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Identification of the di-pyridyl ketone isonicotinoyl hydrazone (PKIH) analogues as potent iron chelators and anti-tumour agents

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1 In an attempt to develop chelators as potent anti-tumour agents, we synthesized two series of novel ligands based on the very active 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) group. Since lipophilicity and membrane permeability play a critical role in Fe chelation efficacy, the aldehyde moiety of the PCIH series, namely 2-pyridylcarboxaldehyde, was replaced with the more lipophilic 2-quinolinecarboxaldehyde or di-2-pyridylketone moieties. These compounds were then systematically condensed with the same group of acid hydrazides to yield ligands based on 2-quinolinecarboxaldehyde isonicotinoyl hydrazone (QCIH) and di-2-pyridylketone isonicotinoyl hydrazone (PKIH). To examine chelator efficacy, we assessed their effects on proliferation, Fe uptake, Fe efflux, the expression of cell cycle control molecules, iron-regulatory protein-RNA-binding activity, and ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine incorporation.

2 Despite the high lipophilicity of the QCIH ligands and the fact that they have the same Fe-binding site as the PCIH series, surprisingly none of these compounds were effective. In contrast, the PKIH analogues showed marked anti-proliferative activity and Fe chelation efficacy. Indeed, the ability of these ligands to inhibit proliferation and DNA synthesis was similar or exceeded that found for the highly cytotoxic chelator, 311. In contrast to the PCIH and QCIH analogues, most of the PKIH group markedly increased the mRNA levels of molecules vital for cell cycle arrest.

3 In conclusion, our studies identify structural features useful in the design of chelators with high anti-proliferative activity. We have identified a novel class of ligands that are potent Fe chelators and inhibitors of DNA synthesis, and which deserve further investigation.
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Keywords: Iron (Fe) chelators; Fe metabolism; anti-tumour agents; chemotherapy

Abbreviations: DFO, desferrioxamine; FAC, ferric ammonium citrate; IRE, iron-responsive element; IRP, iron regulatory proteins; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; PCIH, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone; PIH, pyridoxal isonicotinoyl hydrazone; PKIH, di-2-pyridylketone isonicotinoyl hydrazone; QCIH, 2-quinolinecarboxaldehyde isonicotinoyl hydrazone; Tf, transferrin; 311, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone

Introduction

Tumour cells are sensitive to the effects of iron (Fe) chelators because of the critical requirement for Fe in proteins that play essential roles in DNA synthesis (e.g., ribonucleotide reductase) and energy production (e.g., cytochromes; Hershko, 1994). At present, the only chelator in widespread clinical use is desferrioxamine (DFO). However, this ligand suffers from a number of serious problems including: it is extremely expensive, has limited membrane permeability, it is not orally available and requires long subcutaneous infusions (12–24 h day, 5–7 days week) (Hershko, 1994; Olivieri & Brittenham, 1997). In terms of the use of chelators for the treatment of cancer, DFO has shown significant anti-tumour activity (Becton & Bryles, 1988; Blatt & Stitely, 1987; Dezza *et al*., 1989; Donfrancesco *et al*., 1990; 1992; 1995; Estrov *et al*., 1987; Kemp *et al*., 1992; 1995). However, the short half-life of DFO and its low efficacy at permeating cell membranes limits its anti-proliferative activity (Bottomley *et al*., 1985; Richardson *et al*., 1994). Indeed, these factors may have resulted in its failure to inhibit tumour growth in some studies (Blatt, 1994; Selig *et al*., 1998).

Apart from DFO, for many years it was known that ligands and metal complexes of the thiosemicarbazone class are potent anti-tumour agents (Antholine *et al*., 1977; Cory *et al*., 1994; Sartorelli *et al*., 1977; Thelander & Gräslund, 1983). Several of these compounds have reached clinical trials (Deconti *et al*., 1972; Krakoff *et al*., 1974), and recently, 3-aminopyridine-2-carboxyaldehyde thiosemicarbazone (Triapine) has been assessed in animals and humans (Finch *et al*., 1999; 2000). Chelators from other structural classes have also been shown to possess anti-proliferative activity (Kicic *et al*., 2001; Rakba *et al*., 2000; Torti *et al*., 1998). Hence, the development of chelators as anti-tumour agents is promising and requires further exploration.

One class of ligands with high Fe chelation efficacy are those of the pyridoxal isonicotinoyl hydrazone (PIH) class (Brittenham, 1990; Hershko *et al*., 1981; Ponka *et al*., 1979a, b). Our laboratory recently designed and patented a new

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series of chelators that are related to PIH, namely, the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) analogues (Becker & Richardson, 1999; Richardson *et al*., 2001). Several of these ligands showed higher Fe chelation activity than PIH (Becker & Richardson, 1999; Richardson *et al*., 2001), and low anti-proliferative activity, and demonstrate potential for the treatment of Fe-loading diseases (Becker & Richardson, 1999; Richardson *et al*., 2001). We also developed a range of lipophilic PIH analogues that show far greater efficacy than DFO at inhibiting proliferation (Darnell & Richardson, 1999; Richardson & Milnes, 1997; Richardson *et al*., 1995).

Considering the high Fe chelation efficacy of the PCIH analogues (Becker & Richardson, 1999; Richardson *et al*., 2001), in the present study we synthesized new and potentially more effective ligands based upon their structure (Figure 1). Since lipophilicity plays an important role in the chelation of intracellular Fe (Porter *et al*., 1988), we prepared analogues of the PCIH class where the relatively hydrophilic aldehyde, 2-pyridylcarboxaldehyde, was replaced with the more hydrophobic 2-quinolinecarboxaldehyde or 2-dipyridyl ketone moieties. Hence, three series of chelators were examined: the PCIH analogues, the 2-quinolinecarboxaldehyde isonicotinoyl hydrazone (QCIH) analogues, and the di-2-pyridylketone isonicotinoyl hydrazone (PKIH) analogues (Figure 1). Systematic condensation of a number of acid hydrazides with each of the formerly mentioned aldehydes or ketone moieties resulted in 18 novel substituted ligands (Figure 2) with a range of lipophilicities (Table 1).

We show that the above mentioned alterations in chelator structure result in marked changes in biological activity relative to the PCIH analogues. In fact, despite all ligands having a very similar Fe-binding site, the QCIH group of chelators showed no significant Fe-chelation activity. In contrast, the PKIH analogues were among the most potent inhibitors of ^{3}H-thymidine incorporation that we have identified. These latter chelators show considerable potential as anti-proliferative agents and deserve further investigation.

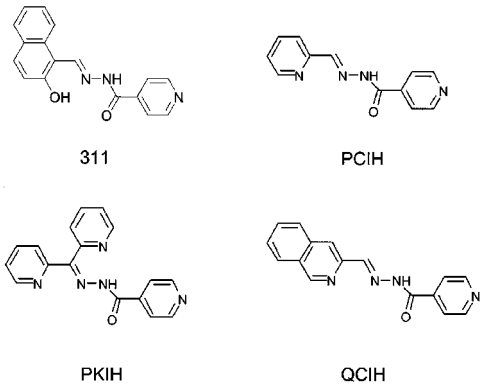

Figure 1 Schematic illustration showing the structure of the cytotoxic Fe chelator, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311), compared to the three groups of aroylhydrazone chelators assessed in this study: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), di-2-pyridylketone isonicotinoyl hydrazone (PKIH), and 2-quinolinecarboxaldehyde isonicotinoyl hydrazone (QCIH).

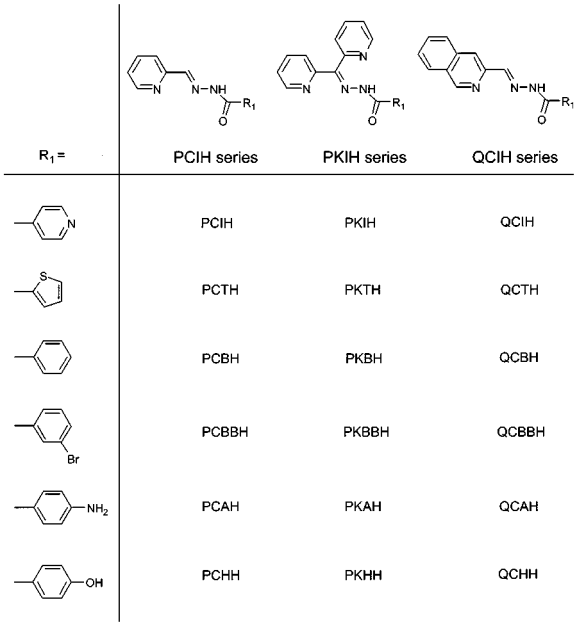

Figure 2 Schematic illustration showing the three groups of aroylhydrazone chelators, the substituents (R1), and the nomenclature used to identify each chelator.

Methods

Chelator synthesis and characterization

The PCIH and QCIH analogues were synthesized by Schiff base condensation with either 2-pyridylcarboxaldehyde or 2-quinolinecarboxaldehyde and their respective acid hydrazides (Johnson *et al*., 1982). The PKIH series of ligands were synthesized by condensing 2-di-pyridyl ketone with the acid hydrazides using the method of Bacchi *et al*. (1996). These chelators were characterized using established procedures (Bernhardt *et al*., 2001; Richardson & Bernhardt, 1999). The Fe complexes of these ligands were prepared using standard techniques described previously (Richardson & Bernhardt, 1999). Both PIH and its cytotoxic analogue, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311; Figure 1), were synthesized and characterized as reported before (Richardson & Bernhardt, 1999). DFO was purchased from Novartis (Basel, Switzerland). All of the aroylhydrazone chelators were dissolved in dimethyl sulphoxide (DMSO) as 10 mM stock solutions immediately prior to an experiment and then diluted in 10% FCS so that the final DMSO concentration was equal to or less than 0.5 % (v v^{-1}) (Richardson *et al*., 1995).

Cell culture

The human SK-N-MC neuroepithelioma cell line and human MRC-5 fibroblast cell line were purchased from the American Type Culture Collection (ATCC), Rockville, MD, U.S.A. These cell types were employed as they have been widely used to assess the activity of chelators in our previous studies and their Fe metabolism is well characterized (Lovejoy & Richardson, 2002; Richardson & Milnes, 1997; Richardson *et al*., 1995). Hence, the use of these cells enables useful comparisons to our past investigations. Cells

Table 1 The calulated n-octanol partition coefficients (log P_{calc}) for the chelators

	Crippens' fragmentation	*Viswanadhans' fragmentation*	*Broto's method*	*average log P_{calc}*
PIH	0.43	0.63	−0.16	0.30
311	2.63	2.83	2.17	2.54
PCIH	1.11	1.20	0.56	0.96
PCTH	2.43	2.54	*	**
PCBH	2.45	2.51	1.68	2.21
PCBBH	3.28	3.30	2.56	3.05
PCAH	1.65	1.73	0.86	1.41
PCHH	2.06	2.23	1.29	1.86
PKIH	1.66	1.83	0.93	1.47
PKTH	2.98	3.17	*	**
PKBH	3.00	3.15	2.05	2.73
PKBBH	3.83	3.94	2.93	3.57
PKAH	2.20	2.36	1.23	1.93
PKHH	2.61	2.86	1.66	2.38
QCIH	2.11	2.20	1.68	2.00
QCTH	3.43	3.54	*	**
QCBH	3.45	3.52	2.80	3.26
QCBBH	4.28	4.31	3.68	4.09
QCAH	2.65	2.73	1.98	2.45
QCHH	3.06	3.23	2.41	2.90

*Unable to be calculated by Broto's method. **Unable to calculate. Log P_{calc} values were calculated using the program Chem Draw Pro V 4.5.

were cultured as described previously (Richardson & Baker, 1990, 1992).

Effect of the chelators on cellular proliferation

The effect of the chelators on the proliferation of cells was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay by essentially the same method as described previously (Richardson *et al*., 1995). The effect of the chelators was always assessed using cells in exponential growth.

Labelling of transferrin with ^{59}Fe

Apotransferrin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was labelled with ^{59}Fe (as ferric chloride in 0.1 M HCl, Dupont NEN, MA, U.S.A.) to produce ^{59}Fe-transferrin (^{59}Fe-Tf) using standard procedures described previously (Richardson & Baker, 1990; 1992). In all studies, Tf at 0.75 μM was used ([^{59}Fe] = 1.5 μM) which is well above the saturation of the transferrin receptor in SK-N-MC cells (Richardson & Ponka, 1994), allowing efficient Fe uptake.

Effect of the chelators on ^{3}H-thymidine, ^{3}H-leucine, and ^{3}H-uridine incorporation

After a 20 h incubation with the chelators, ^{3}H-thymidine, ^{3}H-leucine, or ^{3}H-uridine (1 μCi ml^{-1}) were then added for 2 h at 37°C. The cells were detached from the 96 well plate using 1 mM EDTA in Ca(II)/Mg(II)-free PBS and collected and washed using a cell harvester (Tomtec Harvester 96, Linbrook, Queensland, Australia). Radioactivity was measured on a β-scintillation counter (LKB Wallace, Turku, Finland).

Iron uptake and iron efflux experiments

The effect of chelators on ^{59}Fe uptake from ^{59}Fe-Tf and ^{59}Fe release from prelabelled cells was studied using standard procedures (Richardson *et al*., 1995). The amount of ^{59}Fe internalized by cells was measured by incubation with the general protease, Pronase (1 mg ml^{-1}), for 30 min at 4°C to remove membrane-bound ^{59}Fe and Tf (Iacopetta & Morgan, 1983; Karin & Mintz, 1981). To assess the ability of the chelator to permeate cell membranes and bind intracellular Fe pools, standard techniques described previously were used (Richardson *et al*., 1995).

Iron regulatory protein gel-retardation assay

The gel-retardation assay was used to measure the interaction between the iron regulatory proteins (IRPs) and iron-responsive element (IRE) using established techniques (Abboud & Haile, 2000; Darnell & Richardson, 1999; Hirling *et al*., 1994; Müllner *et al*., 1989).

Northern blot analysis

Northern blot analysis was performed by isolating total RNA using the Total RNA Isolation Reagent (Advanced Biotechnologies Ltd, Surrey, U.K.) as described in our previous studies (Darnell & Richardson, 1999).

Statistics

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

Results

Comparison of the effect of the PCIH, QCIH and PKIH chelators on iron mobilization from prelabelled SK-N-MC neuroepithelioma cells

The Fe chelation efficacy of the QCIH and PKIH ligands were examined by comparing their activity to DFO and the corresponding PCIH analogue (Becker & Richardson, 1999; Richardson *et al*., 2001). The ability of the chelators to mobilize ^{59}Fe from SK-N-MC neuroepithelioma cells was assessed after a 3 h labelling period with ^{59}Fe-Tf (0.75 μM) at 37°C. The cells were then washed and reincubated for 3 h at 37°C in the presence and absence of the internal standard DFO (100 μM) or the other chelators at 50 μM (Figures 3 and 4). We used DFO at a concentration of 100 μM due to the low efficacy of this chelator at mobilizing ^{59}Fe from cells and preventing ^{59}Fe uptake from ^{59}Fe-Tf (Becker & Richardson, 1999; Darnell & Richardson, 1999).

In terms of their ability to mobilize ^{59}Fe from prelabelled cells, nearly all of the PKIH series showed similar or greater activity than the corresponding PCIH analogue (Figure 3a). The only exception was PKBBH, which showed less activity than PCBBH at causing ^{59}Fe release (viz. 21 ± 2% compared to 35 ± 1% respectively; Figure 3a). The greatest difference in activity between the PKIH and PCIH groups was found when comparing the more hydrophilic compounds from these series (see Table 1), i.e., PCAH and PKAH or PCHH and PKHH.

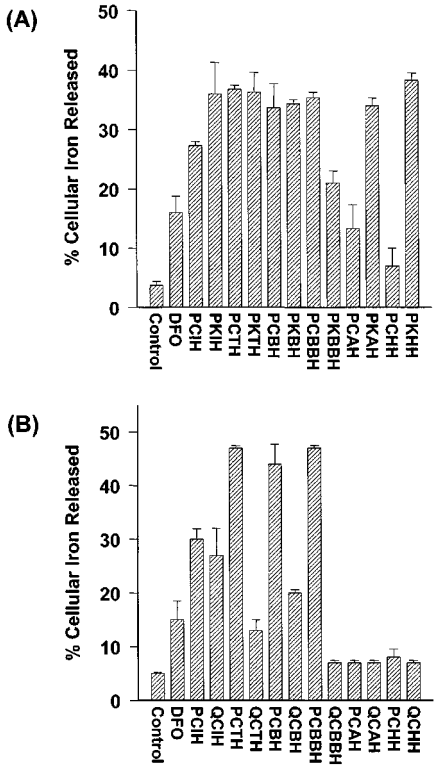

Figure 3 The effect of the chelators on ^{59}Fe mobilization from prelabelled SK-N-MC neuroepithelioma cells. (A) Comparison of the PKIH and PCIH ligands; (B) Comparison of the QCIH and PCIH ligands. Cells were labelled with ^{59}Fe-transferrin (0.75 μM) for 3 h at 37°C, washed, and then reincubated for 3 h at 37°C in the presence of medium alone (control) or medium containing DFO (100 μM) or the other chelators (50 μM). Results are expressed as the mean ± s.d. of three replicates in a typical experiment of two experiments performed.

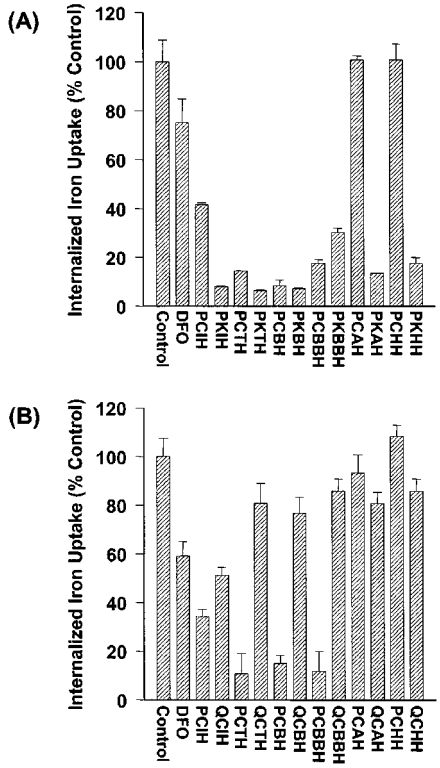

Figure 4 The effect of the chelators on ^{59}Fe uptake from ^{59}Fe-transferrin (^{59}Fe-Tf) by SK-N-MC neuroepithelioma cells. (A) Comparison of the PKIH and PCIH ligands; (B) Comparison of the QCIH and PCIH ligands. The cells were incubated for 3 h at 37°C in media containing ^{59}Fe-Tf (0.75 μM) and either DFO (100 μM) or the other chelators (50 μM), washed, and then incubated with pronase (1 mg ml) for 30 min at 4°C. Results are expressed as the mean ± s.d. of three replicates in a typical experiment of two experiments performed.

In general, the QCIH ligands did not behave in a similar manner to the PCIH or PKIH ligands, and showed little activity in terms of inducing ^{59}Fe mobilization (Figure 3b). The most effective chelator of the QCIH series was the parent compound, QCIH, that caused comparable cellular ^{59}Fe mobilization to PCIH (Figure 3b). The reason for the Fe chelation activity of QCIH compared to the remainder of the QCIH analogues remains unclear.

Analysis of the ^{59}Fe mobilization data for all three groups of chelators suggested that the dipyridyl substituent of the PKIH series appears to confer on the chelators high Fe chelation activity irrespective of the hydrazide substitution (Figure 3a). In contrast, the inclusion of the quinoline ring appears to result in low Fe chelation activity (Figure 3b).

Comparison of the effect of the PCIH, QCIH and PKIH chelators on iron uptake from transferrin

To determine the ability of the novel QCIH and PKIH series of chelators to inhibit ^{59}Fe uptake from ^{59}Fe-Tf (0.75 μM), SK-N-MC cells were incubated for 3 h at 37°C with ^{59}Fe-Tf (0.75 μM) and either DFO (100 μM) or the other chelators (50 μM) and the cells then washed. As found for studies examining ^{59}Fe mobilization (Figure 3a), all PKIH ligands were very effective at inhibiting ^{59}Fe uptake from ^{59}Fe-Tf (Figure 4a). It is of interest that the chelators PKAH and PKHH showed marked activity while the corresponding PCIH analogues, PCAH and PCHH, showed little effect (Figure 4a). As also shown in ^{59}Fe mobilization studies (Figure 3a), PKBBH was not as effective as PCBBH at preventing cellular ^{59}Fe uptake (Figure 4a). Almost all of the QCIH chelators were less effective than the PCIH and PKIH ligands at preventing ^{59}Fe uptake from ^{59}Fe-Tf (Figure 4b).

Effect of chelator concentration on cellular iron mobilization and iron uptake from transferrin

Considering the high activity of the PKIH group of chelators (Figures 3a and 4a), it was important to further assess their efficacy as a function of concentration (Figure 5a). To examine the effect of ligand concentration on ^{59}Fe mobilization, cells were prelabelled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C, washed, and then re-incubated for 3 h at 37°C with the chelators at a range of concentrations (0.5–50 μM). The effect of chelator concentration on ^{59}Fe uptake from ^{59}Fe-Tf was assessed by incubating the chelators (0.5–50 μM) with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C and the cells then washed. We used PCTH as a relevant internal control as it has shown very high Fe chelation activity in this study (Figures 3 and 4) and our previous work (Becker & Richardson, 1999).

Three of the PKIH ligands, namely PKIH, PKTH and PKBH, were more effective than PCTH at mobilizing cellular

^{59}Fe at concentrations less than 10 μM (Figure 5a). For instance, at a concentration of 1 μM, PKTH released 26% of cellular ^{59}Fe, whereas PCTH only released 10% (Figure 5a). Similar to the Fe efflux studies, three of the PKIH chelators, namely PKIH, PKBH and PKTH, had higher efficacy than PCTH at preventing ^{59}Fe uptake from Tf (Figure 5b). The lower efficacy of PKAH and PKHH in ^{59}Fe uptake and ^{59}Fe mobilization assays could be due to their greater hydrophilicity (Table 1) because of the amino and hydroxyl substituents respectively (Figure 2). However, the reason for the lower activity of PKBBH remains unclear.

Effect of the iron chelators on the proliferation of SK-N-MC neuroepithelioma cells

We compared the effect of the PCIH, QCIH and PKIH series of chelators on the proliferation of the SK-N-MC neuroepithelioma cell line (Table 2). This cell type was employed as it has been widely used to assess the anti-proliferative activity of chelators in our previous studies and enables a useful comparison (Becker & Richardson, 1999; Richardson & Milnes, 1997; Richardson *et al*., 1995). In all experiments, the well characterized chelators 311 and DFO (Richardson & Milnes, 1997; Richardson *et al*., 1995) were used as relevant internal controls (Table 2). Both the PCIH and QCIH ligands had little effect on cellular proliferation (Figure 2). All chelators of the PCIH and QCIH series had IC_{50} values of 46 μM to greater than 50 μM, except PCBBH which had an IC_{50} of 40 μM (Table 2). These results were in strong agreement with examination of cell morphology by phase contrast microscopy and Trypan blue staining which demonstrated these chelators showed little cytotoxic activity.

In contrast to the PCIH and QCIH series of chelators, the PKIH ligands showed very high anti-proliferative activity even at very low chelator concentrations (Table 2). The chelators, PKIH, PKTH, PKBH and PKBBH showed very similar anti-proliferative activity to the potent cytotoxic Fe chelator 311 (Table 2). In contrast, PKHH and PKAH showed far less activity, having IC_{50} values of 38 and 42 μM, respectively (Table 2).

We also performed experiments to determine whether the chelators are still effective at inhibiting cellular proliferation when complexed with Fe(III). At a ligand to Fe molar ratio of 1 : 1 and 2 : 1, all of the PKIH-Fe complexes showed a similar level of anti-proliferative activity as that found for the ligands (data not shown). As a relevant control in the same studies, the 1 : 1 and 2 : 1 ligand to Fe complexes of 311 were also prepared and assessed. As found previously (Richardson & Bernhardt, 1999; Richardson *et al*., 1995), these Fe complexes of 311 had no significant effect on proliferation at concentrations up to 50 μM.

Effect of the most active PKIH chelators on the proliferation of fibroblasts

As an initial assessment of selective anti-proliferative activity, we compared the effect of the most active PKIH analogues (PKIH, PKTH, PKBH and PKBBH) on the growth of SK-N-MC neuroepithelioma cells and MRC-5 fibroblasts. The MRC-5 cell type is a normal human lung fibroblast cell strain that is mortal and senesces after a number of passages *in vitro* (Zhu *et al*., 2001). In comparison we examined the SK-N-MC neuroepithelioma cell type that is immortal. The effects of

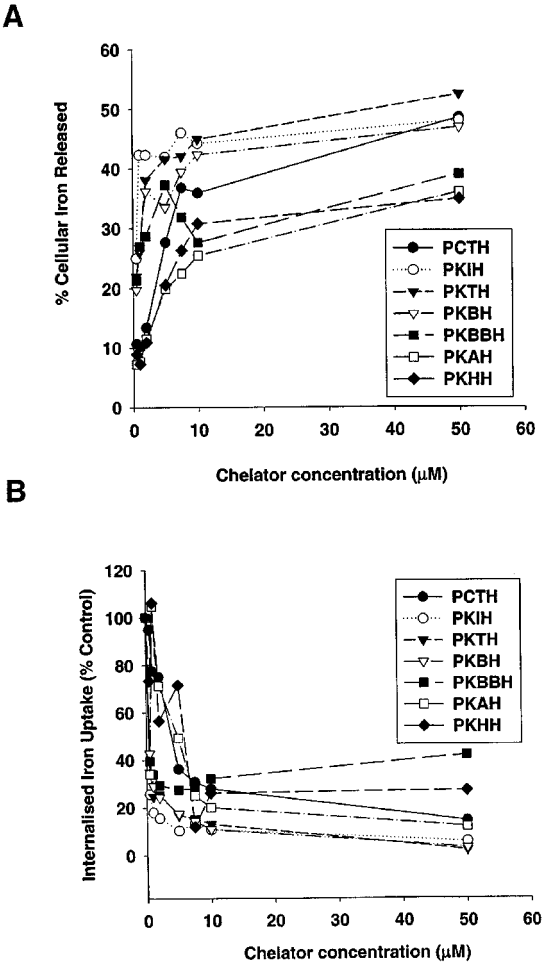

Figure 5 The effect of concentration of the PKIH analogues compared to the highly active PCTH chelator on: (A) cellular ^{59}Fe mobilization and (B) internalized ^{59}Fe uptake from ^{59}Fe-transferrin (^{59}Fe-Tf). (A) Cells were labelled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C, washed, and then reincubated for 3 h at 37°C in the presence of medium alone (control) or medium containing the chelators (0.5–50 μM). (B) The cells were incubated for 3 h at 37°C in media containing ^{59}Fe-Tf (0.75 μM) alone (control) or with the chelators (0.5–50 μM), washed, and then incubated with pronase (1 mg ml) for 30 min at 4°C. Results are expressed as the mean ± s.d. of three replicates in a typical experiment of two experiments performed.

Table 2 The effect of the PCIH, QCIH and PKIH series of Fe chelators on the proliferation of SK-N-MC neuropithelioma cells using the MTT assay

IC_{50} values of chelators (μM)					
PCIH analogues		*QCIH analogues*		*PKIH analogues*	
DFO	>50	DFO	>50	DFO	>50
311	3±2	311	3±2	311	3±2
PCIH	>50	QCIH	49±3	PKIH	2±1
PCTH	48±4	QCTH	>50	PKTH	3±1
PCBH	46±7	QCBH	50±1	PKBH	3±1
PCBBH	40±12	QCBBH	49±3	PKBBH	1±1
PCAH	>50	QCAH	>50	PKAH	42±9
PCHH	>50	QCHH	>50	PKHH	38±10

Data is expressed as mean ± s.d. from four experiments.

these chelators at inhibiting proliferation were more marked in SK-N-MC cells than fibroblasts. For instance, while these ligands had IC_{50} values of 1–3 μM in SK-N-MC cells (Table 2), the IC_{50} values in fibroblasts for PKBBH, PKBH, PKTH and PKIH were 8, 11, 16 and >25 μM respectively. These results suggest that PKIH shows the greatest selectivity against tumour cells. Hence, this ligand may be the lead compound for future studies *in vivo*. Since the anti-proliferative activity of the four PKIH analogues showed similarities, the effects on growth of SK-N-MC and MRC-5 cells of only one PKIH analogue (i.e., PKIH) is illustrated in Figure 6 compared to DFO.

The relationship between lipophilicity and the activity of the PCIH, QCIH and PKIH analogues

To assess the relationship between lipophilicity and either anti-proliferative activity or Fe chelation efficacy, average log *P* values (*n*-octanol-water partition coefficients) for all chelators were calculated. Log *P* values were estimated (Table 1) according to Broto's method (Broto *et al*., 1984), Crippen's fragmentation procedure (Ghose & Crippen, 1987), or Viswanadhan's fragmentation procedure (Viswanadhan *et al*., 1987) using Chem Draw Pro. (v. 4.5, Cambridge Software, 1997). The use of three methods of estimating log *P* values was felt to be more appropriate than using just one that could introduce bias towards a particular calculation scheme. The values calculated from the three methods were averaged to prevent this. The average calculated log *P* values (Table 1) were then plotted against either their IC_{50} values, or their ability to induce ^{59}Fe mobilization or prevent ^{59}Fe uptake. These plots demonstrated no strong correlation to anti-proliferative activity or Fe chelation efficacy (all correlations: r <0.52) (data not shown).

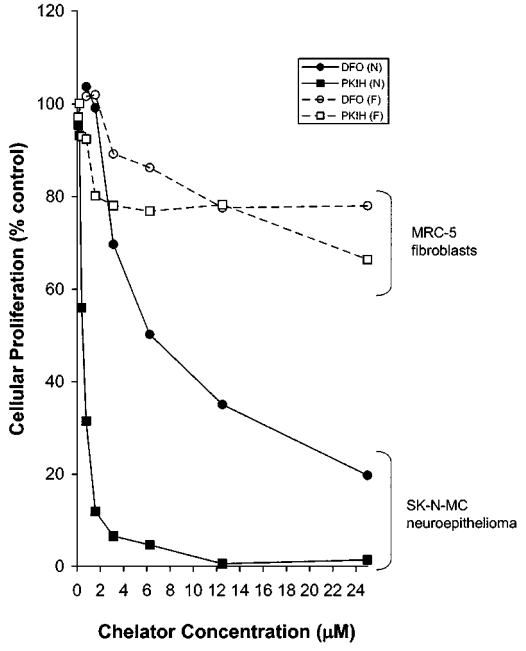

Figure 6 The influence of one of the most effective PKIH analogues (PKIH) compared to desferrioxamine (DFO) on the proliferation of SK-N-MC neuroepithelioma cells (N) compared to MRC-5 fibroblasts (F). Cells were incubated in the presence and absence of the chelators (0–25 μM) for 72 h at 37°C. After this incubation period, cellular density was measured via the MTT assay (see Methods for details). Each data point represents the mean of three separate experiments with duplicate determinations in each experiment.

Effect of the PCIH, QCIH and PKIH series of chelators on ^{3}H-thymidine incorporation

To further understand the mechanisms of action of the QCIH and PKIH ligands, we investigated the effect of these chelators on ^{3}H-thymidine incorporation compared to the PCIH analogues and also to DFO and 311 that acted as relevant controls (Table 3). This was important to assess as the ability of a chelator to bind cellular Fe could result in the inhibition of the Fe-containing enzyme, ribonucleotide reductase, which plays a critical role in deoxyribonucleotide synthesis (Thelander & Reichard, 1979). The effect of the PCIH chelators on ^{3}H-thymidine incorporation was reported previously (Becker & Richardson, 1999), and this data is shown for comparison to the QCIH and PKIH analogues (Table 3).

Four PKIH ligands, namely PKIH, PKTH, PKBH, and PKBBH, were the most effective chelators examined in terms of their ability to inhibit ^{3}H-thymidine incorporation, showing similar or slightly greater activity than the potent ribonucleotide reductase inhibitor, 311 (Green *et al*., 2001) (Table 3). In fact, there was almost undetectable levels of ^{3}H-thymidine in cells treated with these latter ligands. For instance, PKBBH reduced ^{3}H-thymidine incorporation to 0.02% of the control (Table 3). Several of the more hydrophilic chelators of the PKIH series, namely PKAH and PKHH (Table 1), showed significantly ($P<0.0001$) less activity, inhibiting ^{3}H-thymidine incorporation to 23% and 25% of the control respectively (Table 3). However, the relatively low efficacy of these latter chelators at inhibiting ^{3}H-thymidine incorporation cannot solely be attributed to their hydrophilicity, as the most hydrophilic ligand of this series (PKIH; Table 1) had very high activity (Table 3).

In comparison to 311 and the PKIH chelators, the QCIH ligands showed considerably less activity at inhibiting ^{3}H-thymidine incorporation (Table 3). However, despite the lack of effect of the QCIH series at mobilizing ^{59}Fe from cells (Figure 3b), inhibiting ^{59}Fe uptake from ^{59}Fe-Tf (Figure 4b), or preventing proliferation (Table 2), these ligands showed some activity at inhibiting ^{3}H-thymidine incorporation,

Table 3 The effect of the PCIH, QCIH and PKIH ligands on ^{3}H-thymidine incorporation by SK-N-MC neuroepithelioma cells

^{3}H-thymidine incorporation (% Control)					
PCIH analogues		*QCIH analogues*		*PKIH analogues*	
Control	100±7	Control	100±7	Control	100±7
DFO	29±13	DFO	29±13	DFO	29±13
311	0.1±0.1	311	0.1±0.1	311	0.1±0.1
PCIH	43±1	QCIH	77±9	PKIH	0.06±0.02
PCTH	64±14	QCTH	83±6	PKTH	0.04±0.01
PCBH	72±16	QCBH	64±5	PKBH	0.03±0.01
PCBBH	33±6	QCBBH	81±10	PKBBH	0.02±0.01
PCAH	12±1	QCAH	43±2	PKAH	23±10
PCHH	11±2	QCHH	41±2	PKHH	25±4

Data is expressed as mean±s.d. from four experiments.

reducing it to 41–83% of the control (Table 3). The most active of these compounds were QCHH and QCAH, which reduced ^{3}H-thymidine incorporation to 41% and 43% of the control value respectively (Table 3). Interestingly, the hydrophilic PCIH analogues (PCHH and PCAH; Table 1) were also the most active of that series in terms of their efficacy at inhibiting ^{3}H-thymidine incorporation (Table 3). In contrast, their corresponding PKIH analogues (PKHH and PKAH) were the least effective of the PKIH group (Table 3). The low activity of the QCIH group of compounds in terms of Fe chelation efficacy (Figures 3–5) or anti-proliferative activity (Table 2) precluded them from further investigation in the present study.

Comparison of the PCIH and PKIH series of chelators on ^{3}H-leucine and ^{3}H-uridine incorporation

Considering the marked effects of the PKIH group of ligands on ^{3}H-thymidine incorporation, the effect of these chelators were assessed on ^{3}H-leucine (Table 4) and ^{3}H-uridine (Table 5) incorporation. These results were compared to the previously reported effects of the analogous PCIH series of ligands which show high Fe chelation activity and low anti-proliferative effects (Becker & Richardson, 1999).

The most active of the PKIH analogues in terms of inhibiting ^{3}H-thymidine incorporation, namely PKIH, PKTH, PKBH and PKBBH, were significantly ($P<0.0001$) less effective at preventing the incorporation of ^{3}H-leucine and ^{3}H-uridine into protein and RNA respectively (Tables 4 and 5). Further, these PKIH analogues had significantly ($P<0.001$) less effect than 311 on ^{3}H-leucine incorporation (Table 4). In general, the PKIH series of chelators were more effective than the PCIH analogues at preventing ^{3}H-leucine or ^{3}H-uridine incorporation (Tables 4 and 5). Of all the PKIH analogues, PKBBH showed the greatest activity at inhibiting ^{3}H-leucine or ^{3}H-uridine incorporation, having slightly less or similar efficacy to 311, respectively.

Table 4 The effect of the PCIH and PKIH ligands on ^{3}H-leucine incorporation by SK-N-MC neuroepithelioma cells

^{3}H-leucine incorporation (% Control)			
PCIH analogues		*PKIH analogues*	
Control	100±7	Control	100±7
DFO	9±1	DFO	9±1
311	2±1	311	2±1
PCIH	74±7	PKIH	26±1
PCTH	21±6	PKTH	19±8
PCBH	30±13	PKBH	21±2
PCBBH	16±5	PKBBH	8±1
PCAH	76±2	PKAH	25±2
PCHH	80±10	PKHH	22±1

Data is expressed as mean±s.d. from four experiments.

Table 5 The effect of the PCIH and PKIH ligands on ^{3}H-leucine incorporation by SK-N-MC neuroepithelioma cells

^{3}H-uridine incorporation (% Control)			
PCIH analogues		*PKIH analogues*	
Control	100±2	Control	100±2
DFO	34±5	DFO	34±5
311	5±2	311	5±2
PCIH	50±8	PKIH	19±1
PCTH	43±2	PKTH	5±1
PCBH	31±9	PKBH	5±1
PCBBH	47±2	PKBBH	3±1
PCAH	40±9	PKAH	28±1
PCHH	41±11	PKHH	21±1

Data is expressed as mean±s.d. from three experiments.

Effect of the PKIH analogues on the expression of WAF1 *and* GADD45 *mRNA*

Considering the marked Fe chelation efficacy (Figures 3–5) and high anti-proliferative activity (Table 2) of the PKIH ligands, it was important to determine their effect on the expression of the genes *WAF1* and *GADD45* that encode cell cycle inhibitors essential for G_1-S arrest (El-Deiry *et al*., 1993; Kastan *et al*., 1992). As shown previously (Becker & Richardson, 1999; Darnell & Richardson, 1999; Lovejoy & Richardson, 2002), the internal controls DFO, PCBBH, and most notably 311, caused up-regulation of *GADD45* and *WAF1* mRNA expression, while PCBH and PCIH had little effect (Figure 7). In good correlation with their marked anti-proliferative activity, PKIH, PKTH, PKBH and PKBBH had a pronounced effect, significantly ($P<0.0001$) increasing *GADD45* and *WAF1* mRNA levels when compared to the control. Interestingly, these latter chelators were significantly ($P<0.01$) more potent at inducing *WAF1* and *GADD45* mRNA expression than 311 (Figure 7). In contrast, the less cytotoxic analogues of this series (i.e., PKAH and PKHH) had far less effect (Figure 7).

The effect of the chelators on iron regulatory protein RNA-binding activity

To further assess the effect of the PKIH series of chelators on intracellular Fe metabolism, their effect on IRP-RNA-binding activity was determined (Figure 8). This was important to examine as the IRP-IRE mechanism is a major regulator of cellular Fe homeostasis that responds to Fe chelation (Hentze & Kühn, 1996). In these studies, SK-N-MC cells were incubated with the chelators (25 μM) for 20 h at 37°C. The Fe chelators, DFO (100 μM) and 311 (25 μM), or the Fe donor ferric ammonium citrate (FAC; 100 μg/ml), were used as relevant controls in all experiments (Becker & Richardson, 1999; Darnell & Richardson, 1999). The chelator, PCAH, was used as another control as it was shown to reduce IRP-RNA-binding activity probably *via* its ability to redistribute intracellular Fe (Becker & Richardson, 1999).

As expected, a significant ($P<0.005$) increase in IRP-RNA binding occurred in cells incubated with DFO and 311, while FAC and PCAH significantly ($P<0.001$) decreased RNA-binding (Figure 8) as shown previously (Becker & Richardson, 1999; Darnell & Richardson, 1999). Compared to the control, there was a significant ($P<0.01$) increase in IRP-RNA binding activity after treatment of cells with the PKIH series (Figure 8), reflecting their ability to bind cellular Fe pools. Addition of β-mercaptoethanol and FeCN to the cell lysates demonstrated that no change occurred in the total amount of IRP-RNA binding activity after incubation with all the chelators (data not shown).

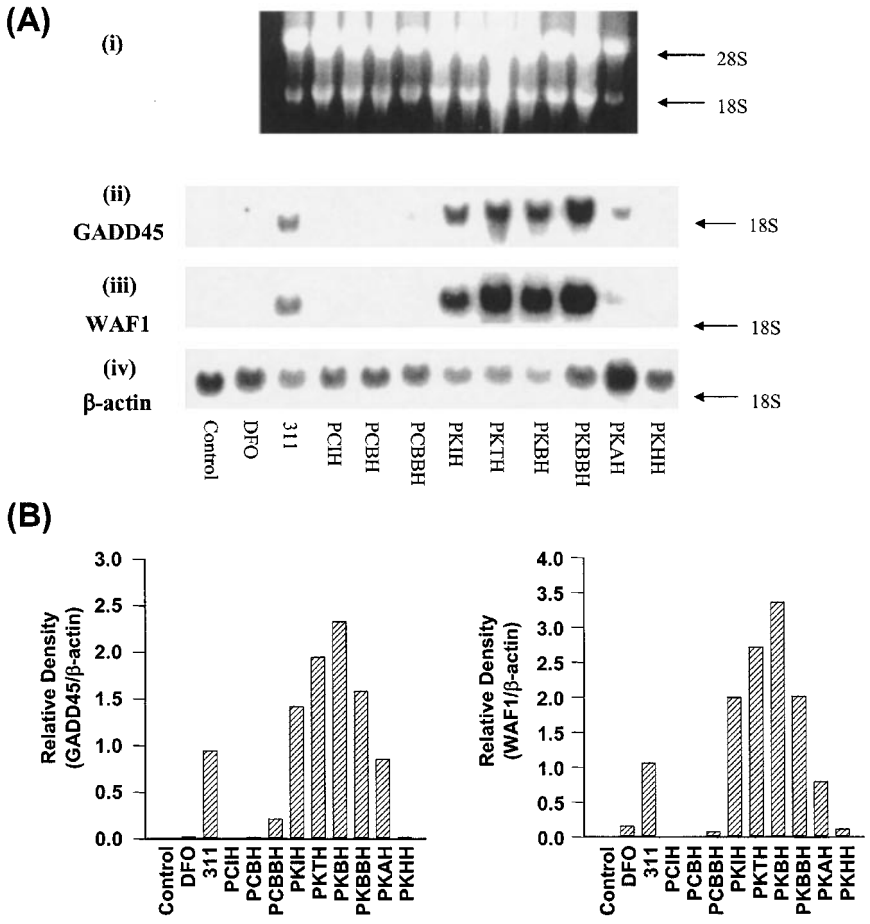

Figure 7 Many of the PKIH series of Fe chelators markedly increase *WAF1* and *GADD45* mRNA expression in SK-N-MC neuroepithelioma cells. The effect of the PKIH group of chelators are compared to DFO, 311 and some chelators of the PCIH series. (A) (i) Ethidium bromide staining of the agarose gel, (ii) *GADD45*, (iii) *WAF1*, and (iv) *β-actin* mRNA levels; (B) Densitometric analysis of the results in (A) normalized to the *β-actin* loading control. Total RNA was extracted from cells after a 20 h incubation at 37°C with medium alone (control) or medium containing DFO (100 μM) or the other chelators (25 μM). Northern blotting was performed using standard procedures (see Methods). The result illustrated is a typical experiment from three experiments performed.

Discussion

Aroylhydrazones are highly effective in terms of their ability to bind Fe, and these ligands have been designed for the treatment of Fe overload disease and cancer (Baker *et al*., 1992; Ponka *et al*., 1979a, b; Richardson *et al*., 1995). In this study, we examined the structure-activity relationships of 18 ligands belonging to three related groups of novel aroylhydrazone chelators, namely the PCIH, QCIH and PKIH series. Despite the fact that each of these analogues have a very similar Fe-binding site (see Figures 1 and 2), the activity of these chelators differed substantially. The PCIH group of ligands had high Fe chelation activity but low anti-proliferative effects, the QCIH group had little Fe chelation or anti-proliferative activity, and some of the PKIH series were amongst the most effective Fe chelators and anti-proliferative agents yet examined in our laboratory.

Previous studies have shown that the lipophilicity of a ligand has a marked effect on their ability to bind intracellular Fe and result in anti-proliferative activity (Porter *et al*., 1988; Richardson *et al*., 1995). In the present study, this latter observation led to the systematic development of chelators with a range of lipophilicities (Table 1). Compared to each member of the PCIH and PKIH group, the QCIH ligands were the most lipophilic, due to the presence of the quinoline ring (Table 1). However, surprisingly, the Fe chelation efficacy and anti-proliferative activity of the QCIH series were very low. This was notable, as increased lipophilicity has been associated with marked aroylhydrazone cytotoxicity (Johnson *et al*., 1982). Other ligands derived from quinoline heterocycles such as 8-hydroxyquinoline show high Fe-binding activity (Henry *et al*., 2001), and the reason for the lack of activity of the QCIH group of chelators is unclear. However, it is possible that the QCIH group may be too lipophilic, resulting in partitioning into membranes and ineffective targeting of intracellular Fe pools. It is of interest that similar problems have been noted for other very lipid soluble chelators such as cholylhydroxamic acid (Baker *et al*., 1985).

It is relevant to note that in contrast to the PCIH group of chelators (Becker & Richardson, 1999), the addition of Fe(III) to the PKIH series had no effect on their anti-proliferative activity. This could indicate that at least part of the anti-proliferative activity of the PKIH series was due to

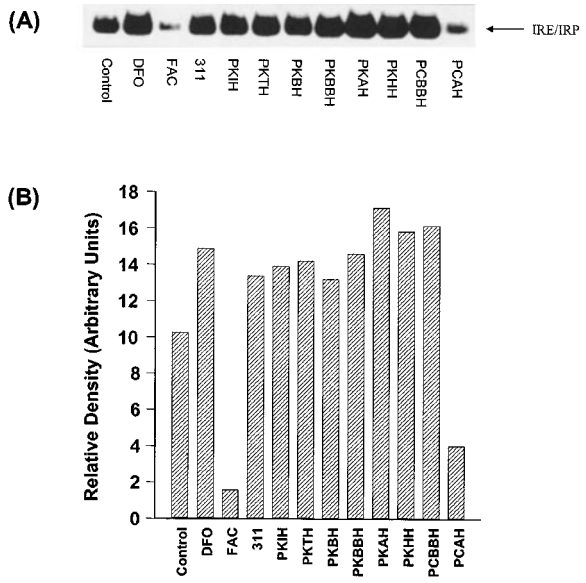

Figure 8 All of the PKIH analogues increase iron-regulatory protein (IRP)-RNA-binding activity in SK-N-MC neuroepithelioma cells. (A) Active IRP-RNA-binding activity; (B) Densitometric analysis of the results in (A). The effect of the PKIH analogues were compared to several chelators that acted as internal controls (ie., DFO, 311, PCBBH, and PCAH) and also the Fe donor, ferric ammonium citrate (FAC). Cells were incubated with DFO (100 μM), FAC (100 μg ml) and the remaining chelators (25 μM) for 20 h at 37°C. The IRP-RNA-binding activity was then assessed by the gel-retardation assay using standard techniques (see Methods). The result illustrated is a typical experiment from three experiments performed.

the generation of an Fe complex that mediates their cytotoxicity. This activity could be due to the production of cytotoxic free radicals from the complex or the ability of the Fe complex to directly inhibit the activity of essential target molecules. Relevant to these mechanisms of action, it is known that the cytotoxic agents, doxorubicin and bleomycin, bind Fe or Cu and redox cycle to generate cytotoxic free radicals (Burger *et al*., 1981; Muller *et al*., 1998). Considering a direct effect of the Fe complex on molecular targets, previous studies have shown that the Fe complex of some thiosemicarbazones can inhibit the activity of ribonucleotide reductase (Thelander & Gräslund, 1983).

One of the most significant findings in this study was the ability of some of the PKIH group of chelators, particularly PKIH, PKTH, PKBH and PKBBH, to markedly inhibit the incorporation of ^{3}H-thymidine into DNA (Table 3). In fact, the activity of these compounds was similar or slightly greater than that found for 311 (Table 3), an Fe chelator which markedly inhibits proliferation (Richardson & Milnes, 1997) and ribonucleotide reductase activity (Green *et al*., 2001). Furthermore, it was of interest that these PKIH analogues, while being very effective at inhibiting ^{3}H-thymidine incorporation, had far less effect than 311 on ^{3}H-leucine incorporation (Table 4). These results could suggest that the PKIH analogues are more specific than 311 in terms of their effect on ribonucleotide reductase. Considering the important catalytic role of Fe in ribonucleotide reductase activity (Thelander & Reichard, 1979), and the high Fe chelation activity of the PKIH series (Figures 3–5), it is possible that the decrease in ^{3}H-thymidine incorporation was due to the effect of these chelators or their Fe complexes on this enzyme.

An important issue regarding the use of Fe chelators for the treatment of cancer is their selectivity at inhibiting the growth of neoplastic compared to normal cells. Indeed, all cells require Fe, but neoplastic cells are more sensitive to the action of chelators due to their greater ribonucleotide reductase activity and higher rates of proliferation (Hershko, 1994; Takeda & Weber, 1981). Preliminary investigations in the present study, comparing neuroepithelioma cells and fibroblasts, demonstrate that the most effective PKIH series show some selective activity against neoplastic cells (Figure 6). This is probably because fibroblasts are more slowly proliferating than the neuroepithelioma cells (Lovejoy & Richardson, 2002). Indeed, as shown previously, the doubling time of the MRC-5 fibroblasts (22 h) was longer compared to that of the SK-N-MC neuroepithelioma cells (16 h) (Lovejoy & Richardson, 2002). Further studies *in vivo* in animal tumour models are vital, and should provide essential information regarding the therapeutic potential of these chelators. The fact that other chelators such as DFO (Hershko, 1994), Triapine (Finch *et al*., 1999; 2000) and aroylhydrazones (Johnson *et al*., 1982; Pickart *et al*., 1983; Sah, 1954), show selective anti-proliferative activity in animal models and clinical trials, indicates the existence of an exploitable therapeutic 'window'.

It is known that Fe depletion has multiple effects (apart from the inhibition of ribonucleotide reductase), including changing the expression of molecules that play major roles in cell cycle progression (Darnell & Richardson, 1999; Gao & Richardson, 2001). Our previous studies have suggested that cytotoxic chelators, in contrast to those that do not have such activity, markedly up-regulate the mRNA levels of *GADD45* and *WAF1* (Becker & Richardson, 1999; Darnell & Richardson, 1999). The protein products of these genes are involved in inhibiting cell cycle progression (El-Deiry *et al*., 1993; Kastan *et al*., 1992), and are relevant to assess in terms of the potential anti-proliferative activity of a chelator. In good correlation with their potent ability to inhibit proliferation, PKIH, PKBH, PKBBH and PKTH, markedly increased the expression of both *GADD45* and *WAF1* mRNA to levels greater than 311 (Figure 7). In agreement with their lower anti-proliferative activity, PKAH and PKHH were found to have far less effect on *WAF1* and *GADD45* mRNA expression. Hence, together with our previous studies (Becker & Richardson, 1999; Darnell & Richardson, 1999), these observations suggest that the expression of these genes appears to provide a useful indicator of the anti-proliferative potential of Fe chelators.

Some of the PCIH and PKIH analogues showed high Fe chelation efficacy (Figures 3–5). Apart from the ability of chelators to permeate membranes to bind intracellular Fe, another possible mechanism to decrease Tf-bound-^{59}Fe uptake is by directly removing Fe from the Fe-binding sites of this protein (Baker *et al*., 1992). Experiments with the PCIH analogues demonstrated that like chelators of the PIH class (Baker *et al*., 1992), these compounds do not remove significant amounts of ^{59}Fe directly from ^{59}Fe-Tf (manuscript in preparation). Considering the structural similarity of the QCIH and PKIH chelators to those of the PCIH class (Figure 1), it can be suggested that these ligands may also be ineffective at removing ^{59}Fe from ^{59}Fe-Tf. Further studies are currently underway to address this issue.

In conclusion, we have identified key structure-activity relationships essential for the design of clinically useful Fe chelators. Ligands of the PCIH class generally show high high chelation efficacy and low anti-proliferative effects, while chelators of the QCIH series lack appreciable Fe chelation activity. Importantly, we have also determined that the more lipophilic members of the PKIH class of chelators are potent Fe chelators that possess marked anti-proliferative activity. Indeed, these latter ligands are among the most active inhibitors of DNA synthesis yet identified in our laboratory, and deserve further investigation as potential anti-tumour agents.

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