

Increasing the Tumoricidal Activity of Daunomycin-pHPMA Conjugates Using Vitamin B₁₂ as a Targeting Agent

Gregory Russell-Jones^{1,3,*}, Kirsten McTavish¹, John McEwan¹ and Bruce Thurmond²

¹Access Pharmaceuticals Australia Pty Ltd. Roseville, NSW, Australia 2069; ²Access Pharmaceuticals Inc, Dallas, Tx; ³23 Greenfield Ave, Middle Cove, NSW 2068 Australia

Abstract: Many different types of tumour have previously been shown to over-express cell-surface receptors involved in the uptake of Vitamin B₁₂ (VB₁₂). We have examined the potential of using VB₁₂ as a targeting agent for the delivery of pHPMA-conjugated daunomycin in four murine tumour models. VB₁₂-targeted-daunomycin-HPMA conjugates were found to increase both the number of survivors and the survival time of tumour-bearing mice. The data indicate that VB₁₂ may be highly effective in enhancing the efficacy of polymer-bound cytotoxins, particularly in those tumours that over-express receptors involved in vitamin B₁₂ uptake.

Keywords: Vitamin B12, cyanocobalamin, cancer targeting, HPMA, daunomycin.

INTRODUCTION

Many workers have endeavored to increase the circulating half-life of cytotoxic drugs used in cancer treatment by linking the drugs to polymers. This has the advantage of reducing the toxicity of the drug, as well as the additional advantage of targeting the altered tumour vasculature and restricted/occluded lymphatic drainage often found in tumour masses, a phenomenon called "the enhanced permeability and retention (EPR) effect" [1]. Polymer-based targeting has been used with doxorubicin conjugated to *N*-(2-Hydroxypropyl) methacrylamide (HPMA) copolymer, which was used in Phase I/II clinical trials to varying effects [2-7]. Less success was found with HPMA conjugates of paclitaxel or camptothecin [4, 5], however encouraging results have been found from Platinum-HPMA chelates [8-13].

Whilst linkage of small cytotoxic molecules to a polymer such as pHPMA increases the amount of drug that reaches and is retained by some tumour tissue, the polymer-bound chemotherapeutic does not preferentially accumulate equally in all tumour types [7], or in small-non-vascularized metastases, nor does it result in increased rates of internalization of the chemotherapeutic into the tumour cells. Additionally, the increased size of the drug-polymer conjugate can restrict the exit of the conjugate from the vasculature. Targeted chemotherapy for cancer treatment offers potential advantages in tumour treatment due to greater specificity of delivery, and the ability to utilize mechanism for vascular transcytosis thus increasing the amount of drug which reaches the extracellular

space, and subsequently increasing the uptake of the cytotoxin into the tumour cells. One problem with this approach is, however, to identify generic markers that are over-expressed on the surface of tumour cells but not on normal tissue. In order to overcome both of these restrictions we have been examining the potential of using targeting agents, such as vitamins to increase the uptake and retention of polymer-bound cytotoxins into the tumour mass.

Previous studies by various workers have shown that many aggressive tumours over-express surface receptors involved in the uptake of vitamin B₁₂ [14-31]. The degree of over-expression has been found to correlate with the stage of tumour growth, with the highest levels found on stage IV carcinomas [1, 25, 30, 31]. Thus increased uptake of the vitamin B₁₂-targeted Rhodamine-pHPMA copolymer has been observed in Colo-26 (Colon carcinoma), P815 (Mastocytoma), M109 (Lung), RENCA and RD995 (Renal), and 4T1, JC and MMT06056 (Breast) cancers. Little or no uptake was observed in BW5147 (lymphoma), B16 (melanoma), LL-2 (Lung), CHT-116 (Colon), L1210 (leukaemia), and Ov2008 and ID8 (Ovarian) cancers. Over-expression of surface receptors involved in the uptake of vitamin B₁₂ has enabled several workers to successfully image malignant, but not benign tumours, through the use of imaging agents such as ¹¹¹In-DTPA-vitamin B₁₂ [25-27, 32].

In the studies reported here we have endeavored to combine the passive tumour targeting obtainable with polymer-bound daunomycin (Dnm) with the active tumour targeting of vitamin B₁₂ and have been able to increase the effectiveness of treatment of the tumours with polymer-bound Dnm when compared to treatment with free Dnm. For these studies Dnm was linked to the

*Address corresponding to this author at the Access Pharmaceuticals Australia Pty Ltd. Roseville, NSW, Australia 2069, 23 Greenfield Ave, Middle Cove, NSW 2068 Australia; Tel: 61-(2)-94176707; Fax: ?????????????????????; E-mail: mentor127@optusnet.com.au

polymer *via* a well established tetrapeptide biodegradable linker, Gly-Phe-Leu-Gly, which permits release of drug by the action of lysosomal enzymes [4]. These studies have shown that the effectiveness of passive targeting is highly variable between tumours and that the vitamin B₁₂-targeted polymers may be able to greatly increase the uptake of drug-polymer conjugates into the tumour mass and increase the effectiveness of the conjugated Dnm cytotoxin without increasing by-stander toxicity.

EXPERIMENTAL METHODS

VB₁₂ was obtained from Rousell-Uclaf whilst all other reagents were from Sigma-Aldrich, Sydney Australia. Gly-Phe-Leu-Gly was obtained from Peptec, Melbourne, Australia. Tumour cell lines were obtained from the following sources: JC, 4T1, ATCC; Colo-26, Access Pharmaceuticals, Dallas; Renca, Chuck Grissom, University of Utah. Animals were obtained from the Animal Resources Centre, Perth, Western Australia. All animal experiments were carried out following approval from the institutional Animal Ethics Committee. PolyHPMA was prepared according to methods described previously [33, 34] and size purified on Sepharose 6B according to the method of Kissel and co-workers [35] to yield high and low molecular weight preparations (see Table 1). The resultant polymer was modified with Gly-Phe-Leu-Gly-Dnm to yield pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm (Dnm-HPMA) using a modification of methods described previously [11]. Briefly, pHPMA dissolved at 20 mg/ml in dry DMSO, was activated with an equal weight of CDT, added as a powder, and reacted overnight with an equal volume of a complex of lysine and copper (Lys-Cu-Lys), dissolved at 100 mg/ml in DMSO containing 50 mg/ml DIEA, to yield pHPMA-*N*- ϵ -Lys-Cu-Lys. The free alpha amino and carboxyl groups on the complex were exposed following treatment with EDTA and extensive dialysis. Succinylated Gly-Phe-Leu-Gly was prepared by dissolving the tetrapeptide at 100 mg/ml in

DMSO and reacting it with an equal weight of succinic anhydride. The resultant Succinyl-TP was purified by RP-HPLC on a Vydac C18 column, eluting with a gradient of 0-80% acetonitrile in 0.1% TFA. The eluted peptide was lyophilized until used. The peptide was dissolved in DMF and DCC/NHS was used to activate the free carboxyl group on succinylated-Gly-Phe-Leu-Gly-Dnm, which was then reacted with pHPMA-*N*- ϵ -Lys, to yield pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm (Dnm-HPMA). Glycyl-N-vitamin B₁₂ was made using standard methods *via* CDT activation of the 5'hydroxyl-group on vitamin B₁₂ [36, 37]. Briefly, dry vitamin B₁₂ was dissolved in DMSO at 100 mg/ml and stirred rapidly. CDT (3.5 molar excess) was added as a powder and used to activate the vitamin B₁₂ for 20 minutes. A ten-fold molar excess Glycine was dissolved at 100 mg/ml in DMSO containing 1% triethylamine and added to the activated VB₁₂. The reaction was allowed to proceed overnight at room temperature, at which time the VB₁₂-conjugate was precipitated by the addition of acetone to 80% and left to stand. The supernatant was decanted and the pellet dissolved in distilled water. The product was then purified by binding to Dowex 1 x 2, and elution with 2.5% acetic acid. The VB₁₂-*N*-Gly-COOH derivative was activated with DCC/NHS in DMSO and used to link to the free alpha amino groups on pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm to yield VB₁₂-[Dnm-HPMA], and the product purified *via* extensive dialysis. Representative analysis of VB₁₂-[Dnm-HPMA] is shown in Table 1.

Tumour Growth Inhibition Studies

Four tumour cell lines, which had previously been shown to have VB₁₂-dependent uptake of VB₁₂-bound pHPMA Rhodamine polymer, were injected subcutaneously at 2×10^6 cells/mouse. These included two breast cell lines, 4T1 and JC, plus one colon cancer line, Colo-26 and a renal cell line, RENCA. Tumours were monitored until they had reached approximately 50 mg in weight, at which time the mice (n=10 per group) received 3 daily intravenous

Table 1: Analysis of VB₁₂-[Dnm-HPMA]. Dnm Substitution was Calculated from its Spectrum using Published Extinction Co-Efficients (OD₂₉₀, OD₄₈₀, OD₄₉₅, and OD₅₃₂ nm). VB₁₂ Substitution was also Obtained from OD₂₇₈, OD₃₆₁ and OD₅₅₀ after Making Appropriate Deductions for Dnm Spectra. M_w, M_n and PDI were Determined Using a Malvern Zetasizer

Polymer	# VB ₁₂ (wt %)	# DNM (wt %)	M _w	M _n	PDI
Dnm-HPMA (LMW)	0 (0%)	4.1 (7.9%)	27132	13800	1.5
VB ₁₂ -Dnm-HPMA	3 (15.6 %)	4.1 (7.5%)	31200	17900	1.74
HMW-HPMA		11.5 (7.8%)	80050	65080	1.65

injections of either 5 mg/kg daunomycin (Dnm), 20 mg Dnm/kg of polymer-bound Dnm, or saline (untreated controls). Animal weights, tumour size and animal survival were then measured at least three times per week. Statistical analysis was performed using Students Ttest.

RESULTS AND DISCUSSION

Initial dosing studies were performed in mice bearing the Colo-26 tumours using non-targeted pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm (Dnm-HPMA) in order to establish the maximum tolerated dose (MTD) to be used in subsequent studies (Figure 1). These studies compared low molecular weight (LMW) (22 kDa) Dnm-HPMA at 22.5, 45 and 90 mg/kg Dnm, with high molecular weight (80 kDa) Dnm-HPMA (HMW-Dnm-HPMA) (20 mg/kg). It was not possible to use higher doses of the HMW-Dnm-HPMA due to the viscous nature of the high molecular weight material. These studies established that the MTD for the LMW Dnm-HPMA was around 20 mg/kg, which was therefore used in further studies. Despite its apparently greater efficacy, further experimentation using the HMW Dnm-HPMA was precluded due to its highly viscous nature, which made it extremely difficult to administer.

Administration of either the LMW or HMW Dnm-HPMA was found to dramatically improve the survival rate of tumour bearing mice with 4 out of 10 mice in either group surviving to over 200 days, with no evidence of tumours being found at sacrifice at Day 200. In contrast, all mice were dead by day 45 in either the control or Dnm-treated mice. Treatment with the HMW (20 mg/kg) Dnm-HPMA lead to a more effective reduction in tumour weight than treatment with LMW (22.5 mg/kg) Dnm-HPMA ($P < 0.05$, days 3, 8, 13, 27, 31-41)

i) Treatment of Various Tumours with [pHPMA-Lys-N-Su-TP-Dnm] (Dnm-HPMA)

In the current studies, injection of Dnm-HPMA polymer conjugates (20 mg Dnm/kg) into tumour-bearing mice was found to be highly effective in treatment of Colo-26, and RENCA tumour-bearing cell lines, with full regression of Dnm-TP-HPMA-treated Colo-26 (4 of 10, day 183; Figure 1a, b, c) and RENCA (7 of 10, day 80; Figure 4a, b, c) tumours. In contrast, treatment with these conjugates in 4T1 and JC breast cancer-bearing mice had a partial effect on tumour growth and increased survival time from 30-35 days (4T1, JC) in control mice to 40-45 days (4T1, JC,

respectively) in treated mice (Figures 2a, b and 3a, b, respectively). A significant reduction in tumour mass was found in both 4T1 ($P < 0.05$, days 5-29) and JC ($P < 0.05$, days 6-24) following treatment with Dnm-HPMA. Dnm-HPMA polymer conjugate was also tested against P815 mastocytoma (data not shown), however, there was only a small increase in the effectiveness of polymer-bound drug over free Dnm, possibly due to a lack of sensitivity of this cell line to Dnm. Apart from the known difference in sensitivity of various cancer lines to different chemotherapeutic agents, the variability of response to the Dnm-HPMA in the different cell lines could also be due to differences in expression of drug resistance genes such as the P-glycoprotein, known to be induced by doxorubicin in breast cancer cell lines [38-40], or other factors such as uneven drug distribution in solid tumour masses [41], or non-uniform processing of the polymers inside the cell [42], or differences in tumour vasculature [7]. Whatever the reason for the variability, similar results to those reported above have also been reported in clinical trials of Dox-TP-pHPMA (PK1) [7].

ii) Treatment with VB₁₂-N-Glycyl-[pHPMA-Lys-N-Su-TP-Dnm]

In vitro experiments using targeted Rhodamine-Lysyl-pHPMA have shown it is possible to greatly increase the uptake of Rho-Lysyl-pHPMA into many tumour lines, through the use of vitamin B₁₂ as a targeting agent [11, 31]. In order to examine the potential of targeting with vitamin B₁₂ *in vivo*, studies were performed comparing non-targeted pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm to vitamin B₁₂ targeted pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm prepared by direct linkage of *N*-Glycyl-5'-O-vitamin B₁₂ to free α -amino-groups on preformed pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm polymer conjugates.

Treatment of RENCA tumour-bearing mice with both targeted and non-targeted pHPMA-Lys-Su-TP-*N*-Dnm (Dnm-HPMA) copolymer conjugate was highly effective in reducing the size of the tumour mass and also in increasing the long-term survivors. Complete tumour regression was achieved following treatment with the non-targeted Dnm-polymer complex (7 of 10) and the VB₁₂-targeted polymer (8 of 10) at day 80, with the group receiving the VB₁₂-targeted polymer marginally more effective at controlling tumour growth (days 28-52) (Figure 4a), than the non-targeted Dnm-Polymer. In contrast, all of the control mice were dead by day 45 (Figure 4b). Free Dnm administered at the maximum tolerated dose (5 mg, MTD) (Figure 4c) had

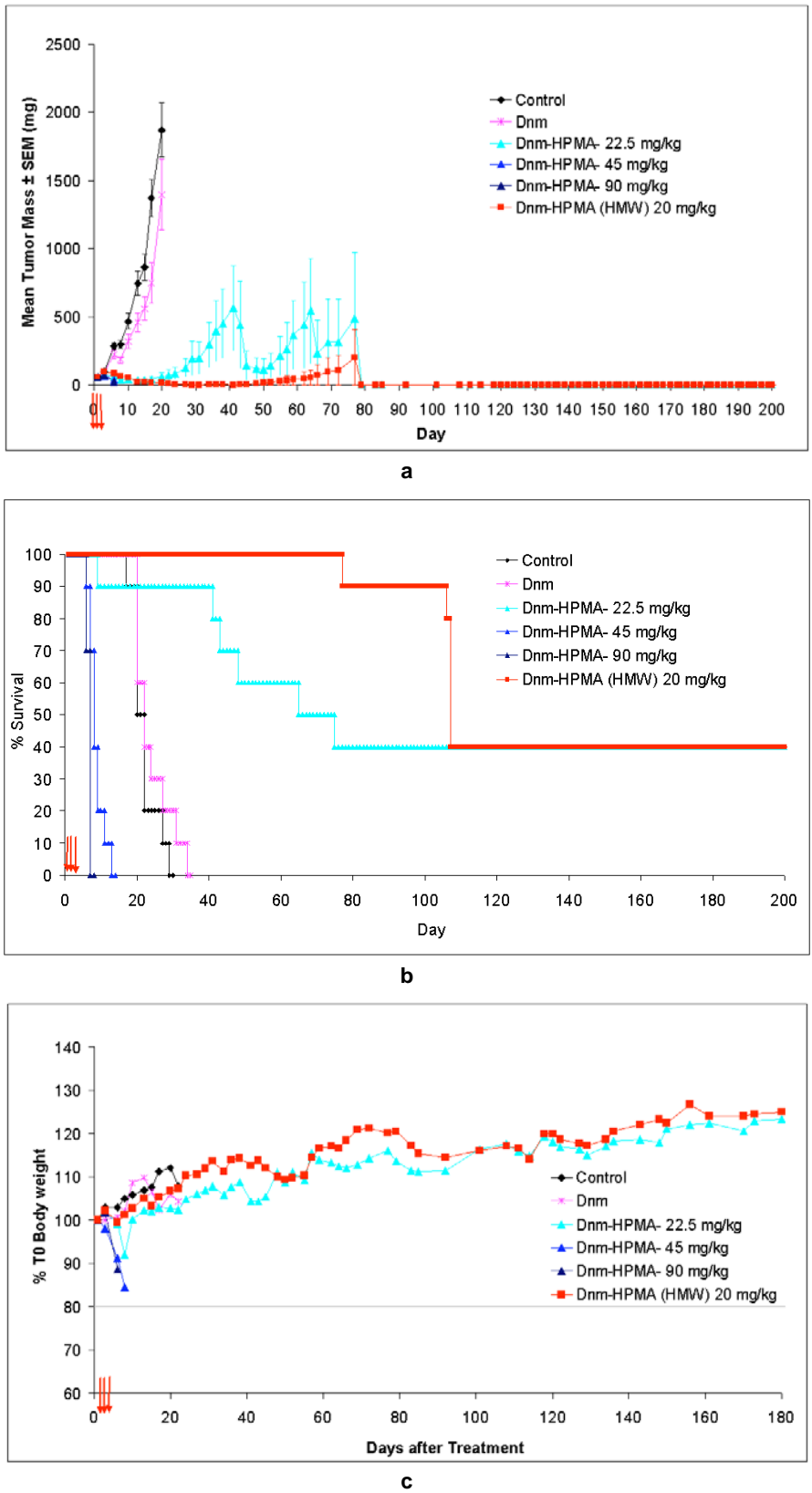


Figure 1: Survival of Colo-26 tumour-bearing mice following treatment with Daunomycin (Dnm) or Dnm-HPMA. The tumour size of each mouse was monitored until the tumour reached approximately 50 mg in weight. Mice were then injected iv with 3 doses (day 1, 2, 3) of either Dnm (5 mg/kg) or Dnm- HPMA (22.5, 45, or 90 mg/kg Dnm) or HMW Dnm- HPMA (20 mg/kg Dnm).
a. Top Panel Tumour Growth; **b.** Middle Panel Kaplan-Meier Plot of survivors; **c.** Bottom Panel Modification in body weight following treatment.

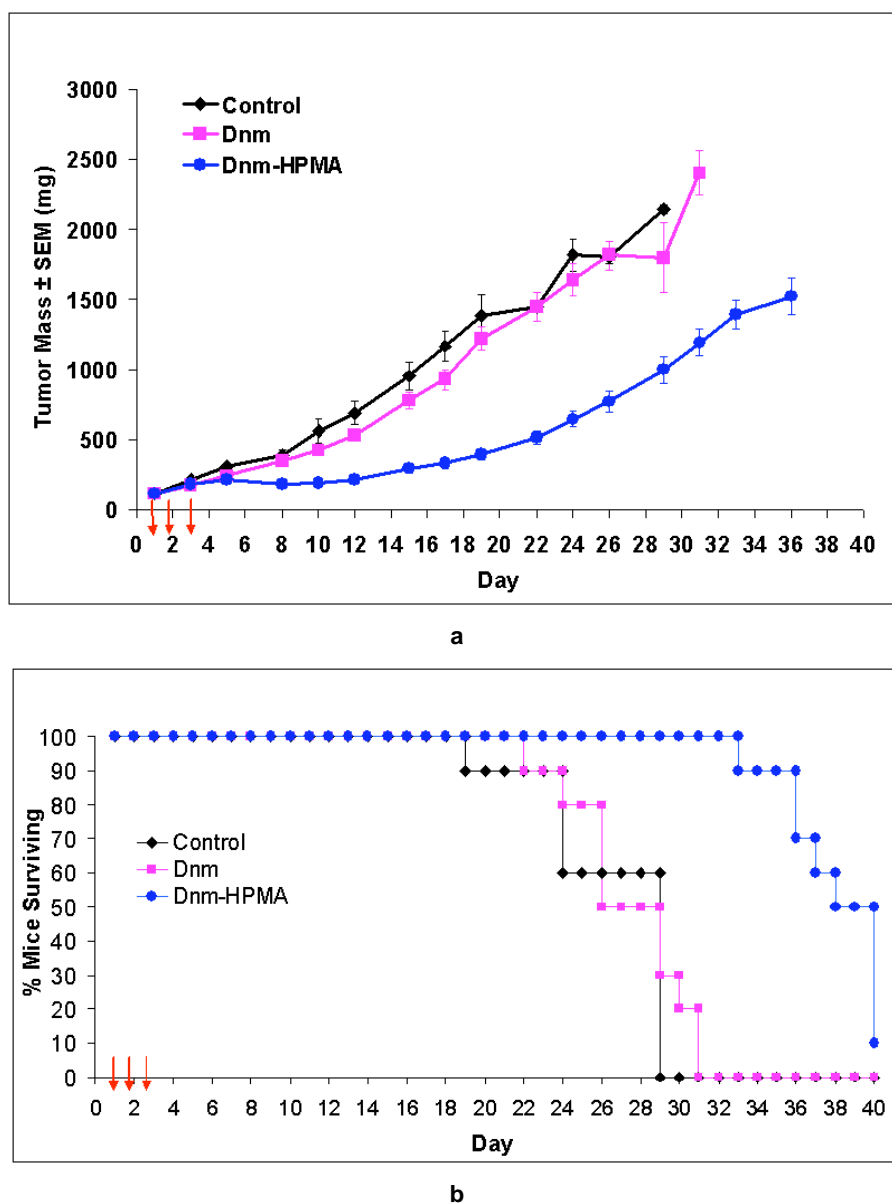


Figure 2: Survival of 4T1 tumour-bearing mice following treatment with Daunomycin (Dnm) or Dnm-HPMA. The tumour size of each mouse was monitored until the tumour reached approximately 50 mg in weight. Mice were then injected iv with 3 doses (day 1, 2, 3) of either Dnm (5 mg/kg) or Dnm-HPMA (20 mg/kg Dnm).

a. Top Panel Tumour Growth; **b.** Bottom Panel Kaplan-Meier Plot of survivors.

no effect upon tumour control. Similar results were found when 4T1 (Figure 5) bearing mice were treated with both targeted and non-targeted Dnm-HPMA copolymer conjugate.

Polymer-bound Dnm was found to be much more effective in tumour treatment than equitoxic doses of free Dnm in various tumour models. The effectiveness of treatment with the pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm was increased still further in RENCA and 4T1 tumour models, which are known to over-express receptors involved in VB₁₂ uptake, when these mice were treated with VB₁₂-targeted polymer-drug complex. Treatment

with VB₁₂-targeted and untargeted pHPMA-*N*- ϵ -Lys-*N*- α -Su-TP-Dnm resulted in increased numbers of survivors, reduction in tumour mass, and reduced toxicity over free Dnm given under the same treatment regime. In two of the cell lines, Colo-26 colon carcinoma and RENCA renal carcinoma, treatment resulted in greatly increased survival rates and reduction in tumour mass to undetectable levels 183 days after initial treatment (40%) in Colo-26 bearing tumour mice, and at day 82 (60% Dnm-HPMA, 80% VB₁₂-[Dnm-HPMA]) in RENCA tumour bearing mice. This is in contrast to control animals, or those treated with MTD levels of Dnm, where all animals were

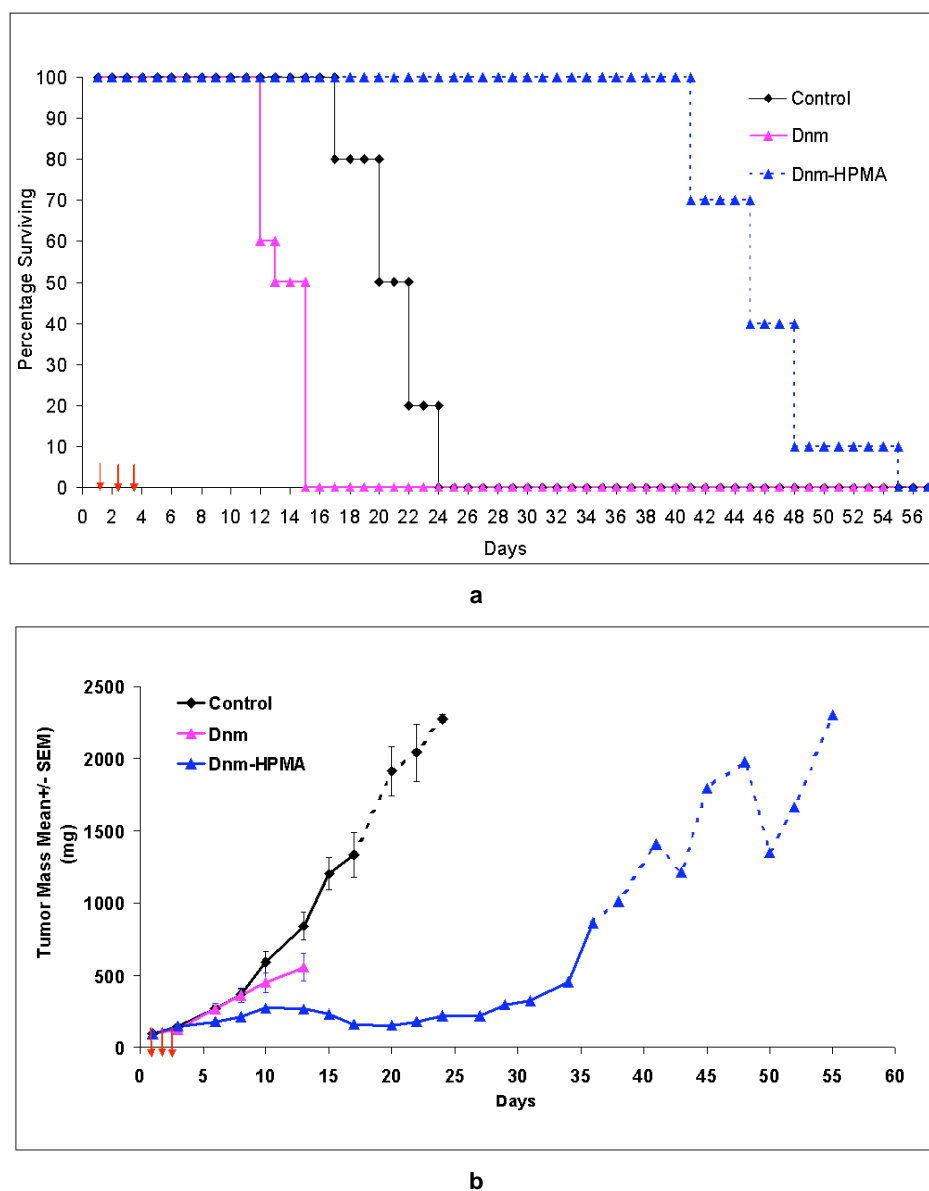


Figure 3: Survival of JC tumour-bearing mice following treatment with Daunomycin (Dnm) or Dnm-HPMA. The tumour size of each mouse was monitored until the tumour reached approximately 50 mg in weight. Mice were then injected iv with 3 doses (day 1, 2, 3) of either Dnm (5 mg/kg) or Dnm-HPMA (20 mg/kg Dnm).

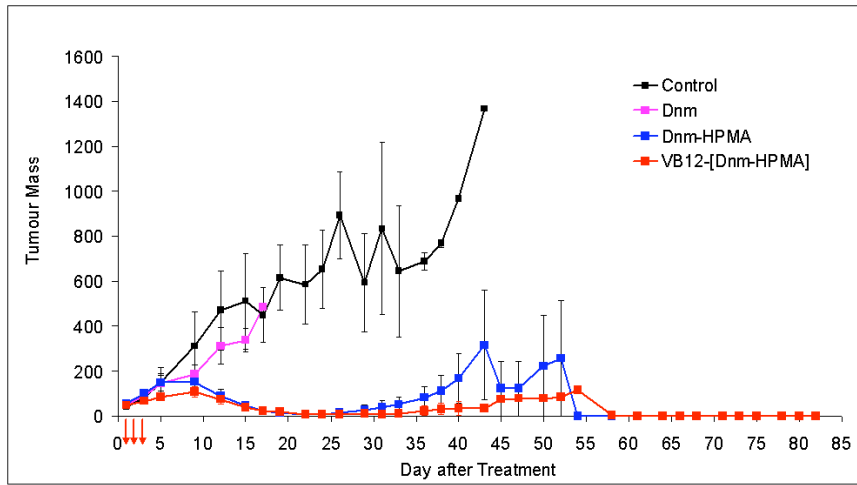
a. Top Panel Tumour Growth; **b.** Bottom Panel Kaplan-Meier Plot of survivors.

moribund by day 34, or 42 respectively. This dramatic improvement in survival rate of treated mice is even more impressive when one considers that the long term survivors only received 3 doses of polymer-bound Dnm, at sub-MTD levels.

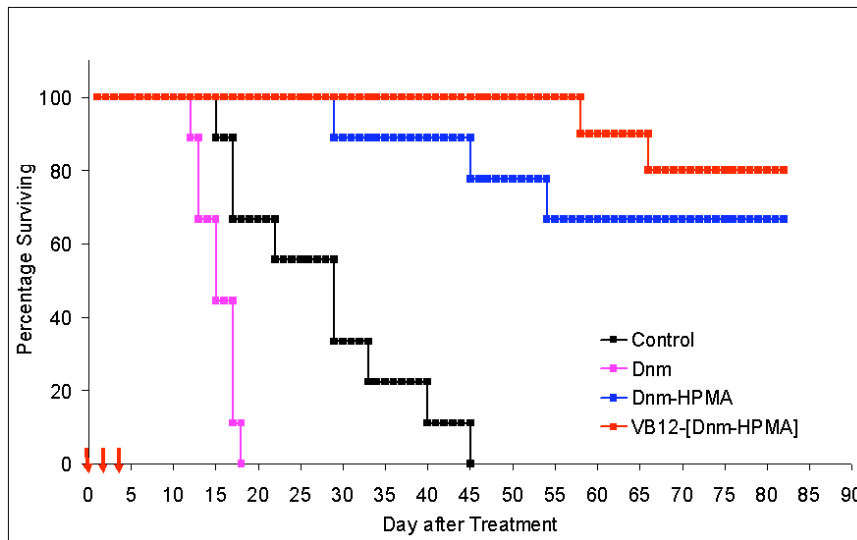
CONCLUSIONS

Administration of Dnm-HPMA polymer conjugates to different tumour-bearing cell lines showed highly variable control of tumour growth, which ranged from retardation of growth to long term elimination of the tumours, depending upon the tumour cell line. There was no evidence of any effect on tumour growth when

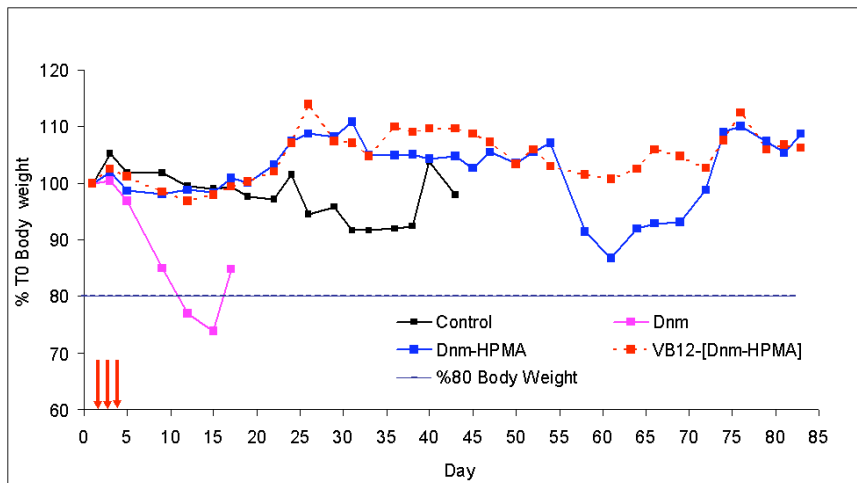
free Dnm was administered. The efficacy of polymer-conjugated Dnm was greatly increased when the polymer conjugate was targeted with VB₁₂ and administered to mice bearing tumours that over-express receptors involved in vitamin B₁₂ uptake. These data confirm the observations on increased uptake of the polymer-bound fluorophore, rhodamine, when the polymer is targeted with VB₁₂ and demonstrate that this increased uptake leads to more effective Dnm delivery, and resultant anti-tumour activity. The results of these experiments have enormous potential in increasing the efficacy of drug treatment in colon, renal and breast cancers.



a



b



c

Figure 4: Treatment of RENCA tumour-bearing mice with Daunomycin (Dnm), Dnm-HPMA or VB₁₂-[Dnm-HPMA]. The tumour size of each mouse was monitored until the tumour reached approximately 50 mg in weight. Mice were then injected iv with 3 doses (day 1, 2, 3) of either Dnm (5 mg/kg) or Dnm-HPMA (20 mg/kg Dnm).
a. Top Panel Tumour Growth; **b.** Middle Panel Kaplan-Meier Plot of survivors; **c.** Bottom Panel Modification in body weight following treatment.

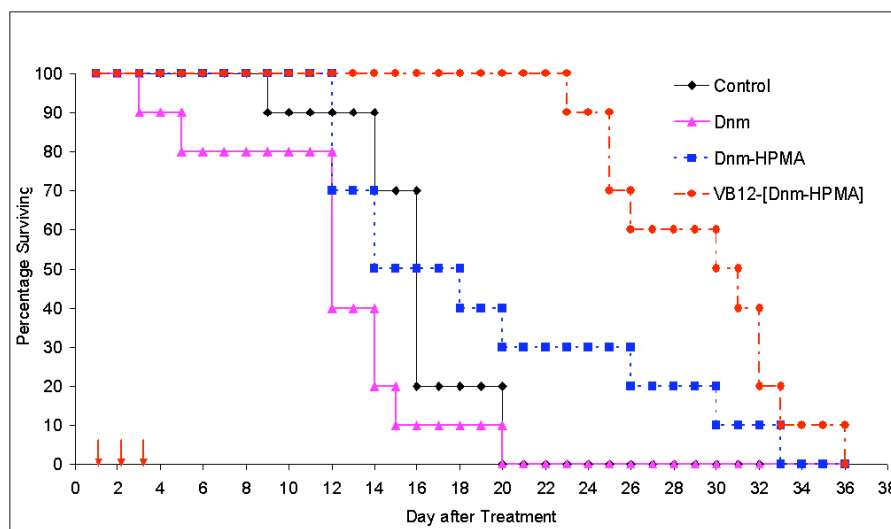


Figure 5: Survival of 4T1 tumour-bearing mice following treatment with Daunomycin (Dnm), Dnm-HPMA or VB₁₂-[Dnm-HPMA]. The tumour size of each mouse was monitored until the tumour reached approximately 50 mg in weight. Mice were then injected iv with 3 doses (day 1, 2, 3) of either Dnm (5 mg/kg), Dnm-HPMA (20 mg/kg Dnm) or VB₁₂-[Dnm-HPMA] (20 mg/kg Dnm). Data presented is a Kaplan-Meier Plot of survivors.

Further work is underway to examine alternative VB₁₂-conjugation chemistry and to optimize the dose regime for tumour treatment.

REFERENCES

- Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumorotropic accumulation of proteins and the anti-cancer agent SMANCS. *Can Res* 1986; 44: 6357-92.
- Vasey PA, Kaye SB, Morrison R, *et al*. Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl) methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents-drug-polymer conjugates. *Cancer Research Campaign Phase I/II Committee. Clin Cancer Res* 1999; 5: 83-94.
- Thomson AH, Vasey PA, Murray LS, *et al*. Population pharmacokinetics in phase I drug development: a phase I study of PK1 in patients with solid tumours. *Br J Cancer* 1999; 81: 99-107. <http://dx.doi.org/10.1038/sj.bjc.6690657>
- Duncan R, Vicent MJ. Do HPMA copolymer conjugates have a future as clinically useful nanomedicines? A critical overview of current status and future opportunities. *Adv Drug Deliv Rev* 2010; 62: 272-82. <http://dx.doi.org/10.1016/j.addr.2009.12.005>
- Duncan R. Development of HPMA copolymer-anticancer conjugates: clinical experience and lessons learnt. *Adv Drug Deliv Rev* 2009; 61: 1131-48. <http://dx.doi.org/10.1016/j.addr.2009.05.007>
- Seymour LW, Ulbrich K, Steyger PS, *et al*. Tumor tropism and anti-cancer efficacy of polymer-based doxorubicin prodrugs in the treatment of subcutaneous murine B16F10 melanoma. *Br J Cancer* 1994; 70: 636-41. <http://dx.doi.org/10.1038/bjc.1994.363>
- Seymour LW, Ferry DR, Kerr DJ, *et al*. Phase II studies of polymer-doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer. *Int J Oncol* 2009; 34: 1629-36. http://dx.doi.org/10.3892/ijo_00000293
- Rademaker-Lakhai JM, Terret C, Howell SB, *et al*. A Phase I and pharmacological study of the platinum polymer AP5280 given as an intravenous infusion once every 3 weeks in patients with solid tumors. *Clin Cancer Res* 2004; 10(10): 3386-95. <http://dx.doi.org/10.1158/1078-0432.CCR-03-0315>
- Campone M, Rademaker-Lakhai JM, Bennouna J, *et al*. Phase I and pharmacokinetic trial of AP5346, a DACH-platinum-polymer conjugate, administered weekly for three out of every 4 weeks to advanced solid tumor patients. *Cancer Chemother Pharmacol* 2007; 60: 523-33. <http://dx.doi.org/10.1007/s00280-006-0397-0>
- Joly F, Madroszyk A, Floquet A, Gedouin D, Bourdel F, Campone M. AP5346 (ProLindac™), a pH-dependent polymer-vectorized DACH platinum, is active in borderline potentially platinum-sensitive ovarian cancer (OC) patients: results from an ongoing Phase I/II trial. *Proceedings AACR-NCI-EORTC 2008 Molecular Targets and Cancer Therapeutics Conference*, Geneva, Switzerland.
- Russell-Jones GJ, McTavish K, McEwan J, Rice JR, Nowotnik D. Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumours. *J Inorg Biochem* 2004; 98: 1625-33. <http://dx.doi.org/10.1016/j.jinorgbio.2004.07.009>
- Nowotnik DP, Cvitkovic E. ProLindac (AP5346): a review of the development of an HPMA DACH platinum Polymer Therapeutic. *Drug Deliv Rev* 2009; 61: 1214-9. <http://dx.doi.org/10.1016/j.addr.2009.06.004>
- Rice JR, Gerberich JL, Nowotnik DP, Howell SB. Preclinical efficacy and pharmacokinetics of AP5346, a novel diaminocyclohexane-platinum tumor-targeting drug delivery system. *Clin Cancer Res* 2006; 12: 2248-54. <http://dx.doi.org/10.1158/1078-0432.CCR-05-2169>
- Rachmilewitz B, Sulkes A, Rachmilewitz M, Fuks A. Serum transcobalamin II levels in breast carcinoma patients. *Israel J Med Sci* 1981; 17: 874-79.
- Rachmilewitz B, Rachmilewitz M, Moshkowitz B, Gross J. Serum transcobalamin II in myeloid leukemia. *J Lab Clin Med* 1971; 78: 275-79.
- Rachmilewitz B, Sulkes A, Rachmilewitz M, Fuks Z. Serum transcobalamin II levels in breast carcinoma patients. *Isr J Med Sci* 1981; 17(9-10): 874-8.
- Flodh J. Accumulation of labeled vitamin B-12 in some transplanted tumors. *Acta Radiol* 1968; (Suppl. 284): 55-60.
- Flodh J, Ullberg S. Accumulation of labeled vitamin B₁₂ in

- some transplanted tumours. *Int J Cancer* 1968; 3: 694-99.
<http://dx.doi.org/10.1002/ijc.2910030518>
- [19] Blomquist L, Flodh H, Ullberg S. Uptake of labeled vitamin B₁₂ and 4-Iodophenylalanine in some tumours of mice. *Specialia* 1969; 15: 294-96.
- [20] Amagasaki T, Green R, Jacobsen DW. Expression of transcobalamin II receptors by human leukemia K562 and HL-60 cells. *Blood* 1990; 76: 1380-6.
- [21] McLean GR, Pathare PM, Wilbur DS, *et al.* Cobalamin analogues modulate the growth of leukemia cells *in vitro*. *Cancer Res* 1997; 57: 4015-22.
- [22] McLean GR, Quadros EV, Rothenberg SP, Morgan AC, Schrader JW, Ziltener HJ. Antibodies to transcobalamin II block *in vitro* proliferation of leukemic cells. *Blood* 1997; 89: 235-42.
- [23] McLean GR, Williams MJ, Woodhouse CS, Ziltener HJ. Transcobalamin II and *in vitro* proliferation of leukemic cells. *Leuk Lymphoma* 1998; 30: 101-9.
- [24] Warnock SH, Collins DA, Morton KA. Comparison of tumor uptake and nuclear medicine images of gallium-67, Thallium-201, and [Cobalt-57]-vitamin B-12 in sarcoma bearing mice. *Clin Res* 1992; 40: 7A.
- [25] Collins DA, Hogenkamp HPC. Transcobalamin II receptor imaging *via* radiolabelled diethylene-triaminepentaacetate cobalamin analogues. *J Nucl Med* 1997; 38: 717-23.
- [26] Collins DA, Hogenkamp HPC, O'Connor MK, *et al.* Biodistribution of radiolabelled adenosylcobalamin in patients diagnosed with various malignancies. *Mayo Clin Proc* 2000; 75: 568-80.
<http://dx.doi.org/10.4065/75.6.568>
- [27] Collins DA, Hogenkamp HPC, Gebhard MK. Tumor imaging *via* indium 111-labeled DTPA-adenosylcobalamin. *Mayo Clin Proc* 1999; 74: 687-91.
<http://dx.doi.org/10.4065/74.7.687>
- [28] Cannon MJ, McGreevy JM, Holden JA, West FG, Grissom CB. The uptake and distribution of fluorescently labelled cobalamin in neoplastic and healthy breast tissue. *Lasers in Surgery. Advanced Characterization, Therapeutics and Systems. Proc SPIE* 2000; 3907: 612-15.
<http://dx.doi.org/10.1117/12.386306>
- [29] Bauer JA, Morrison BH, Grane RW, Jacobs BS, Dabney S, Gamero AM. Effects of interferon beta on transcobalamin II-receptor expression and antitumor activity of nitrosylcobalamin. *J Natl Cancer Inst* 2002; 94: 1010-9.
<http://dx.doi.org/10.1093/jnci/94.13.1010>
- [30] Russell-Jones GJ, McTavish K, McEwan JF. Preliminary studies on the selective accumulation of vitamin-targeted polymers within tumours. *J Drug Targ* 2011; 19(2): 133-9. Epub 2010 May 6.
<http://dx.doi.org/10.3109/10611861003734027>
- [31] Russell-Jones GJ. Cellular Targeting. In CRC press. "Biodrug Delivery Systems: Fundamentals, Applications and Clinical Development". Ed Mariko Morishita and Kinam Park 2011 (Manuscript accepted).
- [32] Waibel R, Treichler H, Schaefer NG, *et al.* New derivatives of vitamin B12 show preferential targeting to tumors. *Cancer Res* 2008; 68: 2904-11.
<http://dx.doi.org/10.1158/0008-5472.CAN-07-6771>
- [33] Duncan R, Rejmanova P, Kopecek J, Lloyd JB. Pinocytic uptake and intracellular degradation of N(2-hydroxypropyl) methacrylamide copolymers, a potential drug delivery system. *Biochim Biophys Acta* 1981; 678: 143-50.
[http://dx.doi.org/10.1016/0304-4165\(81\)90058-1](http://dx.doi.org/10.1016/0304-4165(81)90058-1)
- [34] Pinciroli V, Rizzo V, Angelucci F, Tatò M, Vigevani A. ¹H-NMR characterization of methacrylamide polymer conjugates with the anti-cancer drug doxorubicin. *Magn Reson Chem* 1997; 35: 2-8.
[http://dx.doi.org/10.1002/\(SICI\)1097-458X\(199701\)35:1<2::AID-OMR24>3.0.CO;2-D](http://dx.doi.org/10.1002/(SICI)1097-458X(199701)35:1<2::AID-OMR24>3.0.CO;2-D)
- [35] Kissel M, Peschke P, Subr V, *et al.* Synthetic macromolecular drug carriers: biodistribution of poly[(N-2-hydroxypropyl)methacrylamide] copolymers and their accumulation in solid rat tumors. *PDA J Pharm Sci Technol* 2001; 55(3): 191-201
- [36] McEwan JF, Veitch HS, Russell-Jones GJ. Synthesis and Biological Activity of ribose-5'-carbamate derivatives of vitamin B₁₂. *Bioconj Chem* 1999; 10: 1131-36.
<http://dx.doi.org/10.1021/bc990086p>
- [37] Russell-Jones GJ, McEwan JF. Vitamin B12 derivatives and methods for their preparation. 1999; USP 6150341.
- [38] Mealey KL, Barhoumi R, Burghardt RC, Safe S, Kochevari, DT. Doxycyclin induces expression of P glycoprotein in MCF-7 breast carcinoma cells. *Antimicrobiol Agents Chemother* 2002; 46: 755-61.
<http://dx.doi.org/10.1128/AAC.46.3.755-761.2002>
- [39] Peer D, Dekel Y, Melikhov D, Margalit R. Fluoxetine inhibits multidrug resistance extrusion pumps and enhances responses to chemotherapy in syngeneic and in human xenograft mouse tumor models. *Canc Res* 2004; 64: 7562-69.
<http://dx.doi.org/10.1158/0008-5472.CAN-03-4046>
- [40] Riganti C, Miraglia E, Viarisio D, *et al.* Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. *Canc Res* 2005; 65: 516-25.
- [41] Lankelma J, Dekker H, Luque RF, *et al.* Doxorubicin gradients in human breast cancer. *Clin Cancer Res* 1999; 5: 1703-707.
- [42] Jensen KD, Kopečková P, Bridge JHB, Kopeček J. The cytoplasmic escape and nuclear accumulation of endocytosed and microinjected HPMA copolymers and a basic kinetic study in Hep G2 cells. *AAPS PharmSci* 2001; 3: article 32.
<http://dx.doi.org/10.1208/ps030432>