Preliminary studies on the selective accumulation of vitamin-targeted polymers within tumors

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Abstract
Many different cancer types have previously been found to show increased uptake of the vitamins folate, vitamin B12, and biotin; however, it is not known whether these tumor lines show increased uptake of one or more of the vitamins. The current study was designed to examine the relative uptake of the three vitamins in 10 different types of cell lines. Rhodamine-labeled hydroxypropyl-methacrylamide (HPMA) was targeted with vitamin B₁₂, folate, or biotin, and the uptake of the labeled polymer was compared both in vitro cell cultures and in mice-bearing tumors from a variety of tumor cell lines. Fluorescent microscopy of cell cultures and histological examination of tumor sections showed greatly increased uptake of the fluorescently labeled polymer in many tumors when the polymer was targeted with folate, biotin, or vitamin B₁₂. Tumors with enhanced uptake of vitamin B₁₂- or folate-targeted rhodamine-HPMA also showed increased uptake of biotin-Rho-HPMA. In contrast, tumors with increased uptake of folate-Rho-HPMA did not show increased uptake of vitamin B₁₂ (VB₁₂)-HPMA and vice versa. These findings suggest that vitamin-targeted polymers may greatly increase the uptake of drug–polymer complexes in certain tumors, which may result in an increased efficacy of antitumor agents, and which may allow for easier imaging of both the primary and metastatic tumors.

Keywords: Folate; vitamin B12; cyanocobalamin; biotin; cancer targeting; tumor imaging

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 additional advantage of reducing the toxicity of the drug, as well as taking advantage of altered tumor vasculature often found in tumor masses. Although this strategy may increase the amount of drug that reaches some tumor tissue, the polymer-bound chemotherapeutic does not preferentially accumulate in small nonvascularized metastases, nor does it result in increased rates of internalization of the chemotherapeutic into the tumor cells. In order to overcome both of these restrictions, we examined the potential of vitamins to act as targeting agents for polymer-bound cytotoxins used in cancer therapy. It is now well established that many aggressive tumors overexpress surface receptors involved in the uptake of vitamin B₁₂ (Flodh, 1968; Flodh & Ullberg, 1968; Blomquist et al., 1969; Rachmilewitz et al., 1971; Rachmilewitz et al., 1981; Collins et al., 1999; Collins et al., 2000) or folate (Boerman et al., 1991; Weitman et al., 1992; Garin-Chesa et al., 1993; Gottschalk et al., 1994; Toffoli et al., 1997; Toffoli et al., 1998; Reddy & Low, 1998; Reddy et al., 1999). Additionally, it has also been shown that there are increased levels of vitamin B₁₂-binding proteins in the serum of hepatocellular carcinoma patients (Kane et al., 1978). The degree of overexpression of the folate receptor has been found to correlate with the stage of tumor growth, with the highest levels found on stage IV carcinomas (Reddy & Low, 1998). In addition, there are reports of increased uptake of biotin-targeted nanoparticles into HepG2 cells (Na et al., 2003) and uptake of ¹³¹Sm-DTPA-bis-Biotin into AS-30D hepatoma grown in Wistar rats (Correa-González et al., 2003).

In the aforementioned studies, the uptake of only one of the three vitamins, folate, vitamin B₁₂, or biotin was examined, but these workers did not compare relative

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uptake of biotin, folate, or vitamin B₁₂ in the various cell lines. We have previously reported preliminary studies on comparative uptake of the three vitamins (Russell-Jones et al., 2004). In the present study, we have extended these studies and have compared the accumulation of vitamin-targeted rhodamine-labeled hydroxypropylmethacrylamide (HPMA) within several tumors of varying histological types both in vitro and in vivo. We have found that vitamin-targeted polymers have the potential to greatly increase the uptake of drug–polymer conjugates into tumor cells and tumor metastases with little uptake into normal tissue. The subcellular location of biotin-targeted polymers appears ideal for imaging of tumors, as well as for treatment with α-particle-emitting radionuclide.

Materials and methods

Biotin-Qdots 655 (10–12 nm size) were obtained from Invitrogen, Mulgrave, Victoria, Australia and were used at 2.5 µl per 500 µl tissue culture well. Lysine-modified-HPMA polymer (molecular weight ~22 kDa) was prepared in-house and was modified with rhodamine isothiocyanate (Sigma–Aldrich, Sydney, Australia) using standard methods (Lindner et al., 1994). Biotin-Qdots 655 were obtained from Australian Biosearch, Balcatta, Western Australia, Australia. A single batch of rhodamine–HPMA (prepared as described above) was dissolved at 20 mg/ml in DMSO and was then modified with folate, glycyln–vitamin B₁₂ or biotin, each of which had been activated with 1.5-fold molar excess of N-hydroxysuccinimide (Sigma Pharmaceuticals, South Croydon, Victoria, Australia) and TSTU (N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate, Fluka; Sigma). All preparations were diazoyed extensively against distilled water before use. Tumor cell lines were obtained from the following sources: B16-F10, BW5147.3, CaCo-2, CCL8, HCT-116, JC, 4T1, P815, ATCC; Colo-26, Ov2008, Access Pharmaceuticals, Dallas, TX; ID8, L1210, L1210-FR, M109, Endocyte, West Lafayette, IN; MCF-7, Ovcar-3, RD995, RENCA, Chuck Grissom, University of Utah; 0157, TECRA International, Frenchs Forest, New South Wales, Australia; and were housed in climate-controlled rooms at 22°C. The frozen tumors were sectioned prior to freezing and storage at −18°C. The frozen tumors were sectioned prior to examination with a Zeiss Axiosplan fluorescent microscope.

Results and discussion

Specific targeting and increased uptake of folate–Rho-HPMA was shown by accumulation of the rhodamine-labeled polymer in L1210-FR (leukemia), Ov2008 and ID8 (ovarian) cell lines (Table 1; Figures 1–4; Russell-Jones et al., 2004), whereas increased uptake of vitamin B₁₂–Rho-HPMA polymer was observed in Colo-26 (colon), P815 (mastocytoma), M109 (lung), RENCA, RD995 (renal), 4T1, JC, and MMT06056 (mammary) cell lines (Table 1; Figures 1–4; Russell-Jones et al., 2004). In addition, increased uptake of biotin–Rho-HPMA was seen in all tumors showing enhanced uptake of either folate- or vitamin B₁₂-targeted rhodamine-HPMA, but not in L1210, 0157, BW5147, B16, LL-2, or HCT-116 cell lines (Table 1; Figures 1–4). Thus, tumor cells that displayed an increase in the uptake of vitamin B₁₂ (VB₁₂) or folate also had increased levels of receptors involved.

Table 1. Receptor status of various cell lines as determined by uptake of vitamin-targeted rhodamine-labeled HPMA into different tumors grown in vitro.

<table>
<thead>
<tr>
<th>Receptor status</th>
<th>Cell line</th>
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<tbody>
<tr>
<td>FR, TCIIR, BBP/BBPR</td>
<td>0157, BW5147, B16, LL-2, HCT-116, L1210</td>
</tr>
<tr>
<td>FR++, TCIIR+, BBP/BBPR+++</td>
<td>L1210-FR, Ov2008, ID8</td>
</tr>
<tr>
<td>FR, TCIIR++, BBP/BBPR+++</td>
<td>Colo-26, M815, 4T1, JC, RENCA, M109, RD995, MMT06056</td>
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Uptake of Rho-pHPMA was determined after 5 h incubation with the targeted polymers and compared to uptake of the unlabeled Rho-pHPMA. Representative tumor types were lymphoma (0157, BW5147), lung (M109, LL-2), melanoma (B16), colon (HCT-116, Colo-26), leukemia (L1210, L1210-FR), ovarian (Ov2008, ID8), mastocytoma (P815), renal (RENCA, RD995), or breast (JC, 4T1, MMT06056). Results are representative of multiple observations by different viewers, with similar levels of uptake seen upon multiple viewing. Similarly, the intracellular location of rhodamine-HPMA with the different targeting agents was characteristic of the targeting agent (See also Figure 3).
Preliminary studies on accumulation of vitamin-targeted polymers in biotin binding and uptake. Similar findings were found in vivo, but in addition, the vastly increased level of uptake of biotin-targeted rhodamine-HPMA polymer in tumor cells leads to easy identification of metastatic tissue (Figure 2). The results for folate receptor expression are in good agreement to the level of folate receptor observed by Parker and co-workers (2005) for L1210-FR, M109, RENCA, and 4T1. The biotin-binding activity of the cell lines tested has not been described before.

High-power histological examination of culture-well slides of the tumor showed fluorescence accumulation in vesicle-like structures within tumors grown in vitro and incubated with biotin-targeted polymers, suggesting endosomal uptake of targeted polymers (Figure 3). Time course experiments using Biotin-Qdots (Figure 4) showed surface clustering of fluorescent material at 60 min, with little evidence of internalization at that time, this was followed by internalization and sorting to many similarly sized and shaped intracellular organelles (Figures 3 and 4), possibly mitochondria, the ultimate fate of many of the biotin coenzymes.

The water-soluble vitamins folate, vitamin B12, and biotin do not readily cross either the vasculature epithelium or cell walls. Separate cellular uptake mechanisms have been described for both free folate (the reduced folate carrier) and free biotin [sodium-dependent multivitamin transporter (SMVT)]; however, these uptake systems are not able to transport folate or biotin conjugates such as those used in these studies (Baur & Baumgartner, 2000; Park & Sinko, 2005). Low and others (Coney et al., 1991; Leamon & Low, 1991; Turek et al., 1993; Toffoli et al., 1997; Wang & Low, 1998) have shown that an alternative receptor, the folate receptor is

Figure 1. Increased uptake of vitamin B12- and biotin-targeted rhodamine-HPMA by Ov2008 tumor cells grown in vitro. Top left, rhodamine-HPMA; top right, folate-rhodamine-HPMA; bottom left, vitamin B12-rhodamine-HPMA; bottom right, biotin-rhodamine-HPMA.

Figure 2. Increased uptake of biotin-targeted rhodamine-HPMA by M109 metastatic tumor cells grown in vivo. Top, BisB-stained cells; middle, biotin-rhodamine-HPMA; bottom, biotin-rhodamine-HPMA + BisB-stained cells.
overexpressed on several cancer cell types, and has the capability of binding and internalizing folate-targeted ligands. Cellular uptake of vitamin B12 occurs after binding of VB12 to transcobalamin II (TCII) followed by binding of the VB12–TCII complex to the multipurpose-binding protein megalin (Birn et al., 2002). The increased uptake of VB12-Rho-HPMA observed was presumably due to either increased binding of TCII-VB12-Rho-HPMA to the cells, increased production of TCII by the tumor cells, or a combination of both. In this regard, it has previously been shown that there can be elevated levels of plasma TCII in patients with breast cancer, renal cancer, or multiple myeloma (Carmel & Hollander, 1978; Rachmilewitz et al., 1981; Jensen et al., 1983). Cellular uptake of biotin has been shown in many cases to be due to SMVT (Prasad et al., 1998; Prasad et al., 1999); however, the observed uptake of biotin-targeted Rho-HPMA cannot be due to the SMVT, as this transporter requires the presence of a free carboxyl on the biotin molecule. Uptake via the SMVT is inhibited by molecules such as pantothenic acid, desthiobiotin and lipoic acid, each of which has a free carboxyl group, but is not inhibited by biotin methyl ester, biocytin, or diamino biotin (see Table 2) (Said et al., 1987). During the preparation of the biotin-targeted imaging agents, the free carboxyl group on biotin is utilized in the formation of a “peptide bond,” thus making the biotin unavailable for transport via the SMVT (see Table 2). Moreover, transport via the SMVT does not require SMVT clustering and vesicle formation, which was a feature of the observed uptake of biotinylated-phMA or biotinylated Qdots (see Figures 1–4). In the current studies, receptor clustering on the surface of RD995 cells was observed 60 min after administration of Biotin-Qdots (10–12 nm particles) (Figure 4), with little evidence of internalization at that time. By 5 h after administration, surface-bound material appears to have entered the cells and could be seen to have accumulated in subcellular vesicles located in clusters around the nucleus of the cell (Figure 4). Similar subcellular location was also shown for biotin-Rho-HPMA (22 kDa) (Figures 1 and 3), in ID8, MMT, Ov2008, and RENCA cell lines. Furthermore, the relatively large size of both the biotin-Rho-HPMA (22 kDa) and the biotin-Qdot (10–12 nm) would preclude uptake through small molecule transporters such as SMVT. Additionally, SMVT expression has been shown to be highest in tissues such as placenta, liver, brain, kidney, lung, and heart in rats, rabbits, and humans (Stanley et al., 2002), with evidence of SMVT-mediated transport of biotin in the duodenum, jejunum, ileum, and colon. Fluorescent imaging using biotin-Rho-HPMA injected into M109-bearing mice showed high levels of uptake of the fluorescent probe into tumor metastases growing immediately adjacent to intestinal tissue, with no evidence of uptake of the fluorescent probe into the adjacent “normal” tissue (Figure 2), once again suggesting that uptake into this tissue was not due to the SMVT process. Similarly, studies in humans using 111In-biotin, in a three-step monoclonal-antibody-biotin/avidin/111In-biotin procedure showed very low levels of imaging activity of normal tissue (Paganelli et al., 1991; Casalini et al., 1997; Boerman et al., 2003). Studies using 99mTc-DOTA–biotin complexes in pretargeted therapy showed very little systemic toxicity, also suggesting very low levels of uptake into normal tissue (Axworthy et al., 2000; Breitz et al., 2000). These studies are in contrast to those using 111In and 99mTc-Cbl.

Figure 3. Punctate staining of internalized biotin-targeted rhodamine-HPMA by various tumor cell lines grown in vivo. Top left, ID8; top right, MMT; bottom left, Ov2008; bottom right, RENCA.

Figure 4. Surface binding and internalization of biotin-Qdots HPMA by RD995 cell line grown in vivo. Left, 1 h; middle, 1 h; right, 5 h. Blue color BisB-stained nuclei. Red color biotin-Qdot.
chelates, which showed considerable uptake into liver, kidney, and salivary glands (Collins et al., 2000).

Apart from SMVT, there are at least four other proteins that have been shown to bind to biotin and which could possibly be involved in the biotin-mediated uptake of Rho-HPMA; these are avidin, biotin-binding protein I (BBPI), and BBPII (all from eggs) (Murthy & Adiga, 1984; White, 1985; White & Whitehead, 1987) and biotinidase, which is present in serum (Seshagiri & Adiga, 1987a; Seshagiri & Adiga, 1987b; Chauhan & Dakshinamurti, 1988). BBPI has also been found in the serum of pregnant rats (Seshagiri & Adiga, 1987a). BBPs analogous to egg yolk BBPs have also been found in the serum of egg laying chickens (Mandella et al., 1978; White & Whitehead, 1978; Hytönen et al., 2007). Although normally, serum biotinidase is derived from synthesis in the liver, biotinidase has been found in the majority of mammalian cells with 25% of the cellular biotinidase activity being located in the nuclear fraction (Pispa, 1965). An important intracellular role for biotinidase may be the biotinylation of histones that is essential for DNA transcription and replication. The increased synthesis of DNA, RNA, and proteins required for rapid cellular division may be “helped” by increased levels of intracellular biotin, with a resultant upregulation in the processes involved in biotin uptake.

The finding that the receptors involved in uptake of biotin by tumor cells is coexpressed with those involved in either vitamin B\textsubscript{12} or folate has tremendous potential in the imaging and treatment of a wide range of highly aggressive tumor cell types including breast, ovary, lung, colon, kidney, and leukemias. As such, targeting with the small molecular weight, water-soluble vitamin, biotin, with its ease of chemical modification and low cost has the potential to readily surpass the more common targeting agents such as monoclonal antibodies and small peptides. The observed perinuclear accumulation of the biotinylated imaging agents suggests that this targeting agent may be ideal for delivery of α-emitting radionuclides such as \textsuperscript{90}Y, \textsuperscript{211}At, \textsuperscript{225}Ac, or \textsuperscript{212}Bi, which have been shown to be highly effective in radionuclide therapy of cancers (Grana et al., 2002).
Studies are continuing to identify the molecules involved in biotin uptake and in the development of targeting and treatment agents.

Conclusions

Vitamins have an essential role in many of the processes involved in the rapid cell division of many highly aggressive tumors. As a result, such tumors, in many instances, upregulate the processes involved in the uptake of several of these vitamins, namely folate, vitamin B12, and biotin. Rhodamine-modified HPMA targeted with these vitamins have been shown to be selectively accumulated within many different tumor types using both in vitro and in vivo models. These vitamins, particularly biotin, have an immense potential to be used as imaging agents and targeting moieties for the delivery of cytotoxic agents to a wide variety of tumors. There is also an enormous potential to use biotin in combination with folate or vitamin B12 as dual targeting for tumor cells.

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Declaration of interest

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References


Preliminary studies on accumulation of vitamin-targeted polymers


