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Extra View

Bacterially-Derived Nanocells for Tumor-Targeted Delivery of Chemotherapeutics and Cell Cycle Inhibitors

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minicell, nanovector, nanoparticle, cancer, molecularly targeted drugs, cytotoxic drugs

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ABSTRACT

Chemotherapeutic drug therapy in cancer is seriously hampered by severe toxicity primarily due to indiscriminate drug distribution and consequent collateral damage to normal cells. Molecularly targeted drugs such as cell cycle inhibitors are being developed to achieve a higher degree of tumor cell specificity and reduce toxic side effects. Unfortunately, relative to the cytotoxics, many of the molecularly targeted drugs are less potent and the target protein is expressed only at certain stages of the cell cycle thus necessitating regimens like continuous infusion therapy to arrest a significant number of tumor cells in a heterogeneous tumor mass. Here we discuss targeted drug delivery nanovectors and a recently reported bacterially-derived 400 nm sized minicell that can be packaged with therapeutically significant concentrations of chemotherapeutic drugs, targeted to tumor cell surface receptors and effect intracellular drug delivery with highly significant anti-tumor effects in vivo. We also report that molecularly targeted drugs can also be packaged in minicells and targeted to tumor cells with highly significant tumor growth-inhibition and regression in mouse xenografts despite administration of minute amounts of drug. This targeted intracellular drug delivery may overcome many of the hurdles associated with the delivery of cytotoxic and molecularly targeted drugs.

PRINCIPLE APPROACHES TO IMPROVE THE THERAPEUTIC INDEX OF ANTI-CANCER DRUGS

Current chemotherapeutic drugs are constrained by severe systemic toxicity due to indiscriminate drug distribution and narrow therapeutic indices. Dose-limiting toxicity, rapid clearance necessitating frequent administration of high doses of chemotherapeutics, and drug resistance prevents a satisfactory clinical response.[1] Consequently over the past decade a significant global effort has focused on the discovery and development of molecularly targeted drugs.

For example, many tumor-associated mutations result in the abnormal regulation of protein kinases involved in progression through the cell division cycle. The cyclin-dependent kinase (CDK) family has received special attention due to their central role in cell proliferation and upregulation in many human cancers. A plethora of small-molecule CDK inhibitors have been characterized and some of them are currently in clinical development.[2,3] Other serine-threonine protein kinases such as the Aurora proteins (mostly Aurora A and B) or Polo-like kinases (Plk1) are also receiving increased attention as putative cancer targets.[4-7]

Another approach to reduce collateral damage to normal cells is to encapsulate the chemotherapeutic drug in a nanovector and target it to the tumor microenvironment. This approach is exemplified by Doxil (doxorubicin encapsulated in pegylated liposomes: Doxil, Alza Pharmaceuticals; Caelyx, Schering-Plough).[8,9] These formulations have a long circulation time, and the liposomes eventually extravasate through the abnormally permeable vessels (passive targeting) characteristic of many tumors and accumulate in tumor tissue due to the poor lymphatic drainage. This phenomenon is termed the enhanced permeation and retention effect (EPR)[10,11] and is a consequence of the dysregulated nature of tumor angiogenesis, resulting in endothelial fenestrations and hyperpermeability. Nanovector-based passive targeting of tumor interstitium is thought to occur via convective and diffusive transport within the vasculature.[12]

Once concentrated in the tumors, the liposomes breakdown and deliver high concentrations of the drug to the tumor. However, despite the significant reduction in toxicity compared to free drug administration, pegylated liposomes still suffer from side effects such as skin toxicity including hand-foot syndrome and mucositis,[13-15] myelosuppression

and myocardial damage.[16] Other nanovector systems include synthetic biodegradable nanoparticles,[12,17] polymer micelles[18,19] and several others.[20] 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.

A third approach is to encapsulate a drug in a nanovector and target the package intracellularly within tumor cells. Active targeting requires a ligand on the vector directed against a receptor at the tumor cell surface. The ligand-receptor interaction results in endocytosis of the nanovector and intracellular release of the drug. This approach potentially avoids the toxic side effects of non-targeted drug carriers and achieves a higher concentration of drug within cancer cells resulting in a significant improvement in the therapeutic index. Such a strategy has been explored for a number of different nanovectors such as immunoliposomes,[21-26] polymeric nanoparticles,[27-30] immunomicelles[31] and nanoparticle-aptamer bioconjugates.[32] A number of different over-expressed tumor cell-surface receptors have been utilized for nanovector targeting such as EGFR,[33] HER2/*neu*,[34] folic acid,[35-40] asialoglycoprotein,[41] prostate specific membrane antigen,[42] transferrin[43,44] and others.

BACTERIALLY-DERIVED MINICELLS FOR TARGETED DELIVERY OF CHEMOTHERAPEUTIC DRUGS

We recently described another approach for targeted and intracellular delivery of chemotherapeutic drugs.[45] The approach relies on using a bacterially-derived minicell to package chemotherapeutic drugs and target them to tumor cells in vivo via bispecific antibodies where one arm of the antibody attaches to the O-polysaccharide component of the lipopolysaccharide (LPS) found on the minicell surface and the other arm can be directed to any tumor cell-surface receptor.

Minicells were first observed and described by Howard Adler and colleagues in 1967, who also coined the term "minicell".[46] They are anucleate, non-living nano-sized cells (400 nm in diameter) and are produced as a result of mutations in genes that control normal bacterial cell division[47-49] thereby de-repressing polar sites of cell fission. To more accurately describe the particle, we propose the new term "nanocell" instead of "minicell" since the size of the vector is 400 nm and is not in the mini- or micro-range.

It was demonstrated that a range of chemotherapeutic drugs with differing structure, charge, hydrophobicity and solubility such as doxorubicin, paclitaxel, irinotecan, 5-fluorouracil, cisplatin, carboplatin and vinblastine, could be readily packaged within the minicells.[45] Interestingly, the method of drug packaging was as simple as coincubating minicells with each drug for as little as 2 hrs. Hydrophobic drugs required small concentrations of cosolvents in the incubation reaction to ensure that the drug remained in solution during coincubation with minicells. The functional integrity of minicells was not compromised with the use of small concentrations of solvents like DMSO, Cremophor or ethanol. The solvent was then easily washed away prior to attaching bispecific antibodies to drug-packaged minicells. The drug-packaged minicells did not leak drug when incubated in buffer or serum for over 24 hrs. Drug-packaging in minicells was shown to be dependent on both the concentration of drug in the loading solution, and time of incubation.[45] Drug loading of minicells possibly occurs by diffusion down a concentration gradient with entry via non-specific porin channels[50] in the outer membrane. 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 fast one-dimensional diffusion process.[51] Nonspecific diffusion of hydrophobic solutes across the outer membrane is thought to occur through other channels such as the FadL family of outer membrane proteins[52,53] and OmpW.[54] In addition to providing a barrier to solute entry, bacterial membranes contain a plethora of transport proteins involved in exporting solutes across their phospholipid bilayer-membranes, against a concentration gradient.[55] Thus, retention of drug in minicells, after loading, is possibly due to the metabolic inactivity that results from their lack of bacterial genome.

CONCENTRATION OF CHEMOTHERAPEUTIC DRUGS PACKAGED IN MINICELLS

It was discovered that an unprecedented concentration of 1 million to 10 million drug molecules can be packaged within a minicell.[45] In contrast, other nanovectors such as liposomes have been shown to package ~10,000 molecules of drug within each liposome.[56] Similarly, armed antibodies can conjugate only four to ten drug molecules per antibody. The potency of observed anti-tumor effects[45] may depend on the concentration of a drug that is delivered intracellularly within cancer cells.

PASSIVE AND ACTIVE TARGETING OF DRUG-PACKAGED MINICELLS TO TUMOR CELLS IN VIVO

The biodistribution of i.v. administered ^{125}I-labeled-minicells in nude mice with EGFR over-expressing breast cancer xenografts revealed that at 2 hrs post-treatment ~30% of the ^{EGFR}minicells were localized in the tumor.[45]

This rapid appearance of ^{EGFR}minicells within the tumor microenvironment suggests extravasation of ^{EGFR}minicells from the circulation due to the tumor-associated leaky vasculature. There is considerable debate regarding the pore size or fenestrations associated with abnormal tumor vasculature and the size limitation of a nanovector to enable passive targeting. For example, some of the data suggests pore cutoff size ranging from 200 nm to 1.2 μm,[57] or from 100 nm to 780 nm,[58] or from 100 nm to 2 μm depending on the tumor type, malignancy, and stage of the disease.[59] Apart from size, extravasation of nanovectors into the tumor interstitium relies on a large number of physical factors and this has been described in an excellent review (ref. 60).

Following passive targeting, the BsAb-targeted minicells achieve active targeting of the tumor cells via receptor engagement, endocytosis, intracellular breakdown of drug-packaged minicells and drug delivery.[45]

Biodistribution studies in tumor-bearing mice showed that within 6 hrs post-i.v. administration of ^{EGFR}minicells$_{Dox}$, ~30% of the injected dose of Dox was found in the tumor.[45] Thus targeted minicell delivery provides at least a 30-fold enrichment in tumor drug delivery.

BsAb linkage to the surface of minicells via the cell-surface exposed O-polysaccharide is extremely robust; a factor that likely accounts, in part, for the efficiency of this cell-targeting approach. As a consequence, targeted minicell-mediated drug-delivery was shown to result in highly significant inhibition and even regression of tumor

growth in vivo, in mice with either human breast, ovarian, leukemia and lung cancer xenografts.[45] Interestingly, 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 ~1,875-fold and ~8,000-fold lower amounts of Dox and Pac respectively, delivered to xenografts via minicells compared with the respective free drugs.[45]

Although it has been shown that the abnormal tumor microenvironment is characterized by interstitial hypertension, and that this phenomenon may limit access of anti-cancer antibody therapeutics, this does not appear to be an absolute barrier at least in the rodent xenograft model as is exemplified by minicells (both in mouse xenografts and in dogs diagnosed with non-Hodgkin's lymphoma),[45] immunoliposomes[61] and antibody conjugated to Quantum Dots.[62]

TARGETED MINICELL-MEDIATED DRUG DELIVERY IN DOGS WITH NON-HODGKIN'S LYMPHOMA

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 targeted $minicells_{Dox}$.[45] 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 8,470 μg and 39,300 μg of Dox per dose respectively (30 mg/m^2) as part of multi-drug combination chemotherapy. Thus the treatment with $^{CD3}minicells_{Dox}$ required 1,764- and 471-fold less Dox per dose respectively, to achieve highly significant tumor regression.

TARGETED DELIVERY OF MOLECULARLY TARGETED DRUGS TO TUMOR CELLS VIA THE MINICELL VECTOR

While many of the drugs being developed against molecular targets appear promising as anticancer drugs, several such candidates are faced with some serious hurdles such as low pK and potency[63] which necessitates high drug doses to achieve a therapeutic effect. This results in dose limiting toxicities. Many of the inhibitors of molecular targets are also found to be reversible. Absence of covalent binding to the molecular target may result in weak activity and again necessitates high drug dosing to achieve therapeutic effect. Additionally, some of the targets only appear at certain stages of the cell cycle and since the tumor cells in a patient are a heterogeneous population, such drugs would require dosing for prolonged periods of time e.g., continuous infusion,[64] in order to ensure that the plasma drug concentration is maintained over a sufficient period of time to catch cancer cells when the target molecule is expressed. Such a regimen of treatment may result in serious toxic side effects and development of drug resistance.

The kinesin spindle protein (KSP), also termed kinesin-5 or Eg5, is a microtubule motor protein that is essential for the formation of bipolar spindles and the proper segregation of sister chromatids during mitosis.[65,66] Inhibitors of KSP, like monastrol, cause the formation of monopolar mitotic spindles, activates the spindle assembly checkpoint, and arrests cells at mitosis, which leads to subsequent cell death.[65,67-70] Several structurally unrelated chemical compounds that function as mitotic inhibitors have been identified[71-74] and several are in clinical studies. Monastrol, the first Eg5 inhibitor identified, induces mitotic arrest without affecting interphase microtubules, and has been a useful tool for dissecting the mechanisms underlying spindle assembly. However, its clinical potential is limited because of its weak Eg5 inhibitory activity (IC_{50}, 14 μm).[67]

In order to determine whether a targeted minicell vector could package and enhance the therapeutic index of a weak molecularly targeted drug like monastrol, we carried out an experiment where a human breast cancer xenograft was established in nude mice and treated with EGFR-targeted, monastrol-packaged minicells ($^{EGFR}minicells_{Monastrol}$) and compared the anti-tumor effects with the administration of free monastrol. Minicells were generated from an *S. Typhimurium min*CDE- mutant strain and were purified using gradient centrifugation/filamentation/filtration/endotoxin removal procedure as previously described.[45] Monastrol (Sigma-Aldrich, St. Louis, MO, USA) was packaged into the minicells by creating a concentration gradient of monastrol between the extracellular and intracellular compartments and drug concentration packaged within minicells was determined by LC-MS/MS. An anti-O-polysaccharide/anti-human EGFR BsAb was constructed by linking the Fc parts of the two respective monoclonal antibodies (MAbs) with protein A/G.[45] The anti-EGFR MAb was selected because the target MDA-MB-468 cells are known to over-express EGFR on the cell surface. The BsAb was used to coat the monastrol-packaged minicells ($minicells_{Monastrol}$) via the anti-O-polysaccharide linkage to result in EGFR-targeted, $minicells_{Monastrol}$ ($^{EGFR}minicells_{Monastrol}$). LC-MS/MS results showed that 10^8 $^{EGFR}minicells_{Monastrol}$ carried ~520 ng of the drug. Balb/c *nu/nu* mice were purchased from Animal Resources Centre (Perth, WA, Australia), and all animal experiments were performed in compliance with the guide of care and use of laboratory animals and with Animal Ethics Committee approval. Human breast adenocarcinoma cells (MDA-MB-468, ATCC; human mammary epithelial cells) were grown and established as a xenograft between the shoulder blades of each mouse and tumor volume was measured twice a week as previously described.[45] Eighteen days post-implantation, the tumors reached ~80mm^3, and mice were randomized to seven different groups (n = 8 per group).

$^{EGFR}minicells_{Monastrol}$ treatment of the mice was compared with non-targeted $minicells_{Monastrol}$ and free monastrol treatments as shown in Figure 1A. The results showed a highly significant anti-tumor effect with $^{EGFR}minicells_{Monastrol}$ treatment (G_7 *vs* G_1 to G_5; $p < 0.0004$) while free monastrol and non-targeted $minicells_{Monastrol}$ showed no anti-tumor effects. Failure to see tumor growth-inhibition with $minicells_{Monastrol}$ corroborates previous results[45] which indicate that BsAb-mediated targeting is essential. The highly significant anti-tumor effects with $^{EGFR}minicells_{Monastrol}$ was despite a 240-fold lower dose of monastrol compared to free drug treatment (compare groups G_6 or G_7 vs. G_2).

Thymidylate synthase (TS) inhibitors form another class of new targeted drugs in development.[75-78] This effort has been necessary since first-line cytotoxic drugs for metastatic colorectal cancer such as 5-Fluorouracil, which is a TS inhibitor,[79] suffers from drawbacks of severe toxicity and rapid development of drug resistance. TS expression has been reported to be cell cycle dependent[80,81] and its activity levels are higher in proliferating cells than in non-proliferating cells.[82]

OSI-7904L is the liposomal formulation of OSI-7904 [(S)-2-[5-[1,2-dihydro-3-methyl-1-oxobenzo[*f*]quinazolin-9-yl)methyl]amino-1-oxo-2 isoindolynl]-glutaric acid], a potent selective non-competitive TS inhibitor. It consists of small (20-80 nm) unilamellar vesicles containing OSI-7904 within their aqueous cores. The liposomal

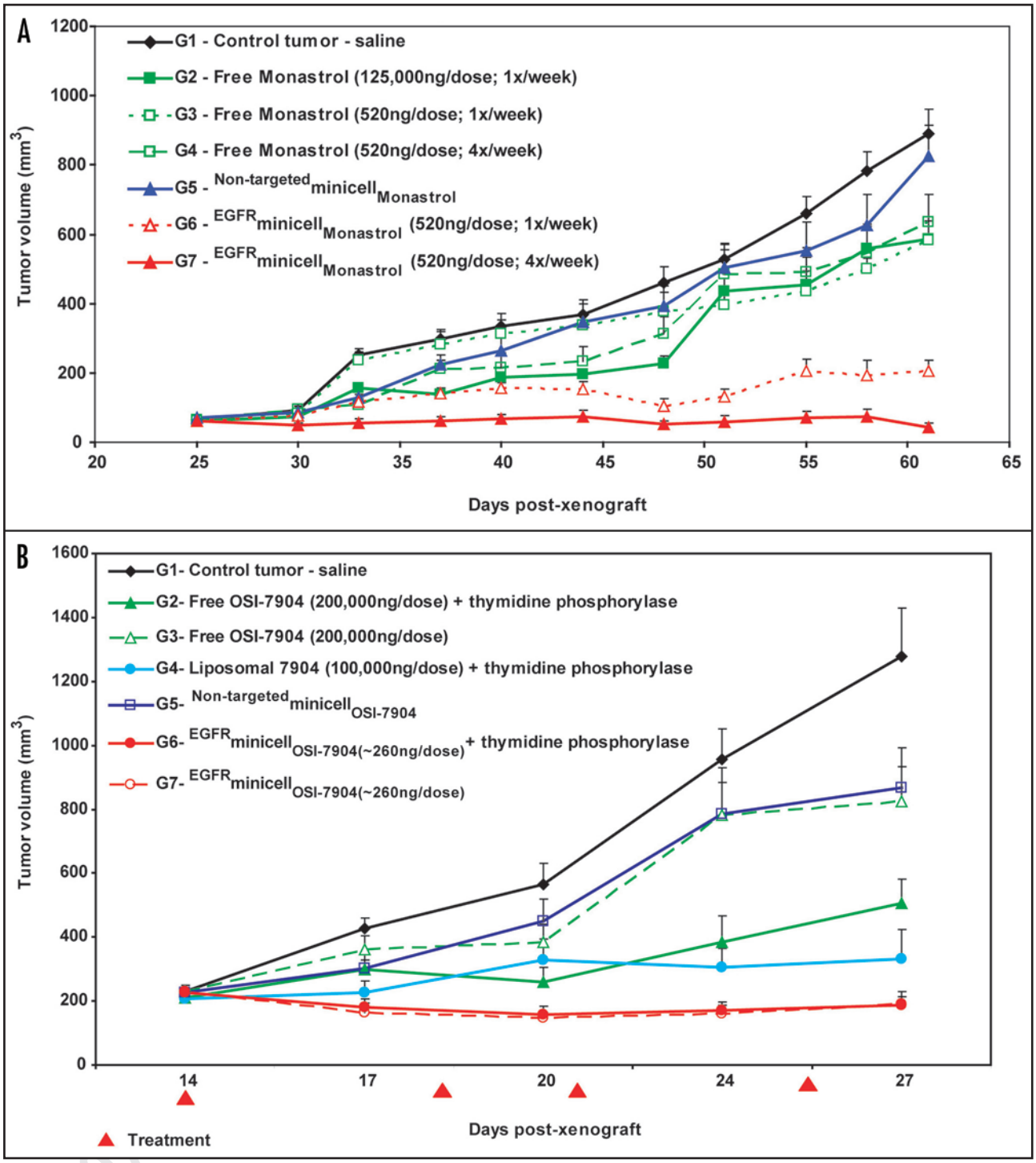

Figure 1. Inhibition/regression of tumor growth in mice treated with receptor-targeted minicells packaged with molecularly targeted drugs. (A) Human breast cancer (MDA-MB-468) xenografts in Balb/c *nu/nu* mice (n = 8 per group) treated with free monastrol (G_2 to G_4), $^{non\text{-}targeted}minicells_{Monastrol}$ (G_5) or $^{EGFR}minicells_{Monastrol}$ (G_6 and G_7). All doses were administered via a tail vein injection. All minicell treatments received 10^8 minicells per dose. The result shows mean tumor volume (*y*-axis) in various groups of mice *vs.* days post-establishment of tumor xenografts (*x*-axis). (B) Human colon cancer (HT29) xenografts in Balb/c *nu/nu* mice (n = 8 per group) were administered i.v. with the various treatments shown in the figure. All minicell treatments received 10^8 minicells per dose. Treatment days are shown below the x-axis (red triangles). Error bars for both graphs; ± SEM.

encapsulation greatly increases plasma, tissue and tumor exposure to OSI-7904.[83]

We carried out a xenograft study in nude mice (*n* = 8 mice per group) to compare the anti-tumor effects of free OSI-7904, liposomally encapsulated OSI-7904 (OSI7904L; both a kind gift from Neil Gibson, OSI Pharmaceuticals Inc., Melville, NY, USA) and either EGFR-targeted or non-targeted minicells packaged with OSI-7904 (designated $^{EGFR}minicells_{OSI\text{-}7904}$ and $minicells_{OSI\text{-}7904}$ respectively). Since circulating levels of thymidine in rodents is relatively high,[84] it can ameliorate the cytotoxicity of TS inhibitors. To bypass the thymidine

salvage pathway, efficacy studies in rodents are often performed by intraperitoneal (i.p.) administration of thymidine phosphorylase, which lowers circulating thymidine levels by metabolizing thymidine to thymine and deoxyribose-5-phosphate. Therefore, in this study we also included additional treatment groups of free OSI-7904, OSI-7904L and ^{EGFR}minicells$_{OSI-7904}$ where thymidine phosphorylase was administered i.p. (Fig. 1B, Groups 2, 4 and 6 respectively).

The study was carried out in HT29 human colon cancer xenografts and tumors were allowed to grow to 200 mm^3 to 250 mm^3 before the various treatments were administered i.v. via the tail vein.

The results showed (Fig. 1B) a highly significant anti-tumor effect following ^{EGFR}minicells$_{OSI-7904}$ treatment (G_7 mice). Additionally, the anti-tumor effects were identical in mice treated with ^{EGFR}minicells$_{OSI-7904}$ or ^{EGFR}minicells$_{OSI-7904}$ with thymidine phosphorylase (G_7 and G_6 mice respectively). This is in contrast to the groups treated with free OSI-7904 where some reduction in tumor growth rate was only seen in the thymidine phosphorylase pre-treated group (G_2 *vs* G_3 mice). Presumably, since the drug was encapsulated in the minicells and only released intracellularly, the ^{EGFR}minicells$_{OSI-7904}$ treatment may not be subject to circulating levels of thymidine and hence the thymidine salvage. OSI-7904L formulation was effective in stabilizing tumour growth but not as effective as ^{EGFR}minicells$_{OSI-7904}$. More importantly, ^{EGFR}minicells$_{OSI-7904}$ was more effective (G_7 mice, 260 ng drug/dose) at a dose that was ~385-fold less than the liposomal formulation OSI-7904L (G_4 mice, 100,000 ng drug/dose). The minicell delivery vector thus dramatically increased the therapeutic index.

AMELIORATION OF TOXICITY USING MINICELLS AS A TARGETED DELIVERY VECTOR

Minicells are stable, and can be targeted to cancer cells in vivo with high specificity and can, thus, be delivered in high concentration in vivo without toxicity. This was evident by the lack of a febrile response, weight loss, or skin/fur changes etc. in the murine xenograft model. Importantly, minicells were well tolerated with no adverse side-effects or deaths in any of the actively-treated animals, despite repeat dosing.

Since minicells 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.[85] A minicell purification procedure to eliminate free endotoxin and free bacterial components has been developed to minimize the potential for toxic side effects.[45]

Interestingly, in the two dogs and three pigs studied only the latter demonstrated a very short lived and mild TNFα response.[45] This contrasts with TNFα levels as high as 20,000 pg/ml after i.v. injection of 2 μg/kg LPS in pigs.[86] Neither a TNFα response nor an increase in IL-6, another inflammatory cytokine, was observed in the tumor-bearing dogs despite repeat i.v. administration of high doses of minicells. Additionally, neither the pigs or the dogs showed adverse effects in terms of their hematological indices, serum chemistries, body weight, temperature, urine analysis, food intake or growth.

O-polysaccharide is the main antigen exposed on the minicell surface and it is well recognized from the large body of work on bacterial vaccines that during natural or experimental infections with Gram negative bacteria, anti-O-polysaccharide humoral antibody response is predominant[87-89] and is a T-cell independent response.[90,91] Yet surprisingly, the anti-O-polysaccharide antibody titers remained at background levels despite repeat administration of the ^{CD3}minicells$_{Dox}$.[45] It is well recognized that in late stage cancer, the immune system is partly compromised[92,93] and this may account for the absence of an anti-O-polysaccharide antibody response in these dogs. Although case studies in the two dogs is very encouraging, the data is anectodal and further dog clinical trial studies would be required.

CONCLUDING REMARKS

These anucleate minicells can be readily produced in high yield from both Gram+ and Gram- organisms and purified free of parental bacteria, membrane blebs, nucleic acids, cellular debris and free endotoxin, using commercially available filters.

In recent work, we have tested the Dox-packaged, monkey-EGFR-targeted minicells in 60 rhesus monkeys (two full toxicology trials) and these minicells were administered via the i.v. route in five repeat doses (weekly) and in escalating concentrations. Extensive analysis of various parameters revealed no signs of toxicity despite doses of minicells as high as 2 x 10^{10} (manuscript in preparation). This data is highly encouraging for the potential progression into human studies.

The use of molecularly targeted minicell nanovectors affords multiple potential advantages over conventional cancer therapy, some of which include; (a) the ability to easily package therapeutically significant concentrations of different cytotoxic or molecularly targeted drugs into the minicell, (b) the ability to readily attach different BsAbs on the minicell surface in order to target a receptor found on the surface of a tumor cell i.e., ability to target many different solid tumors, (c) the ability to deliver the drug intracellularly within a tumor cell and without leakage of drug from the vector during systemic circulation, (d) the ability to provide a dramatic increase in the therapeutic index with minimal to no toxic side effects. This also enables the use of potent cytotoxics that have failed toxicity trials but have the potential to be highly potent anti-cancer drugs, (e) minicells are easily purified to homogeneity and the long standing pharmaceutical industry experience in bacterial fermentation and production of bacterial vaccines shows that such processes are relatively cheap. Currently there is considerable international pressure to make life-saving medicines like anti-cancer therapies more affordable[94] but the very high cost of goods to make such medicines e.g., monolonal antibodies, makes it very difficulty for pharmaceutical companies to meet such demands. The minicell nanovector has the potential to significantly reduce cost of goods particularly since a minicell-based anti-cancer therapeutic would carry tiny fractions of the drug and the targeting antibody compared to free drug or free antibody therapy, (f) intra-cytoplasmic drug delivery may also partly overcome obstacles in anticancer therapy such as multi-drug resistance.

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