Minicells: Versatile vectors for targeted drug or si/shRNA cancer therapy
Jennifer A MacDiarmid and Himanshu Brahmbhatt

Effective cancer therapy continues to be a daunting challenge due mainly to considerable tumor cell heterogeneity, drug-resistance, and dose-limiting toxicity of therapeutics. Here we review a versatile nano-cellular (minicell) delivery vehicle that can be packaged with therapeutically effective concentrations of chemotherapeutic drugs, siRNAs or shRNAs and can be targeted to tumors via minicell-surface attached bispecific antibodies. A range of minicell-based therapeutics have shown highly effective tumor stabilization/regression in the murine xenograft model and in case studies in canines with late-stage endogenous tumors. Repeat intravenous dosing shows absence of toxicity or immunogenicity in both species. The minicell-based therapeutic has potential applications in personalized cancer medicine.

Address
EnGeneIC Ltd, Cancer Therapeutics, Building 2, 25 Sirius Road, Lane Cove West, Sydney, NSW 2066, Australia

Corresponding author: Brahmbhatt, Himanshu (hbrahmbhatt@engeneic.com)

Introduction
Cancer remains the major cause of death in most advanced countries and the incidence of cancer increases as populations age. Despite considerable advances in development of cancer therapeutics, the disease continues to be plagued with a highly significant mortality rate.

Chemotherapeutic drugs lack cancer-cell selectivity and indiscriminate drug distribution results in severe toxicity and limits anti-tumor efficacy. Owing to the intrinsic genetic diversity and the ability to rapidly mutate, tumors develop multi-drug resistance which contributes to the high rate of treatment failure. To overcome these drawbacks, efforts are being made to develop targeted drug delivery systems such as stealth liposomes [1], micelles [2], nanoparticles [3,4], and polymer–drug conjugates [5]. The very large number of different nanocarriers being currently developed, several of which are FDA approved, in clinical trials and marketed is reviewed [6**]. However, these technologies are also hampered by shortcomings, such as drug leakage in vivo, lack of versatility in terms of packaging a diverse range of different drugs without significant derivatization, thereby reducing drug potency, and difficulties in production scale-up, particularly for nanoparticles.

Anti-cancer antibodies directed to over-expressed receptors on cancer cells, such as the epidermal growth factor receptor (EGFR; [7,8]) and HER2/neu [9**] are less toxic, but do not possess the potency and wide spectrum anti-tumor activity of chemotherapeutic drugs. Additionally, some for example HER2/neu are only over-expressed in ~25% of breast cancer resulting in HER2/neu negative breast cancer patients, meaning that the majority of patients do not benefit from antibody therapy. The high rate of mutations in the tumors also results in receptor mutations [10] and consequent development of resistance to antibody therapeutics.

In recent times, RNA interference (RNAi) has emerged as a powerful technology for the development of cancer therapeutics. RNAi is a natural phenomenon resulting in potent post-transcriptional gene silencing produced by double-strand RNAs that occur in most eukaryotes [11**]. This multi-step process is initiated by double stranded 20–30 nucleotide small interfering RNAs (siRNAs) or microRNAs (miRNAs) resulting in highly efficient and sequence-specific knockdown of the targeted gene’s expression. siRNAs can also be expressed from plasmid DNAs as short hairpin RNAs (shRNA) using an RNA polymerase III promoter. RNAi has considerable potential in the treatment of cancer [12**] with several ongoing clinical trials. Nevertheless, several challenges need to be overcome for exogenous siRNA to be widely used as a cancer therapeutic. These include: firstly, lability of siRNAs, resulting in rapid degradation by serum nucleases, secondly, poor membrane permeability of siRNAs limiting cellular uptake, thirdly, need for effective design of active siRNAs to ensure optimal gene silencing activity with minimal ‘off-target’ effects, and fourthly, need to achieve efficient intracellular delivery to target cells in vivo [13,14]. Several promising strategies have been developed for systemic siRNA delivery such as nanoparticles [15], aptamer-siRNA conjugates [16], nanimmune-noliposomes [17], cationic polymer and lipid-based
siRNA complexes [18] etc., but several hurdles, including delivery, still need to be overcome.

In this article we review a versatile tumor-targeted, nanocellular carrier, a bacterially derived minicell, capable of carrying therapeutically significant concentrations of drugs or siRNAs or shRNAs and can be targeted to tumors via attachment of bispecific antibodies (BsAbs) to the minicell-surface O-polysaccharide.

Bacterially derived minicells were first observed, described, and termed “minicells” by Howard Adler and colleagues in 1967 [19]. They are anucleate, nonliving nano-sized cells (400 nm in diameter) and are produced as a result of mutations in genes that control normal bacterial cell division [20,21,22] thereby de-repressing polar sites of cell fission.

Previously we had reported that minicells can be packaged with therapeutically significant concentrations of a range of chemotherapeutics [23] or siRNAs and shRNAs [24]. These minicells selectively targeted to cancer cells via BsAbs, effect highly significant tumor stabilization/regression in a variety of tumor xenograft models, as well as in case studies of dogs with endogenous tumors.

Packaging chemotherapeutic drugs or siRNAs or shRNAs into minicells and tumor targeting via attachment of bispecific antibodies

Minicells are derived from a minCDE-chromosomal deletion mutant of Salmonella enterica serovar Typhimurium (S. Typhimurium) and purified as described previously [23].

A range of chemotherapeutic and molecularly targeted drugs with differing structure, charge, hydrophobicity, and solubility such as doxorubicin, paclitaxel, irinotecan, 5-fluorouracil, cisplatin, carboplatin, gemcitabine, vinblastine, and monastrol, could be readily packaged within the minicells ([23,24]; schematic shown in Figure 1). This was accomplished by co-incubating each drug with intact minicells for a period of time that was optimized for each drug [23].

Drug-packaging in minicells was shown to be dependent on both the concentration of drug in the loading solution, and time of incubation [23]. Drug permeation into

Figure 1

Schematic showing bispecific antibody-targeted, drug/siRNA-packaged minicells. Schematic showing the packaging of anti-cancer drugs or siRNAs into empty minicells and targeting them to tumor cell-surface receptor using bispecific antibodies where one arm of the antibody has minicell-surface O-polysaccharide specificity and the other arm has specificity for the tumor cell-surface receptor for example EGFR.
minicells possibly occurs via non-specific porin channels [25] in the outer membrane. High resolution structural analysis of some bacterial porins suggests that permeation of chemicals through the porin channel is driven by molecular interactions with the surface rather than by free diffusion [26,27]. Detailed studies of porins have revealed charged residues within the channels resulting in a transversal electric field that separates polar and non-polar solutes. Polar solutes are thought to be oriented in the field during permeation which therefore becomes a rapid one-dimensional entry process [28]. Nonspecific movement of hydrophobic solutes across the outer membrane possibly occurs through other channels such as the FadL family of outer membrane proteins [29,30] and OmpW [31]. Thus it is likely that drug entry into minicells is through a facilitated entry process. Leakage of the drug from the minicells was not observed following incubation in buffer or serum for over 24 hours.

Quantitation of drug-packaged in minicells showed that approximately a million molecules of doxorubicin were loaded per minicell. In contrast, other nanoparticles such as liposomes have been shown to package ~10,000 molecules of drug per liposome [32]. Similarly, armed antibodies can conjugate only less than 10 drug molecules per antibody. The potency of anti-tumor effects may depend on the concentration of a drug that is delivered intracellularly within cancer cells.

siRNAs are also packaged in minicells through co-incubation ([24]; schematic shown in Figure 1). shRNA encoding plasmids are initially transformed into the minicell CDE-3. Typhimurium strain and the plasmid segregates into the minicells. When the minicells are purified, they are therefore pre-packaged with therapeutically significant copies of the plasmid. PCR quantitation studies showed that ~50 copies of plasmid or ~14,000 copies of siRNA are packaged per minicell.

Targeting of minicells to tumor cells was achieved using bispecific antibodies in which one arm recognizes the O-polysaccharide component of the minicell surface lipopolysaccharide (LPS) and the other, a cell-surface receptor specific for the mammalian cell to be targeted,
for example EGFR [33] or HER2/neu receptor [34**] on breast and ovarian cancer cells, respectively. Linkage of these two antibodies via their Fc regions was achieved using protein A/G. This ensures absence of complement-mediated in vivo toxicity since the Fc part of each monoclonal antibody is blocked by protein A/G. The tumor targeting antibody can be varied depending on the desired tumor target.

**Mechanism of passive targeting of solid tumors post-i.v. administration of drug/si/shRNA-packaged, BsAb-targeted minicells**

Post-intravenous administration, the BsAb-targeted, drug/siRNA/shRNA-packaged minicells appear to rapidly fall out of the vascular circulation and into the tumor microenvironment possibly due to the leaky vasculature associated with solid tumors, a phenomenon recognized as the enhanced permeation and retention effect (EPR; [35*,36,37,38*,6**]; schematic shown in Figure 2).

These investigators demonstrated that most solid tumors have blood vessels with defective architecture and usually produce extensive amounts of various vascular permeability factors. Most solid tumors therefore exhibit enhanced vascular permeability thus ensuring nutrient and oxygen supply to tumor tissues. There is general agreement that the fenestrations associated with abnormal tumor vasculature can range from 10 nm to 1000 nm or more depending on the tumor type, malignancy, and stage of disease [39*,40,41**]. Additionally, it is also recognized that proliferating cancer cells in tumor tissue compress lymphatic vessels particularly at the center of the tumor [42*] resulting in inefficient drainage of fluid from the tumor center and this coupled with vascular content leakage from tumor vessels causes interstitial hypertension which is thought to reduce the delivery of therapeutic agents to solid tumors [43*,44**].

Although the EPR effect has provided an excellent opportunity for the development of a large number of different nanoparticle delivery systems for cancer therapy, in recent times, new hurdles have been identified. For example, the larger the tumor, the greater the pathophysiological heterogeneity and the central area of these tumors do not appear to exhibit the EPR effect [45*].

This unique phenomenon in solid tumors — the EPR effect — is considered to be a landmark principle in tumor-targeting chemotherapy and is becoming increasingly important for anticancer drug development. For example, Doxil®, a PEGylated (polyethylene glycol-coated) doxorubicin-packaged liposome, DaunoXome®, liposomal daunorubicin, and Abraxane®, a nanoparticle albumin-bound paclitaxel have all been approved for treatment of several different cancers.

**Mechanism of intracellular delivery of drug/si/shRNA via drug/si/shRNA-packaged, BsAb-targeted minicells**

Once in the tumor microenvironment, the BsAb-targeted minicells actively target tumor cells via binding of the tumor receptor-specific antibody to the tumor cell surface receptor. *In vitro* studies demonstrated that the minicells are endocytosed, degraded in late endosomes/lysosomes and the payload (drug or siRNA or shRNA) is released ([23**,24**]; schematic shown in Figure 3). These studies also showed that the payload appears to escape into the tumor-cell cytoplasm in therapeutically significant concentrations although the mechanism of lysosomal membrane escape is not understood. Interestingly, payloads like shRNA encoding plasmids also escape the lysosomal membrane and are translocated into the tumor cell nucleus where shRNA expression occurs. *In vivo* xenograft studies reveal that therapeutically significant concentrations of shRNA are expressed in the tumor xenograft to enable tumor stabilization and even regression. This has been observed following the expression of shRNAs or delivered siRNAs targeting mRNAs encoding multi-drug resistance (MDR1, P-glycoprotein; [46]) and cell cycle-associated proteins implicated in tumor cell proliferation, such as polo-like kinase 1 (PLK1; [47*]), kinesis spindle protein (KSP; [48]) and cyclin-dependent kinase 1 (CDK1; [49]). siRNAs or shRNAs delivered via BsAb-targeted minicells to tumor cells were shown to effect potent G2 arrest and apoptosis both *in vitro* and *in vivo*.

These cell-cycle associated proteins are of interest for cancer therapy, since they are critically involved in cell proliferation. Thus, PLK1 and CDK1 play an essential role in the control of mitotic progression in proliferating cells. KSP provides the propulsive forces required to separate centrosomes during prophase, enabling them to migrate to opposite poles and establish a functional bipolar spindle.

Interestingly, chemotherapeutic payloads that produced these potent anti-tumor effects were achieved with the delivery of amounts of drug that are markedly smaller than those required with systemic delivery of free drug. For example, highly significant anti-tumor effects were observed with ~1875-fold and ~8000-fold lower amounts of Dox and Pac respectively delivered to xenografts via minicells compared with the respective free drugs.

This was evident in dog case studies where rapid tumor regression was evident in two dogs diagnosed with advanced (stage IV) T-cell non-Hodgkin’s lymphoma (NHL) when treated i.v. with *anti-canine-CD3* minicellsDox [23**]. One dog (4 kg) received a total of five doses over 35 days, and the other (40 kg), seven doses over 48 days providing an average of 4.8 μg and 83.4 μg of Dox per dose respectively. Interestingly, conventional chemotherapy in
these dogs would require the administration of 8470 μg and 39,300 μg of Dox per dose respectively (30 mg/m²) as part of multi-drug combination chemotherapy. Thus the treatment with CDsminicellsDox required 1764-fold and 471-fold less Dox per dose respectively, to achieve highly significant tumor regression.

**Treatment of drug resistant tumor via sequential minicell-mediated delivery of siRNA followed by drugs**

Drug-resistance is a major limitation to effective long-term cancer treatment [50]. Proof-of-concept studies in *vivo* to treat drug-resistant cancers via minicell-delivered therapies was demonstrated [24**] using a dual sequential treatment protocol in which the first treatment targets a known drug resistance mechanism (e.g. overexpression of the multi-drug resistance protein MDR1) via BsAb-targeted, si/shRNA-packaged minicells. After allowing for sufficient time to achieve highly significant knockdown of the drug-resistance mediating protein, a second i.v. administration is carried out with BsAb-targeted, cytotoxic drug-packaged minicells where the tumor was known to be highly resistant to the cytotoxic drug. This resulted in a dramatic reversal of drug resistance *in vivo* in various xenograft murine models and even highly aggressive multi-drug resistant uterine cancer xenografts were eliminated with 100% survival in mice [24**] treated with the dual-minicell protocol. These results show that drug resistance can be effectively reversed in previously highly resistant tumor xenografts using minicell-mediated si/shRNA delivery, and that such tumors are then exquisitely sensitive to the second wave of minicells packaged with the cytotoxic drug. This dual treatment strategy may assist in the development of personalized treatment of cancer particularly in the treatment of late-stage cancers where current treatment options are seriously limited.

**Biodistribution of minicells in mouse xenograft studies**

The biodistribution of i.v. administered 125I-labeled-minicells in nude mice with EGFR overexpressing breast cancer (MDA-MB-468) xenografts revealed that at two hours post-treatment, ~30% of the EGFR minicells were localized in the tumor [23**]. This was in contrast to ~3% of the anti-EGFR/O-polysaccharide BsAb that accumulated in the tumor at two hours post-i.v. administration. By 6 and
24 hours, ~4.6% and ~0.5%, respectively, of specifically targeted EGFRminicells remained in the tumors. This data suggested that the EGFRminicells rapidly extravasate from the tumor-associated leaky vasculature and are trapped in the tumor microenvironment likely due to the EPR effect.

Biodistribution of micell-packaged drug following i.v. administration of EGFRminicellsDox or free Dox to nude mice with breast cancer xenografts (tumor volume between 140 and 170 mm³) showed that at six hours, ~30% of the Dox dose administered by the EGFRminicellsDox was found in the tumors, as compared to only ~1% of free Dox.

Similar results were observed in mice with much larger tumors (400–600 mm³), in which ~28.1% of the Dox dose in EGFRminicellsDox was found in the tumors at six hours, as compared to only ~0.21% of that in non-targeted, minicellDox, and ~1.8% for free Dox. Plasma concentration of Dox at both time-points was undetectable.

At six hours, biodistribution to the liver, spleen, and lungs was higher with the EGFRminicellsDox and minicellsDox compared to free Dox and showed a rapid decrease by 24 hours.

Thus targeted micell delivery provides at least a 30-fold enrichment in tumor drug delivery.

**Immune and cytokine/interferon response to drug/si/shRNA-packaged, bispecific antibody targeted micelles**

In mouse xenograft studies there was no evidence of any toxicity [23**,24**] despite repeat dosing of BsAb-targeted, drug/si/shRNA-packaged micelles (15–20 doses have been administered in several mouse xenograft experiments). This was evident by the lack of a febrile response, weight loss, or skin/fur changes etc. in the murine xenograft model. Importantly, micelles were well tolerated with no adverse side-effects or deaths in any of the treated animals.

Since micelles are of bacterial origin, it is necessary to be cautious with systemic administration as bacterial products are known to elicit potent inflammatory responses activated by Toll-like receptors [51]. A micell purification procedure to eliminate free endotoxin and free bacterial components was developed to minimize the potential for toxic side effects [23**,24**].

Recent studies have questioned whether the anti-tumor effects of siRNA are due to specific knockdown of target mRNA or, rather, are non-specific and merely due to siRNA-mediated activation of the innate immune response [52*]. To address this issue, HCT116 xenograft experiment was carried out [24**] with mice being treated with EGFRminicellssiPlk1, EGFRminicellsiiNonS or saline (controls). The treatments were administered four times, 24 hours apart. Groups of mice were sacrificed at 4 (early response) and 24 (late response) hours post-treatment and serum was collected and assayed for mouse and human Type I and II interferons, and for inflammatory cytokines produced by cells of the immune system. These cytokines are known to be elicited following siRNA delivery in mice [52*].

The results showed that at both time points, human interferon and cytokine levels were low (range of ~10 to ~30 pg/ml) and the levels elicited by EGFRminicells-siPlk1 were indistinguishable from those observed with saline or EGFRminicellsiiNonS. In contrast, mouse interferon and cytokine responses were higher, being in the range of ~50 to ~150 pg/ml for IFN-α, IFN-β and IFN-γ, ~300 to ~1200 pg/ml for TNF-α, and ~10 to ~100 pg/ml for IL-6. Both IFN-γ and IL-6 showed a spike at four hours but returned to baseline (saline control level) by 24 hours. Moreover, the TNF-α response was greater at 24 hours than at four hours. Importantly, however, there were no significant differences in any of the interferon or cytokine levels in mice treated with EGFRminicells-siPlk1 compared to EGFRminicellsiiNonS. The significant rise in mouse TNF-α and IL-6 at 24 hours may be attributed to the micell vector itself since it is well known that systemic administration of bacterial cells does activate the Toll-like receptors resulting in TNF-α and IL-6 responses. However, with micell administrations in mice, these responses have been self-limiting and have not resulted in toxicity to the mice.

The above results indicate that the potent anti-tumor effects observed in mouse xenografts are unlikely to be due to interferon or inflammatory cytokine responses, since treatment with EGFR-targeted iiNonS-packaged micelles did not produce any anti-tumor effects despite similar interferon and cytokine responses as those in mice treated with EGFRminicellsiiPlk1.

**Conclusions**

The micell vector has quite remarkable properties in being able to package, in therapeutically significant concentrations, a range of different drugs, siRNAs or shRNAs. Each of these loadings is carried out following a simple co-incubation of the therapeutic moiety with the intact micelles in an appropriate buffer. The size of the micell (~400 nm) ensures retention within the reticuloendothelial system and prevents it from penetration into normal tissues which is a hurdle faced by drug-conjugated monoclonal antibody therapeutics. For example, extravasation of Cetuximab® (anti-EGFR monoclonal antibody) into the skin causes severe skin toxicity. The micell vector is too large to enter into normal skin, gastrointestinal or liver tissue although these organs also exhibit leaky vasculature. However, the fenestrations in these normal tissues range from 50 nm to 100 nm, thus preventing micell-based
therapeutics from entering into these tissues. This was demonstrated by MacDiarmid and colleagues in a rhesus monkey trial (n = 36) where anti-monkey EGFR monoclonal antibody-targeted, doxorubicin-packaged minicells were administered i.v. in monkeys in a dose-escalation toxicity trial. Five repeat doses (one per week) were administered. The results showed complete absence of toxicity in the monkeys and inflammatory cytokine and immune responses were also normal (unpublished data).

These results paved the way for the first-in-man, Phase I/IIa multi-center clinical trial in cancer patients and this trial is currently in progress.

Acknowledgement

We thank Martin Hale, Animated Biomedical Productions, Sydney, for the artwork in Figures 1–3.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest
•• of outstanding interest


10. Bardelli A, Siena S: Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol 2010, 28:1254-1261. Discusses the evidence that metastatic colorectal cancers respond differently to EGFR-targeted agents and that the tumor-specific response has a genetic basis The role of oncogenic activation of EGFR downstream effectors on response to therapy is also discussed.


Study shows the mechanism by which an antibiotic like ampicillin penetrates a bacterial membrane protein channel and how the process of entry is facilitated by complementary charges between the antibiotic and the channel architecture.


Discusses three-dimensional crystal structures of porins and suggests possible mechanisms by which porins may be able to discriminate between polar and non-polar solutes.


Describes the possible mechanisms by which the FadL family of membrane-associated proteins may transport hydrophobic compounds into bacterial cells.


Discusses progress made with liposomal carriers for breast cancer treatment and suggests future improvements with immunoliposomes.


Shows that HER-2/neu was found to be amplified from 2- to greater than 20-fold in 30% of the 189 primary human breast cancers investigated. Amplification of the HER-2/neu gene was a significant predictor of both overall survival and time to relapse in patients with breast cancer.


Early studies showing that accumulation of macromolecules in tumor tissue was likely due to enhanced permeability and retention effect.


Discusses molecular mechanisms of factors related to the EPR effect, the unique anatomy of tumor vessels, limitations and techniques to avoid such limitations.


Early studies analyzing the functional limits of transvascular transport of macromolecules and its modulation by the tumor microenvironment.


Using a range of microscopy techniques, tumor architecture and the morphology of defective blood vessels along with the size of fenestrae are clearly shown in different tumors.


Provides evidence that proliferating cancer cells cause intratumour vessels to compress and collapse.


Reviews evidence supporting a hypothesis that certain antiangiogenic agents can transiently “normalize” the abnormal structure and function of tumor vasculature to make it more efficient for oxygen and drug delivery.


Reviews current knowledge on the tumor microenvironment generated barriers which limit extravasation of drugs and focuses on modalities for overcoming these barriers using multi-functional polymeric carriers.


Provides the first clinical proof that modulations of vascular pathophysiology can uniquely accomplish enhanced tumor selective delivery of polymeric drugs and thus yielded better clinical outcome.


Reviews the biology of polo-like kinases and its potential as an anti-cancer target.


This study cautions regarding interpretations of therapeutic benefits following siRNA delivered in vivo in mice since unmodified siRNAs can activate the innate immune response and elicit nonspecific therapeutic benefits.