Membrane Transport and Intracellular Sequestration of Novel Thiosemicarbazone Chelators for the Treatment of Cancer

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ABSTRACT
Iron is a critical nutrient for DNA synthesis and cellular proliferation. Targeting iron in cancer cells using specific chelators is a potential new strategy for the development of novel anticancer agents. One such chelator, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT), possesses potent and selective anticancer activity (*J Med Chem* 50:3716–3729, 2007). To elucidate the mechanisms of its potent antitumor activity, Bp4eT was labeled with 14C. Its efficacy was then compared with the 14C-labeled iron chelator pyridoxal isonicotinoyl hydrazone (PIH), which exhibits low anticancer activity. The ability of these ligands to permeate the cell membrane and their cellular retention was examined under various conditions using SK-N-MC neuroepithelioma cells. The rate of 14CPIH uptake into cells was significantly (p < 0.001) lower than that of 14CBP4eT at 37°C, indicating that the increased hydrophilicity of 14CPIH reduced membrane permeability. In contrast, the influx of 14CPIH was significantly (p < 0.05) higher than that of 14CBP4eT, leading to increased cellular retention of 14CBP4eT. In addition, the uptake and release of the 14C-labeled chelators was not reduced by metabolic inhibitors, indicating that these processes were energy-independent. No significant differences were evident in the uptake of 14CBP4eT at 37 or 4°C, demonstrating a temperature-independent mechanism. Furthermore, adjusting the pH of the culture medium to model the tumor microenvironment did not affect 14CBP4eT membrane transport. It can be concluded that 14CBP4eT more effectively permeated the cell membrane and evaded rapid efflux in contrast to 14CPIH. This property, in part, accounts for the more potent anticancer activity of BP4eT relative to PIH.

Introduction
Cancer remains one of the main underlying causes of morbidity and mortality (Curado et al., 2007). Current treatments are far from optimal, having undesirable side effects because of their nonselective nature (Grahame-Smith and Aronson, 2006). Thus, new, improved chemotherapeutic strategies are urgently required.

Iron is an essential cofactor for the catalytic activity of many enzymes including ribonucleotide reductase (RR), which catalyzes the rate-limiting step in DNA synthesis (Kolberg et al., 2004). Because neoplastic cells are generally more metabolically active than their normal counterparts, they require larger amounts of iron (Pahl and Horwitz, 2005). In fact, cancer cells express high levels of transferrin receptor 1, which is responsible for iron uptake from the iron transport protein transferrin (Tf) (Richardson and Baker, 1990; Trinder and Baker, 2003). High expression of transferrin receptor 1 has been demonstrated to correlate with tumor growth and metastasis in animal models (Cavanaugh et al., 1999). In addition, cancer cells have been shown to express greater levels of RR in comparison with normal cells (Elford et al., 1970). Considering this, cancer cells could be expected to be more sensitive to iron deprivation than normal cells (Le and Richardson, 2002).

In the search for more effective anticancer agents, iron chelators have emerged as a novel class of chemotherapeutics worthy of investigation. The chelator desferrioxamine (DFO; Fig. 1A) is typically used for the treatment of iron-overload disease but has also been assessed for anticancer activity (Kalinowski and Richardson, 2005). However, its poor membrane-permeability, because its relative hydrophilicity, short

**ABBREVIATIONS:** RR, ribonucleotide reductase; 2,4-DNP, 2,4-dinitrophenol; BpT, 2-benzoylpyridine thiosemicarbazone; Bp4eT, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone; DFO, desferrioxamine; DMEM + GLU, Dulbecco’s modified Eagle’s medium with glucose; DMEM – GLU, Dulbecco’s modified Eagle’s medium without glucose; HUVEC, human umbilical vein endothelial cells; MES, 4-morpholine ethanesulfonic acid; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen species; Tf, transferrin; MEM, minimal essential medium; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
half-life, and relatively low antiproliferative activity has resulted in mixed outcomes in clinical trials (Kalinowski and Richardson, 2005). This has led to studies examining iron chelators of greater lipophilicity.

In comparison with hydrophilic DFO, the relatively hydrophobic iron chelator pyridoxal isonicotinoyl hydrazone (PIH; Fig. 1B) is able to bind Fe(III) with high selectivity, inducing the mobilization of intracellular iron from a number of cell types (Huang and Ponka, 1983; Richardson and Ponka, 1994; Richardson, 1997). It is noteworthy that although PIH can effectively mobilize iron from cells, it seems to target iron pools that are not necessary for proliferation, demonstrating poor antiproliferative activity in cell culture (IC₅₀ 75 μM) (Richardson et al., 1995).

A series of structure-activity relationship studies of the PIH analogs over the last 15 years has assessed both antiproliferative efficacy and iron chelation activity (Richardson et al., 1995; Becker et al., 2003; Yuan et al., 2004; Whitnall et al., 2006). This work has led to the development of the 2-benzoylpyridine thiosemicarbazone (BpT) chelators, which demonstrate potent and selective anticancer activity (Kalinowski et al., 2007). These ligands have the ability to sequester cellular iron and the capacity to form iron complexes that are redox-active (Kalinowski et al., 2007). Hence, the BpT analogs are able to cause iron mobilization and generate cytotoxic reactive oxygen species (ROS), providing a novel approach for the inhibition of cancer cell growth (Kalinowski et al., 2007).

Although the BpT series possess antiproliferative activity in SK-N-MC neuroepithelioma cells (IC₅₀ 0.002–0.005 μM; Kalinowski et al., 2007), they show 1250- to 3000-fold less efficacy for inhibiting the proliferation of normal MRC-5 fibroblasts (IC₅₀ > 6.25 μM) (Kalinowski et al., 2007). Moreover, BpT chelators are effective in vivo, selectively inhibiting the growth of human tumor xenografts in nude mice (Y. Yu and D. R. Richardson, unpublished results). Within the BpT series, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT; Fig. 1C), showed marked antiproliferative activity with an IC₅₀ of 0.002 μM and was a clear leading candidate for further studies (Kalinowski et al., 2007).

Although Bp4eT possessed potent and selective anticancer activity, the exact mechanisms behind these important properties remain unclear. Thus, the aim of the current study was to investigate the mechanisms of action of the potent antiproliferative activity of Bp4eT in comparison with PIH, which exhibits poor antiproliferative efficacy. To do this, ¹⁴C-labeled chelators were used. Because chemotherapeutic cytotoxicity depends on a variety of factors, including drug transport across membranes and retention within cells (Gottesman et al., 2002), both the cellular uptake and release of ¹⁴C chelators were examined. In this investigation, the effects of temperature, metabolic inhibitors, pH, and extra- cellular protein on the cellular retention of the chelators were measured. In addition, the cellular uptake of ¹⁴C chelators was analyzed in a variety of cultured neoplastic and normal cell types. It can be concluded that Bp4eT has efficient membrane transport and retention within tumor cells compared with PIH, resulting in greater drug delivery and maximal cytotoxicity.

Fig. 1. Chemical structure of the iron chelators DFO (A), PIH (B), and Bp4eT (C).

Materials and Methods

¹⁴C Chelators. [¹⁴C]PIH and [¹⁴C]Bp4eT were synthesized by The Institute of Isotopes Ltd. (Budapest, Hungary), incorporating the ¹⁴C label at the imine carbon, which represents a highly stable site for incorporating the isotope. The final purity of both compounds was determined by high-performance liquid chromatography using UV detection. The certificate of analysis indicated the purities of [¹⁴C]PIH and [¹⁴C]Bp4eT were 95.5 and 100%, respectively, with a final specific radioactivity of 75 μCi/mg. All compounds were prepared as their hydrochloride salts to maximize solubility. Chelators were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) as 10 mM stock solutions and diluted in complete medium so that the final dimethyl sulfoxide concentration was < 0.5% (v/v).

Cell Culture. The human SK-N-MC neuroepithelioma cell line (American Type Culture Collection, Manassas, VA) was grown as described previously (Richardson et al., 1995). In brief, the cells were grown in minimal essential medium (MEM; Invitrogen, Mulgrave, VIC, Australia) with 10% (v/v) fetal calf serum (Sigma-Aldrich) and supplemented with the following additions from Invitrogen: 1% (v/v) sodium pyruvate, 1% (v/v) nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glucose, and 0.28 ng/ml Fungizone. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air in a water-jacketed incubator (Forma Series II; Thermo Fisher Scientific, Marietta, OH). The SK-N-MC cell line was chosen for the majority of these investigations because its iron metabolism and the effect of a variety of chelators on this cell type is well characterized (Richardson and Ponka, 1994; Richardson et al., 1995).

The human SK-Mel-28 melanoma, DMS-53 lung carcinoma, MCF-7 breast cancer, and MRC-5 fibroblast cell lines were also obtained from the American Type Culture Collection (Manassas, VA). These cell lines were cultured in a manner similar to that of the SK-N-MC cell line described above. Human umbilical vein endothelial cells (HUVECs) were kindly donated by Pat Psansarakit (Heart Research Institute, Sydney, Australia). HUVECs were cultured in M199 media (SAFC Biosciences, St Louis, MO) with the standard
supplementation described above for MEM alone and 0.5% vascular endothelial growth factor (Starrate, Bethungra, Australia).

**General 14C Chelator Cellular Uptake Procedure.** The 14C chelator uptake into cells was examined using the SK-N-MC cell line by implementing standard protocols (Huang and Ponka, 1983; Richardson, 1997). A cell suspension of SK-N-MC cells at 10^6 cells/ml was added to tissue culture plates and incubated for 24 h at 37°C to produce a confluent monolayer. In experiments designed to examine 14C chelator uptake, the cells were incubated with complete media containing [14C]Bp4eT (5–250 μM), [14C]PH (5–250 μM), or no chelator (control) at 37°C for 120 min. After these studies, a 14C chelator concentration of 25 μM was selected because it provided appropriate labeling efficiency of cells that led to a highly sensitive assay. In experiments measuring the effect of temperature on 14C chelator uptake, the cells were incubated in media containing 14C chelator at 4 or 37°C for up to 120 min.

**Studies examining the influence of the Warburg effect** (Warburg, 1956) on 14C chelator uptake used pH-adjusted complete media. These media were altered to final pH 5.5 or 6.5 using MES (25 mM; Sigma-Aldrich) and to pH 7.4 and 8.0 (control media) using HEPES (25 mM; Sigma-Aldrich).

During all incubations with chelators, cells remained viable as judged by cellular morphology, adherence to the culture substratum, and the exclusion of trypsin blue. This was determined using a Countess Automated Cell Counter (Invitrogen, Carlsbad, CA). At the conclusion of the incubation period, the media were aspirated, and the cells were washed four times on ice with ice-cold phosphate-buffered saline (PBS) to remove extracellular 14C chelator. Then, PBS (1 ml) was added to each plate, and the cells were removed from the substratum using a plastic spatula. This cell suspension was placed into counting tubes to quantitate 14C chelator uptake into cells. Scintillation fluid (2.5 ml; PerkinElmer Life and Analytical Sciences, Melbourne, VIC, Australia) was added to the cell suspension, and the radioactivity of each sample, including backgrounds and 14C chelator standards, was counted using a MicroBeta Counter (PerkinElmer Life and Analytical Sciences). Results were expressed as molecules of chelator per cell as a function of chelator concentration or incubation time.

In some experiments, 14C chelator uptake in a variety of normal and cancer cell types was compared and calculated as a function of the surface area of the cells. This was performed because the relative size of the cells varied markedly. The surface area (measured in squared micrometers) of each cell type was determined using ImageJ 1.42 software (http://rsbweb.nih.gov/ij/) analysis of phase-contrast images taken with a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam camera and AxioVision Release 4.7 Software (Carl Zeiss GmbH, Jena, Germany). The average cell surface area was calculated after measuring 100 cells per cell type.

**The Effect of Metabolic Inhibitors on 14C Chelator Uptake.** The effect of metabolic inhibitors on the uptake of 14C chelators was studied using five well characterized inhibitors (Sigma-Aldrich), including sodium azide (NaN₃; 30 mM), oligomycin (30 μM), sodium cyanide (NaCN; 5 mM), sodium fluoride (NaF; 15 mM) and 2,4-dinitrophenol (2,4-DNP; 2 mM). In brief, SK-N-MC neuroepithelioma cells were preincubated with inhibitors or media alone for 30 min at 37°C. The media were removed and replaced with media containing [14C]PH (25 μM) or [14C]Bp4eT (25 μM) in the presence or absence of inhibitors, and the cells were subsequently incubated for 60 min at 37°C. The remainder of the experiment was conducted using the general uptake procedure described above. During these studies, Dulbecco’s modified Eagle’s medium without glucose (DMEM – GLU; Invitrogen) was used to aid the ATP-depletion induced by the metabolic inhibitors (Richardson, 1997). Results were expressed as a percentage of the control, namely DMEM + GLU.

**General 14C Chelator Cellular Efflux Procedure.** The release of the 14C chelators from prelabeled SK-N-MC cells was performed using well established techniques (Huang and Ponka, 1983; Richardson et al., 1995; Richardson, 1997). In brief, SK-N-MC cells were prelabeled with either [14C]Bp4eT (25 μM), [14C]PH (25 μM), or media alone for 120 min at 37°C. The cells were then placed on ice, the media were aspirated, and the cell monolayer was washed four times with ice-cold PBS. For experiments examining the effect of temperature on 14C chelator release, complete media (1 ml; 4 or 37°C) were added to each plate, and the cells were incubated at 4 or 37°C for up to 180 min. There was no significant decrease in viability throughout these incubations as measured by the Trypan blue exclusion assay. Experiments conducted to analyze the effect of pH on 14C chelator efflux were incubated at 37°C for up to 120 min in pH-adjusted media (pH 5.5, 6.5, 7.4, or 8.0), as described for uptake experiments above. In some studies, the effect of the extracellular proteins Tf and albumin on 14C chelator release was assessed. In these experiments, cells were incubated at 37°C in MEM without fetal calf serum (control) or in this medium containing Tf (5 or 40 mg/ml) or albumin (5 or 40 mg/ml).

At the end of each incubation period, the cells were placed on ice, and the overlying media were placed into counting tubes to estimate the level of extracellular 14C chelator. Then, PBS (1 ml) was added to the cells, which were subsequently scraped from the plates using a plastic spatula. This suspension was placed into counting tubes to represent the fraction of intracellular 14C chelator. Scintillation fluid (2.5 ml) was added to each sample and to the backgrounds and standards, and the tubes were counted using a MicroBeta Counter.

**The Effect of Metabolic Inhibitors on 14C Chelator Efflux.** SK-N-MC cells were preincubated with [14C]PH (25 μM) or [14C]Bp4eT (25 μM) in DMEM + GLU for 90 min at 37°C. The cells were then placed on ice, and the metabolic inhibitors described previously or media alone were added and incubated for 30 min at 37°C. After four washes with ice-cold PBS, the plates were reincubated in DMEM – GLU for 5, 15, 30, and 60 min at 37°C in media in the presence or absence of inhibitors. The remainder of the experiment was completed in accordance with the general efflux methods described above.

It should be noted that the inhibitor 2,4-DNP was not used in efflux studies because of problems associated with its intense color that led to quenching and thus problems associated with quantification of 14C by β-counting. On the other hand, in uptake experiments, the inhibitors were washed off cells before β-counting, and thus, quenching did not affect the counting efficiency of 2,4-DNP-treated samples.

**Cellular ATP Determination.** Parallel ATP determinations were performed simultaneously in uptake and efflux experiments involving the use of metabolic inhibitors to confirm their inhibitory effects on energy metabolism. ATP levels were quantitatively analyzed using an ATP bioluminescence assay kit (Sigma-Aldrich) following the manufacturer’s instructions. Throughout the experiment, assay samples were kept on ice. In brief, to lyse cells, the samples underwent three freeze-thaw cycles, and plates were then scraped in distilled water (140 μl). The samples were then centrifuged at 4°C for 15 min at 14,000 rpm. The ATP assay mix (100 μl) was added to a 96-well plate, mixed, and allowed to stand for 3 min at room temperature to remove any endogenous ATP. The sample supernatant (100 μl) was then added to the ATP assay mix, shaken, and the fluorescence was measured on a microplate reader (560 nm). A new standard curve was generated with each assay using the ATP standard supplied.

**Statistical Analysis.** Results are expressed as mean ± S.E.M. Statistical significance was determined using the Student’s t test, one-way ANOVA, or two-way ANOVA with replication. Results were considered statistically significant when p < 0.05.

**Results**

**Rate of 14C Chelator Uptake.** The Bp4T series of ligands have shown high antiproliferative activity and selectivity against cancer cells (Kalinoeiski et al., 2007). To further
understand their mechanism of action, the current studies have assessed the ability of one of the most potent members of this series of compounds, namely Bp4eT, to permeate cancer cell membranes. For comparison with Bp4eT, we have assessed the uptake of the chelator, PIH, which shows some structural similarity (Fig. 1), but demonstrates poor antiproliferative activity (Richardson et al., 1995). By determining the cellular uptake and subsequent release of these ligands, information relevant to their marked differences in antiproliferative activity should be obtained.

In initial studies, the uptake of [14C]Bp4eT and [14C]PIH were measured after a 120-min incubation at 37°C to evaluate the rate of entry of the ligands into SK-N-MC neuroepithelioma cells (Fig. 2A). The uptake of both chelators increased as a linear function of chelator concentration \(r^2 = 0.99\) in the range of 5 to 250 \(\mu\)M without any evidence of saturation. Higher concentrations of chelator were not used because of the potential toxic effects on cells, leading to spurious results, and also because of limitations in the solubility of these relatively hydrophobic compounds. Although both 14C chelators possessed linear uptake kinetics, the rate of [14C]PIH uptake \(1.45 \times 10^6\) molecules of chelator/cell/min) was significantly \(p < 0.001\) lower than that of [14C]Bp4eT (3.02 \(\times 10^6\) molecules of chelator/cell/min). These results demonstrated that [14C]Bp4eT had an increased rate of entry into SK-N-MC cells than [14C]PIH (Fig. 2A).

The Effect of Temperature on 14C Chelator Uptake. The effect of temperature and time on 14C chelator uptake was investigated to determine whether cellular uptake of [14C]Bp4eT was markedly and significantly \(p < 0.001\) greater than [14C]PIH uptake was investigated to determine whether [14C]Bp4eT had an increased rate of entry into SK-N-MC cells than [14C]PIH (Fig. 2A).

Within 15 min of reincubation at 37°C, the release of [14C]Bp4eT was significantly \(p < 0.05\) greater than that of [14C]PIH released from cells at 37°C. After the 180-min reincubation, \(57 \pm 3\%\) of [14C]Bp4eT was released at 37°C. In comparison, significantly \(p < 0.01\) lower levels of [14C]Bp4eT were released at 4°C (20 ± 2%) after 180 min (Fig. 2C). Thus, [14C]Bp4eT release from cells was temperature-dependent in contrast to its uptake (Fig. 2B).

Within 15 min of reincubation at 37°C, the release of [14C]PIH was significantly \(p < 0.05\) greater than that of [14C]Bp4eT, with \(80 \pm 2\%\) of [14C]PIH released from cells after 180 min (Fig. 2C). In addition, the percentage of cellular [14C]PIH released was significantly \(p < 0.001\) lower at 4°C (17 ± 2% at 180 min) compared with 37°C (80 ± 2% at 180 min). In fact, the release of [14C]PIH at 4°C was similar to [14C]Bp4eT at 4°C (Fig. 2C). Therefore, the release of intra-

Fig. 2. A, the rate of [14C]Bp4eT and [14C]PIH uptake by SK-N-MC cells. The cells were incubated in media containing [14C]Bp4eT or [14C]PIH at 5 to 250 \(\mu\)M for 120 min at 37°C. The cells were then plated on ice, washed four times using ice-cold PBS, and radioactivity was quantified. B, the effect of temperature and time on 14C chelator uptake by SK-N-MC cells. Cells were incubated with media containing [14C]Bp4eT (25 \(\mu\)M) or [14C]PIH (25 \(\mu\)M) at 37 or 4°C for up to 120 min. The remainder of the experiment was performed as for A, C and D, the effect of temperature and time on 14C chelator efflux. Cells were prelabeled with [14C]Bp4eT (25 \(\mu\)M) or [14C]PIH (25 \(\mu\)M) for 120 min at 37°C. Cells were then plated on ice and washed four times with ice-cold PBS and reincubated in media for up to 180 min at 37 or 4°C. Results are expressed as mean ± S.E.M. from triplicate determinations of three experiments.
cellular $[^{14}\text{C}]{\text{PIH}}$ was also found to be temperature-dependent, as identified for its uptake (Fig. 2B).

Considering the greater intracellular concentrations of $[^{14}\text{C}]{\text{Bp4eT}}$ within cells (Fig. 2B) and its lower release relative to $[^{14}\text{C}]{\text{PIH}}$ (Fig. 2C), it is clear that $[^{14}\text{C}]{\text{Bp4eT}}$ was becoming sequestered within cells (Fig. 2D). In fact, after a 180-min reincubation at 37°C, $6.4 \pm 1.2 \times 10^7$ molecules per cell of $\text{Bp4eT}$ were found. This was equal to $39 \pm 7\%$ of the total $[^{14}\text{C}]{\text{Bp4eT}}$ initially found intracellularly when the reincubation began. In contrast, 32-fold less $[^{14}\text{C}]{\text{PIH}}$ remained associated within the cell after a 180-min reincubation at 37°C (i.e., $0.2 \pm 0.01 \times 10^7$ molecules/cell; Fig. 2D). This represented $12 \pm 0.2\%$ of the $[^{14}\text{C}]{\text{PIH}}$ found initially within the cell at the start of the reincubation. Hence, far more $[^{14}\text{C}]{\text{Bp4eT}}$ remained sequestered within the cell relative to $[^{14}\text{C}]{\text{PIH}}$.

The Effect of Metabolic Inhibitors on $[^{14}\text{C}]{\text{Chelator Uptake.}}$ Considering the results described above, experiments were then performed to determine whether chelator uptake was energy-dependent (Fig. 3, A and B). The effect of five well characterized metabolic inhibitors, namely NaN$_3$, oligomycin, NaCN, 2,4-DNP, and NaF (Henderson and Zevely, 1984; Svec, 1985; Qian and Morgan, 1991; Richardson, 1997), on $[^{14}\text{C}]{\text{Bp4eT}}$ and $[^{14}\text{C}]{\text{PIH}}$ uptake into SK-N-MC cells was investigated at 37°C. In addition, in these studies, ATP assays were performed in parallel to $[^{14}\text{C}]{\text{ligand}}$ uptake experiments to assess the effects of the inhibitors on energy metabolism (Fig. 3, C and D). Studies compared the effects on $[^{14}\text{C}]{\text{ligand}}$ uptake in DMEM $\pm$ GLU (Fig. 3). Furthermore, in all of these experiments, the metabolic inhibitors were added to DMEM – GLU to ensure ATP depletion of cells (Richardson, 1997).

Although the metabolic inhibitors NaN$_3$, oligomycin, NaCN, 2,4-DNP and NaF significantly ($p < 0.01$) reduced cellular ATP levels compared with control medium (namely DMEM + GLU; Fig. 3C), they did not significantly ($p > 0.05$) decrease $[^{14}\text{C}]{\text{Bp4eT}}$ uptake (Fig. 3A). In fact, 2,4-DNP caused a significant ($p < 0.01$) increase in intracellular $[^{14}\text{C}]{\text{Bp4eT}}$ uptake compared with control medium (Fig. 3A). The increased uptake of $[^{14}\text{C}]{\text{Bp4eT}}$ in the presence of 2,4-DNP could be consistent with nonspecific adsorption of the $^{14}\text{C}$-labeled ligand to the cell because of membrane damage, as observed for other molecules (Baker et al., 1992). In contrast to 2,4-DNP, the inhibitors NaN$_3$, oligomycin, NaCN, and NaF had no significant ($p > 0.05$) effect on $[^{14}\text{C}]{\text{Bp4eT}}$ uptake compared with the control (DMEM + GLU). Overall, all five metabolic inhibitors had no inhibitory effects on $[^{14}\text{C}]{\text{Bp4eT}}$ uptake, suggesting that $[^{14}\text{C}]{\text{Bp4eT}}$ uptake is an energy-independent process.

In addition, no significant ($p > 0.05$) inhibitory effect of the metabolic poisons was evident on the uptake of $[^{14}\text{C}]{\text{PIH}}$ by cells compared with the controls (Fig. 3B). This was despite the effect of these inhibitors to significantly ($p < 0.01$) reduce ATP levels relative to the control (DMEM + GLU; Fig. 3D). Both 2,4-DNP and NaF increased $[^{14}\text{C}]{\text{PIH}}$ uptake relative to the control (Fig. 3B). Again, this may be due to membrane damage induced by the inhibitor, leading to increased nonspecific adsorption of $[^{14}\text{C}]{\text{PIH}}$. In summary, these results suggest that energy-dependent mechanisms may also be responsible for the uptake of $[^{14}\text{C}]{\text{PIH}}$.

The Effect of Metabolic Inhibitors on $[^{14}\text{C}]{\text{Chelator Efflux.}}$ Further studies then examined the effect of metabolic inhibitors on the cellular release of $[^{14}\text{C}]{\text{Bp4eT}}$ and $[^{14}\text{C}]{\text{PIH}}$ to determine whether this was an energy-dependent process (Fig. 4, A and B). Again, cellular ATP levels were assessed simultaneously to confirm the inhibitory effects of the metabolic inhibitors (Fig. 4, C and D). Although inhibitor-treated cells possessed significantly ($p < 0.001$) lower levels of ATP than control medium (DMEM $\pm$ GLU; Fig. 4, C and D), no significant ($p > 0.05$) effect was observed on the cellular release of $[^{14}\text{C}]{\text{Bp4eT}}$ and $[^{14}\text{C}]{\text{PIH}}$ (Fig. 4, A and B). These results indicated that the cellular release of $[^{14}\text{C}]{\text{Bp4eT}}$ and $[^{14}\text{C}]{\text{PIH}}$ occurred through an energy-independent mechanism, involving the passive diffusion of both of these ligands across the cell membrane.

The Effect of pH on $[^{14}\text{C}]{\text{Chelator Uptake.}}$ A common hallmark of malignant neoplasms is the Warburg effect, in which the tumor possesses a slightly acidic (pH 6.92–7.24) microenvironment (Warburg, 1956; Helmlinger et al., 1997). Because the thiosemicarbazone classes of chelators are...
polyprotic (Richardson et al., 1990, 2006), their net charge is dependent on the pH of the solution. This can markedly alter membrane permeability, and thus it was important to understand the potential influence the Warburg effect has on the cellular uptake of $^{14}$C]Bp4eT and $^{14}$C]PIH. Considering this, the pH of the medium was altered using a variety of buffers (see Materials and Methods) so that the final pH was 5.5, 6.5, 7.4, or 8.0 (Fig. 5, A–D). These experiments demonstrated that the initial cellular uptake of $^{14}$C]Bp4eT was significantly ($p < 0.05$) lower at pH 5.5 and 6.5 than at pH 7.4 between 5 and 30 min at 37°C (Fig. 5A). However, for the remainder of the incubation up to 120 min at 37°C, there was no significant ($p > 0.05$) difference in uptake compared with pH 7.4 (Fig. 5A). At pH 8.0, the uptake of $^{14}$C]Bp4eT was not significantly different from that found at pH 7.4. In summary, after a 2-h incubation, intracellular levels of $^{14}$C]Bp4eT were not altered by the pH range examined.

In contrast to the results found for $^{14}$C]Bp4eT, the cellular uptake of $^{14}$C]PIH demonstrated considerable dependence on pH (Fig. 5B). At time points of 30 min or greater, significantly ($p < 0.01$) higher levels of intracellular $^{14}$C]PIH were evident at pH values of 5.5 and 6.5 compared with control, at pH 7.4. It is noteworthy that the cellular uptake of $^{14}$C]PIH was greatest at pH 5.5, and this was found to be approximately double that found at pH 7.4 (Fig. 5B). On the other hand, no significant ($p > 0.05$) difference in $^{14}$C]PIH uptake was evident at pH 8.0 compared with pH 7.4. Therefore, the cellular uptake of $^{14}$C]PIH was enhanced under slightly acidic conditions.

The Effect of pH on $^{14}$C Chelator Efflux. Additional experiments assessed the effect of extracellular pH on the cellular release of $^{14}$C]Bp4eT and $^{14}$C]PIH to analyze the influence the Warburg effect may have on tumor drug retention in vivo (Fig. 5, C and D). The cellular release of $^{14}$C]Bp4eT and $^{14}$C]PIH was not significantly ($p > 0.05$) altered by extracellular pH, with values of 5.5, 6.5, and 8.0, compared with the control, at pH 7.4.

The Effect of Proteins on $^{14}$C Chelator Efflux. In general, tumors possess leaky, immature and tortuous blood vessels (Carmeliet and Jain, 2000), resulting in high levels of protein within the tumor interstitium (Greish, 2007). Furthermore, interactions of drugs with proteins may also affect the bioavailability of a drug (van der Veld et al., 2008). Thus, the cellular release of $^{14}$C]Bp4eT and $^{14}$C]PIH was performed in the presence and absence of proteins, namely human Tf and albumin, at concentrations of 5 or 40 mg/ml (Fig. 6, A and B) to determine whether the protein microenvironment of tumors affects chelator retention. The cellular release of $^{14}$C]Bp4eT was significantly ($p < 0.05$) lower in protein-free media (control) compared with media containing Tf (5 or 40 mg/ml) or albumin (5 or 40 mg/ml; Fig. 6A). However, no significant ($p > 0.05$) difference in $^{14}$C]Bp4eT release was evident among the varying concentrations of albumin and Tf analyzed, suggesting that the release of the ligand from cells was independent of the specific protein type and concentration. These results indicate that the proteins could act as an extracellular “sink” to facilitate the release of $^{14}$C]Bp4eT from cells. In contrast, no significant ($p > 0.05$) difference in the release of $^{14}$C]PIH was observed in the presence or absence of proteins (Fig. 6B).

$^{14}$C Chelator Uptake in Normal and Cancer Cells. Our previous studies have shown that Bp4eT markedly inhibits the growth of cancer cells relative to normal cells both in vitro (Kalinowski et al., 2007) and in vivo (Y. Yu and D. R. Richardson, unpublished data). Moreover, it is essential that clinically used chemotherapeutic agents preferentially target cancer cells as opposed to normal tissue to minimize side effects (Graham-Smith and Aronson, 2006). Thus, the following experiments were designed to examine the differences in the uptake of $^{14}$C]Bp4eT and $^{14}$C]PIH between cancer (SK-N-MC neuroepithelioma, MCF-7 breast cancer, and SK-Mel-28 melanoma cells) and normal cells (MRC-5 fibroblasts and endothelial cells (HUVECs)). These studies were performed using confluent (100%) and subconfluent (50%) plates.
of cells to examine the effect of the degree of confluence on cellular uptake. Results were expressed as molecules of chelator per total surface area per cell. This was necessary because the surface area of different cell types varied markedly, and this allowed the accurate quantification of the uptake of the chelators for comparison between cells.

Under confluent conditions, MCF-7 breast cancer cells possessed significantly (p < 0.05) higher intracellular levels of \([14C]Bp4eT\) compared with MRC-5 and HUVEC cell types (Fig. 7A). In addition, the SK-N-MC cell line also showed significantly (p < 0.05) increased intracellular levels of \([14C]Bp4eT\) compared with MRC-5 cells, although no significant (p > 0.05) differences were evident compared with HUVEC cells. Furthermore, there was no significant (p > 0.05) increase in the cellular uptake of \([14C]Bp4eT\) in SK-Mel-28 melanoma cells relative to normal cell types (Fig. 7A). It is noteworthy that the uptake of \([14C]Bp4eT\) differed in subconfluent cells, and this may be due to the adherence of the ligand to the plastic substratum of the culture plates under subconfluent conditions (Fig. 7B). Under these conditions, similar levels of \([14C]Bp4eT\) uptake were observed in SK-N-MC and SK-Mel-28 cells compared with normal cell lines (Fig. 7B). In contrast, significantly (p < 0.05) higher intracellular levels of \([14C]Bp4eT\) were present in MCF-7 cells relative to that in normal cells. Together, these results demonstrate that generally there is no clear difference in the uptake of \([14C]Bp4eT\) between normal and neoplastic cells. However, uptake of this ligand by MCF-7 breast cancer cells was greater than that found for the normal and other neoplastic cells.

No significant increase in \([14C]PIH\) uptake by cancer cell lines relative to normal cells was observed under confluent conditions (Fig. 7C). However, the uptake of \([14C]PIH\) was markedly and significantly (p < 0.001) greater by HUVECs than any other neoplastic or normal cell type (Fig. 7C). In addition, under subconfluent conditions, \([14C]PIH\) uptake into normal MRC-5 cells was significantly (p < 0.05) lower than all other cancer and normal cells (Fig. 7D), demonstrating that \([14C]PIH\) showed no selective uptake under subconfluent conditions.

**Discussion**

The toxicity of anticancer drugs and tumor resistance remain major problems in oncology (Grahame-Smith and Aronson, 2006). Common causes for resistance and toxicity to chemotherapeutic agents include insufficient drug delivery to the tumor, inadequate drug uptake, and/or rapid efflux (Gottesman et al., 2002). As a consequence, the current studies were undertaken to examine the membrane transport of the potential antitumor agent Bp4eT.

**Bp4eT Rapidly Enters Cells via a Metabolic Energy-Independent Mechanism Consistent with Diffusion.** As part of this study, the uptake of \([14C]Bp4eT\), which possesses marked anticancer activity (Kalinowski et al., 2007), was compared with \([14C]PIH\), a ligand with low antiproliferative activity (Richardson et al., 1995). The rate of \([14C]Bp4eT\) uptake by cells was markedly greater than that of \([14C]PIH\) (Fig. 2A), illustrating that \([14C]Bp4eT\) can more easily and rapidly permeate cells. This can be attributed to the lipophilic nature of Bp4eT (logP 4.02) (Kalinowski et al., 2007), in contrast to the relatively more hydrophilic ligand, PIH (logP −2.11) (Richardson et al., 1995). Numerous studies have shown that hydrophilic chelators possess poor antiproliferative activity, whereas compounds with relatively high lipophilicity have potent antiproliferative effects (Richardson et al., 1995; Hodges et al., 2004). As a consequence, the higher intracellular levels of \([14C]Bp4eT\), because of its greater membrane permeability, may be partly responsible for its potent anticancer activity (Kalinowski et al., 2007). In contrast, the relatively hydrophilic \([14C]PIH\) led to lower membrane permeability, correlating with its poor antiproliferative activity (Richardson et al., 1995).

Because some transporters can influence cellular drug retention, such as the multidrug resistance-associated protein and P-glycoprotein (Gottesman et al., 2002), it was important...
to examine the mechanisms involved in membrane transport of \(^{14}\text{C}\)Bp4eT and \(^{14}\text{C}\)PIH. The current results indicate that Bp4eT permeates cells via passive diffusion. The following three observations support this conclusion. First, results obtained from \(^{14}\text{C}\) chelator uptake studies as a function of ligand concentration (Fig. 2A) demonstrated that uptake occurred by zero-order kinetics and remained unsaturated throughout the broad concentration range used. Second, \(^{14}\text{C}\)Bp4eT uptake by cells was not reduced despite depleting ATP stores using metabolic inhibitors (Fig. 3, A and C). The third piece of evidence demonstrating that \(^{14}\text{C}\)Bp4eT permeates membranes through a passive process can be deduced by comparing its uptake at 37 and 4°C. For the duration of the steady state (Fig. 2B), there was no significant difference in intracellular \(^{14}\text{C}\)Bp4eT at 37 and 4°C, demonstrating temperature-independent uptake. Together, Bp4eT permeates cells via passive diffusion rather than active transport.

Bp4eT and PIH Are Released from Cells by a Temperature-Dependent Mechanism, but Hydrophobic Bp4eT Is Sequestered in Cells Relative to PIH. Although it is important for adequate quantities of drug to permeate cells, it is also essential that they evade efflux to allow sufficient quantities to be retained to cause cytotoxicity. It is therefore essential that they evade efflux to allow sufficient quantities to be retained to cause cytotoxicity. The temperature-dependent mechanism, but hydrophobic Bp4eT is sequestered in cells relative to PIH. In these studies, Bp4eT and PIH demonstrated a significant reduction in \(^{14}\text{C}\) chelator release at 4°C than at 37°C (Fig. 2C). These findings are consistent with a temperature-dependent release mechanism and may result from a change in membrane fluidity that occurs upon decreasing temperature affecting drug transport.

The Warburg Effect Does Not Influence Bp4eT Uptake or Release by Tumor Cells. Tumors often contain a slightly acidic microenvironment because of enhanced glycolysis, which has been referred to as the Warburg effect (Warburg, 1956). Considering the chelators used are polyprotic compounds, their charge is influenced by pH, which affects membrane transport (Richardson et al., 2006). This latter compound predominantly exists in its neutral form at pH 4 to 11 (Richardson et al., 2006). This result is consistent with the species distribution of PIH as a function of pH, in which its neutral species predominates at pH 6 (Richardson et al., 1990; Doungdee et al., 1995).

In contrast, alterations in pH had no significant effect on \(^{14}\text{C}\)Bp4eT uptake (Fig. 5A). These data indicate that \(^{14}\text{C}\)Bp4eT is present largely as a neutral ligand between pH 4 and 8.0, resulting in facile passage across the cell membrane. This suggestion is in accordance with the predicted protonation constants of Bp4eT based on the structure of very similar DpT ligands, particularly Dp4eT (Richardson et al., 2006). This latter compound predominantly exists in its neutral form at pH 4 to 11 (Richardson et al., 2006). Accordingly, although the tumor milieu varies with respect to pH in accordance with the Warburg effect (Warburg, 1956), it is unlikely that it would influence Bp4eT uptake.

Extracellular Protein Increases Efflux of Bp4eT but Not PIH. Another factor in tumors that could affect the retention of these compounds is the quantity of protein in the interstitial fluid. Tumors possess more permeable blood vessels (Carmeliet and Jain, 2000), which result in higher levels of proteins that favor the release of \(^{14}\text{C}\)Bp4eT but not \(^{14}\text{C}\)PIH, due to the higher permeability of Bp4eT compared to PIH.

Fig. 7. The uptake of \(^{14}\text{C}\)Bp4eT (A and B) and \(^{14}\text{C}\)PIH (C and D) as a function of time in 100% confluent (A and C) and 50% subconfluent (B and D) cancer and normal cells. Confluent and subconfluent cancer cell lines, including SK-N-MC, MCF-7, and SK-Mel-28, as well as normal cells, such as MRC-5 and HUVeCs, were incubated with \(^{14}\text{C}\)Bp4eT (25 \(\mu\text{M}\)) or \(^{14}\text{C}\)PIH (25 \(\mu\text{M}\)) containing media at 37°C for 15 or 120 min. Cells were then placed on ice and washed four times with ice-cold PBS, and the radioactivity was quantified. Results are expressed as mean \(\pm\) S.E.M. from triplicate determinations in three experiments.
notable that low levels of extracellular protein (5 mg/ml) resulted in increased retention of intracellular [14C]Bp4eT (Buss et al., 2002, 2003). It is also notable that low levels of extracellular protein (5 mg/ml) resulted in increased efflux of [14C]Bp4eT at high concentrations of protein (40 mg/ml). Thus, the higher protein levels within tumor interstitial fluid as a result of increased permeability of tumor vasculature (Carmeliet and Jain, 2000) will probably not promote [14C]Bp4eT release to any greater extent than in normal tissues. Conversely to Bp4eT, the presence of protein in medium had no marked effect on [14C]PIH efflux at either protein concentration examined (Fig. 6B). These observations could be interpreted to suggest the added proteins preferentially bind the hydrophobic ligand, Bp4eT, to a greater extent than the more hydrophilic chelator, PIH.

All Cancer Cell Lines Did Not Preferentially Accumulate Higher Intracellular Levels of Bp4eT Relative to Normal Cells. Effective chemotherapy requires compounds to preferentially target cancer cells over normal cells to minimize systemic toxicity (Grahame-Smith and Aronson, 2006). Previous studies demonstrated that Bp4eT has selective anti-proliferative activity in vitro (Kaliniowski et al., 2007) and in vivo (Y. Yu and D. R. Richardson, unpublished results). Furthermore, closely related compounds of the DpT class showed selective antitumor activity in vivo (Whittall et al., 2006). Therefore, 14C chelator uptake was compared between cancer and normal cells. Although increased [14C]Bp4eT uptake was evident in MCF-7 cancer cells compared with normal cells, this difference was not apparent in all tumor cell types (Fig. 7, A and B). Thus, other factors may be responsible for the selective anticancer activity of Bp4eT. These could include the greater sensitivity of cancer cells to iron deprivation and their effect on other iron-dependent targets, including RR, the metastasis suppressor NDRG1, and cyclin D1 (Le and Richardson, 2004; Yu et al., 2007).

In conclusion, [14C]Bp4eT and [14C]PIH uptake occurs through passive diffusion. It is noteworthy that these results reveal the lipophilic chelator [14C]Bp4eT can rapidly permeate cells, reaching higher intracellular levels than the more hydrophilic ligand, [14C]PIH. In addition, [14C]Bp4eT was retained within cells to a greater extent than [14C]PIH. Thus, [14C]Bp4eT easily enters cancer cells and sufficient levels of the ligand or its redox-active iron complex are retained to facilitate cytotoxicity. In contrast, [14C]PIH was efficiently released, which probably contributes to its low antiproliferative effects. These studies also demonstrate that [14C]Bp4eT membrane transport was not adversely affected by pH. Hence, it is unlikely that the mildly acidic tumor microenvironment would hinder Bp4eT uptake, enabling maximum efficacy.

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References


Richardson DR (1997) Mobilization of iron from neoplastic cells by some iron chelators is an energy-dependent process. Biochim Biophys Acta 1320:45–57.


van der Veldt AA, Luurtsema G, Luitertink M, Lammertsma AA, and Hendriks NH.
(2008) Individualized treatment planning in oncology: role of PET and radiola-
abeled anticancer drugs in predicting tumour resistance. Curr Pharm Des 14:
2914–2931.
with a wide spectrum of potent antitumor activity that overcomes resistance to
Yu Y, Kovacevic Z, and Richardson DR (2007) Tuning cell cycle regulation with an
chelators with marked and selective antitumor activity: in vitro and in vivo
Zimmer G and Schirmer H (1974) Viscosity changes of erythrocyte membrane and
membrane lipids at transition temperature. Biochim Biophys Acta 345:314–
320.

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