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

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Abbreviations: 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; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; HUVECs, human umbilical vein endothelial cells; MES, 4-morpholine ethanesulfonic acid; NaCN, sodium cyanide; NaF, sodium fluoride; NaN₃, sodium azide; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen species; RR, ribonucleotide reductase; Tf, transferrin; TfR1, transferrin receptor 1.
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 anti-cancer agents. One such chelator, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT), possesses potent and selective anti-cancer activity (Kalinowski, D.S. et al., (2007) J. Med. Chem. 50:3716-3729). To elucidate the mechanisms of its potent anti-tumor activity, Bp4eT was labeled with $^{14}$C. Its efficacy was then compared to the $^{14}$C-labeled iron chelator, pyridoxal isonicotinoyl hydrazone (PIH), which exhibits low anti-cancer 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 $^{14}$C-PIH uptake into cells was significantly ($p<0.001$) lower than that of $^{14}$C-Bp4eT at $37^\circ C$, indicating that the increased hydrophilicity of $^{14}$C-PIH reduced membrane permeability. In contrast, the efflux of $^{14}$C-PIH was significantly ($p<0.05$) higher than that of $^{14}$C-Bp4eT, leading to increased cellular retention of $^{14}$C-Bp4eT. Additionally, the uptake and release of the $^{14}$C-chelators was not reduced by metabolic inhibitors, indicating these processes were energy-independent. No significant differences were evident in the uptake of $^{14}$C-Bp4eT at $37^\circ C$ or $4^\circ C$, demonstrating a temperature-independent mechanism. Furthermore, adjusting the pH of the culture medium to model the tumor microenvironment did not affect $^{14}$C-Bp4eT membrane transport. It can be concluded that $^{14}$C-Bp4eT more effectively permeated the cell membrane and evaded rapid efflux in contrast to $^{14}$C-PIH. This property, in part, accounts for the more potent anti-cancer 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 due to their non-selective nature (Grahame-Smith and Aronson, 2006). Thus, new improved chemotherapeutic strategies are urgently required.

Iron is an essential co-factor for the catalytic activity of many enzymes including ribonucleotide reductase (RR), which catalyzes the rate-limiting step in DNA synthesis (Kolberg et al., 2004). As 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 (TfR1), which is responsible for iron uptake from the iron transport protein, transferrin (Tf) (Richardson and Baker, 1990; Trinder and Baker, 2003). High expression of TfR1 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 to 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 anti-cancer agents, iron chelators have emerged as a novel class of chemotherapeutics worthy of investigation. The chelator, desferrioxamine (DFO; Figure 1A), is classically used for the treatment of iron overload disease, but has also been assessed for anti-cancer activity (Kalinowski and Richardson, 2005). However, its poor membrane permeability due to its relative hydrophilicity, short half-life and relatively low anti-proliferative 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 to hydrophilic DFO, the relatively hydrophobic iron chelator, pyridoxal isonicotinoyl hydrazone (PIH; Figure 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, 1997; Richardson and Ponka, 1994). Interestingly, although PIH can effectively mobilize iron from cells, it appears to target iron pools that are not necessary for proliferation, demonstrating poor anti-proliferative activity in cell culture (IC_{50}: 75 µM) (Richardson et al., 1995).

A series of structure-activity relationship studies of the PIH analogues over the last 10 years has assessed both anti-proliferative efficacy and iron chelation activity (Becker et al., 2003; Richardson et al., 1995; Whitnall et al., 2006; Yuan et al., 2004). This work has led to the development of the 2-benzoylpyridine thiosemicarbazone (BpT) chelators, which demonstrate potent and selective anti-cancer activity (Kalinowski et al., 2007). These ligands have the ability to sequester cellular iron, as well as the capacity to form iron complexes that are redox-active (Kalinowski et al., 2007). Hence, the BpT analogues 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 anti-proliferative activity in SK-N-MC neuroepithelioma cells (IC_{50}: 0.002-0.005 µM; Kalinowski et al., 2007), they show 1,250-3,000-fold less efficacy for inhibiting the proliferation of normal MRC-5 fibroblasts (IC_{50} > 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 (Yu, Y. and Richardson, D.R., unpublished results). Within the BpT series, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT; Figure 1C), showed marked anti-proliferative activity with an IC_{50} of 0.002 µM and
was a clear leading candidate for further studies (Kalinowski et al., 2007).

While Bp4eT possessed potent and selective anti-cancer 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 anti-proliferative activity of Bp4eT in comparison to PIH, which exhibits poor anti-proliferative efficacy. In order to do this, $^{14}$C-labeled chelators were employed. Since 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 $^{14}$C-chelators were examined. In this investigation, the effects of temperature, metabolic inhibitors, pH and extracellular protein on the cellular retention of the chelators were measured. In addition, the cellular uptake of $^{14}$C-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 in comparison to PIH, resulting in greater drug delivery and maximal cytotoxicity.
Materials and Methods

\(^{14}\text{C}-\text{Chelators}\)

\(^{14}\text{C}-\text{PIH}\) and \(^{14}\text{C}-\text{Bp4eT}\) were synthesized by The Institute of Isotopes Ltd (Budapest, Hungary) incorporating the \(^{14}\text{C}\) label at the imine carbon which represents a highly stable site for incorporating the isotope. The final purity of both compounds was determined by HPLC using UV detection. The certificate of analysis indicated the purity of \(^{14}\text{C}-\text{PIH}\) and \(^{14}\text{C}-\text{Bp4eT}\) were 98.5\% and 100\%, respectively, with a final specific radioactivity of 75 \(\mu\text{Ci/mg}\). All compounds were prepared as their hydrochloride salts to maximize solubility. Chelators were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) as 10 mM stock solutions and diluted in complete medium so that the final [DMSO] < 0.5\% (v/v).

Cell Culture

The human SK-N-MC neuroepithelioma cell line (American Type Culture Collection (ATCC), Manassas, VA) was grown as previously described (Richardson et al., 1995). Briefly, the cells were grown in minimal essential medium (MEM; Invitrogen, Victoria, Australia) with 10\% (v/v) fetal calf serum (Sigma-Aldrich, St. Louis, USA) and supplemented with the following additions from Gibco (Victoria, Australia): 1\% (v/v) sodium pyruvate, 1\% (v/v) non-essential amino acids, 100 U/mL penicillin, 100 \(\mu\text{g/mL}\) streptomycin, 2 mM glutamine and 0.28 ng/mL fungizone. The cells were then incubated at 37\(^\circ\)C in a humidified atmosphere of 5\% \(\text{CO}_2/95\%\) air in a water-jacketed incubator (Thermo Scientific, Forma Series II, Marietta, OH, USA). The SK-N-MC cell line was chosen for the majority of these investigations as 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 ATCC. These cell lines were cultured similarly to that of the SK-N-MC cell line described above. Human umbilical vein endothelial cells (HUVECs) were kindly donated by Mr. Pat Pisansarakit (Heart Research Institute, Sydney, Australia). HUVECs were cultured in M199 media (SAFC Biosciences, St Louis, MO, USA) with the standard supplementation described above for MEM alone and 0.5% vascular endothelial growth factor (Starrate, Bethungra, Australia).

**General $^{14}$C-Chelator Cellular Uptake Procedure**

The $^{14}$C-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 $1 \times 10^6$ 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 $^{14}$C-chelator uptake, the cells were incubated with complete media containing $^{14}$C-Bp4eT (5-250 µM), $^{14}$C-PIH (5-250 µM) or no chelator (control) at 37°C for 120 min. Subsequent to these studies, a $^{14}$C-chelator concentration of 25 µM was selected as it provided appropriate labeling efficiency of cells that led to a highly sensitive assay. In experiments measuring the effect of temperature on $^{14}$C-chelator uptake, the cells were incubated in media containing $^{14}$C-chelator at 4°C or 37°C for up to 120 min.

Studies examining the influence of the Warburg effect (Warburg, 1956) on $^{14}$C-chelator uptake utilized pH-adjusted complete media. This media was altered to a final pH of 5.5 or 6.5 using MES (25 mM; Sigma-Aldrich) and to a pH of 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 trypan blue. This was determined using a Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA, USA). At the conclusion of the incubation period, the media was aspirated and the cells were washed 4 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; Perkin-Elmer, Melbourne, Victoria, Australia) was added to the cell suspension and the radioactivity of each sample, including backgrounds and 14C-chelator standards, were counted using a Micro-Beta Counter (Perkin Elmer, Waltham, MA, USA). Results were expressed as molecules of chelator/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 as the relative size of the cells varied markedly. The surface area (µm²) of each cell type was determined using Image J 1.42 software (National Institutes of Health, USA) analysis of phase-contrast images taken with a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam camera and AxioVision Release 4.7 Software (Zeiss, Munich, Germany). The average cell surface area was calculated after measuring 100 cells/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 pre-
incubated with inhibitors or media alone for 30 min at 37°C. The media was removed and replaced with media containing 14C-PIH (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 pre-labeled SK-N-MC cells was performed using well established techniques (Huang and Ponka, 1983; Richardson, 1997; Richardson et al., 1995). Briefly, SK-N-MC cells were pre-labeled with either 14C-Bp4eT (25 µM), 14C-PIH (25 µM), or media alone for 120 min at 37°C. The cells were then placed on ice, the media aspirated and the cell monolayer washed four times with ice-cold PBS. For experiments examining the effect of temperature on 14C-chelators release, complete media (1 mL; 4°C or 37°C) was added to each plate and the cells were incubated at 4°C 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 FCS (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 was placed into counting tubes to estimate the level of extracellular $^{14}$C-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 $^{14}$C-chelator. Scintillation fluid (2.5 mL) was added to each sample as well as the backgrounds and standards and the tubes were counted using a Micro-Beta Counter.

**The Effect of Metabolic Inhibitors on $^{14}$C-Chelator Efflux**

SK-N-MC cells were pre-incubated with $^{14}$C-PIH (25 µM) or $^{14}$C-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. Following four washes with ice-cold PBS, the plates were re-incubated 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 due to problems associated with its intense color that led to quenching and thus problems associated with quantification of $^{14}$C by $\beta$-counting. On the other hand, in uptake experiments, the inhibitors were washed off cells prior to $\beta$-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 in order 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. Briefly, 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 45 min at 14,000 rpm. The ATP assay mix (100 µL) was added to a 96-well plate, mixed and allowed to stand 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 $^{14}$C-Chelator Uptake

The BpT series of ligands have shown high anti-proliferative activity and selectivity against cancer cells (Kalinowski 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 to Bp4eT, we have assessed the uptake of the chelator, PIH, which shows some structural similarity (Figure 1), but demonstrates poor anti-proliferative activity (Richardson et al., 1995). By determining the cellular uptake and subsequent release of these ligands, information relevant to their marked differences in anti-proliferative activity should be obtained.

In initial studies, the uptake of $^{14}$C-Bp4eT and $^{14}$C-PIH were measured after a 120 min incubation at 37ºC 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 5-250 µM without any evidence of saturation. Higher concentrations of chelator were not used due to 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 $^{14}$C-chelators possessed linear uptake kinetics, the rate of $^{14}$C-PIH uptake ($1.45 \times 10^6 \pm 0.96$ molecules of chelator/cell/min) was significantly ($p<0.001$) lower than that of $^{14}$C-Bp4eT ($3.02 \times 10^6 \pm 0.3$ molecules of chelator/cell/min). These results demonstrated that $^{14}$C-Bp4eT had an increased rate of entry into SK-N-MC cells than $^{14}$C-PIH (Fig. 2A).

The Effect of Temperature on $^{14}$C-Chelator Uptake

The effect of temperature and time on $^{14}$C-Bp4eT and $^{14}$C-PIH uptake was investigated in
order to determine whether these processes could potentially be carrier-mediated. At 37°C and 4°C, a biphasic mode of uptake as a function of time was evident for 14C-Bp4eT and 14C-PIH (Fig. 2B). The uptake of 14C-Bp4eT was markedly and significantly \( p < 0.001 \) greater than 14C-PIH at all time points at both 37°C and 4°C. The cellular uptake of 14C-Bp4eT increased rapidly with time and reached a plateau within 30 min of incubation at both 4°C and 37°C. Between 5 to 15 min of incubation, the cellular uptake of 14C-Bp4eT was significantly \( p < 0.01 \) greater at 37°C than at 4°C (Fig. 2B). Additionally, at 37°C, 14C-Bp4eT uptake peaked at the 15 min time point suggesting that the initial rate of uptake of the chelator may have exceeded the rate of simultaneous efflux from the cell. However, the remainder of the time points (30-120 min) demonstrated no significant \( p > 0.05 \) difference in 14C-Bp4eT uptake between 4°C and 37°C. Therefore, after 30 min, it appeared the rate of simultaneous uptake and efflux equilibrated to an extent similar to that observed at 4°C (Fig. 2B).

In contrast to 14C-Bp4eT uptake, the uptake of 14C-PIH was significantly \( p < 0.01 \) lower in cells incubated at 4°C than at 37°C (Fig. 2B), demonstrating a dependence on temperature and a different mechanism of uptake to that found with 14C-Bp4eT.

**The Effect of Temperature on 14C-Chelator Efflux**

The release of 14C-Bp4eT and 14C-PIH from SK-N-MC cells was also examined as a function of time at 4°C and 37°C (Fig. 2C) to investigate its dependence on temperature. In these experiments, cells were labeled for 120 min with the 14C-chelators, washed and then reincubated for up to 180 min at 4°C or 37°C. After the 180 min reincubation, 57 ± 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 $^{14}$C-PIH was significantly $(p<0.05)$ greater than that of $^{14}$C-Bp4eT, with 80 ± 2% of $^{14}$C-PIH released from cells after 180 min (Fig. 2C). Additionally, the percentage of cellular $^{14}$C-PIH released was significantly $(p<0.001)$ lower at 4°C (17 ± 2% at 180 min) when compared to 37°C (80 ± 2% at 180 min). In fact, the release of $^{14}$C-PIH at 4°C was similar to $^{14}$C-Bp4eT at 4°C (Fig. 2C). Therefore, the release of intracellular $^{14}$C-PIH was also found to be temperature-dependent, as identified for its uptake (Fig. 2B).

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

The Effect of Metabolic Inhibitors on $^{14}$C-Chelator Uptake

Considering the results described above, experiments were then performed to determine whether chelator uptake was energy-dependent (Fig. 3A and B). The effect of five well characterized metabolic inhibitors, namely NaN$_3$, oligomycin, NaCN, 2,4-DNP and NaF (Henderson and Zevely, 1984; Qian and Morgan, 1991; Richardson, 1997; Svec, 1985), on $^{14}$C-Bp4eT and $^{14}$C-PIH uptake into SK-N-MC cells was investigated at 37°C. In addition, in
these studies, ATP assays were performed in parallel to $^{14}$C-ligand uptake experiments to assess the effects of the inhibitors on energy metabolism (Fig. 3C and D). Studies compared the effects on $^{14}$C-ligand uptake in DMEM ± 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 in comparison to control medium (namely DMEM + GLU; Fig. 3C), they did not significantly ($p>0.05$) decrease $^{14}$C-Bp4eT uptake (Fig. 3A). In fact, 2,4-DNP caused a significant ($p<0.01$) increase in intracellular $^{14}$C-Bp4eT in comparison to control medium (Fig. 3A). The increased uptake of $^{14}$C-Bp4eT in the presence of 2,4-DNP could be consistent with non-specific adsorption of the $^{14}$C-labeled ligand to the cell due to 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 $^{14}$C-Bp4eT uptake compared to the control (DMEM + GLU). Overall, all five metabolic inhibitors had no inhibitory effects on $^{14}$C-Bp4eT uptake, suggesting that $^{14}$C-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 $^{14}$C-PIH by cells as compared to 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 $^{14}$C-PIH uptake relative to the control (Fig. 3B). Again, this may be due to membrane damage induced by the inhibitor, leading to increased non-specific adsorption of $^{14}$C-PIH. In summary, these results suggest that energy-independent mechanisms may also be responsible for the uptake of $^{14}$C-PIH.
The Effect of Metabolic Inhibitors on $^{14}$C-Chelator Efflux

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

The Effect of pH on $^{14}$C-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 (Helmlinger et al., 1997; Warburg, 1956). Since the thiosemicarbazone classes of chelators are polyprotic (Richardson et al., 2006; Richardson et al., 1990), their net charge is dependent upon 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 values were equal to 5.5, 6.5, 7.4 and 8.0 (Fig. 5A-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 5-30 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 to pH 7.4 (Fig. 5A). At pH 8.0, the uptake
of $^{14}$C-Bp4eT was not significantly different to 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 5.5 and pH 6.5 compared to control (pH 7.4). Interestingly, 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). Conversely, no significant ($p>0.05$) difference in $^{14}$C-PIH uptake was evident at pH 8.0 in comparison to 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 in order to analyze the influence the Warburg effect may have on tumor drug retention *in vivo* (Fig. 5C and D). The cellular release of $^{14}$C-Bp4eT and $^{14}$C-PIH was not significantly ($p>0.05$) altered by extracellular pH (pH 5.5, 6.5 and 8.0) in comparison to the control (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 Veldt 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. 6A and B) to determine if 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) in comparison to 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 (Yu, Y. and Richardson, D.R. unpublished data). Moreover, it is essential that clinically used chemotherapeutic agents preferentially target cancer cells as opposed to normal tissue to minimize side effects (Grahame-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 sub-confluent (50%) plates of cells to examine the effect of the degree of confluency on cellular uptake. Results were expressed as molecules of chelator/total surface area per cell. This was necessary as 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 \(^{14}\text{C}-\text{Bp4eT} \) in contrast to the normal MRC-5 and HUVEC cell types (Fig. 7A). Additionally, the SK-N-MC cell line also showed significantly \(p<0.05\) increased intracellular levels of \(^{14}\text{C}-\text{Bp4eT} \) compared to MRC-5 cells, although no significant \(p>0.05\) differences were evident in comparison to HUVEC cells. Furthermore, there was no significant \(p>0.05\) increase in the cellular uptake of \(^{14}\text{C}-\text{Bp4eT} \) in SK-Mel-28 melanoma cells relative to normal cell types (Fig. 7A). Interestingly, the uptake of \(^{14}\text{C}-\text{Bp4eT} \) differed in sub-confluent cells and this may be due to the adherence of the ligand to the plastic substratum of the culture plates under sub-confluent conditions (Fig. 7B). Under these conditions, similar levels of \(^{14}\text{C}-\text{Bp4eT} \) uptake were observed in SK-N-MC and SK-Mel-28 cells compared to normal cell lines (Fig. 7B). In contrast, significantly \(p<0.05\) higher intracellular levels of \(^{14}\text{C}-\text{Bp4eT} \) were present in MCF-7 cells relative to that in normal cells. Collectively, these results demonstrate that generally there is no clear difference in the uptake of \(^{14}\text{C}-\text{Bp4eT} \) 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 also other neoplastic cells.

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

The toxicity of anti-cancer drugs as well as 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). Consequently, the current studies were undertaken to examine the membrane transport of the potential anti-tumor agent, Bp4eT.

Bp4eT Rapidly Enters Cells via a Metabolic Energy-Independent Mechanism Consistent with Diffusion

As part of this study, the uptake of $^{14}$C-Bp4eT, which possesses marked anti-cancer activity (Kalinowski et al., 2007), was compared to $^{14}$C-PIH, a ligand with low anti-proliferative activity (Richardson et al., 1995). The rate of $^{14}$C-Bp4eT uptake by cells was markedly greater than that of $^{14}$C-PIH (Fig. 2A), illustrating that $^{14}$C-Bp4eT can more easily and rapidly permeate cells. This can be attributed to the lipophilic nature of Bp4eT (log $P$ 4.02; Kalinowski et al., 2007), in contrast to the relatively more hydrophilic ligand, PIH (log $P$ -2.11) (Richardson et al., 1995). Numerous studies have shown that hydrophilic chelators possess poor anti-proliferative activity, while compounds with relatively high lipophilicity have potent anti-proliferative effects (Hodges et al., 2004; Richardson et al., 1995). Consequently, the higher intracellular levels of $^{14}$C-Bp4eT, owing to its greater membrane permeability, may be partly responsible for its potent anti-cancer activity (Kalinowski et al., 2007). In contrast, the relatively hydrophilic $^{14}$C-PIH led to lower membrane permeability, correlating with its poor anti-proliferative activity (Richardson et al., 1995).

Since some transporters can influence cellular drug retention, such as the multi-drug resistance associated protein and P-glycoprotein (Gottesman et al., 2002), it was important to
examine the mechanisms involved in membrane transport of $^{14}$C-Bp4eT and $^{14}$C-PIH. The current results indicate that Bp4eT permeates cells via passive diffusion. The following three observations support this conclusion. First, results obtained from $^{14}$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 employed. Second, $^{14}$C-Bp4eT uptake by cells was not reduced despite depleting ATP stores using metabolic inhibitors (Fig. 3A, C). The third piece of evidence demonstrating that $^{14}$C-Bp4eT permeates membranes through a passive process can be deduced by comparing its uptake at $37^\circ$C and $4^\circ$C. For the duration of the steady state (Fig. 2B), there was no significant difference in intracellular $^{14}$C-Bp4eT at $37^\circ$C and $4^\circ$C, demonstrating temperature-independent uptake. Collectively, Bp4eT permeate 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 they evade efflux to allow sufficient quantities to be retained to cause cytotoxicity (Gottesman et al., 2002). Therefore, to understand why Bp4eT was a more potent anti-cancer drug than PIH, $^{14}$C-chelator efflux was investigated. In these studies, Bp4eT and PIH demonstrated a significant reduction in $^{14}$C-chelator release at $4^\circ$C than $37^\circ$C (Fig. 2C). These findings are consistent with a temperature-dependent release mechanism and may result from: (1) a change in membrane fluidity that occurs upon decreasing temperature affecting drug transport (Zimmer and Schirmer, 1974); or (2) a decrease in the efficacy of an energy-dependent transporter. To clarify this, additional efflux experiments performed with metabolic inhibitors (Fig. 4A and B) showed no difference in the release of intracellular $^{14}$C-chelator compared to
the control, despite a marked decrease in ATP. This indicated the mechanism of ligand release was energy-independent, but temperature-dependent. Hence, Bp4eT transport occurs via temperature-dependent passive diffusion, where membrane fluidity can affect membrane transport.

Importantly, the percentage of $^{14}$C-PIH released from cells was significantly higher than that of $^{14}$C-Bp4eT at 37°C (Fig. 2C). These results together with the rapid uptake of $^{14}$C-Bp4eT (Fig. 2B), resulted in 32-fold higher levels of $^{14}$C-Bp4eT relative to $^{14}$C-PIH within cells (Fig. 2D). The marked permeability and increased retention of $^{14}$C-Bp4eT may be partly responsible for its potent anti-cancer effects, resulting in increased intracellular ROS and cell death (Kalinowski and Richardson, 2005; Kalinowski et al., 2007). This is facilitated by the difference in the iron-binding site of Bp4eT ($N,N,S$) relative to PIH ($O,N,O$) (Figure 1), which leads to the ability of Bp4eT to bind metals and redox cycle to generate ROS (Kalinowski et al., 2007).

**The Warburg Effect Does Not Influence Bp4eT Uptake or Release by Tumor Cells**

Tumors often contain a slightly acidic microenvironment due to 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 permeability (Richardson et al., 2006; Richardson et al., 1990). Thus, the extracellular pH was modified to model variations within the tumor microenvironment that might influence chelator uptake and efflux. The results demonstrated that alterations in the extracellular pH to those encountered in tumors (pH 6.9-7.2; Helminger et al., 1997) had little effect on $^{14}$C-chelator efflux (Fig. 5C and D). Interestingly, greater intracellular uptake of $^{14}$C-PIH was evident under acidic conditions (pH 5.5 and 6.5) in comparison to the control (pH 7.4) (Fig.
5B). These results suggest a greater amount of $^{14}$C-PIH was present in its neutral form at a pH of 5.5-6.5, allowing higher permeability than at pH 7.4. This concept is consistent with the species distribution of PIH as a function of pH, where its neutral species predominates at pH 6 (Doungdee, 1995; Richardson et al., 1990).

In contrast, alterations in pH had no significant effect on $^{14}$C-Bp4eT uptake (Fig. 5A). These data indicate $^{14}$C-Bp4eT is present largely as a neutral ligand between pH 5.5-8.0, 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 in the pH range of 4-11 (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 it would influence Bp4eT uptake.

**Extracellular Protein Increases Efflux of Bp4eT, but not PIH**

Another factor in tumors which 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 within the tumor interstitium (Greish, 2007; Maeda, 2001). Hence, studies were performed in the presence of common proteins present in the interstitial fluid, namely Tf and albumin, to determine whether the protein microenvironment of tumors affects the cellular retention of the $^{14}$C-labeled ligands. These investigations demonstrated $^{14}$C-Bp4eT release was increased in the presence of proteins compared to protein-free media and was independent of the protein type (Fig. 6A). Since lipophilic ligands have an affinity for proteins, these macromolecules may act as an extracellular “sink”, increasing release of intracellular $^{14}$C-Bp4eT (Buss et al., 2002; Buss et...
al., 2003). It is also notable that low levels of extracellular protein (5 mg/mL) resulted in similar efflux of \(^{14}\)C-Bp4eT as high concentrations of protein (40 mg/mL). Thus, the higher protein levels within tumor interstitial fluid due to increased permeability of tumor vasculature (Carmeliet and Jain, 2000), will probably not promote \(^{14}\)C-Bp4eT release to any greater extent than in normal tissues. Conversely to Bp4eT, the presence of protein in medium had no marked effect on \(^{14}\)C-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 \textit{in vitro} (Kalinowski et al., 2007) and \textit{in vivo} (Yu, Y. and Richardson, D.R. unpublished results). Furthermore, closely related compounds of the DpT class showed selective anti-tumor activity \textit{in vivo} (Whitnall et al., 2006). Therefore, \(^{14}\)C-chelator uptake was compared between cancer and normal cells. Although increased \(^{14}\)C-Bp4eT uptake was evident in MCF-7 cancer cells in comparison to normal cells, this difference was not apparent in all tumor cell types (Fig. 7A and B). Thus, other factors may be responsible for the selective anti-cancer activity of Bp4eT. These could include the greater sensitivity of cancer cells to iron-deprivation, as well as their effect on other iron-dependent targets, including: RR, the metastasis suppressor NDRG1, cyclin D1 \textit{etc} (Le and Richardson, 2004; Yu et al., 2007).

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

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Footnotes

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**Figure Legend**

**Figure 1.** Chemical structure of the iron chelators: (A) desferrioxamine (DFO), (B) pyridoxal isonicotinoyl hydrazone (PIH) and (C) 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT).

**Figure 2.** (A): The rate of $^{14}$C-Bp4eT and $^{14}$C-PIH uptake by SK-N-MC cells. The cells were incubated in media containing $^{14}$C-Bp4eT or $^{14}$C-PIH at 5-250 µM for 120 min at 37°C. The cells were then placed on ice, washed 4 times using ice-cold PBS and radioactivity was quantified. (B): The effect of temperature and time on $^{14}$C-chelator uptake by SK-N-MC cells. Cells were incubated with media containing $^{14}$C-Bp4eT (25 µM) or $^{14}$C-PIH (25 µM) at 37°C 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 $^{14}$C-chelator efflux. Cells were pre-labeled with $^{14}$C-Bp4eT (25 µM) or $^{14}$C-PIH (25 µM) for 120 min at 37°C. Cells were then placed on ice and washed 4 times with ice-cold PBS and re-incubated in media for up to 180 min at 37°C or 4°C. Results are expressed as mean ± S.E.M. from triplicate determinations of 3 experiments.

**Figure 3.** (A) and (B): The effect of metabolic inhibitors on (A) $^{14}$C-Bp4eT and (B) $^{14}$C-PIH uptake by SK-N-MC cells after 60 min at 37°C. Cells were pre-incubated with inhibitors: NaN₃ (30 mM), NaF (15 mM), 2,4-DNP (2 mM), oligomycin (30 µM) or NaCN (5 mM), or media alone (DMEM ± glucose [GLU]) for 30 min at 37°C. The media was removed and the cells then incubated with media containing $^{14}$C-PIH (25 µM) or $^{14}$C-Bp4eT (25 µM) with or without inhibitors for 60 min at 37°C. The cells were then placed on ice and washed 4 times with ice-cold PBS and the radioactivity was quantified. Results are expressed as mean ± S.E.M. from triplicate determinations of 6 experiments. * vs DMEM ± GLU, p<0.05; ** DMEM ± GLU, p<0.01; using one-way ANOVA. (C) and (D): The effect of metabolic inhibitors...
inhibitors on ATP levels in (C) $^{14}$C-Bp4eT and (D) $^{14}$C-PIH uptake experiments that were performed in (A) and (B), respectively. Results are mean ± S.E.M. from triplicate determinations of 2 experiments and are expressed as a % of the control (DMEM + GLU). * vs DMEM ± GLU, p<0.05; ** DMEM ± GLU, p<0.01; using one-way ANOVA.

Figure 4. (A) and (B): The effect of metabolic inhibitors and time on (A) $^{14}$C-Bp4eT and (B) $^{14}$C-PIH efflux from SK-N-MC cells. Cells were pre-labeled with media containing $^{14}$C-Bp4eT (25 µM) or $^{14}$C-PIH (25 µM) for 90 min at 37°C. The cells were then placed on ice and metabolic inhibitors, namely NaN$_3$ (30 mM), NaF (15 mM), oligomycin (30 µM) or NaCN (5 mM), were added to appropriate plates and the cells incubated for a further 30 min at 37°C. After this incubation, the cells were placed on ice, washed 4 times with ice-cold PBS and then reincubated for up to 60 min at 37°C in media with or without inhibitors. Results are expressed as mean ± S.E.M. calculated from triplicates of 4 experiments. Statistical significance was determined using two-way ANOVA with replication. (C) and (D): The effect of metabolic inhibitors on ATP levels in (C) $^{14}$C-Bp4eT and (D) $^{14}$C-PIH efflux experiments that were performed in (A) and (B), respectively. Results are mean ± S.E.M. of triplicates in a typical experiment of 3 experiments performed and are expressed as a % of the control (DMEM + GLU).

Figure 5. The effect of altering the extracellular pH on (A) $^{14}$C-Bp4eT and (B) $^{14}$C-PIH uptake into SK-N-MC cells. Cells were incubated with $^{14}$C-Bp4eT (25 µM) or $^{14}$C-PIH (25 µM) in media at a pH of 5.5, 6.5, 7.4 or 8.0 for up to 120 min at 37°C. The cells were then placed on ice, washed 4 times using ice-cold PBS and the radioactivity was quantified. The effect of altering the extracellular pH on (C) $^{14}$C-Bp4eT and (D) $^{14}$C-PIH efflux from SK-N-MC cells. Cells were pre-labeled with media (pH 7.4) containing $^{14}$C-Bp4eT (25 µM) or $^{14}$C-
PIH (25 µM) for 120 min at 37°C, placed on ice and washed 4 times with ice-cold PBS, and then re-incubated in media at a pH of 5.5, 6.5, 7.4 or 8.0 for up to 180 min at 37°C. Results are expressed as mean ± S.E.M. from triplicate determinations in 3 experiments.

**Figure 6.** (A) and (B): The effect of proteins on (A) $^{14}$C-Bp4eT and (B) $^{14}$C-PIH efflux from SK-N-MC cells. Cells were pre-labeled with media containing $^{14}$C-Bp4eT (25 µM) or $^{14}$C-PIH (25 µM) for 120 min at 37°C. Cells were then placed on ice, washed 4 times with ice-cold PBS and then re-incubated in protein-containing media or protein-free media at 37°C for up to 180 min. Results are expressed as mean ± S.E.M. from triplicate determinations in 3 experiments.

**Figure 7.** (A-D): The uptake of $^{14}$C-Bp4eT ((A) and (B)) and $^{14}$C-PIH ((C) and (D)) as a function of time in 100% confluent ((A) and (C)) and 50% sub-confluent ((B) and (D)) cancer and normal cells. Confluent and sub-confluent 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 $^{14}$C-Bp4eT (25 µM) or $^{14}$C-PIH (25 µM) containing media at 37°C for 15 min or 120 min. Cells were then placed on ice and washed 4 times with ice-cold PBS and the radioactivity was quantified. Results are expressed as mean ± S.E.M. from triplicate determinations in 3 experiments.
Figure 1

(A) DFO

(B) PIH

(C) Bp4eT
Figure 6

(A) \(^{14}\text{C-\text{Bp4eT}}\)

(B) \(^{14}\text{C-PIH}\)

- Control
- 5 mg/mL Albumin
- 40 mg/mL Albumin
- 5 mg/mL Tf
- 40 mg/mL Tf

% Cellular Chelator Released

Time (min)