_3–BSA–NS) were synthesized using chemical cross-linking and were shown to release As_2O_3 at a much slower rate than pure As_2O_3 alone in buffer [61].

As_2O_3 has been formulated with other forms of nanoparticles with success in treating cancer cells. In 2005, Zhou *et al.* developed another form of albumin immunonanosphere treatment containing As_2O_3 to specifically target bladder cancer cells. The nanosphere consisted of a monoclonal antibody to bind specifically to BDI-1 bladder cancer cells. It was shown that the nanosphere treatments bound to and induced apoptosis in these bladder cancer cells *in vitro* [62]. Furthermore, Yang *et al.* developed poly(lactic-*co*-glycolic acid)–$MgFe_2O_4$ magnetic nanoparticles containing As_2O_3 in 2009. They developed spherical nanoparticles that, once loaded, could be magnetically targeted to specific cells. In-vitro studies using these nanoparticles demonstrated time-dependent and dose-dependent growth inhibition by means of MTT assays on human osteocarcinoma cells.

An interesting study in 2009 used nanoparticulate liposomes termed 'nanobins' to encapsulate As_2O_3. These nanoparticles were specifically targeted towards folate receptors (FRs), which occur naturally on certain cell surfaces. FR-positive human nasopharyngeal (KB) and cervix (HeLa) cells as well as FR-negative human breast cancer cells, MCF-7, were used to test the efficacy of this drug delivery method. Results from cocultures of these cell types indicated that folate-mediated As_2O_3 showed specificity for FR-positive cells and increased uptake of As_2O_3, particularly where the FR number was high. In addition, coencapsulation of nickel ions potentiated As_2O_3 efficacy [63]. Further studies from the same group on nanobins in 2010 developed a lipid-encapsulated version of As_2O_3 to target breast cancer. In-vivo and in-vitro testing of this formulation revealed that nanobin encapsulation of As_2O_3 increased delivery potential to tumour sites, pharmacokinetics and efficacy of tumour apoptosis induction [64]. Ahn *et al.* concluded that this method was a potential therapeutic platform for harnessing the benefits of As_2O_3 in solid tumours.

Arsenic trioxide and albumin nanoparticles

The advantages of using nanoparticles as a mode of drug delivery for specific chemotherapeutics are many. The use

of specific macromolecule conjugations has shown specific advantages particularly in increasing biocompatibility and uptake of the drug at the cellular level. BSA is a protein that is easily taken into the cell by a normal cellular mechanism and has been shown to be nontoxic, nonimmunogenic and highly biodegradable [65]. Macromolecules, such as BSA, are also advantageous conjugates due to their ability to avoid passive diffusion into highly circulated tissues, preventing high toxicity and high levels of distribution of drugs [65].

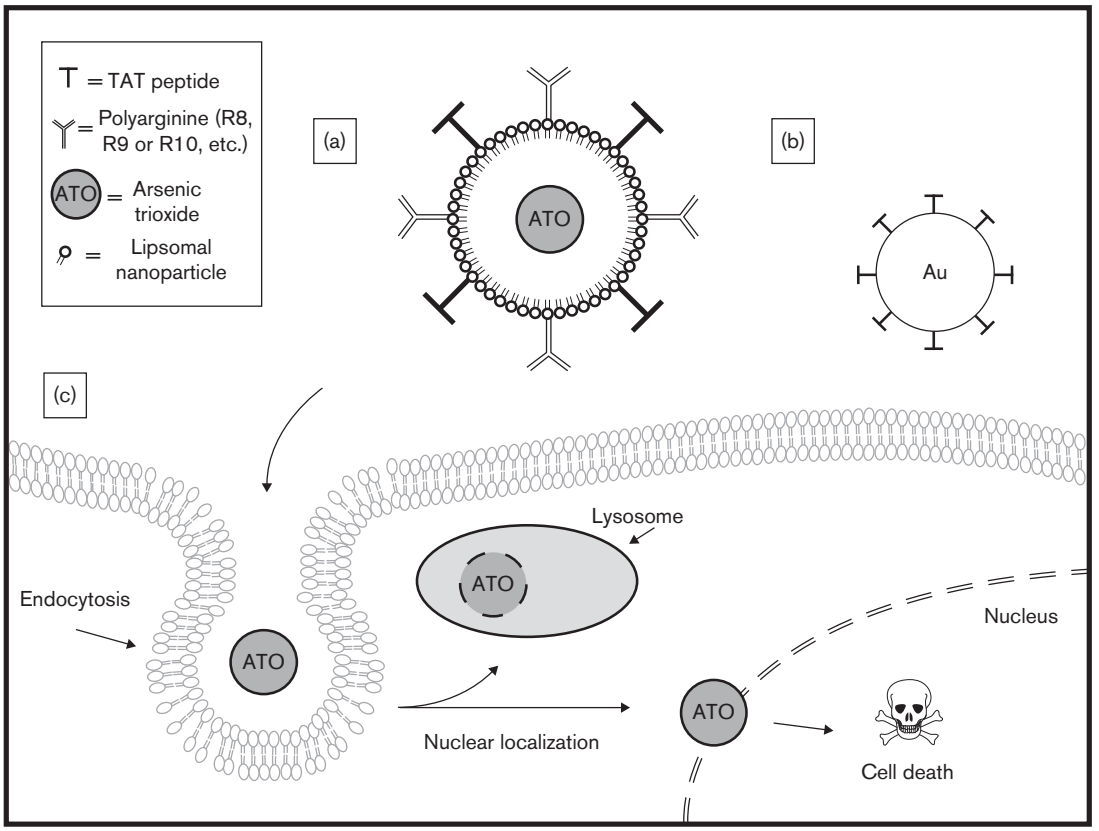
A very important albumin nanoformulation to target cancer is that of human serum albumin and paclitaxel. Most importantly, this nanoformulation, known as ABI-007, has been used in the treatment of metastatic breast cancer [66], and is commercially known as Abraxane. Abraxane is significantly less toxic than paclitaxel due to the absence of cremophore, which is required to solubilize paclitaxel for administration. When bound to albumin, paclitaxel is soluble in the body and can be administered without the increased, dose-limiting toxic cremophore [67]. Similar formulations using chemotherapeutic drugs include BSA microspheres conjugated to Gemzar, a known anticancer drug. These microspheres demonstrated an increased effect on renal cancer cells *in vitro* compared with the same molar concentration of pristine Gemzar [65].

Studies conducted on As_2O_3-loaded BSA nanoparticles have also shown positive results. In 2008, Yang *et al.* characterized nanoparticles loaded with As_2O_3 with a drug-loading capacity of around 27% and an average size of 734 nm. In-vitro testing demonstrated cell cytotoxicity induction in an APL cell line and antitumour effects in H22 hepatoma cells *in vivo* [68]. Loaded nanoparticles demonstrated a slow drug release and a cumulative release of around 95%. In addition, loaded nanoparticles showed increased antitumour affects compared with the drug in solution alone [69]. This is an important characteristic for drug delivery of As_2O_3 due to its high toxicity level; a slower and specific release could allow for less systemic toxicity (Table 1).

Transactivator of transcription protein and intracellular delivery of arsenic trioxide as a cancer treatment

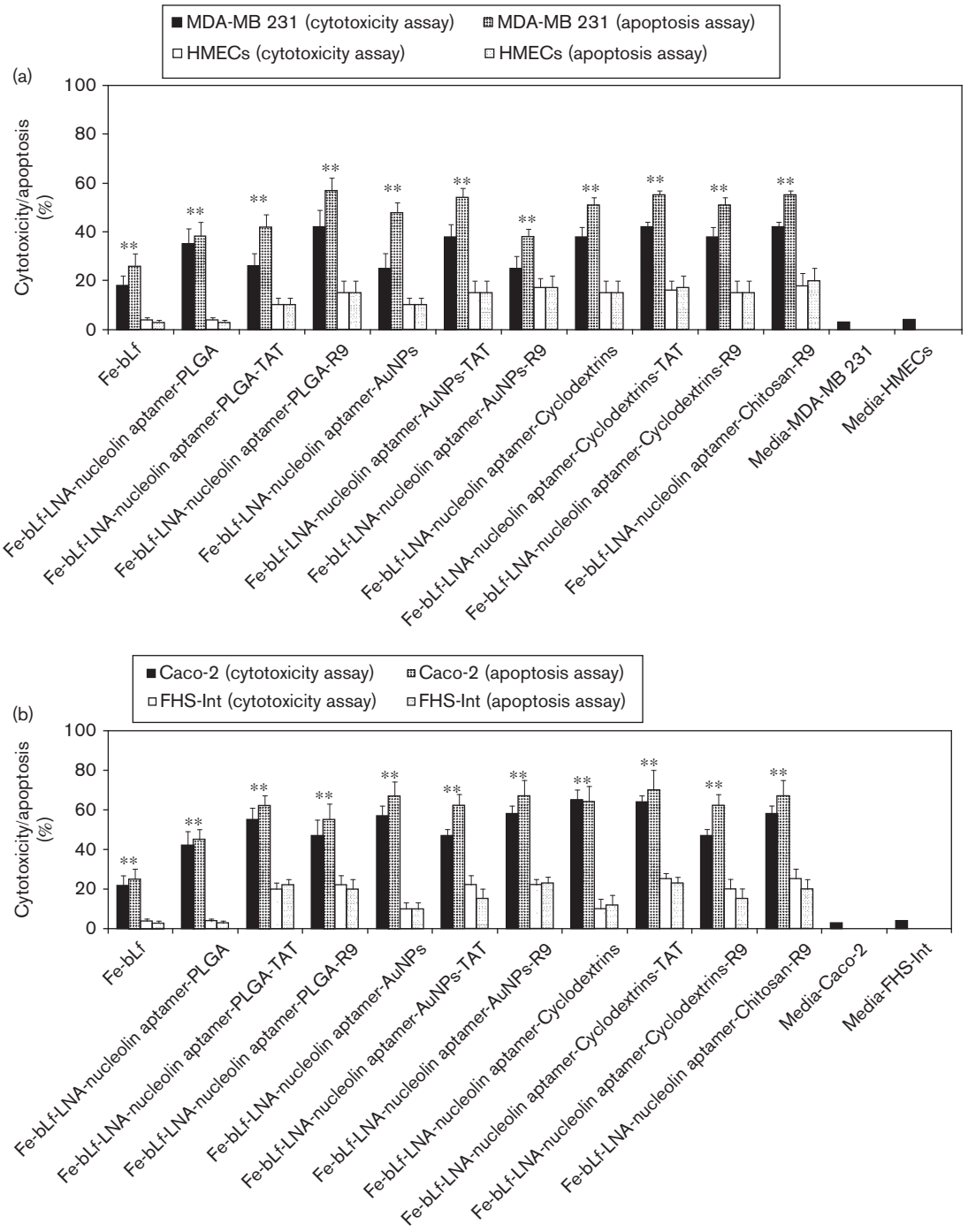
To date, there are no published data on the conjugation of TAT to As_2O_3 formulations. The proposed concept

Fig. 1



Proposed mechanism of a multifunctional nanoparticle. (a) Multifunctional liposomal nanoparticles with transactivator of transcription (TAT) and polyarginine cell-penetrating peptides on the surface to achieve cellular internalization and specificity with an arsenic trioxide drug load. (b) Gold nanoparticle with the TAT peptide. (c) Mechanism for cellular internalization (endocytosis) of arsenic trioxide leading to nuclear localization and cell death.

Fig. 2



Cell cytotoxicity determined by lactate dehydrogenase (LDH) release assay and cell death (apoptosis) by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay of (a) breast cancer cells and (b) colon cancer cells and compared with normal cells following treatment with 1600 μg/ml of iron-saturated lactoferrin (Fe-bLf) and other nanocarriers as mentioned. Cells were treated for 24 h with different nanoformulations and stained by TUNEL analysis for apoptotic cells. Cell death is shown here in terms of apoptotic (% apoptosis) and LDH release (% cytotoxicity). All treatments were performed in triplicate and assay was repeated three times independently, with similar results. The mean for the representative experiment was calculated and presented as mean ± SD values. **Highly significant $P < 0.001$ value from the normal control cell lines and with media only. *Significant $P < 0.05$ value from the normal control cell lines and control with media only.

(Fig. 1), however, has many interesting prospects. As_2O_3 is a very potent anticancer drug with the ability to cause apoptosis in different types of cancer cells lines (*in vitro*) and tumours (*in vivo*). The negative side effects of this drug, however, are great, with significant dose-limiting restraints in treatment options. In recent times, the

option for drug encapsulation and delivery through nanomedicine technologies has provided a new life to otherwise limited anticancer compounds such as As_2O_3 (Table 2) [64–69]. The nature and characteristics of nanoparticles and their delivery mechanisms make them ideal solution to the increased toxicity caused by many chemotherapeutics. First, drug encapsulation in nanoparticles allows for protection of both the drug and its function until it reaches the target site as well as protection of normal tissues, whose exposure to the drug would be undesirable. This increases bioavailability and decreases systemic toxicity and accumulation. Second, nanoparticles are advantageous in their ability to enter a cell to deliver its drug load; again, this enhances the effect on the target cell while protecting nontargeted cells. This function is greatly enhanced by the use of CPPs such as the TAT peptide. With this in mind, it is possible to conceive that the well performing As_2O_3 nanoparticles synthesized from albumin could also be conjugated to the TAT peptide for greater therapeutic advantage, overcoming the heightened toxicity caused by As_2O_3 and increasing the level of entry into specific cancer cells by harnessing the activity of CPPs.

We have shown that 100% iron-saturated bovine lactoferrin (Fe-bLf) acts as a potent natural adjuvant and fortifying agent for augmenting cancer chemotherapy with a broad utility in the treatment of cancer [70]. The key findings of the study revealed that when introduced into mice, Fe-bLf bound to the intestinal epithelium and was preferentially taken up within Peyer's patches. Fe-bLf also enhanced antitumour activity in combination with major anticancer drugs (paclitaxel, doxorubicin, epirubicin or fluoracil), the combination being capable of completely eradicating large tumours of EL4 lymphoma, Lewis lung carcinoma and B16 melanoma that were otherwise completely insensitive to chemotherapy. Fe-bLf increased leucocyte infiltration (CD4 +, CD8 +, natural killer and dendritic cells) to tumours, lamina propria and the spleen. Importantly, Fe-bLf also restores both red and white blood cell numbers depleted by chemotherapy, potentially fortifying the mice against cancer [71–73]. Fe-bLf was prepared and loaded onto different poly(lactic-*co*-glycolic acid, gold, liposomal, polymeric, chitosan and magnetic (Fe_3O_4) nanoparticles. These nanoparticles were conjugated with an LNA-nucleolin-modified DNA aptamer (a molecule present on cancer cell surfaces and translocated to the nucleus) [71,74] and an LNA-EpCAM-modified RNA aptamer [74], known to be overexpressed on the apical side of the cancer cell surfaces [75]. The average size of these nanocarriers was found to be (80 ± 15 nm) as determined by dynamic light scattering, scanning electron microscope, transmission electron microscope, Fourier transform infrared spectroscopy, differential scanning calorimeter, thermo gravimetric analysis and X-ray diffraction. These LNA–aptamer-loaded Fe-bLf nanocarriers were used to treat human cancer cell lines Caco-2 (colon cancer), MDA-MB-231 (breast cancer), normal human mammary epithelial cells and normal FHs 74 Int (adherant primary fetal small intestinal) obtained from the American Type Culture Collections (ATCC, Rockville, Maryland, USA). These LNA–aptamer complexes of Fe-bLf nanocarriers induce LDH release or cytotoxicity and cell death through apoptosis measured by TUNEL and Annexin-V cell death assays in breast and colon cancer cells *in vitro* and spare normal human mammary epithelial cells and FHs 74 Int cells in an in-vitro coculture model (Fig. 2a and b). This indicates that nanocarriers loaded with modified LNA–aptamers have the potential to kill cancer cells more specifically and effectively while sparing normal cells.

Conclusion

CPPs (TAT and polyarginine) play a major role in transferring protein, DNA, RNA, siRNA and miRNA from intracellular spaces to the cytoplasm and cell organelles within a cell. Here, we reviewed the roles of these peptides in the internalization of nanoparticles. These peptides can be used to cargo complex molecules in the field of nanotechnology and nanomedicine. In the future, these CPPs can be used for nanodiagnosis, imaging and monitoring of the effects of drugs in human diseases. New-generation vaccines and improved DDSs are in clinical trials and soon new disease-targeted therapy will be available in hospitals to increase the efficacy of delivery and bioavailability of drugs while lowering the toxicity. This technology will also facilitate monitoring of chronic diseases in real time.

Acknowledgements

The work was supported by grants from the Institute of Biotechnology, Institute for Technology, Research and Innovation and the Australian-India Strategic Research Fund (AISRF BF030016), Australia.

Conflicts of interest

There are no conflicts of interest.

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