These observations indicated a saturable transport mechanism using SK-N-MC neuroepithelioma cells (ethyl-3-thiosemicarbazone (14C-Bp4eT). In marked contrast to the fact that 14C-Dp44mT uptake was temperature-dependent and significantly as a function of concentration, 14C-Dp44mT uptake was saturable using SK-N-MC neuroepithelioma cells (14C-PIH) and the thiosemicarbazone 14C-2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (14C-Bp4eT). In marked contrast to the cellular uptake of 14C-PIH and 14C-Bp4eT, which were linear as a function of concentration, 14C-Dp44mT uptake was saturable using SK-N-MC neuroepithelioma cells (Bmax, 4.28 ± 107 molecules of chelator/cell; and Kd, 2.45 μM). Together with the fact that 14C-Dp44mT uptake was temperature-dependent and significantly (P < 0.01) decreased by competing unlabeled Dp44mT, these observations indicated a saturable transport mechanism consistent with carrier/receptor-mediated transport. Other unlabeled ligands that shared the saturated N4 structural moiety with Dp44mT significantly (P < 0.01) inhibited 14C-Dp44mT uptake, illustrating its importance for carrier/receptor recognition. Nevertheless, unlabeled Dp44mT most markedly decreased 14C-Dp44mT uptake, demonstrating that the putative carrier/receptor shows high selectivity for Dp44mT. Interestingly, in contrast to 14C-Dp44mT, uptake of its Fe complex [Fe(14C-Dp44mT)2] was not saturable as a function of concentration and was much greater than the ligand alone, indicating an alternate mode of transport. Studies examining the tissue distribution of 14C-Dp44mT injected intravenously into a mouse tumor model demonstrated the 14C label was primarily identified in the excretory system. Collectively, these findings examining the mechanism of Dp44mT uptake and its distribution and excretion have clinical implications for its bioavailability and uptake in vivo.

Introduction
Intensive research has established that cancer cells have distinctly altered signaling pathways, enabling uncontrolled proliferation (Hanahan and Weinberg, 2011). Interestingly, emerging evidence demonstrates that iron (Fe) homeostasis is perturbed in cancer cells (Merlot et al., 2013). For instance, due to their high metabolic requirement for Fe, malignant cells are sensitive to iron deprivation using chelators (Buss et al., 2003; Kalinowski and Richardson, 2005). One of the most potent groups of chelators with anticancer activity compared with normal cells, tumor cells have higher levels of the transferrin receptor 1 (TfR1) that is responsible for Fe uptake from the Fe transport protein transferrin (Tf) (Soyer et al., 1987). This increased TfR1 expression also correlates with tumor grade and stage (Seymour et al., 1987; Soyer et al., 1987). Additionally, the Fe-dependent enzyme ribonucleotide reductase, which catalyzes the rate-limiting step in DNA synthesis, is upregulated in cancer cells (Elford et al., 1970; Takeda and Weber, 1981). Changes in Fe trafficking proteins have also been observed in cancer cells, including ferritin and ferroportin 1 (Pinnix et al., 2010; Merlot et al., 2013). Together, these alterations in Fe metabolism impart neoplastic cells with a “high intracellular Fe” phenotype (Pinnix et al., 2010).

Due to their high metabolic requirement for Fe, malignant cells are sensitive to iron deprivation using chelators (Buss et al., 2003; Kalinowski and Richardson, 2005). One of the most potent groups of chelators with anticancer activity

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ABBREVIATIONS: 44mT, 4,4-dimethyl-3-thiosemicarbazide; Ap4eT, 2-acetylpyridine 4-ethyl-3-thiosemicarbazone; Ap4mT, 2-acetylpyridine 4-methyl-3-thiosemicarbazone; Ap4pT, 2-acetylpyridine 4-phenyl-3-thiosemicarbazone; ApT, 2-acetylpyridine thiosemicarbazone; Bp4mT, 2-benzoylpyridine 4-methyl-3-thiosemicarbazone; Bp4eT, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone; Bp4pT, 2-benzoylpyridine 4-phenyl-3-thiosemicarbazone; Bp4T, 2-benzoylpyridine thiosemicarbazone; Dp4mT, di-2-pyridylketone 4-methyl-3-thiosemicarbazone; Dp4pT, di-2-pyridylketone 4-phenyl-3-thiosemicarbazone; Dp4T, di-2-pyridylketone 4-thiosemicarbazone; Dp4eT, di-2-pyridylketone 4-ethyl-3-thiosemicarbazone; Dp4pT, di-2-pyridylketone 4-phenyl-3-thiosemicarbazone; DpC, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone; DpT, di-2-pyridylketone thiosemicarbazone; FCS, fetal calf serum; ID, injected dose; PBS, phosphate-buffered saline; PIH, pyridoxal isonicotinoyl hydrazone; Tf, transferrin; TfR1, transferrin receptor 1.

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include the di-2-pyridylketone thiosemicarbazone (DpT) series, in particular di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1; Yuan et al., 2004; Whitnall et al., 2006). The broad and potent antiproliferative activity of Dp44mT has been shown in >30 tumor cell types, including drug-resistant and p53 mutant cells (Whitnall et al., 2006; Rao et al., 2009; Tian et al., 2010).

In terms of its effects in vivo, Dp44mT inhibited tumor growth in multiple tumor mouse models, including human pancreatic, melanoma, and neuroepithelioma xenografts (Whitnall et al., 2006; Kovacevic et al., 2011), as well as reducing metastasis in a breast cancer model (Liu et al., 2011). Relative to the thiosemicarbazone, Triapine (Nanotherapeutics, Alachua, FL; Fig. 1), which has been examined in >20 phase I and II international clinical trials (Merlot et al., 2013), a 30-fold lower dose of Dp44mT was necessary to induce the same antitumor activity in vivo (Whitnall et al., 2006).

Interestingly, Dp44mT has a variety of molecular targets. The ligand can potently mobilize intracellular Fe and also prevent cellular Fe uptake from Tf (Yuan et al., 2004). Furthermore, Dp44mT increases the expression of the potent metastasis suppressor, N-myc downstream-regulated gene 1 (Le and Richardson, 2004; Kovacevic et al., 2011). Due to its ability to deplete cellular Fe (Yuan et al., 2004), Dp44mT causes cell cycle arrest at the G1/S phase (Noulsri et al., 2009; Rao et al., 2009) and inhibits the activity of the Fe-dependent enzyme ribonucleotide reductase, impeding cellular DNA synthesis and proliferation (Yu et al., 2011). Interestingly, Dp44mT was observed to accumulate within lysosomes, where it forms redox-active copper (Cu) complexes that induce oxidative damage and lysosomal permeabilization (Lovejoy et al., 2011). This latter event results in the release of cathepsins that lead to the cleavage of proapoptotic Bid and apoptosis (Lovejoy et al., 2011).

While much has been revealed concerning the intracellular targets of Dp44mT, the mechanisms involved in the membrane transport of the drug remain undeciphered and are the subject of this investigation. In fact, little is known regarding the membrane transport mechanisms of iron chelators in mammalian cells. It has been demonstrated that ligand lipophilicity plays a critical role in their uptake and activity (Baker et al., 1985; Porter et al., 1988). Hydrophilic ligands such as desferrioxamine show slow uptake relative to more lipophilic chelators (Richardson et al., 1994). Indeed, a direct correlation is found between increased lipophilicity and greater iron chelation efficacy and antiproliferative activity (Richardson et al., 1995). A previous study demonstrated that the cellular uptake of thiosemicarbazone 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT; Fig. 1) occurred via an energy-independent and temperature-independent uptake mechanism consistent with passive diffusion (Merlot et al., 2010).

In this investigation, Dp44mT was labeled with 14C to assess membrane transport in tumor cells. These studies were performed in comparison with several chelators that show structural similarity, namely Bp4eT and the arylhydrazine pyridoxal isonicotinoyl hydrazide (PIH; Fig. 1), whose uptake mechanisms were previously characterized and act as internal controls (Merlot et al., 2010). Our results demonstrate that in contrast to Bp4eT and PIH, Dp44mT is transported into cells by a saturable carrier/receptor–mediated mechanism.

Materials and Methods

Chelators and Other Reagents. 14C-Bp4eT, 14C-PIH, and 14C-Dp44mT were synthesized and characterized by the Institute of Isotopes Ltd (Budapest, Hungary) with the isotope 14C incorporated at the imine carbon (see asterisk, Fig. 1). The certificate of analysis of the labeled 14C-chelators indicated the compounds were 98.5–100% pure and had a final specific activity of 75 µCi/µg. The Fe(III) and Cu(II) 14C-complexes were prepared by adding ferric chloride (FeCl3; Sigma-Aldrich, St. Louis, MO) or cupric sulfate (CuSO4; Sigma-Aldrich), respectively, at a 2:1 (14C-chelator/metal) molar ratio. Human Tf was purchased from Sigma-Aldrich. The following radionuclides were purchased from PerkinElmer (Waltham, MA): 59FeCl3, dissolved in 0.5 M HCl (cat. no. NEZ037) and “carrier-free” Na125I dissolved in 10 mM NaOH (specific activity = 17.4 mCi/mg; cat. no. NEZ033A). In competition experiments with the 14C-labeled ligands above, nonlabeled chelators or their precursors were used (for protocol see “14C-Chelator Cellular Uptake” below). The synthesis and characterization of these compounds have been described in detail in previous publications (Yuan et al., 2004; Richardson et al., 2006; Kalinowski et al., 2007; Jansson et al., 2010) and include:

Chelators of the DpT series (Supplemental Fig. 1A), namely: DpT; di-2-pyridylketone 4-methyl-3-thiosemicarbazone (Dp4mT); Dp44mT; di-2-pyridylketone 4-ethyl-3-thiosemicarbazone (Dp4eT); di-2-pyridylketone 4-allyl-3-thiosemicarbazone (Dp4aT); di-2-pyridylketone 4-phenyl-3-thiosemicarbazone (Dp4pT); and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (Dp4c).

Dp44mT precursors (Supplemental Fig. 1A), namely: di-2-pyridylketone (DpK); and 4,4-dimethyl-3-thiosemicarbazide (44mT).

The 2-benzoylpyridine thiosemicarbazone (BpT) series (Supplemental Fig. 1B), namely: BpT; 2-benzoylpyridine 4-methyl-3-thiosemicarbazone (Bp4mT); 2-benzoylpyridine 4,4-dimethyl-3-thiosemicarbazone (Bp4pT); 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT); and 2-benzoylpyridine 4-allyl-3-thiosemicarbazone (Bp4aT).

The 2-acetylpyridine thiosemicarbazone (ApT) series (Supplemental Fig. 1C), namely: ApT; 2-acetylpyridine 4-methyl-3-thiosemicarbazone (Ap4mT); 2-acetylpyridine 4,4-dimethyl-3-thiosemicarbazone (Ap4pT); 2-acetylpyridine 4-ethyl-3-thiosemicarbazone (Ap4eT); and 2-acetylpyridine 4-phenyl-3-thiosemicarbazone (Ap4pT).

Chelators were dissolved in dimethylsulfoxide (Sigma-Aldrich) and diluted in complete medium so that the final dimethylsulfoxide concentration was <0.5% (v/v).

Cell Culture. The human SK-N-MC neuroepithelioma, DMS-53 lung carcinoma, SK-Mel-28 melanoma, and MRC-5 fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA). The SK-N-MC, SK-Mel-28, and MRC-5 cell types...
were cultured in minimal essential medium (Life Technologies, Mulgrave, VIC, Australia), while the DMS-53 cell type was grown in RPMI 1640 (Life Technologies). These media were supplemented with 10% (v/v) fetal calf serum (FCS; Sigma-Aldrich) and the following additives from Life Technologies: 1% (v/v) sodium pyruvate, 1% (v/v) nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glucose, and 0.28 mg/ml Fungizone. The cells were then incubated at 37°C in 5% CO2 and 95% atmosphere, as previously described (Merlot et al., 2010).

**Preparation of 59Fe–125I-Transferrin.** Human apo-Tf (Sigma-Aldrich) was labeled with 59Fe to produce the 59Fe trace–labeled dioxiferri protein 59Fe2-Tf using standard procedures (Richardson and Baker, 1990). The 59Fe2-Tf was further labeled with 125I (supplied as carrier-free Na125I; PerkinElmer) using an adaptation of the iodine monochloride method of McFarlane (1958) to produce doubly labeled 59Fe2-125I-Tf (Richardson and Baker, 1990). Unbound 59Fe and 125I were thoroughly removed by exhaustive vacuum dialysis (Richardson and Baker, 1990).

**14C-Chelator Cellular Uptake.** Uptake of the 14C-chelator by cells was performed as described previously (Merlot et al., 2010) for up to 120 minutes at 4°C or 37°C in medium that, unless specified otherwise, contained the 14C-chelator (25 μM), 10% FCS, and all of the supplements described above. Notably, the medium used throughout our investigation contains only trace levels of Fe, i.e., <1.8 μM Fe (personal communication, Technical Support, Life Technologies). Moreover, as already stated, our studies implement this media supplemented with 10% FCS, which contains the avid iron-binding protein Tf at a final concentration of 2 μM (personal communication, Technical Support, Life Technologies). This means that any low-molecular-weight iron will be effectively bound to Tf, which has an extremely high Fe-binding affinity (Ke, 1024 M⁻¹; Chung, 1984). Similarly, it is also known that Tf binds copper ion with appreciable avidity (Chung, 1984) and would also lead to media that is depleted of this metal ion. Since we are generally using ligands at a concentration of 25 μM, the formation of Fe or Cu complexes by the 14C-chelators in the medium prior to cellular uptake would be insignificant. Additionally, in the serum of mammals, almost all the iron and copper is bound to Tf and ceruloplasmin, respectively (Hsieh and Frieden, 1975; Chung, 1984), and is not in a low-molecular-weight form that can be easily chelated by these types of arylhydrazine and thiosemicarbazon ligands (Richardson et al., 2009).

In competition experiments, unlabeled agents were incubated with cells for 120 minutes at 37°C at concentrations of 2.5, 5, 25, 50, 100, 150, or 250 μM in the presence of labeled 14C-chelator (25 μM). The unlabeled chelators and precursors used included those described above in "Chelators and Other Reagents" (Supplemental Fig. 1), as well as Triapine and PiH (Fig. 1). At each time point, uptake was inhibited by chilling cells in Petri dishes to 4°C on a tray of ice and washing the monolayer four times with ice-cold phosphate-buffered saline (PBS), as per standard labeling protocols (Merlot et al., 2010). Cells were then removed from the plates using a Teflon spatula in 1 ml of PBS and added to scintillation vials. Scintillation fluid (2.5 ml; PerkinElmer, Melbourne, VIC, Australia) was added to these cell suspensions and the radioactivity measured using a Wallac 1420 MicroBeta TriLux Counter (PerkinElmer) with appropriate calibration standards and backgrounds. Results were expressed as molecules of chelator/cell or as uptake (% control). Nonlinear regression analysis was performed on data obtained from studies examining cellular uptake as a function of concentration. Binding curves were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) to determine the maximum number of binding sites (Bmax) and the equilibrium dissociation constant (Kd).

**Effect of Metabolic Inhibitors on 14C-Dp44mT Uptake.** The effect of two well-characterized metabolic inhibitors (Sigma-Aldrich), namely, sodium azide (NaN3; 30 mM) and sodium cyanide (NaCN; 5 mM) were assessed on the uptake of 14C-Dp44mT by cells. In brief, SK-N-MC neuroepithelioma cells were preincubated with inhibitors or media alone for 30 minutes/37°C. The media were then removed and replaced with media containing 14C-Dp44mT (25 μM) or 59Fe–125I-transferrin (0.75 μM) in the presence or absence of inhibitors, and incubated for 60 minutes/37°C. The remainder of the experiment was conducted using the general uptake procedure above. During these studies, Dulbecco’s modified Eagle’s medium without glucose (Invitrogen, Carlsbad, CA) was used to aid the ATP depletion induced by the metabolic inhibitors (Richardson, 1997; Merlot et al., 2010). As a positive control for ATP depletion and inhibition of receptor-mediated endocytosis, ATP levels and the uptake of 59Fe–125I-transferrin were examined concurrently under these incubation conditions as they are well known to be markedly reduced by these inhibitors (Morgan and Baker, 1969; Iacopetta and Morgan, 1983; Merlot et al., 2010). ATP levels were quantitatively analyzed using an ATP bioluminescence assay kit (Sigma-Aldrich) following the manufacturer’s instructions, as described previously (Merlot et al., 2010). Results were expressed as a percentage of the control.

**Tumor Xenograft Model in Nude Mice.** Six-week-old, female BAL/Bc nu/nu mice were obtained from the Animal Resources Centre (Canning Vale, Perth, WA, Australia) and used for studies after acclimatization within the facility until 7 weeks of age. The mice were housed in sterile conditions in the University of Sydney, Bosch Animal House, where they received food and water ad libitum. All in vivo experiments were approved by the Animal Ethics Committee (University of Sydney). To generate tumor xenografts, DMS-53 lung carcinoma cells were harvested and suspended in RPMI medium devoid of added supplements. The viable cell number was determined by Tripan-blue dye exclusion using a Countess Automated Cell Counter (Life Technologies, Carlsbad, CA) and was always >95%. A final density of 106 cells/ml was obtained and mixed with an equal volume of cold Matrigel (BD Biosciences, San Jose, CA). Matrigel was kept on ice at 4°C to prevent polymerization and solidification.

Prior to implantation, mice were anesthetized with isoflurane (Sigma-Aldrich). DMS-53 cells (5 × 106 cells; 100 μl) were subcutaneously injected into the right hind flank of each mouse (Whitnall et al., 2006; Yu et al., 2012). Tumor growth was calculated in cubic millimeters by measuring the two perpendicular diameters of the tumors with Vernier calipers. Tumor volume was estimated using the formula (L/2 × W2), where L was the longest diameter (length) and W was the shortest diameter (width) perpendicular to L (Balsari et al., 2004). Measurements and mouse body weight changes were made and recorded twice weekly.

A single dose of radiolabeled chelator was initiated 3 weeks after implantation, once the mean tumor volume reached ~400 mm³. Experimental groups were randomized and each group (n = 16) received either 14C-Bp4eT, 14C-PiH, or 14C-Dp44mT supplemented with unlabeled Bp4eT, PiH, or Dp44mT, respectively, to yield a final dose of 2 mg/kg of body weight, and a final specific activity of 1 μCi (chelator specific radioactivity, 75 μCi/mg). Chelators were dissolved in a vehicle consisting of 30% sterile propylene glycol (Sigma-Aldrich) and 70% sterile saline (0.9% NaCl; Baxter, Old Toongabbie, NSW, Australia) and administered intravenously via the tail vein (100 μl/mouse). Chelator-treated groups (n = 16) were further subdivided into four groups (n = 4) according to the following time points: 0.5, 1, 4, and 24 hours. Control mice (n = 4) were treated with vehicle alone. Animals were placed in metabolic cages during these studies in groups of four to collect urine and feces.
At 0.5, 1, 4, and 24 hours postadministration, mice were anesthetized with isoflurane and blood samples were collected by cardiac puncture. To eliminate residual radioactivity of blood in tissues, mice underwent intracardiac perfusion with 10 ml of PBS injected into the left ventricle with the right atrium being incised to allow release of blood. Following perfusion, mice were euthanized by cervical dislocation and were immediately dissected. The liver, gallbladder, kidneys, lungs, spleen, brain, bladder, large intestine, small intestine, bone (left femur), skeletal muscle (left thigh), and perirenal adipose tissue were removed and weighed. Femurs were cleaned of connective tissue and muscle. Feces and urine were collected from metabolic cages for each group (n = 4) during the specified time points.

Sample Combustion. Tissue, blood, fecal, and urine samples from in vivo administration experiments with 14C-labeled ligands were combusted using a Sample Oxidizer Model 307 (PerkinElmer). Combustion of the tissues, etc., was essential to prevent radioactive quenching and the inaccurate quantification of 14C (Moore, 1981). Wet weights of tissue and whole organs were recorded prior to combustion and large tissues or organs (>400 mg) were homogenized and a 300-mg aliquot was combusted due to the sample weight limitations of the machine. Blood samples were placed in a heparinized tube and centrifuged to allow the sedimentation of blood cells from plasma. A 10-μl aliquot of urine and a 100-μl aliquot of blood and serum were combusted. Samples were placed within combustion cones with the combustion process aided using 250 μl of Combustaid (PerkinElmer) per sample with combustion being performed for 2 minutes. After combustion, 14CO2 was trapped by 10 ml of Carbosorb E (PerkinElmer) and mixed with 10 ml of Permafluor E scintillant (PerkinElmer). The combustion efficiency of the oxidizer was determined using 14C standards before each run of the instrument and was always >97%.

Radioactive samples from the combustion process were analyzed using the β-counter described above. Background radioactivity was measured using corresponding samples from control animals that were administered the vehicle alone. Results were expressed as percentage of injected dose (ID)/g of organs, tissues, and feces or percentage of ID/μl of urine.

Statistical Analysis. Results are expressed as mean ± S.E.M. Statistical comparisons were made using the Student’s t-test and were considered statistically significant when P < 0.05.

Results

14C-Dp44mT Uptake as a Function of Concentration Is Saturable and Temperature-Dependent. The cellular uptake of 14C-Dp44mT, 14C-Bp4eT, or 14C-PIH by SK-N-MC neuroepithelioma cells was examined over a range of concentrations for 2 hours at 4°C and 37°C (Fig. 2). This cell type was initially used because its response to these chelators has been well characterized in our laboratory (Richardson and Punka, 1994; Richardson et al., 1995, 2006; Kalinowski et al., 2007; Merlot et al., 2010). Notably, at 37°C, cycling carriers or receptors (i.e., internalized and membrane-bound) can be detected, while at 4°C endocytosis and exocytosis are inhibited and only membrane-bound carriers/receptors are found (Morgan, 1981; Harding et al., 1983). Importantly, a 2-hour incubation was used because its response to these chelators has been well characterized in our laboratory (Richardson and Punka, 1994; Richardson et al., 1995, 2006; Kalinowski et al., 2007; Merlot et al., 2010). Notably, at 37°C, cycling carriers or receptors can be detected, while at 4°C endocytosis and exocytosis are inhibited and only membrane-bound carriers/receptors are found (Morgan, 1981; Harding et al., 1983).

Consistency with a saturable transport mechanism. A nonlinear regression fit for a one-binding-site model reported a Bmax value of 4.28 × 107 ± 0.26 molecules of chelator/cell (n = 9) and a Kd value of 2.45 ± 0.67 μM (n = 9) at 37°C (Table 1). This model was used because ligand uptake as a function of concentration was a single exponential curve. In contrast, at 4°C, analysis of the data yielded a lower Bmax value of 2.49 × 107 ± 0.13 molecules of chelator/cell and a higher Kd value of 4.71 ± 1 μM. Since ligand uptake at 37°C represents total uptake (namely, membrane and intracellular; Harding et al., 1983), while uptake
at 4°C represents membrane uptake only (Morgan, 1981; Iacopetta and Morgan, 1983), this enables calculation of the percentage of putative cycling carriers/receptors internalized. This analysis revealed that 62 ± 16% (Table 1) of binding sites were internalized, which is in general agreement with a variety of other cycling receptors, e.g., TR1 (Ciechanover et al., 1983; Mulford and Lodish, 1988). Notably, similar saturable 14C-Dp44mT uptake was also evident in other cell types, including DMS-53 lung carcinoma and SK-Mel-28 melanoma cells, as well as mortal MRC-5 fibroblasts (Supplemental Fig. 2), suggesting expression of the carrier/receptor was widespread. The B\text{max} and K\text{d} values from the binding data in these latter cell types (Supplemental Table 1) were generally similar to those obtained using SK-N-MC cells (Table 1). However, it is notable that the K\text{d} from MRC-5 binding data were 2- to 5-fold higher than that found for the other three cell types.

As demonstrated in our previous studies (Merlot et al., 2010) and in marked contrast to 14C-Dp44mT, the uptake of 14C-Bp4eT and 14C-PIH increased linearly as a function of concentration in the range of 5–250 μM (Fig. 2, B and C), without showing any evidence of saturation. Unlike 14C-Dp44mT, the cellular uptake of 14C-Bp4eT showed no significant (P > 0.05) difference at 4°C and 37°C (Fig. 2B). This observation suggested different mechanisms were involved in the uptake of these ligands. Additionally, 14C-PIH uptake process was temperature-dependent (Fig. 2C), although, in contrast to 14C-Dp44mT (Fig. 2A), no evidence of saturable binding was evident. In fact, incubating cells at 4°C relative to 37°C significantly (P < 0.05) decreased 14C-PIH uptake by approximately 80%.

The Effect of Metabolic Inhibitors on 14C-Dp44mT Uptake. Considering the data above, studies were then performed to determine whether 14C-Dp44mT uptake was energy-dependent (Fig. 3, A–D). The effect of two well characterized metabolic inhibitors, namely, NaN\text{3} and NaN\text{CN} (Henderson and Zevely, 1984; Qian and Morgan, 1991; Richardson, 1997), on 14C-Dp44mT uptake into SK-N-MC cells was examined at 37°C (Fig. 3A). Importantly, NaN\text{3} (30 mM) and NaN\text{CN} (5 mM) significantly (P < 0.001) reduced 14C-Dp44mT uptake to 56 ± 3% and 68 ± 3% of the control, respectively. Concurrently, as positive controls, these inhibitors significantly (P < 0.01–0.001) decreased ATP levels as well as 55Fe and 125I-transferrin uptake (Fig. 3, B–D), indicating these agents inhibited ATP synthesis and reduced receptor-mediated endocytosis, respectively, as shown previously (Morgan and Baker, 1989; Merlot et al., 2010). Together, with results showing the temperature-dependent uptake of 14C-Dp44mT (Fig. 2A), these studies indicated an energy-dependent mechanism consistent with receptor-mediated endocytosis.

14C-Dp44mT Uptake, but Not 14C-Bp4eT or 14C-PIH Uptake, Is Inhibited by Its Unlabeled Ligand. Competitive binding between a 14C-labeled ligand and the same nonlabeled ligand should reduce uptake of the label if a saturable mechanism is involved. To examine this, the cellular uptake of 14C-Dp44mT, 14C-Bp4eT, and 14C-PIH at 37°C was assessed in the presence of an increasing concentration of their corresponding unlabeled chelator (Fig. 4, A–C). The uptake of 14C-Dp44mT decreased with increasing concentrations of unlabeled Dp44mT (Fig. 4A), with this effect becoming significant (P < 0.001) at an unlabeled ligand concentration of 5 μM. This observation was consistent with competition with a saturable transport mechanism. Conversely, the uptake of 14C-Bp4eT and 14C-PIH was not significantly (P > 0.05) inhibited by their respective unlabeled drug (Fig. 4, B and C). This observation is consistent with the uptake of 14C-Bp4eT and 14C-PIH by a noncarrier-mediated process.

Interestingly, 14C-Bp4eT uptake was significantly (P < 0.001) increased in the presence of high concentrations of unlabeled Bp4eT, namely, at 100–250 μM (Fig. 4B). A similar, although not significant (P > 0.05), increase in 14C-PIH uptake as a function of unlabeled ligand concentration was also observed (Fig. 4C). These data, in Fig. 4, B and C, may be attributed to membrane perturbation by these agents that can increase binding of the radiolabel to cells (Baker et al., 1992).

14C-Dp44mT Uptake Is Not Affected by Its Synthetic Precursors, DpK and 44mT. Considering the potential involvement of a carrier/receptor–mediated process in Dp44mT transport, the uptake of 14C-Dp44mT (25 μM) was examined in the presence of increasing concentrations of its unlabeled precursors, namely, DpK (2.5–250 μM) or 44mT (2.5–250 μM) (Fig. 5, A–C). These studies were performed to decipher the structural features necessary for the transport mechanism involved. As shown in Fig. 4A, 14C-Dp44mT uptake was markedly decreased in the presence of unlabeled Dp44mT (Fig. 5A). In contrast, increasing concentrations of unlabeled DpK or 44mT had no significant effect (P > 0.05) on 14C-Dp44mT uptake in SK-N-MC cells (Fig. 5, B and C). Therefore, neither of the direct precursors used to synthesize Dp44mT, namely, DpK nor 44mT, were able to compete for the putative carrier/receptor responsible for the uptake of Dp44mT by SK-N-MC cells.

Uptake of 14C-Dp44mT Is Only Inhibited by Ligands with Marked Structural Similarity. In view of the competitive uptake of 14C-Dp44mT by its unlabeled counterpart (Fig. 4A), the effect of a range of structurally diverse thiosemicarbazone and aroylhydrazone ligands (100 μM) on 14C-Dp44mT uptake (25 μM) was also assessed to determine carrier/receptor specificity (Fig. 6). Unlabeled ligands of the DpT series (DpT, Dp4mT, Dp44mT, Dp4eT, Dp4pT, and DpC), the BpT series (BpT, Bp4mT, Bp44mT, Bp4eT, and Bp4aT), and the ApT series (ApT, Ap4mT, Ap44mT, Ap4eT, and Ap4pT), as well as Triapine and PIH, were assessed because of the various similarities and differences in their structure (Supplemental Fig. 1, A–C).

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Temperature</th>
<th>B\text{max} (molecules of chelator/cell)</th>
<th>K\text{d} (μM)</th>
<th>% Internalizated</th>
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<tr>
<td>Dp44mT</td>
<td>37°C</td>
<td>4.28 ± 0.26 × 10^7</td>
<td>2.45 ± 0.67</td>
<td>62 ± 16%</td>
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<tr>
<td></td>
<td>4°C</td>
<td>2.49 ± 0.13 × 10^7</td>
<td>4.71 ± 1.00</td>
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<tr>
<td>Cu(14C-PIH)_2</td>
<td>37°C</td>
<td>4.73 ± 0.43 × 10^7</td>
<td>54.33 ± 11.48</td>
<td></td>
</tr>
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</table>
All of the structurally similar unlabeled ligands of the DpT series significantly \( (P < 0.001) \) inhibited \( ^{14} \text{C}-\text{Dp44mT} \) uptake in comparison with the control \( ^{14} \text{C}-\text{Dp44mT} \) alone; Fig. 6). In contrast, only Bp44mT and Bp4mT of the BpT series significantly \( (P < 0.05-0.001) \) decreased \( ^{14} \text{C}-\text{Dp44mT} \) uptake, while other analogs of the BpT series had no significant \( (P > 0.05) \) effect. Furthermore, Ap44mT was the only chelator of the ApT series to significantly \( (P < 0.001) \) decrease \( ^{14} \text{C}-\text{Dp44mT} \) uptake (Fig. 6). The ligands, Triapine and PIH, of the thiosemicarbazone and aroylhydrazone class, respectively, had no significant \( (P > 0.05) \) effect on \( ^{14} \text{C}-\text{Dp44mT} \) uptake.

Unlabeled Dp44mT (100 \( \mu \text{M} \)) most markedly \( (P < 0.001) \) decreased the cellular uptake of \( ^{14} \text{C}-\text{Dp44mT} \) \( \sim 40\% \) of the control \( ^{14} \text{C}-\text{Dp44mT} \) alone; Fig. 6). The ligands, Triapine and PIH, of the thiosemicarbazone and aroylhydrazone class, respectively, had no significant \( (P > 0.05) \) effect on \( ^{14} \text{C}-\text{Dp44mT} \) uptake.

Unlabeled Dp44mT (100 \( \mu \text{M} \)) most markedly \( (P < 0.001) \) decreased the cellular uptake of \( ^{14} \text{C}-\text{Dp44mT} \) \( (25 \mu \text{M}) \) to \( \sim 40\% \) of the control \( ^{14} \text{C}-\text{Dp44mT} \) alone; Fig. 6). Thus, the carrier/receptor involved in the uptake of Dp44mT has particularly high selectivity for Dp44mT in comparison with the range of other ligands tested. Intriguingly, a decrease in \( ^{14} \text{C}-\text{Dp44mT} \) uptake occurred consistently across all series of these \( \alpha-N \)-heterocyclic chelators when the N4 atom of the unlabeled compound was saturated with the same dimethyl substituent, namely, Bp44mT, Ap44mT, and Dp44mT (Fig. 6). These results suggest that ligands containing both the 2-pyridyl and 44mT moiety have affinity for the carrier/receptor site responsible for the uptake of \( ^{14} \text{C}-\text{Dp44mT} \). It is notable that Bp44mT (Supplemental Fig. 1B) shares almost total structural identity to Dp44mT apart from the substitution of 1 nitrogen for a CH moiety (Fig. 1) but shows less activity at reducing \( ^{14} \text{C}-\text{Dp44mT} \) uptake. This observation demonstrates marked specificity in terms of the interaction of the ligand with the putative carrier/receptor.

**Uptake of the Fe and Cu Complexes of the \( ^{14} \text{C} \)-Chelators: Uptake of \( \text{Fe}^{(^{14} \text{C}-\text{Dp44mT})_2} \) Does Not Saturate Relative to That of \( \text{Dp44mT} \).** The uptake of the chelator complexes was also examined, as numerous studies have demonstrated that thiosemicarbazone complexes are redox active, and in particular the Cu complex of Dp44mT is more cytotoxic than the ligand alone (Jansson et al., 2010). Additionally, the anticancer activity of these ligands is attributed to cellular Fe- and Cu-binding (Yuan et al., 2004; Kalinowski et al., 2007; Lovejoy et al., 2011; Merlot et al., 2013). Consequently, the uptake of their Fe and Cu complexes was assessed at 37°C as a function of concentration in comparison with the ligand alone (Fig. 7).

![Fig. 3.](image-url) Incubation of SK-N-MC cells with the metabolic inhibitors, \( \text{NaN}_3 \) (30 \( \text{mM} \)) or \( \text{NaCN} \) (5 \( \text{mM} \)) significantly reduced: (A) \( ^{14} \text{C}-\text{Dp44mT} \) (25 \( \mu \text{M} \)) uptake, (B) cellular ATP levels, (C) \( ^{59} \text{Fe} \) uptake, or (D) \( ^{59} \text{Fe}^{125} \text{I}-\text{transferrin} \) uptake by cells from \( ^{59} \text{Fe}^{125} \text{I}-\text{transferrin} \) \( ([\text{Fe}] = 0.75 \mu \text{M} ; [\text{transferrin}] = 0.375 \mu \text{M}) \). Cells were preincubated with inhibitors or media alone for 30 minutes at 37°C. The media were then removed and replaced with new media containing \( ^{14} \text{C}-\text{Dp44mT} \) or \( ^{59} \text{Fe}^{125} \text{I}-\text{transferrin} \) in the presence or absence of inhibitors, and incubated for 60 minutes at 37°C. The cells were then washed and processed for quantification of \( ^{14} \text{C} \), ATP levels, \( ^{59} \text{Fe} \) or \( ^{125} \text{I} \). Results are expressed as % control ± S.E.M. (three experiments). **\( P < 0.01 \); ***\( P < 0.001 \) versus control.

Importantly, as for the studies examining ligand uptake, all experiments were performed over an incubation time of 2 hours and this was particularly important to minimize any cytotoxic effects.

The uptake of the \( \text{Fe}^{(^{14} \text{C}-\text{Dp44mT})_2} \) complex was linear \((r^2 = 0.95)\) as a function of concentration and markedly and significantly \( (P < 0.01–0.001) \) greater than that found for the relative ligand, \( ^{14} \text{C}-\text{Dp44mT} \) (Fig. 7A). In contrast, as shown in Fig. 2A, the uptake of the ligand alone, \( ^{14} \text{C}-\text{Dp44mT} \), demonstrated clear saturation at 5 \( \mu \text{M} \) (Fig. 7A inset). The marked uptake of the Fe complex relative to the ligand may be attributed, at least in part, to its highly lipophilic nature (Richardson et al., 2006), which results in sequestration in cellular compartments (Lovejoy et al., 2011). Notably, examination of \( \text{Cu}^{(^{14} \text{C}-\text{Dp44mT})_2} \) uptake was attempted, but the extreme toxicity observed even after only a 2-hour incubation at the concentrations used in Fig. 7A precluded assessment. This observation agreed well with the rapid and marked cytotoxic activity of the Cu complex relative to the Fe complex described in previous studies (Lovejoy et al., 2011).

Notably, in the studies above, there are three factors that indicate the complex is being taken up by cells relative to the
free ligand. First, the affinity of Dp44mT for Fe is extremely high (Bernhardt et al., 2009), meaning that in the extracellular milieu there is essentially no free ligand or metal in equilibrium; it is virtually all complexed. Second, ligand uptake alone by cells was markedly and significantly different from its Fe complex (Fig. 7A). This observation can only be accounted for by the fact that it is the complex that is being transported into the cell and not the ligand alone. Third, 14C-Dp44mT uptake saturates at 5 μM, while the uptake of the complex does not saturate and demonstrates a linear relationship as a function of concentration (Fig. 7A). These results demonstrate totally different mechanisms of uptake of 14C-Dp44mT relative to its complex.

Similarly to Fe14C-Dp44mT2, the uptake of Fe14C-Bp4eT2 increased linearly as a function of concentration (r² = 0.98; Fig. 7B). Higher concentrations of Fe14C-Bp4eT2 (i.e., >50 μM) could not be assessed due to its highly cytotoxic nature. In addition, as was evident for Fe14C-Dp44mT2, the Fe14C-Bp4eT2 complex accumulated in cells to significantly (P < 0.001) higher levels than the ligand alone (Fig. 7B). In agreement with the observation utilizing the Cu14C-Dp44mT2 complex above, the
Cu\(^{14}\text{C}-\text{Bp4eT})_2\) complex could not be assessed due to its marked cytotoxicity.

In contrast to the thiosemicarbazones above, the uptake of the Fe and Cu complexes of the aroylhydrazone \(^{14}\text{C}-\text{PIH}\) could be assessed due to its markedly lower cytotoxicity (Richardson et al., 1995). This is because unlike thiosemicarbazones, aroylhydrazone ligands form complexes that are not potently redox active (Chaston et al., 2003). The uptake of the \(\text{Fe}^{(14}\text{C}-\text{PIH})_2\) complex was linear as a function of concentration \((r^2 = 0.99)\) and was significantly \((P < 0.001)\) greater than that found for the ligand at all concentrations above 25 \(\mu\text{M}\) (Fig. 7C). In contrast, as a function of concentration, uptake of...
Cu\(^{14}\text{C-PIH}_2\) plateaued at 100 \(\mu M\). Assessment of the saturable binding using a nonlinear regression fit for a one-binding-site model yielded a \(B_{max}\) value of \(4.73 \pm 0.43 \times 10^3\) ligands/cell \((n = 9)\) and a \(K_D\) value of \(34.33 \pm 11.48 \mu M\) \((n = 9)\) (Table 1). The cellular accumulation of Cu\(^{14}\text{C-PIH}_2\) was significantly \((P < 0.01)\) higher than that of Fe\(^{14}\text{C-PIH}_2\) at all concentrations tested except 250 \(\mu M\), where no significant difference was evident (Fig. 7C).

The Effect of Temperature on the Uptake of the Fe and Cu \(^{14}\text{C-Chelator Complexes}\. The effect of temperature on the uptake of the Fe and Cu \(^{14}\text{C-chelator complexes and their relative ligands as a function of time was examined to determine if cellular uptake was temperature-dependent (Supplemental Fig. 3, A–C). For all ligands and their Fe and Cu complexes, except Cu\(^{14}\text{C-Dp44mT}_2\), biphasic uptake as a function of time was identified (Supplemental Fig. 3, A–C). This observation implied that in the initial phase there was a rapid uptake of the ligand or complex, while in the second phase, the rate of simultaneous uptake and efflux from the cell had equilibrated, leading generally to a plateau in uptake. For some of the complexes [e.g., Fe\(^{14}\text{C-Dp44mT}_2\), Fe\(^{14}\text{C-Bp4eT}_2\), and Cu\(^{14}\text{C-PIH}_2\); Supplemental Fig. 3, A–C], the second phase of uptake did not completely plateau, indicating gradual cellular accumulation. Intriguingly, the Cu complexes accumulated within cells to a greater extent \((P < 0.001)\) than did the Fe complex at 37°C, which may relate to their being trapped in cellular compartments such as the lysosome (Lovejoy et al., 2011).

The cellular uptake of \(^{14}\text{C-Dp44mT}\) and \(^{14}\text{C-PIH} and their complexes as a function of time was temperature-dependent, reaching higher \((P < 0.01)\) intracellular concentrations at 37°C than 4°C (Supplemental Fig. 3, A and C). As discussed above for the results in Fig. 7A, the Cu\(^{14}\text{C-Dp44mT}_2\) complex could not be examined beyond a 30-minute incubation at 37°C due to cytotoxicity (Supplemental Fig. 3A). At 4°C, the Cu\(^{14}\text{C-Dp44mT}_2\) complex was far less cytotoxic than at 37°C, probably because of its lower uptake at 4°C, enabling a 2-hour incubation (Supplemental Fig. 3A).

In contrast to \(^{14}\text{C-Dp44mT}\) and \(^{14}\text{C-PIH}, there was no significant \((P > 0.05)\) difference in \(^{14}\text{C-Bp4eT}\) uptake between 4°C and 37°C (Supplemental Fig. 3B), as evident in previous studies (Merlot et al., 2010), demonstrating \(^{14}\text{C-Bp4eT}\) uptake was temperature-independent. However, in contrast, the cellular uptake of the Fe and Cu complexes of \(^{14}\text{C-Bp4eT}\) was significantly \((P < 0.001)\) higher at 37°C than at 4°C, indicating a temperature-dependent mechanism (Supplemental Fig. 3B).

The Effect of Temperature on the Efflux of Fe \(^{14}\text{C-Chelator Complexes from Cells}\. In view of the fact that the uptake of the \(^{14}\text{C-chelator complexes by cells was greater than that of the ligand (Supplemental Fig. 3, A–C), it was crucial to examine the cellular retention and efflux of the ligand and complexes. The efflux of the Fe\(^{14}\text{C-chelator}_2\) complexes compared with \(^{14}\text{C-chelator}_2\) alone was examined by a 2-hour preincubation with \(^{14}\text{C-chelator complexes or \(^{14}\text{C-ligands alone at 37°C followed by reincubation of cells at 4°C or 37°C for up to 3 hours (Supplemental Fig. 4, A–C). In these experiments, as a result of the 2-hour preincubation period used, the Cu\(^{14}\text{C-chelator}_2\) complexes were too toxic to be studied.}

For all chelators and their Fe complexes, a significantly \((P < 0.05–0.001)\) greater release of cellular radioactivity was evident at 37°C than at 4°C (Supplemental Fig. 4, A–C), demonstrating that the efflux of the \(^{14}\text{C-chelators and}}

Fe\(^{14}\text{C-chelator}_2\) complexes are highly temperature-dependent. Considering this, it is notable that active metabolic processes such as exocytosis are markedly temperature-dependent (Morgan, 1981). Hence, this observation is particularly relevant because Dp44mT uptake was mediated by a process consistent with a saturable carrier/receptor (Figs. 2–6).

Interestingly, the cellular efflux of \(^{14}\text{C-Dp44mT}\) and its Fe complex reached similar levels at 37°C with approximately 70 ± 1% and 67 ± 3% of the radiolabel being released, respectively, after 180 minutes (Supplemental Fig. 4A). Taking into account the higher levels of cell-bound Fe\(^{14}\text{C-Dp44mT}_2\) (Supplemental Fig. 3A) and its similar release from cells relative to the ligand alone (Supplemental Fig. 4A), it can be concluded that there was sequestration of Fe\(^{14}\text{C-Dp44mT}_2\) in the cell. Furthermore, upon reincubation, significantly \((P < 0.05–0.001)\) higher levels of Bp4eT (64 ± 1% after 180 minutes) and PIH (73 ± 4% after 180 minutes) were released in comparison with their Fe complexes (29 ± 2% and 13 ± 1% at 180 minutes, respectively; Supplemental Fig. 4, B and C). In view of the greater cellular uptake of Fe\(^{14}\text{C-Bp4eT}_2\) and Fe\(^{14}\text{C-PIH}_2\) (Supplemental Fig. 3, B and C) and their greater retention (Supplemental Fig. 4, B and C), these complexes also appear to be retained and sequestered within cells, in a way similar to Fe\(^{14}\text{C-Dp44mT}_2\). For all complexes, this sequestration may be related to their increased lipophilicity relative to the ligands alone. This occurs due to the incorporation of the hydrophilic electron-donating groups into the coordination of Fe, decreasing their interaction with the surrounding solvent (water) and leading to greater lipophilicity (Edward et al., 1995).

In Vivo \(^{14}\text{C-Chelator Uptake}\. Considering the distinct saturable uptake of \(^{14}\text{C-Dp44mT}\) in cancer cells in vitro, studies progressed to assessing the uptake of the \(^{14}\text{C-chelators in vivo. The tissue distribution of \(^{14}\text{C-Dp44mT}, \(^{14}\text{C-Bp4eT, and \(^{14}\text{C-PIH was investigated in BALB/c nu-nu mice bearing DMS-35 lung cancer xenografts to assess tumor uptake, biodistribution, and excretion of the chelators. This mouse model was used as DMS-53 cells readily form xenografted tumors and its response to this class of therapeutics has been well characterized by our laboratory (Whitnall et al., 2006; Lovejoy et al., 2012).}

A single intravenous dose (2 mg/kg) of each \(^{14}\text{C-chelator was given 3 weeks after implantation, once the mean tumor volume reached ~400 mm\(^3\). The mice were then sacrificed and tumors collected 0.5, 1, 4, and 24 hours postinjection. The amount of \(^{14}\text{C (% ID/g tissue) in major organs and tissues is presented in Fig. 8. The highest levels of \(^{14}\text{C for all three ligands were detected in organs involved in excretion, namely, the liver, gallbladder, intestines, kidney, and bladder (Fig. 8). In general, the levels of radioactivity in the liver, gallbladder, and small intestine peaked at 0.5–1 hour and then decreased over time. In contrast, levels of \(^{14}\text{C within the large intestine generally increased and peaked at 4 or 24 hours, suggesting the \(^{14}\text{C-label was progressing through the gut to the large intestine for fecal excretion (Fig. 8, A–C). This latter feature of the data was most pronounced for Dp44mT. Unlike fecal excretion, renal excretion occurred more rapidly. The levels of \(^{14}\text{C peaked in the kidneys and bladder at 0.5–1 hours and then declined gradually over 24 hours after the injection.}

Modest levels of \(^{14}\text{C were detected in the lung, spleen, and heart of chelator-treated mice (Fig. 8, A–C). After administration of \(^{14}\text{C-Dp44mT, 6% of the injected dose was present within the heart within 0.5 hours, with this decreasing to}}

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of the three ligands, the uptake of Dp44mT appeared to be sequenced to the greatest extent in adipose tissue (Fig. 8A). In fact, after 0.5 hours, significantly ($P < 0.01$) greater levels of Dp44mT were identified in adipose tissue relative to mice treated with $^{14}$C-Bp4eT or $^{14}$C-PIH viz., $18 \pm 4\%$, $7 \pm 0.6\%$, and $3 \pm 0.7\%$ of the injected dose, respectively. Additionally, there were very minute amounts of radioactivity in brain, bone, and muscle, constituting $<1$, 2, and $3\%$, respectively, of the administered dose at all time-points (Fig. 8, A–C).

After intravenous administration, the amount of radiolabel present within the blood (plasma and blood cells) was relatively low in comparison with other tissues, demonstrating rapid drug clearance (Fig. 8, A–C). Whole blood samples were separated into plasma and blood cells to assess the ratio of radioactivity in the whole blood and plasma. A ratio of whole blood/plasma in ideal circumstances is 1, signifying a lack of drug accumulation in blood cells (Osman et al., 1997). At 0.5 hours postdose, the blood-to-plasma ratio of $^{14}$C-Bp4eT, $^{14}$C-Dp44mT and $^{14}$C-PIH was 1.18, 1.49, and 2.18, respectively (Fig. 8). This pattern was consistently observed at all time-points.

In comparison with the normal tissues, and particularly the gallbladder, small intestine, and large intestine, $^{14}$C-ligand uptake by tumors was minor (Fig. 8A–C). However, it is relevant to note that significantly ($P < 0.005–0.001$) higher levels of Dp44mT were present in the tumor between 0.5 hours and 24 hours postinjection of $^{14}$C-Dp44mT, relative to that found after injection with $^{14}$C-Bp4eT and $^{14}$C-PIH (Fig. 8).

**Routes of Elimination of the Ligands.** In addition to tissue distribution of the $^{14}$C-ligands, urine and feces were collected throughout experimental time points using metabolic cages (Fig. 9). For all compounds, the fecal $^{14}$C excretion increased over the 24-hour period (Fig. 9A), consistent with the general increasing levels of $^{14}$C evident in the large intestine and decreasing levels in the small intestine (Fig. 8). Levels of $^{14}$C in the urine peaked at 0.5–1 hour postdose for all the ligands and then decreased (Fig. 9B). Notably, this observation was consistent with the decreasing levels of radiolabel present in the kidneys and bladder as a function of time (Fig. 8, A–C). For mice administered $^{14}$C-Bp4eT, no urine was produced in the first 0.5 hours after the injection of the label, and thus, the first time point assessing urinary excretion is 1 hour (Fig. 9B).

**Discussion**

**Dp44mT Transport Is via a Carrier/Receptor-Mediated Mechanism.** The studies herein demonstrate that in contrast to the structurally related ligands, Bp4eT and PIH, the cellular uptake of Dp44mT occurs via a carrier/receptor-mediated mechanism. This conclusion is supported by evidence demonstrating that: 1) $^{14}$C-Dp44mT uptake as a function of concentration was saturable in four cell types (Fig. 2A; Supplemental Fig. 2), while uptake of $^{14}$C-Bp4eT and $^{14}$C-PIH was linear (Fig. 2, B and C); 2) $^{14}$C-Dp44mT uptake was temperature- and energy-dependent (Figs. 2A and 3, A–D); and 3) the uptake of $^{14}$C-Dp44mT was subject to competitive inhibition by unlabeled Dp44mT (Fig. 4A) and also by analogs that showed very marked structural similarity (Fig. 6). With respect to temperature, at $4^\circ$C, metabolically active processes such as carrier/receptor-mediated endocytosis and exocytosis are inhibited (Morgan, 1981; Sugano et al., 2010). Thus, temperature-dependent uptake was indicative of this mechanism (Morgan, 1981; Iacopetta and Morgan, 1983; Sugano et al., 2010).

In contrast to $^{14}$C-Dp44mT, the uptake of $^{14}$C-Bp4eT and $^{14}$C-PIH was not saturable as a function of concentration (Fig. 2, A–C) and was not inhibited by metabolic inhibitors (Merlot et al., 2010), suggesting that an energy-dependent carrier/receptor was not involved in the transport of these ligands. Quantitatively, the cellular uptake of $^{14}$C-Dp44mT was significantly lower than $^{14}$C-Bp4eT at $37^\circ$C (cf. Fig. 2, A and B).

Furthermore, the percentage of $^{14}$C-Bp4eT released from cells was lower than that of $^{14}$C-Dp44mT at $37^\circ$C (Supplemental Fig. 4, A and B). Together, these results demonstrate Bp4eT accumulates to a greater extent in cells than Dp44mT and this may be related to its greater lipophilicity (Kalinowski et al., 2007).

The different levels of Bp4eT and Dp44mT in cells may be explained by their differing mode of uptake. The cellular uptake of Dp44mT has characteristics consistent with carrier/receptor-mediated endocytosis (Iacopetta and Morgan, 1983; Richardson and Baker, 1990) that, due to endosomal trafficking, may direct the drug to lysosomes, which are a major target of this agent (Lovejoy et al., 2011). Additionally, greater levels of PIH were also evident in SK-N-MC cells relative to Dp44mT (Fig. 2, A and C). The level of PIH did not correlate with its markedly lower antiproliferative activity in this cell type (PIH IC$_{50}$ value, 75 $\mu$M; Richardson et al., 1995). This observation probably relates to the fact that Fe and Cu complexes of PIH are not redox-active (Richardson et al., 2006; Lovejoy et al., 2011). In contrast, lower cellular levels of PIH were evident relative to the redox-active chelator Bp4eT as shown previously (Merlot et al., 2010). Thus, antiproliferative activity of the agent is not only dependent on cellular retention, but also upon its mode of transport and cytotoxic redox activity.

The saturation of Dp44mT uptake by a number of cell types occurred at $\sim 5–10\, \mu$M. Considering this, it is notable that this level of Dp44mT would be pharmacologically relevant in humans, as the structurally related thiosemicarbazone, Triapine, has been observed at similar concentrations, i.e., 0.5–10 $\mu$M (Wadler et al., 2004; Chao et al., 2012). Moreover, the related chelator, salicylalddehyde isonicotinoyl hydrazone, was shown to reach 100 $\mu$M in rabbit plasma upon i.v. administration at 10 mg/kg (Kovarikova et al., 2005). Thus, in vivo, Dp44mT uptake via the saturable carrier/receptor-mediated mechanism would be pharmacologically relevant and could occur at the concentrations predicted to result after administration.

Recently, our laboratory demonstrated hepatic phase I and II metabolism for a related DpT ligand (Stariat et al., 2013). Critically, it could be suggested that such metabolism may affect the interpretation of the $^{14}$C-Dp44mT uptake results.
However, four lines of evidence argue this is not a significant issue. First, in contrast to our previous study that used concentrated human liver microsomes/S9 fractions (Staritat et al., 2013), the present study implemented nonhepatic tumor cells which are not rich in drug-metabolizing enzymes. Hence, it is unlikely the ligands would be metabolized rapidly. Second, the experiments were specifically designed to minimize metabolism by performing uptake studies over short 30-minute–2-hour/37°C incubations. Third, our studies compared ligand uptake at 37°C and 4°C, at which no active metabolism occurs. In terms of one of the major findings in this investigation, saturable uptake of Dp44mT was still
with a saturated terminal N4 atom (i.e., Dp44mT, Bp44mT, and Ap44mT) resulted in the greatest inhibition of Dp44mT uptake within their respective series and highlight the importance of this moiety in carrier/receptor-mediated uptake. Collectively, both the methyl groups at the R1 and R2 position and the coordinating pyridyl group are key structural moieties for ligand–carrier/receptor binding. Further studies are currently underway to discover the molecular identity of the Dp44mT carrier/receptor.

Saturable carriers/receptors are known for many cytotoxic drugs, including bleomycin (Pron et al., 1999), cisplatin (Ishida et al., 2002), and methotrexate (Deutsch et al., 1989). Furthermore, other thiosemicarbazones are agonists of the c-Mpl thrombopoietin receptor (Duffy et al., 2002). However, it is notable that the carrier/receptor for Dp44mT was very selective for this analog over thiosemicarbazones of the same class (Fig. 6). Consequently, it is unlikely that the c-Mpl thrombopoietin receptor is involved in Dp44mT uptake as they are known to bind other thiosemicarbazones (Duffy et al., 2002).

**Chelator Complexes Are Sequestered in Cells.** In contrast to $^{14}$C-Dp44mT uptake, which was saturable as a function of concentration, uptake of its Fe complex was linear (Fig. 7A), indicating a different mechanism of transport. However, a higher level of uptake of the metal complexes in contrast to the ligands was evident (Fig. 7; Supplemental Fig. 3). This observation together with their marked redox activity may, in part, explain the greater cytotoxicity of the Cu complexes compared with the ligands (Jansson et al., 2010). The greater uptake of the complexes may be attributed to: 1) their increased lipophilicity versus the ligand (Edward et al., 1995); 2) differing modes of uptake; and/or 3) complex sequestration within cellular compartments, e.g., lysosomes (Lovejoy et al., 2011). Interestingly, repeated administration of Dp44mT and a close analog, Bp44mT, to mice bearing tumor xenografts does not lead to tumor Fe depletion, also suggesting that Fe complexes may become sequestered in vivo (Whitnall et al., 2006; Yu et al., 2012).

**In Vivo Distribution and Excretion of $^{14}$C-Chelators in Mice Bearing DMS-53 Xenografts.** Due to their high lipophilicity, all three ligands were excreted in the feces with organs involved in this process being highly labeled (Figs. 8 and 9A). For all chelators, radioactivity peaked in the feces 24 hours after administration. In contrast, renal excretion occurred more rapidly with levels of $^{14}$C in urine peaking at 0.5–1 hour postinjection of $^{14}$C-ligand with the quantity of radiolabel declining over time (Fig. 9B). This is consistent with the decreasing levels of radiolabel present in excretory tissue, namely, the kidneys and bladder (Fig. 8).

Interestingly, at all time points, there was little uptake of the $^{14}$C-chelators in the brain, bone, and muscle of mice (Fig. 8, A–C). This agrees with the limited toxicity found in these organs after treatment (Whitnall et al., 2006; Lovejoy et al., 2012; Yu et al., 2012). Only a minor proportion of $^{14}$C-labeled ligands were detected in the tumors, with significantly more $^{14}$C-Dp44mT being detected relative to the other ligands (Fig. 8A). This difference may be indicative of the anticancer efficacy of the chelators in vivo.

Collectively, Dp44mT is transported into cells by a saturable carrier/receptor–mediated mechanism. Competition experiments demonstrated that the saturated N4 structural moiety and the coordinating pyridyl substituent were critical for carrier/receptor recognition. Studies assessing the tissue distribution of $^{14}$C-Dp44mT injected intravenously into mice bearing observed at 4°C compared with 37°C (Fig. 2A). Hence, even if metabolism occurred at 37°C, major findings of the study were not affected, justifying our protocol. Fourth, competition experiments showed that receptor/carrier–mediated uptake of Dp44mT was highly specific for this ligand (Figs. 4–6). Thus, even if limited Dp44mT metabolism occurred, the metabolites would likely have little effect on uptake. Collectively, our protocol was carefully designed to minimize drug metabolism, and our studies indicate that even if this occurs, it is unlikely to markedly affect the major conclusions.

**Competition for Carrier/Receptor-Binding of $^{14}$C-Dp44mT with Unlabeled Precursors and Analogs.** To elucidate the pharmacophore responsible for transport activity, $^{14}$C-Dp44mT uptake was examined in the presence of its unlabeled synthetic precursors DpK and 44mT (Fig. 5). Increasing concentrations of DpK or 44mT did not alter $^{14}$C-Dp44mT uptake, suggesting that neither moiety is capable of binding the saturable transporter. This suggests that chemical moieties on both substructures are only involved when combined and are necessary for Dp44mT uptake.

A range of structurally related thiosemicarbazones (Supplemental Fig. 1) were also examined to determine competition for the carrier/receptor–mediated transport system responsible for Dp44mT uptake (Fig. 6). As may be expected, unlabeled Dp44mT most markedly reduced $^{14}$C-Dp44mT uptake. Consistently across the ligands tested, thiosemicarbazones...
xenografted tumors demonstrated the ^4^C label was primarily identified in organs associated with fecal and urinary excretion. Collectively, these results have clinical implications for understanding the bioavailability and uptake of Dp44mT in vivo.

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Authorship Contributions

Participated in research design: Merlot, Richardson, Kalinowski. Conducted experiments: Merlot, Pantarat, Menezes, Sahni, Kalinowski. Performed data analysis: Merlot, Pantarat, Menezes, Sahni, Kalinowski.

Wrote or contributed to the writing of the manuscript: Merlot, Richardson, Kalinowski.

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