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Mobilization of iron from neoplastic cells by some iron chelators is an energy-dependent process

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Abstract

Iron (Fe) chelators of the pyridoxal isonicotinoyl hydrazone (PIH) class may be useful agents to treat Fe overload disease and also cancer. These ligands possess high activity at mobilizing ^{59}Fe from neoplastic cells, and the present study has been designed to examine whether their marked activity may be related to an energy-dependent transport process across the cell membrane. Initial experiments examined the release of ^{59}Fe from SK-N-MC neuroblastoma (NB) cells prelabelled for 3 h at 37°C with ^{59}Fe-transferrin (1.25 μM) and then reincubated in the presence and absence of the chelators for 3 h at 4°C or 37°C. Prelabelled cells released 4–5% of total cellular ^{59}Fe when reincubated in minimum essential medium at 4°C or 37°C. When the chelators desferrioxamine (DFO; 0.1 mM) or PIH (0.1 mM) were reincubated with labelled cells at 4°C, they mobilized only 4–5% of cellular ^{59}Fe, whereas as 37°C, these ligands mobilized 21% and 48% of cell ^{59}Fe, respectively. The lipophilic PIH analogue, 311 (2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone; 0.1 mM), which exhibits high anti-proliferative activity, released 10% and 53% of cellular ^{59}Fe when reincubated with prelabelled cells at 4°C and 37°C, respectively. Almost identical results were obtained using the SK-Mel-28 melanoma cell line. These data suggest that perhaps temperature-dependent mechanisms are essential for ^{59}Fe mobilization from these cells. Interestingly, the metabolic inhibitors, 2,4-dinitrophenol, oligomycin, rotenone, and sodium azide, markedly decreased ^{59}Fe mobilization mediated by PIH, but had either no effect or much less effect on ^{59}Fe release by 311. Considering that an ATP-dependent process was involved in ^{59}Fe release by PIH, further studies examined 4 widely used inhibitors of the multi-drug efflux pump P-glycoprotein (P-gp). All of these inhibitors, namely, verapamil (Ver), cyclosporin A (CsA), reserpine (Res) and quinine (Qui), decreased ^{59}Fe mobilization by PIH but had little or no effect on ^{59}Fe release mediated by analogue 311. Further, both CsA and Ver increased the proportion of ethanol-soluble ^{59}Fe within cells in the presence of PIH, suggesting inhibited transport of the ^{59}Fe complex from the cell. However, when PIH-mediated ^{59}Fe release was compared between a well characterized Chinese hamster ovary cell line ($CH^{R}B30$) expressing high levels of P-gp and the relevant control cell line (AuxB1), no appreciable difference in the kinetics of ^{59}Fe release were found. In contrast, it was intriguing that the $CH^{R}B30$ cells released more ^{59}Fe into control medium (i.e., without PIH) than the AuxB1 control line (16.7% compared to 5.9%, respectively). In summary, the results suggest that a temperature- and energy-dependent process was involved in the

Abbreviations: BSS, Hanks' balanced salt solution; CsA, cyclosporin A; DFO, desferrioxamine; MEM, minimum essential medium; NB, neuroblastoma; PBS, phosphate buffered saline; P-gp, P-glycoprotein; PIH, pyridoxal isonicotinoyl hydrazone; Qui, quinine; Res, reserpine; Tf, transferrin; Ver, verapamil; 311, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone.

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efflux of the PIH-^{59}Fe complex from the cells. In contrast, ^{59}Fe release mediated by 311 was temperature-dependent but not energy-dependent, and could occur by simple diffusion or passive transport. Further studies investigating the membrane transport of Fe chelators may be useful in designing regimes that potentiate their anti-neoplastic effects.

Keywords: Iron; Iron chelator; Iron mobilization; Pyridoxal isonicotinoyl hydrazone

1. Introduction

The development of iron (Fe) chelators for clinical use has traditionally been focussed on their ability to remove Fe from patients suffering Fe overload diseases such as thalassemia. However, it has become clear that Fe chelators may also be useful for treating a number of disease states [1]. In fact, these compounds are effective at treating malaria [1,2], for preventing free radical-mediated tissue damage [3], and for inhibiting tumor cell growth [4]. For example, the tris-hydroxamate Fe(III) chelator, desferrioxamine (DFO), is widely used for the treatment of Fe overload, and more recently has been shown to be effective at inhibiting the proliferation of neuroblastoma (NB) cells [5], hepatoma cells [6], leukemia cells [7] and melanoma cells [8]. Apart from DFO, a range of Fe chelators have also been investigated for their anti-proliferative effects in a wide spectrum of cell types [9,10].

The tridentate Fe chelator, pyridoxal isonicotinoyl hydrazone (PIH), is highly effective at mobilizing ^{59}Fe from both normal and neoplastic cells, being far more efficient than DFO [11–13]. Moreover, a clinical trial with PIH showed no evidence of toxicity and resulted in significant Fe excretion [14]. Considering the efficacy of PIH, a range of PIH analogues were synthesized and several showed high activity, these compounds having potential as agents to treat Fe overload [12,15]. Since NB cells appear to be particularly sensitive to DFO [5], we have recently examined the use of the PIH analogues as effective anti-proliferative agents [16–18]. While PIH displayed low anti-proliferative activity, some of its analogues, particularly the lipophilic hydrazones derived from 2-hydroxy-1-naphthylaldehyde, showed high efficacy at inhibiting the growth of NB cells and other neoplastic cells in vitro [17,18]. These compounds were particularly active at mobilizing ^{59}Fe from prelabelled cells, being far more efficient than DFO. The high rate of ^{59}Fe efflux in the presence of the chelators [16–18] suggested that perhaps these ligands were being actively extruded from the cell. Previous studies by Huang and Ponka [19] demonstrated that an energy-dependent process was involved in the release of the PIH-^{59}Fe complex from ^{59}Fe-loaded erythroid cells. However, these studies were performed using rabbit reticulocytes that had been treated with the heme synthesis inhibitor succinylacetone [19]. Treatment of reticulocytes with this latter agent results in an accumulation of ^{59}Fe in the mitochondrion [20], a chelatable pool of ^{59}Fe that is not present under physiological conditions [21]. Hence, the relevance of this work to human neoplastic cells is unclear. In addition, Huang and Ponka only examined the efflux of PIH [19], the mechanism of release of the effective anti-tumor agents derived from 2-hydroxy-1-naphthylaldehyde [17,18] were not assessed.

In the present study, human neoplastic cells have been used to examine the mechanism of PIH-mediated ^{59}Fe release compared to its lipophilic analogue, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311), a chelator that is an active anti-proliferative agent [17,18]. These experiments were undertaken in an attempt to understand the membrane transport of Fe chelators in view of designing regimes that enhance their anti-tumor effects.

2. Materials and methods

2.1. Materials

Iron-59 (as ferric chloride in 0.1 M HCl) was purchased from Dupont, NEN products, Boston, MA. Human transferrin (Tf) was purchased from Boehringer-Mannheim, Mannheim, Germany. Eagle's modified minimum essential medium (MEM), α-MEM containing ribonucleosides and deoxyribonucleosides, Hanks' balanced salt solution (BSS) and

penicillin-streptomycin were obtained from Gibco Laboratories, Canada. Fungizone (amphotericin B) was obtained from Squibb Pharmaceuticals, Montréal, Canada. Fetal calf serum (FCS) was from Wisent, St. Bruno, Québec, Canada. Colchicine, 2,4-dinitrophenol, oligomycin, quinine, reserpine, rotenone, sodium azide, verapamil, and vinblastine, were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A (Sandimmune) was from Sandoz, Dorval, Québec, Canada. Desferrioxamine (desferrioxamine mesylate) was obtained from Ciba-Geigy Pharmaceutical, Summit, NJ. All other chemicals were of analytical reagent quality.

2.2. Preparation of the chelators

Chelators were synthesized by Schiff base condensation between 3 aromatic aldehydes (pyridoxal, '100 series'; salicylaldehyde, '200 series'; 2-hydroxy-1-naphthylaldehyde, '300 series') and a series of acid hydrazides using standard procedures as described previously [15,17]. In the present study we have primarily examined the activity of PIH compared to analogue 311, although in some experiments, the effect of DFO, 206 (salicylaldehyde *p-t*-butylbenzoyl benzoyl hydrazone), 309 (2-hydroxy-1-naphthylaldehyde *m*-fluorobenzoyl hydrazone) and 315 (2-hydroxy-1-naphthylaldehyde 2-thiophenecarboxyl hydrazone) were also examined. The PIH analogues have previously been characterized with respect to their molecular weights, melting points and infrared spectra [22]. The ligands were dissolved in dimethyl sulphoxide (DMSO) as 10 mM stock solutions immediately prior to an experiment and then diluted so that the final concentration of DMSO was equal to 0.1% (v/v). After dilution, the solutions were mixed vigorously to ensure total solubilization. In all experiments with chelators dissolved in MEM or PBS, 1% FCS was added to prevent the compounds from coming out of solution.

In the initial stages of this investigation enquiries were made concerning the ^{14}C-labelling of PIH and its analogues, as this would provide an ideal method for examining their mechanism of uptake and release. However, no ^{14}C-labelled precursors of the chelators were commercially available. This would necessitate custom synthesis of the ^{14}C-labeled ligands which was very expensive, and this prevented their use in the present study.

2.3. Protein purification and labelling

Human apotransferrin was prepared and labelled with ^{59}Fe to produce $^{59}Fe_2$-transferrin (^{59}Fe-Tf), as described by Richardson and Baker [23].

2.4. Cell culture

The human NB cell line, SK-N-MC, and the human melanoma cell line, SK-Mel-28, were obtained from the American Type Culture Collection (Rockville, MD), and were grown in MEM containing 10% FCS, 1% (v/v) non-essential amino acids, 100 μg/ml of streptomycin, 100 U/ml penicillin and 0.28 μg/ml of fungizone. Cells were subcultured and used for experiments as described in previous work [23]. The SK-N-MC and SK-Mel-28 melanoma cell lines were chosen as the Fe metabolism of these cells were characterized in prior investigations [16,23], and both lines were shown to be sensitive to the growth inhibitory effects of the PIH analogues [17,18]. In all studies confluent monolayers of cells were used without cellular overgrowth.

The well characterized CH^RB30 Chinese hamster ovary cells with high P-glycoprotein (P-gp) levels and its parental control, AuxB1, were kindly supplied by Dr. Victor Ling, British Columbia Cancer Agency, Vancouver, Canada. The CH^RB30 CHO cell line was selected by continuous culture in increasing concentrations of colchicine from the CHO cell line CH^RC5 [24], and the CH^RC5 line was clonally selected, stepwise, from Aux B1 [25]. The Aux B1 and CH^RB30 CHO cell lines were grown in α-MEM containing ribonucleosides and deoxyribonucleosides supplemented with 10% FCS. Twenty-four hours after passaging, 30 μg/ml of colchicine was added to the medium of the CH^RB30 cells and the cultures then allowed to grow to near confluence (Kelly Fox and Victor Ling, personal communication). All experiments with these cell lines was perfomed using cultures close to confluence. The CH^RB30 cells have been shown to express very high levels of P-gp and to display multi-drug resistance, while AuxB1 is

drug-sensitive and has only trace amounts of P-gp [24,26].

2.5. The effect of temperature and inhibitors on chelator-mediated iron release

The effect of temperature on chelator-mediated ^{59}Fe release was studied using standard procedures [15–17]. Briefly, cells were prelabelled for 3 h at 37°C with MEM containing ^{59}Fe-Tf (1.25 μM). After this incubation, the cells were placed on a tray of ice and washed 4 times with ice-cold BSS and then reincubated for up to 3 h at either 4°C or 37°C with medium alone or medium containing the chelators (0.1 mM). For reincubation times up to 3 h at 4°C or 37°C, cells remained viable as judged by cellular morphology, adherence to the culture substratum and the exclusion of trypan blue. At the end of the reincubation period, the overlying medium was removed and placed in counting tubes to estimate the release of ^{59}Fe. The cells were then scraped from the plates in 1 ml of BSS using a teflon spatula and put into counting tubes to estimate cellular ^{59}Fe. Radioactivity was measured using a LKB 1282 Compugamma counter. As an estimate of cell density the cell pellet was resuspended in 1 ml of BSS, sonicated for 15 s, and an aliquote used to determine protein concentration by the bicinchoninic acid (BCA) method (Pierce Chemical, Rockford, IL, 61105, USA) [23]. Results are expressed as either the percentage of cellular ^{59}Fe released or as nmoles Fe per gram of protein (gPR).

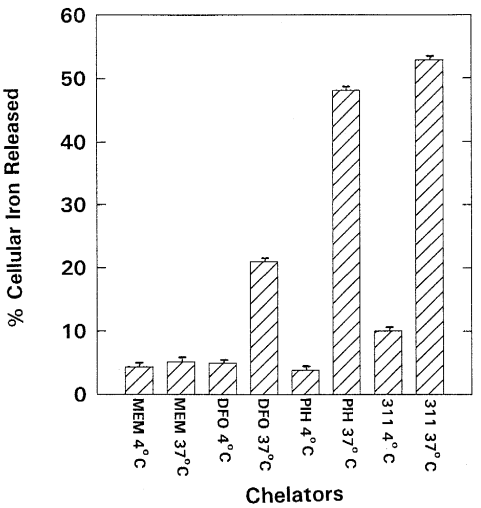


Fig. 1. The effect of incubation temperature on chelator-mediated ^{59}Fe release from prelabelled SK-N-MC neuroblastoma cells. Cells were prelabelled for 3 h at 37°C with ^{59}Fe-transferrin (1.25 μM), washed, and then reincubated in the absence or presence of the chelators (0.1 mM) for 3 h at 4°C or 37°C. Results are presented as the Mean ± S.D. of three replicates in a typical experiment of two experiments performed.

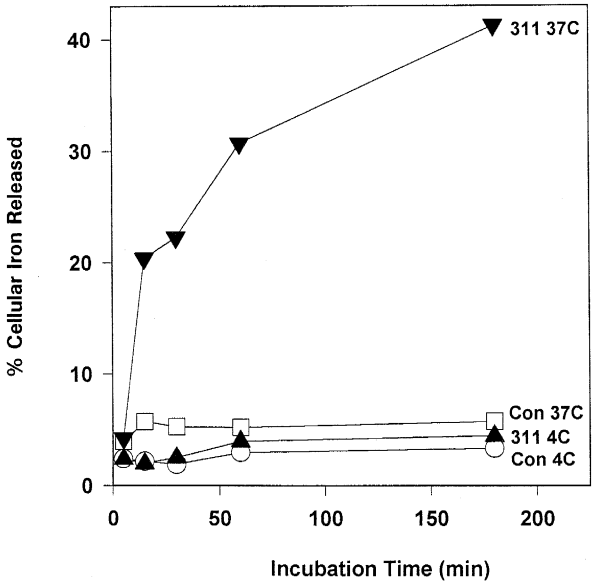


Fig. 2. The effect of incubation temperature on the kinetics of ^{59}Fe release from SK-N-MC neuroblastoma cells in the presence or absence of 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311). Cells were prelabelled with ^{59}Fe-transferrin (1.25 μM) for 3 h at 37°C, washed, and then reincubated for up to 3 h at 4°C or 37°C with control medium alone or medium containing chelator 311 (0.1 mM). Exactly comparable results were obtained for PIH. Results are means of duplicate determinations in a typical experiment of two experiments performed. Variation between replicates was less than 5%.

2.6. The effect of inhibitors on chelator-mediated iron release

Standard methods were implemented to assess the effects of inhibitors on ^{59}Fe release by the chelators [19]. Briefly, cells were prelabelled using a 3 h incubation at 37°C with ^{59}Fe-Tf (1.25 μM) in MEM, then placed on a tray of ice and washed 4 times with ice-cold BSS. The cells were then preincubated with the inhibitor for 30 min or 60 min at 37°C, this medium removed, and replaced with medium containing either the inhibitor alone or the inhibitor and the

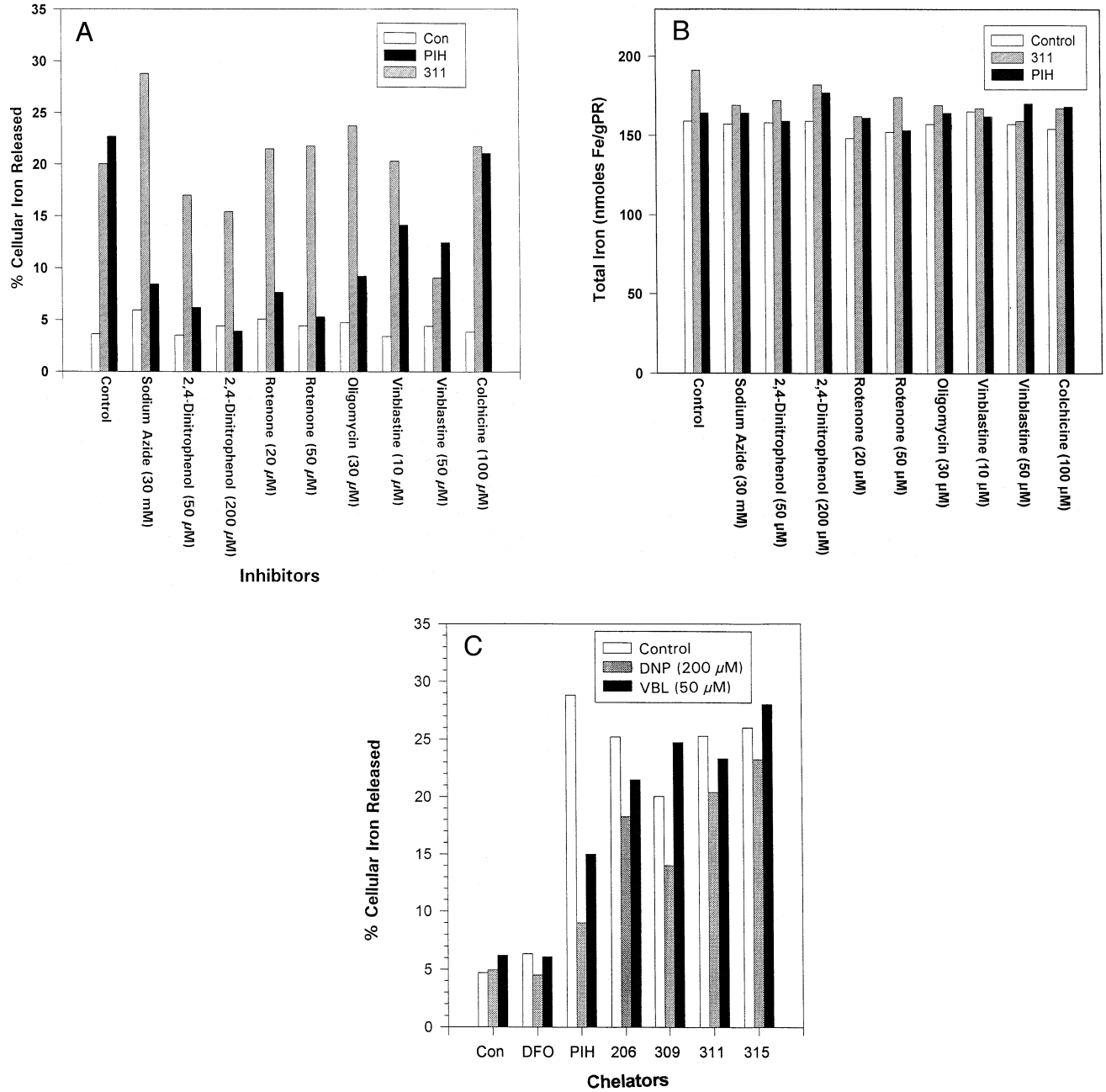


Fig. 3. A: the effect of a range of inhibitors on PIH or analogue 311-mediated ^{59}Fe release from prelabelled SK-N-MC neuroblastoma cells. Cells were labelled with ^{59}Fe-transferrin (1.25 μM) for 3 h at 37°C, washed, and then preincubated for 30 min at 37°C with the inhibitor in PBS. This solution was removed and replaced with PBS containing the inhibitor alone or the inhibitor and the chelator (50 μM) which was incubated with the cells for 60 min at 37°C. B: the effect of the inhibitors on the total amount of ^{59}Fe in the cells. Incubation conditions were the same as described for (A). C: the effect of 2,4-dinitrophenol (DNP; 200 μM) or vinblastine (VBL; 50 μM) on ^{59}Fe release by DFO (100 μM), PIH (50 μM), or the PIH analogues 206, 309, 311 and 315 (50 μM). Incubation conditions were the same as those described for (A). Results are means of duplicate determinations from a typical experiment of 3 experiments performed. Variation between replicates was generally less than 5%.

chelator. Results were compared to when prelabelled cells were preincubated with medium alone for 30 min or 60 min and then exposed to either medium alone or medium containing the chelator. At the end of the reincubation, the overlying medium was removed and placed into counting tubes to estimate ^{59}Fe release. The cells were then scraped from the plates in 1 ml of BSS and put into counting tubes to estimate cellular ^{59}Fe.

For experiments using metabolic inhibitors (sodium azide, 2,4-dinitrophenol, rotenone, oligomycin) and microtubule inhibitors (vinblastine and colchicine), these compounds were prepared in PBS (pH 7.4) and preincubated with cells for 30 min at 37°C. This solution was then removed and replaced with PBS/1% FCS containing either PIH or 311 in the presence of the inhibitors. In these studies, PBS was used instead of MEM, as it does not contain glucose, and hence, aids the metabolic depletion induced by adding sodium azide, 2,4-dinitrophenol, rotenone and oligomycin. As described above, 1% FCS was added with the chelators to prevent them coming out of solution in PBS. In experiments with inhibitors shown to prevent P-glycoprotein transport activity, namely, cyclosporin A (CsA), quinine (Qui), reserpine (Res) and verapamil (Ver), these compounds were prepared in MEM.

2.7. Estimation of chelator-bound ^{59}Fe in cells using ethanol precipitation

The amount of chelator-bound ^{59}Fe within cells was estimated by standard techniques using ethanol precipitation [19]. Briefly, cells were lyzed by scraping them from the petri dishes in 0.5 ml of ice-cold doubly distilled water and the cell suspension transferred to Eppendorf tubes at 4°C. Ice-cold 95% ethanol (1.0 ml) was then added with vigorous mixing and the tubes left on ice for 30 min. After this incubation, the tubes were placed in an IEC MicroMax microcentrifuge (IEC, Canada) and centrifuged at 13200 rpm for 30 min at 4°C. The ethanol-soluble supernatant was then separated from the ethanol-insoluble material using a pasteur pipette and both fractions counted in the gamma counter described above. Previous work has shown that this technique results in the precipitation of ^{59}Fe in ferritin and transferrin, while ^{59}Fe bound to PIH or other chelators was not precipitated [19].

2.8. Statistics

Experimental data were compared using the Student's *t*-test. Results were considered statistically significant when $P < 0.05$.

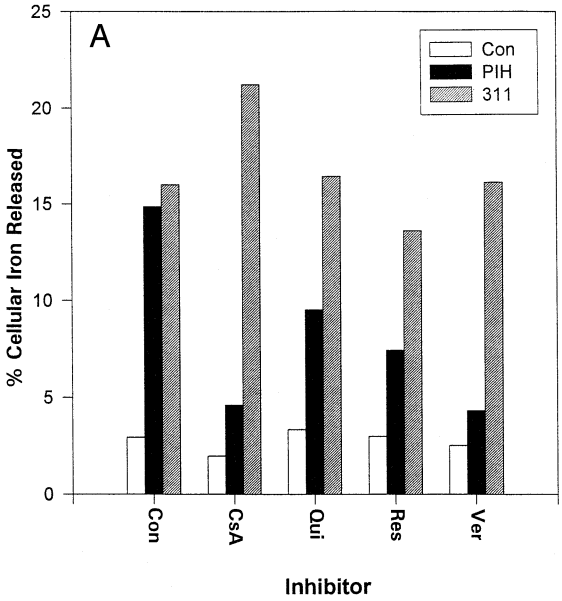
3. Results

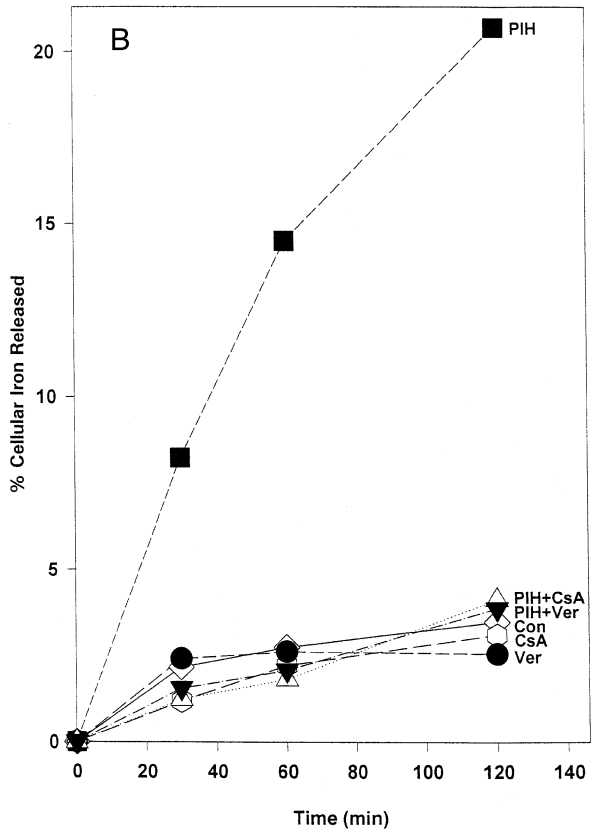
3.1. The effect of temperature and metabolic inhibitors on chelator-mediated iron release

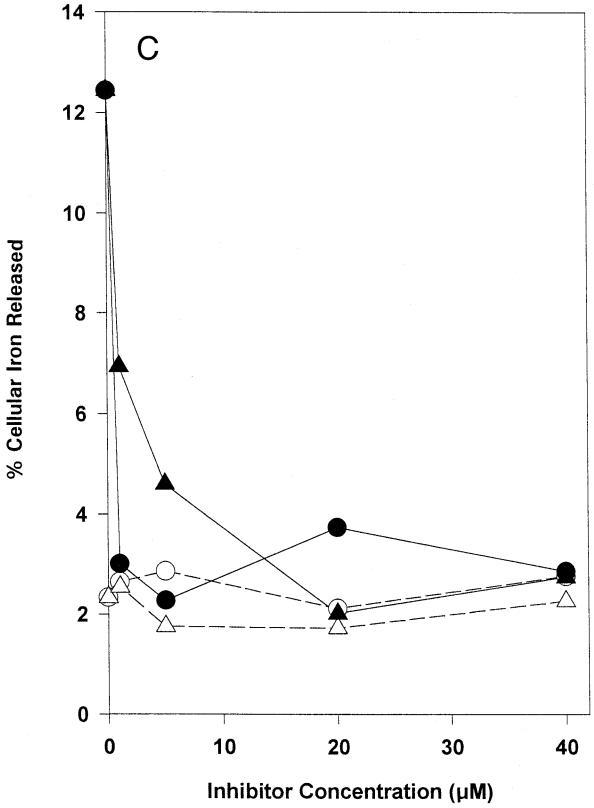
Initial experiments were designed to examine whether ^{59}Fe release mediated by the chelators was temperature-dependent. For the chelators DFO, PIH and 311, ^{59}Fe release from SK-N-MC NB cells at 4°C was far less than that found at 37°C (Fig. 1). For example, when DFO or PIH were reincubated with

Fig. 4. A: the effect of cyclosporin A (CsA), quinine (Qui), reserpine (Res) or verapamil (Ver), on chelator-mediated ^{59}Fe release from SK-N-MC neuroblastoma cells. Cells were labelled with ^{59}Fe-transferrin (^{59}Fe-Tf; 1.25 μM) for 3 h at 37°C, washed, and then preincubated for 60 min at 37°C with medium containing the inhibitors (20 μM). This medium was subsequently removed and replaced with medium containing the inhibitor alone (20 μM) or the inhibitor (20 μM) and the chelator (30 μM) which was incubated with the cells for 60 min at 37°C. B: the effect of CsA and Ver on PIH-mediated ^{59}Fe release as a function of incubation time. Cells were labelled with ^{59}Fe-Tf, washed, and preincubated with medium containing CsA or Ver as described for (A). The cells were then reincubated for 30 min, 60 min or 120 min at 37°C with either medium alone (◇—◇); CsA alone (20 μM; ○---○); PIH (30 μM) and CsA (20 μM) (△ · · · △); Ver (20 μM; ●– – –●); or PIH (30 μM) and Ver (20 μM) (▼– · –▼). C: the effect of CsA and Ver concentration on ^{59}Fe release by PIH. Cells were labelled with ^{59}Fe-Tf and washed as described in (A) and then preincubated with either medium alone or medium containing CsA or Ver (1–40 μM) for 1 h at 37°C. The cells were then reincubated for 1 h at 37°C with either CsA (0–40 μM; △– – –△); CsA (0–40 μM) and PIH (30 μM) (▲—▲); Ver (0–40 μM; ○– – –○); or Ver (0–40 μM) and PIH (30 μM) (●—●). The results are means of duplicate or triplicate determinations in a typical experiment of two experiments performed. The variation between replicates was generally less than 5%.

prelabelled cells at 4°C, they mobilized only 4–5% of cellular ^{59}Fe, whereas as 37°C, these ligands mobilized 21% and 48% of ^{59}Fe, respectively (Fig. 1). The lipophilic PIH analogue, 311, which exhibits high anti-proliferative activity [17,18], released 10% and 53% of cellular ^{59}Fe when reincubated with prelabelled cells at 4°C and 37°C, respectively (Fig. 1). For each chelator the difference in ^{59}Fe release be-







tween 4°C and 37°C was significant ($P < 0.001$). It is important to note that at 4°C PIH in MEM did not mobilize any more cellular ^{59}Fe (3.8%) than MEM only (4.3%) at this temperature (Fig. 1). Hence, the net ^{59}Fe release by PIH at 4°C was zero. In marked contrast, at 37°C ^{59}Fe release by MEM alone was 5.1%, while PIH in MEM mobilized 48.0% of cellular ^{59}Fe at this temperature, resulting in a net ^{59}Fe release of 42.9% by this ligand (Fig. 1). These experiments suggest that temperature-dependent mechanisms are necessary for chelator-mediated ^{59}Fe mobilization from the cells. Almost identical results were obtained with the SK-Mel-28 melanoma cell line (data not shown).

Examination of ^{59}Fe efflux from SK-N-MC NB cells as a function of time at 4°C and 37°C demonstrated that reincubation at 37°C with chelator 311 (0.1 mM) caused a biphasic release of ^{59}Fe. This biphasic release resulted in the efflux of 41% of total cell ^{59}Fe after a 3 h reincubation (Fig. 2). In contrast, when cells were reincubated with 311 at 4°C, very little ^{59}Fe was released for incubation times up to 180 min (4.5% after 180 min). When cells were reincubated with medium alone, slightly more ^{59}Fe was released at 37°C than at 4°C (5.7% compared to 3.4% after 180 min; Fig. 2). Comparable data were obtained when the experiment was repeated using PIH instead of analogue 311 (data not shown).

Further studies examined whether metabolic inhibitors prevented ^{59}Fe release mediated by PIH or analogue 311 (Fig. 3A). The metabolic inhibitors, sodium azide (30 mM), 2,4-dinitrophenol (50 μM and 200 μM), rotenone (20 μM and 50 μM) and oligomycin (30 μM), all markedly prevented the release of ^{59}Fe mediated by PIH, whereas they had either no inhibitory effect (sodium azide, rotenone, oligomycin) or far less effect (2,4-dinitrophenol) on ^{59}Fe release by 311 (Fig. 3A). The microtubule inhibitor vinblastine was previously shown to inhibit the efflux of ^{59}Fe from rabbit reticulocytes by PIH [19]. In the present study using SK-N-MC NB cells, vinblastine (10 μM) also reduced PIH-induced ^{59}Fe mobilization, whereas ^{59}Fe release by analogue 311 was not affected (Fig. 3A). At a higher vinblastine concentration of 50 μM, ^{59}Fe release by 311 was inhibited to a slightly greater extent than ^{59}Fe release by PIH, although this was not a consistent finding at this vinblastine concentration. In some experiments, vinblastine at 50 μM had no effect on 311-mediated ^{59}Fe release, but always decreased ^{59}Fe release by PIH. Colchicine is another microtubule distrupting agent but it had no effect on ^{59}Fe release by PIH or analogue 311 (Fig. 3A). None of the agents examined appreciably affected the total amount of ^{59}Fe present in the cells compared to the relevant control (Fig. 3B).

The effect of some of the inhibitors on ^{59}Fe release by other chelators was also investigated. While vinblastine (50 μM), and especially 2,4-dinitrophenol (200 μM) resulted in a distinct decrease in ^{59}Fe released mediated by PIH, these inhibitors had far less effect on ^{59}Fe release by other PIH analogues shown to have marked anti-proliferative activity [17,18], namely, ligands 206, 309 and 315 (Fig. 3C). In similar studies using sodium azide (30 mM) and oligomycin (30 μM), comparable results showing a marked decrease in PIH-mediated ^{59}Fe release but little effect on ^{59}Fe release by the other chelators (206, 309, 311 and 315) was observed (results not shown). Under the experimental conditions used, DFO-mediated ^{59}Fe release was only slightly greater than the control (Fig. 3C). Hence, it was difficult to assess the effect of the inhibitors on ^{59}Fe release mediated by this latter ligand.

3.2. Effect of inhibitors of P-glycoprotein activity on chelator-mediated iron release

P-glycoprotein (P-gp) has been implicated to play a role in the efflux of a wide variety of lipophilic molecules from normal and neoplastic cells [27–29], and since the activity of this multi-drug efflux pump is ATP-dependent, we decided to examine the role of this molecule in chelator-mediated ^{59}Fe release. Preliminary experiments used 4 widely implemented inhibitors of P-gp activity, namely cyclosporin A (CsA), quinine (Qui), reserpine (Res) and verapamil (Ver) [27–29]. All four inhibitors of P-gp activity decreased PIH-mediated ^{59}Fe release (Fig. 4A), Ver and CsA being the most effective drugs. In contrast to these results, the P-gp inhibitors had little or no effect on ^{59}Fe release mediated by chelator 311 (Fig. 4A), suggesting that this ligand had a different mechanism of release to that found for PIH. The marked effect of CsA and Ver on PIH-mediated ^{59}Fe efflux from SK-N-MC NB cells was evident during reincu-

bation periods with the chelators up to 120 min (Fig. 4B). When the cells were incubated with CsA and Ver alone, these inhibitors had either little or no effect on ^{59}Fe release compared to when medium alone was used (Fig. 4A,B), and both these agents were effective at inhibiting ^{59}Fe release by PIH at concentrations as low as 1 μM (Fig. 4C). A previous investigation using rabbit reticulocytes showed that ethanol-soluble ^{59}Fe increased during incubation with PIH, due to the intracellular accumulation of the PIH-^{59}Fe complex [19]. In the present study, ethanol-soluble ^{59}Fe was examined by prelabelling SK-N-MC cells with ^{59}Fe-Tf (1.25 μM) for 3 h followed by a reincubation period of 15, 30, or 120 min with medium alone (control) or medium containing CsA (30 μM), PIH (30 μM), or these two agents together. There was little ethanol-soluble ^{59}Fe in control cells after a 15, 30, and 120 min reincubation, viz, 7.8%, 7.0% and 4%, respectively. Incubation with PIH resulted in a slight increase in the amount of ethanol-soluble ^{59}Fe compared to control cells (to 11.7%, 10.5% and 5.8% after 15, 30, and 120 min reincubation, respectively). When CsA alone was incubated with cells it slightly decreased the amount of ethanol-soluble ^{59}Fe present compared to the control (to 5.1%, 4.9% and 3.3% after 15, 30, and 120 min). In contrast, incubation of cells with PIH and CsA resulted in a marked increase in the proportion of ethanol-soluble ^{59}Fe to a level greater than that seen with PIH alone (to 17.3%, 26.4% and 15.3% after a 15, 30, and 120 min reincubation). These latter data suggested that in the presence of CsA, the release of the PIH-^{59}Fe complex was inhibited, resulting in an intracellular accumulation. Similar studies using Ver also showed comparable results (data not shown).

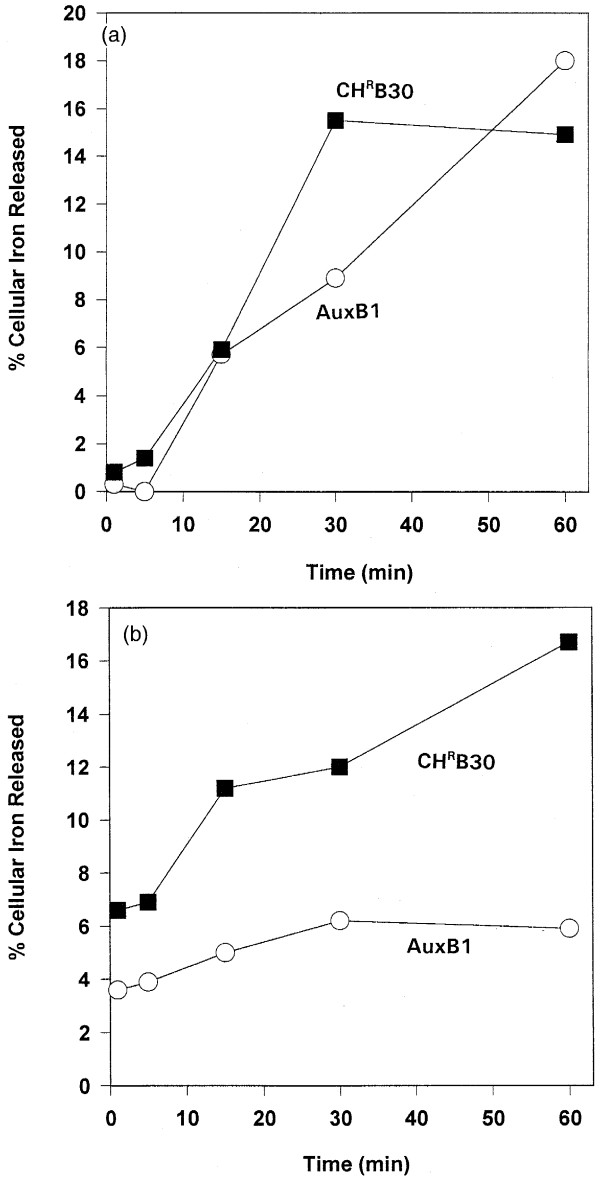


Fig. 5. A: the effect of pyridoxal isonicotinoyl hydrazone (PIH) on ^{59}Fe release from the Chinese hamster ovary cell line CH^{R}B30 which expresses P-glycoprotein at high levels [24], and the control parental cell line AuxB1. The cells were incubated for 3 h at 37°C with ^{59}Fe-transferrin (1.25 μM), washed, and then reincubated for 1–60 min at 37°C with MEM or MEM containing PIH (50 μM). Iron release from control and resistant cells in the presence of medium alone has been subtracted from the respective ^{59}Fe release mediated by PIH in each cell type to give a measure of chelator-mediated release. B: the release of ^{59}Fe from CH^{R}B30 cells compared to the parental control cell line AuxB1 in the presence of medium only. The experiment was performed as described for (A) except the cells were reincubated in medium alone in the absence of PIH. The results are means of duplicate determinations in a typical experiment of two experiments performed. The variation between replicates was generally less than 5%.

3.3. The release of iron from the CH^{R}B30 chinese hamster ovary cell line expressing high levels of P-glycoprotein compared to the control parental line AuxB1

Additional experiments were designed to examine if there was any difference in PIH-mediated ^{59}Fe

release between the CHO cell line CH^RB30 expressing high levels of P-gp and the control cell line Aux B1. No appreciable difference in the kinetics of ^{59}Fe release by PIH was found between these two lines (Fig. 5A). When similar experiments were performed using analogue 311, again no difference was observed in the efflux of ^{59}Fe between the resistant and parental CHO cell lines (data not shown). However, in contrast to these results using PIH or 311, in the CH^RB30 cells with high P-gp levels, appreciably more ^{59}Fe was released into control medium (i.e., without PIH) than the AuxB1 control cells (Fig. 5B), viz after a 60 min reincubation, 5.9% of total cell ^{59}Fe was released from AuxB1 whereas 16.7% was released from CH^RB30 cells.

4. Discussion

The use of Fe chelators for the treatment of cancer is a strategy that has been shown to hold promise for some types of human tumors, including leukemia [4] and neuroblastoma [5]. Recently, our studies using analogues of the tridentate Fe chelator, PIH, have shown that some of these compounds, particularly those derived from 2-hydroxy-1-naphthylaldehyde, are effective anti-proliferative agents that possess high activity at preventing ^{59}Fe uptake from Tf and increasing ^{59}Fe release from cells [16–18]. The high activity of 311 at mobilizing Fe and its potential as a anti-neoplastic agent has prompted us to investigate whether this chelator is actively transported out of the cell. Previous studies have shown that PIH uptake by rabbit reticulocytes occurred by passive diffusion, whereas the PIH-^{59}Fe complex was extruded by an energy-dependent process that was sensitive to the vinca alkaloids vincristine and vinblastine [19].

In the present investigation we have examined the effects of temperature, a number of metabolic inhibitors, anti-microtubule agents, and inhibitors of P-gp activity on ^{59}Fe release from neoplastic cells mediated by PIH compared to 311. In addition, we have compared ^{59}Fe release by these chelators in a CHO cell line expressing high levels of P-gp and a control line. Interestingly, the release of ^{59}Fe by PIH, analogue 311 and DFO, were all temperature-dependent in both SK-N-MC NB cells (Fig. 1) and SK-Mel-28 melanoma cells. When cells were treated with four commonly used metabolic inhibitors, namely, azide, 2,4-dinitrophenol, oligomycin and rotenone, ^{59}Fe release by PIH was markedly inhibited, whereas these agents had either no effect, or far less effect, on ^{59}Fe release mediated by analogue 311 (Fig. 3A). Similar results were also found for a number of other analogues of PIH derived from salicylaldehyde (206) or 2-hydroxy-1-naphthylaldehyde (309 and 315), all of which were not appreciably affected by a range of metabolic inhibitors (Fig. 3C). These results were surprizing, since they suggest that while the efflux of the PIH-^{59}Fe complex requires energy, the release of its structurally related analogues appears to be energy-independent. A previous investigation by Huang and Ponka [19] using reticulocytes demonstrated that the uptake of ^{14}C-PIH into the cell was energy-independent and via passive diffusion, while the release of ^{14}C-labelled PIH was markedly reduced by 2,4-dinitrophenol. Further, it was shown that the inhibited transport of PIH resulted in an accumulation of the ^{14}C-labelled molecule in the reticulocyte [19]. These latter experiments suggested that energy was necessary for PIH release, and substantiate the results of the present study using neoplastic cells.

In the current work, cells have been prelabelled with ^{59}Fe and then reincubated with chelators in the presence and absence of metabolic inhibitors (Fig. 3A–C). Critically, it could be suggested that the decrease in PIH-^{59}Fe mobilization seen in the presence of these agents (Fig. 3A) could be due to the inhibitors altering the total amount of ^{59}Fe, or alternatively, the cellular distribution of ^{59}Fe. However, these agents had no effect on the total amount of ^{59}Fe in the cells compared to the control (Fig. 3B). In addition, if these inhibitors were altering the distribution of ^{59}Fe to prevent PIH-mediated ^{59}Fe release, it would not explain why ^{59}Fe release by 311 was not affected to the same extent as PIH (Fig. 3A), since both PIH and 311 mobilize ^{59}Fe from the same Fe pools [18]. Hence, these results suggest that the inhibitors were not altering the total amount of ^{59}Fe in the cells or the intracellular ^{59}Fe distribution.

Generally, energy-independent transport has been described to occur by either simple diffusion or passive transport involving either channel- or carrier-mediated diffusion [30]. With this in mind, it should be noted that the PIH-analogues are far more lipophilic than PIH [15,17], and this may be a crucial

factor in determining their route of release. It can be suggested that the high lipophilicity of the PIH analogues facilitates rapid diffusion and allows them to bypass the energy-dependent transport route. It is also known that PIH becomes more hydrophilic upon binding Fe [15], which probably accounts for the slight intracellular accumulation seen in the present study using SK-N-MC NB cells and in previous work using rabbit reticulocytes [19]. Perhaps the cellular accumulation of the PIH-^{59}Fe complex aids it in obtaining access to an energy-requiring transporter. Similarly, previous investigations studying the active transport of steroids from cells indicated that the more lipophilic steroids such as progesterone appeared to pass through membranes largely by passive diffusion, whereas the hydrophilic steroid cortisol was transported by an ATP-dependent mechanism [31].

Since ^{59}Fe release by 311 was seemingly energy-independent, it was surprizing that chilling the cells to 4°C inhibited ^{59}Fe mobilization (Fig. 1). This marked decrease in 311-mediated ^{59}Fe release may be explained by changes in the fluidity of the cell membrane that is known to occur upon reducing the temperature from 37°C [32]. This change could possibly effect diffusion of molecules, especially if passive transport was involved, where the appropriate conformation of channels or carriers may be affected by temperature. Another potential explanation is also possible, at 37°C the ^{59}Fe pools within cells may be in a different state than when the cells are chilled to 4°C. For instance, it may be that the chelators access a metabolic pool of ^{59}Fe that is only chelatable at 37°C, at 4°C this pool may not be in a state that can be bound by the ligands. It can also be proposed that the lack of ^{59}Fe mobilization at 4°C by the chelators could be due to the inhibited uptake of the ligands by the cells. However, Huang and Ponka [19] have shown that the uptake of ^{14}C-labelled PIH into cells is via passive diffusion. In the present investigation, the uptake of ^{14}C-ligands could not be examined due to the current lack of commercially available precursors of the chelators. Further studies are obviously necessary to determine the mechanism responsible for temperature-dependent Fe chelation by these ligands. However, it can be suggested that the inhibition of Fe chelation at 4°C appears to be via a different mechanism to that found for the metabolic inhibitors, since energy depletion resulted in a distinct decrease in ^{59}Fe release by PIH only (Fig. 3A), whereas chilling the cells to 4°C markedly prevented ^{59}Fe mobilization by both PIH and 311 (Fig. 1).

In contrast to 311 and the other PIH analogues, PIH was transported by a process that was markedly depressed upon incubating the cells with metabolic inhibitors (Fig. 3A,C). Considering the possible transport mechanism(s) involved, it has been demonstrated that P-gp is an energy-dependent efflux pump that can transport a very diverse variety of organic compounds [27–29]. Further, this transporter is found in a wide variety of normal and neoplastic cell types (including NB cells) that have not been exposed to cytotoxic agents [27–29]. In preliminary studies we examined the effect of 4 widely used inhibitors of P-gp activity on ^{59}Fe release by PIH and 311. All 4 of these compounds, namely CsA, Qui, Res, and Ver, effectively decreased ^{59}Fe mobilization by PIH but had either little or no effect on ^{59}Fe release mediated by analogue 311 (Fig. 4A). Furthermore, when PIH was incubated in the presence of CsA or Ver, the amount of ethanol-soluble ^{59}Fe increased within the cell to a greater extent than that seen with PIH alone, suggesting accumulation of the ^{59}Fe chelate due to inhibited transport. While these inhibitors are extensively used to inhibit P-gp transport activity [27–29], it should be noted they are not specific, and can affect many other cellular processes [27]. In fact, the detergents Tween 80 and Lubrol wx can mimic the effect of Ver on drug transport [27], which indicates the activity of this drug may result from its ability to generally perturb the cell membrane [27]. In this light, when experiments were performed using a well characterized multi-drug resistant CHO cell line ($CH^{R}B30$) with high P-gp expression [24], these cells were no more efficient at releasing the PIH-^{59}Fe complex than the control parental CHO cells (AuxB1; Fig. 5A). The role of P-gp in PIH-mediated ^{59}Fe release could also be questioned from our experiments showing that high concentrations (100 μM) of the P-gp substrate, colchicine, had no appreciable effect on ^{59}Fe release by PIH (Fig. 3A). These latter observations and particularly the experiments using the control and P-gp amplified cell lines suggest that P-gp is not involved in release of the PIH-^{59}Fe complex, and clearly contradict the results using the P-gp inhibitors. These two seemingly conflicting sets

of results could be rationalized by suggesting that another energy-dependent transport mechanism may be responsible for PIH-mediated ^{59}Fe release. It is well known that P-glycoprotein belongs to a family of related transporters that contain ATP-binding cassettes (also known as ABC proteins or traffic ATPases; [28,29]), and the drug efflux activity of some of these proteins can be affected by traditional inhibitors of P-gp [33].

Despite there being no appreciable difference in the efflux of PIH between the control and P-gp amplified cell lines, it was intriguing that the CH^{R}B30 cell line with high P-gp levels, released appreciably more ^{59}Fe into control medium (i.e., without PIH) than the parental control cells, AuxB1 (Fig. 5B). These results could suggest that P-gp may play some role in ^{59}Fe release, and further studies are necessary to examine this intriguing observation further. At present, very little information is known concerning the mechanism of Fe efflux from cells, especially from hepatocytes and macrophages, where the physiological role of Fe release has long been recognized (for review, see [34]). Since P-gp and certain members of this transmembrane pump family have been shown to transport a wide range of drugs [27–29] and natural substrates [35–37], it is not unreasonable to suggest that this protein may be capable of transporting Fe bound to intracellular ligands, and studies have been initiated to examine this further.

The fact that some chelators such as PIH can be transported from cells by energy-dependent mechanisms may actually facilitate their anti-proliferative action by resulting in greater intracellular Fe release. Based on the structure of agents known to be transported by P-gp, it may be possible to design chelators that can be transported by this protein. Hence, the appearance of chemotherapy-resistant tumors due to the amplification of P-gp could be potentially treated with these specially designed ligands that can enter the cell, bind Fe from critically sensitive compartments, and then be pumped out by P-gp. On the other hand, the efficacy of chelators such as 311 that are rapidly transported across the membrane by simple diffusion or passive transport may not be affected by the emergence of cells with increased transporter activity, and will remain highly effective anti-proliferative agents. Thus, additional work examining the membrane transport of Fe chelators may be useful in designing treatment regimes that enhance their anti-proliferative effects.

In conclusion, this study has demonstrated that the mechanism involved in the release of PIH from SK-N-MC NB cells is energy-dependent. In contrast, the closely related PIH analogue, 311, appears to be released from cells by an energy-independent process that is consistent with simple diffusion or passive transport.

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