RESEARCH PAPER

Bp44mT: an orally active iron chelator of the thiosemicarbazone class with potent anti-tumour efficacy

Y Yu, Y Suryo Rahmanto and DR Richardson

Iron Metabolism and Chelation Program, Department of Pathology and Bosch Institute, Blackburn Building (D06), University of Sydney, Sydney, New South Wales, Australia

Correspondence

Dr DR Richardson, Iron Metabolism and Chelation Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, NSW 2006, Australia. E-mail: d.richardson@med.usyd.edu.au

Keywords

iron chelator; anti-tumour chemotherapeutic; oral activity

Received

7 February 2011

Revised

27 April 2011

Accepted

27 May 2011

BACKGROUND AND PURPOSE

Our previous studies demonstrated that a thiosemicarbazone iron chelator (di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; Dp44mT) possesses potent and selective anti-cancer activity but led to cardiotoxicity at non-optimal doses. In this study, we examined the *in vivo* anti-tumour efficacy and tolerability of a new-generation 2-benzoylpyridine thiosemicarbazone iron chelator (2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone; Bp44mT) administered via the oral or i.v. routes.

EXPERIMENTAL APPROACH

BpT chelators were tested *in vitro* against human lung cancer cells (DMS-53) and *in vivo* in DMS-53 tumour xenografts in mice. The toxicity of Bp44mT *in vivo* and its effects on the expression of iron-regulated molecules involved in growth and cell cycle control were investigated.

KEY RESULTS

Administration of Bp44mT by either route resulted in marked dose-dependent inhibition of tumour growth. When administered at 50 mg·kg⁻¹ via oral gavage three times per week for 23 days, the net xenograft growth was inhibited by 75%, compared with vehicle-treated mice. Toxicological examination showed reversible alterations including slight reduction of RBC count, with a decrease of liver and splenic iron levels, which confirmed iron chelation *in vivo*. Importantly, in contrast to Dp44mT, the chelator-treated mice did not show cardiac histological abnormalities. There was also no significant weight loss in mice, suggesting oral administration of Bp44mT was well tolerated.

CONCLUSIONS AND IMPLICATIONS

This is the first study to show that Bp44mT can be given orally with potent anti-tumour efficacy. Oral administration of a novel and effective chemotherapeutic agent provides the benefits of convenience for chronic dosing regimens.

Abbreviations

BpT, 2-benzoylpyridine thiosemicarbazone; Bp44mT, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone; DpT, dipyrindyl thiosemicarbazone; 311, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone; Triapine®, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CDK, cyclin-dependent kinase; DFO, desferrioxamine; Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; PKIH, di-2-pyridylketone isonicotinoyl hydrazone; FTH, ferritin heavy chain; MTD, maximum tolerated dose; NDRG1, N-myc downstream regulated gene-1; RBC, red blood cell; TIBC, total iron-binding capacity; TfR1, transferrin receptor-1; UIBC, unsaturated iron-binding capacity; WBC, white blood cell

Introduction

Chelators bind tightly to metal ions such as iron, which plays many crucial roles in cellular metabolism but which can also be toxic when present in excess (Hershko, 1994; Kalinowski and Richardson, 2005). Important examples of such ligands are desferrioxamine (DFO), 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine®) and deferiprone (Figure 1A) (Hershko, 1994; Kalinowski and Richardson, 2005). In general, iron chelators are used for the treatment of iron overload diseases, although their potential application in cancer treatment is gaining momentum (Kalinowski and Richardson, 2005). For example, DFO is used for the treatment of iron overload but has been studied in clinical trials as an anti-cancer agent (Donfrancesco *et al.*, 1990; Blatt, 1994). However, subsequent phase II studies showed that it was ineffective (Donfrancesco *et al.*, 1993; Blatt, 1994). The failure of DFO is likely to be related to its poor access to intracellular iron pools and its short plasma half-life (Summers *et al.*, 1979; Olivieri and Brittenham, 1997).

As a consequence of its limited membrane permeability, DFO is not orally active (Summers *et al.*, 1979; Olivieri and Brittenham, 1997).

Currently, Triapine® is in clinical trials for cancer treatment and is a potent ribonucleotide reductase inhibitor (Finch *et al.*, 1999; Mackenzie *et al.*, 2007; Attia *et al.*, 2008; Karp *et al.*, 2008; Ma *et al.*, 2008). However, Triapine® causes neutropenia (Mackenzie *et al.*, 2007; Attia *et al.*, 2008), hypoxia and methaemoglobinemia (Ma *et al.*, 2008). Following these observations, a range of chelators have been developed with improved iron chelation efficacy, lipophilicity and anti-cancer activity (Richardson and Milnes, 1997; Lovejoy and Richardson, 2002; Yuan *et al.*, 2004). Among these ligands are those of the hydrazone and thiosemicarbazone classes that demonstrate far greater efficacy than either DFO or Triapine® (e.g. di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone, Dp44mT; Figure 1B) (Kalinowski and Richardson, 2005). This latter chelator belongs to the dipyridyl thiosemicarbazone (DpT) class of ligands and shows marked selectivity against neoplastic cells and pronounced anti-cancer activity in human tumour xenograft models (Yuan *et al.*, 2004; Whitnall *et al.*, 2006; Noulssi *et al.*, 2009). Importantly, at optimal doses, Dp44mT resulted in significant apoptosis in tumours without marked alteration in animal weight or haematological indices (Yuan *et al.*, 2004; Whitnall *et al.*, 2006). However, higher non-optimal doses of Dp44mT (0.7 mg·kg⁻¹) resulted in weight loss and some evidence of myocardial fibrosis in mice (Whitnall *et al.*, 2006).

More recently, a new series of iron chelators derived from Dp44mT known as the 2-benzoylpyridine thiosemicarbazone (BpT) series (Kalinowski *et al.*, 2007) have been generated. The BpT ligands feature substitution of one of the 2-pyridyl moieties with a phenyl ring (Figure 1B) (Kalinowski *et al.*, 2007). Earlier studies *in vitro* found that many BpT chelators were more potent than their DpT counterparts, and their anti-proliferative activity was comparable with that of Dp44mT (Kalinowski *et al.*, 2007). In addition, a number of BpT chelators showed high selectivity (>1500-fold) against cancer cells compared with normal cells (Kalinowski *et al.*, 2007).

As a logical continuation, we investigated the *in vivo* anti-tumour and biological effects of the BpT series. This is the first comprehensive *in vivo* study demonstrating the i.v. and oral activity of the BpT chelator, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone (Bp44mT), against the growth of a human lung cancer xenograft model. Significantly, this chelator did not cause cardiac toxicity or weight loss in mice, compared with the previous generation chelator, Dp44mT (Whitnall *et al.*, 2006). These studies demonstrate the potential of orally active thiosemicarbazone chelators for cancer therapy.

Figure 1

Chemical structures of (A) DFO, deferiprone, Triapine® and PKIH; (B) the DpT and BpT series of chelators.

Methods

Chelators

The BpT chelators, Dp44mT, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311) and di-2-pyridylketone isonicotinoyl hydrazone (PKIH) were synthesized and characterized using standard procedures (Richardson *et al.*, 2006;

Kalinowski *et al.*, 2007). BpT chelators used for *in vivo* administration were synthesized as hydrochloride salts in order to increase their solubility in solution. DFO was obtained from Novartis (Basel, Switzerland), and Triapine® was a gift from Vion Pharmaceuticals (New Haven, CT).

Cell culture

The human DMS-53 lung carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI media (Invitrogen, Carlsbad, CA). The epidermoid carcinoma cell line, KB3-1, and its drug-resistant sub-clone, KB-V1 (maintained in 0.1 µM vinblastine), were a kind gift from R Lock (Children's Cancer Institute Australia, Sydney) and cultured in DMEM (Invitrogen). Cells were grown as described by Richardson and Baker (1990).

Proliferation assays

The effect of chelators on cellular proliferation after a 72 h incubation at 37°C, was assessed using the MTT assay (Richardson *et al.*, 1995). Validation of MTT results was performed using manual cell counts via Trypan blue staining, as described previously (Richardson *et al.*, 1995).

Animal and tumour xenograft model

All animal care and experimental procedures were approved by the University of Sydney Animal Ethics Committee. Female BALB/c *nu/nu* mice were used at 8–10 weeks of age. DMS-53 cells were harvested and re-suspended in a 1:1 ratio of RPMI and Matrigel™ (BD Biosciences, San Jose, CA). Viable cells (5×10^6 cells) were injected s.c. into the right flank of mice. Tumour size was measured using digital Vernier calipers, and volume was calculated as described (Sanceau *et al.*, 2002; Balsari *et al.*, 2004; Whitnall *et al.*, 2006). Chelator treatment began after the tumours had reached a volume of 100–150 mm³.

In vivo chelator administration

Chelators were dissolved in vehicle (30% propylene glycol/0.9% saline). Intravenous administration was via the tail vein (100 µL), while oral administration was given via gavage (300 µL) on alternate days, three times per week. Preliminary studies indicated this oral dosing schedule was optimal. As relevant controls, mice were either i.v. injected or gavaged with the vehicle alone.

Haematology and serum biochemistry

Blood was collected from the hearts of anaesthetized mice by cardiac puncture at the end of the study. Serum clinical and haematological parameters were determined using a Konelab 20i analyser (Thermo-Electron Corporation, Vantaa, Finland) and Sysmex K-4500 analyser (TOA Medical Electronics Co., Kobe, Japan) respectively (Rahmanto and Richardson, 2009).

Histology

Organs were dissected, fixed in 10% formalin, sectioned and stained with either haematoxylin and eosin (H&E), Perl's or Gomori trichrome stain for microscopic examination. These slides were assessed by an independent veterinary patholo-

gist. Splenic iron deposits and liver vacuolation were quantified using the program, ImageJ (version 1.4.3; NIH, Bethesda, MD) (Abramoff *et al.*, 2004).

Tissue iron, copper and zinc determinations

Tissue non-haem iron, copper and zinc concentrations were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES) using standard techniques (Kingston and Jassie, 1988).

Western Blotting

Samples were lysed using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein samples (30–150 µg per lane) were separated on a 4–12% NuPage® Bis-Tris gel (Invitrogen) (Rahmanto and Richardson, 2009). The following antibodies, namely: anti-human mouse transferrin receptor-1 (TfR1; Invitrogen; Cat.#: 136800), anti-human rabbit ferritin heavy chain (FTH; Cat.#: 3398), anti-human rabbit cyclin D1 (Cat.#: 2926), anti-human rabbit p21^{CIP1/WAF1} (Cat.#: 2946; Cell Signaling Technology, Danvers, MA) and anti-human goat N-myc downstream regulated gene-1 (NDRG1; Cat.#: ab37897, Abcam, Cambridge, MA), were incubated at dilutions of 1:1000–1:2500. The secondary antibodies used were anti-rabbit, anti-mouse (Cat. #: 7074, 7076, Cell Signaling Technology) and anti-goat (Cat. #: A5420, Sigma-Aldrich, MO, USA), each at a dilution of 1:10 000. As a control for protein loading, membranes were probed for β-actin. Densitometric analysis was performed using Quantity One software (Bio-Rad, Hercules, CA).

Statistical analysis

Results are expressed as the mean ± SEM. Experimental data were compared using Student's *t*-test. Results were considered statistically significant when *P* < 0.05.

Results

BpT chelators show potent anti-proliferative activity against lung cancer cells and drug-resistant cells

In initial studies, the anti-proliferative activity of the BpT chelators was tested *in vitro* using DMS-53 lung cancer cells over a 72 h incubation using the MTT assay. Our studies demonstrated that formazan product formation was directly proportional to cell number, and our results were also validated using Trypan blue staining and manual cell counts. The DMS-53 cell-type was chosen due to the established anti-proliferative activity of the related ligand Dp44mT against lung cancer cells *in vitro* and *in vivo* (Yuan *et al.*, 2004; Whitnall *et al.*, 2006). The BpT analogues, Bp44mT, Bp4mT, Bp4eT and Bp4aT, showed similar and potent anti-proliferative activity (Table 1) compared with the well known chelators, DFO, 311 and Triapine® (Kalinowski and Richardson, 2005) (Figure 2A). Considering their IC₅₀ values, the BpT chelators showed 19- and 172-fold greater anti-proliferative activity than Triapine® and DFO (Table 1), respectively. In addition, the anti-proliferative efficacy of the BpT chelators was com-

Table 1

IC₅₀ values of chelators in human DMS-53 lung, vinblastine-sensitive (KB3-1) and -resistant (KB-V1) epidermoid carcinoma cells

Treatment	Lung carcinoma DMS-53	Epidermoid carcinoma	
		KB3-1 vinblastine-sensitive	KB-V1 vinblastine-resistant
DFO	6.38 ± 2.71	13.43 ± 1.68	15.85 ± 3.88
Triapine®	0.71 ± 0.17	–	–
Dp44mT	0.04 ± 0.01	0.53 ± 0.28	0.09 ± 0.04
311	0.13 ± 0.06	6.25 ± 3.32	4.79 ± 2.84
Vinblastine	–	0.02 ± 0.01	0.03 ± 0.01
PKIH	–	2.67 ± 0.64	2.23 ± 0.71
Bp4mT	0.04 ± 0.01	–	–
Bp44mT	0.03 ± 0.01	0.76 ± 0.54	0.04 ± 0.01
Bp4eT	0.04 ± 0.01	1.82 ± 1.06	0.07 ± 0.02
Bp4aT	0.04 ± 0.01	1.78 ± 0.94	0.40 ± 0.29

Results are mean ± SEM of three to five separate experiments.

parable with that of Dp44mT (Table 1). The other BpT chelators, namely BpT and Bp4pT (Figure 1B), were not assessed because of their previously demonstrated low anti-proliferative activity (Kalinowski *et al.*, 2007).

Three BpT chelators with the highest anti-proliferative activity (i.e. Bp44mT, Bp4eT and Bp4aT) were then examined for their ability to overcome drug-resistance using the vinblastine-resistant epidermoid KB-V1 cells (Shen *et al.*, 1986). The drug-sensitive, parental KB3-1 cell line was included for comparison. As a control, incubation with vinblastine confirmed that resistance was maintained in KB-V1 cells (Figure 2B). The non-thiosemicarbazone chelators, DFO, PKIH and 311 (Kalinowski and Richardson, 2005), had similar anti-proliferative activity against both vinblastine-sensitive and -resistant cell-types (Figure 2B). In contrast, Bp4eT, Bp4aT and Bp44mT were significantly ($P < 0.05$) more effective at inhibiting proliferation of vinblastine-resistant KB-V1 cells when compared with non-resistant KB3-1 cells (Figure 2B and Table 1). These results are similar to those found for Dp44mT, which also exhibited higher anti-proliferative activity against the resistant cells as shown in Figure 2B and previously (Whitnall *et al.*, 2006). Importantly, other laboratories have also confirmed this effect using other types of thiosemicarbazones (Wu *et al.*, 2007). Hence, the BpT chelators possess potent anti-proliferative activity *in vitro*, and their activity was potentiated in vinblastine-resistant cells.

In vivo anti-tumour efficacy of BpT chelators against the growth of human lung carcinoma xenografts

The potent *in vitro* activity of the BpT chelators warranted *in vivo* evaluation, which was performed using the human DMS-53 tumour xenograft model in nude mice. Before administration, all the chelators were tested for their solubility in the vehicle that was utilized in previous studies (Whitnall *et al.*, 2006). Before oral and i.v. administration, the chelators were subjected to maximum tolerated dose (MTD)

studies to determine the optimal dose. The MTD studies define the dose at which 30% of the cohorts demonstrated deteriorating health, as suggested by 10% weight loss over 2 weeks (Yuan *et al.*, 2004). Unfortunately, the MTD for Bp4eT and Bp4aT were not attained because of poor solubility. Hence, the *in vivo* studies for these thiosemicarbazones were performed at the highest dose possible in this vehicle (30% propylene glycol/saline) by i.v. administration. Nonetheless, Bp44mT had good solubility, and thus, it was possible to deliver this agent at higher doses and a MTD of 15 mg·kg⁻¹ via the i.v. route was determined. However, lower doses of 9 and 12 mg·kg⁻¹ were utilized in i.v. studies because no pronounced weight loss was observed over 2 weeks during MTD studies.

Bp44mT inhibits the growth of lung carcinoma xenografts

Intravenous studies. Studies with Bp4eT and Bp4aT utilizing i.v. administration at 2, 4 and 6 mg·kg⁻¹ (once per day, 5 days per week for 28 days) did not show significant tumour growth inhibition (data not shown). This lack of biological activity was likely to be due to the insufficient dose administered because of the very low solubility of these agents. In fact, these mice remained healthy over 7 weeks without alteration to body or organ weights, haematological indices or organ histology.

In contrast to Bp4eT and Bp4aT, Bp44mT showed pronounced anti-tumour efficacy when administered i.v. at 9 and 12 mg·kg⁻¹ once per day, 5 days per week (Figure 3A). After 28 days, the tumour volumes in mice treated with 9 and 12 mg·kg⁻¹ of Bp44mT were significantly ($P < 0.001$) reduced, compared with vehicle-treated mice. Dp44mT (0.75 mg·kg⁻¹) was included as a positive control and reduced tumour growth to 41% ($n = 7$) of the control, and this was consistent with previous studies (Yuan *et al.*, 2004; Whitnall *et al.*, 2006). The MTD for Bp44mT was much higher than that of Dp44mT (Yuan *et al.*, 2004), and hence, higher doses of Bp44mT were employed.

Figure 2

BpT chelators display pronounced anti-proliferative activity *in vitro* and partially overcome resistance to established chemotherapeutic agents. (A) The BpT chelators, Bp4mT, Bp4eT, Bp4aT and Bp44mT, demonstrate pronounced anti-proliferative efficacy against the DMS-53 lung carcinoma cell type *in vitro* and have comparable activity with Dp44mT and much greater efficacy than DFO, Triapine® and 311. (B) Relative anti-proliferative activity of vinblastine, DFO, 311, PKIH, Dp44mT and the BpT chelators using the drug-resistant KB-V1 cell line as compared with the parental counterpart, namely the KB3-1 cell line. Cells were incubated in the presence or absence of the chelators for 72 h at 37°C. Cellular proliferation was measured using the MTT assay. Results are mean $\pm$ SEM from three experiments. * $P < 0.05$; *** $P < 0.001$.

Figure 3

Bp44mT markedly inhibits the growth of DMS-53 lung carcinoma xenografts in nude mice. (A) Bp44mT inhibits the net tumour growth of DMS-53 lung carcinoma xenografts when administered intravenously once per day, five times per week at 9 or 12 mg·kg⁻¹ for 28 days. Dp44mT (0.75 mg·kg⁻¹) was administered using the same regimen and included as a positive control. (B) Oral gavage administration of Bp44mT was performed once per day, three times per week at 10, 25 or 50 mg·kg⁻¹ for 23 days. The respective controls in (A) and (B) were mice given 30% propylene glycol/0.9% saline solution by i.v. injection or oral gavage respectively. (C) End-point tumour weight (% vehicle control) of mice treated with oral Bp44mT (10, 25 or 50 mg·kg⁻¹) after 23 days of treatment. (D) Photographs of tumour xenografts from the oral administration study after mice were given vehicle control or 50 mg·kg⁻¹ Bp44mT once per day, three times per week for 23 days. **P* < 0.05; ****P* < 0.001.

Oral Bp44mT. Orally active cancer chemotherapeutic agents are preferred because of convenience of administration and this facilitates their use for chronic dosing regimens (Liu *et al.*, 2002; Sparreboom *et al.*, 2002). Considering these factors and the potent activity as shown by the i.v. data with Bp44mT (Figure 3A), we investigated the oral activity of the BpT chelators. Initially, all BpT chelators were subjected to assessment by Lipinski's rules for oral bioavailability (Yu *et al.*, 2009). These rules indicated that all the BpT chelators are likely to have good oral activity (Yu *et al.*, 2009). Hence, Bp44mT was given via oral gavage employing the same human DMS-53 xenograft model used above for the i.v. studies. The MTD studies for oral Bp44mT identified a dose of 75 mg·kg⁻¹ (once per day, 3 days per week). Hence, oral doses were administered at 10, 25 and 50 mg·kg⁻¹. Preliminary experiments using Dp44mT (1–6 mg·kg⁻¹) in oral bioavailability studies showed that mice became moribund after 2–3 days, and hence, Dp44mT was not administered via this route.

In the oral study, Bp44mT administered at 10, 25 and 50 mg·kg⁻¹ (once per day, 3 days per week for 23 days) resulted in marked dose-dependent growth inhibition of the tumours (Figure 3B–C). At the end of the treatment, the tumour volumes in 25 and 50 mg·kg⁻¹ Bp44mT-treated mice were 40% and 25% of that found in vehicle control mice, respectively (Figure 3B). Tumours were weighed at the end of the treatment and these weights were, significantly ($P < 0.05$) less in mice treated with 10, 25 and 50 mg·kg⁻¹ Bp44mT (Figure 3C). The tumours from the control and 50 mg·kg⁻¹ Bp44mT-treated mice were photographed, and all tumours after Bp44mT treatment were smaller than the vehicle control (Figure 3D). Hence, Bp44mT resulted in a significant inhibition of tumour growth using i.v. or oral administration.

Toxicological evaluation of i.v. and oral Bp44mT treatment

Animal body weight and organ/body weight. For toxicological assessment, mouse body weights were monitored throughout the i.v. and oral treatments, and at the end of the study, the organs were collected and their weight expressed as a percentage of total body weight (organ/body weight). Intravenous Bp44mT-treated mice did not show significant ($P > 0.05$) weight loss at doses of 9 and 12 mg·kg⁻¹, as compared with the vehicle-treated mice (Figure 4A). In contrast, Dp44mT-treated (0.75 mg·kg⁻¹) mice lost approximately 10% of their body weight (Figure 4A). Similar to the i.v. studies, oral administration of Bp44mT (10, 25 and 50 mg·kg⁻¹) also did not lead to significant body weight loss (Figure 4B). These results demonstrated that Bp44mT is well tolerated at these doses and showed significant anti-tumour activity (Figure 3A–D).

Both i.v. and oral Bp44mT resulted in a dose-related increase in the liver/body weight ratio (Figure 4C–D). The highest Bp44mT doses of 12 mg·kg⁻¹ (i.v.) and 50 mg·kg⁻¹ (oral) led to a significant ($P < 0.001$) increase in this ratio by 1.3- and 1.5-fold respectively. In addition, there was a slight, but significant ($P < 0.01$) increase in the spleen/body weight ratio (up to 1.2-fold) in the 10- and 50-mg·kg⁻¹ oral treatment groups (Figure 4C–D). The 12 mg·kg⁻¹ Bp44mT i.v. group also showed a significant ($P < 0.05$) increase in spleen weight (1.4-fold; Figure 4C). In agreement with a previous study

(Whitnall *et al.*, 2006), there was a significant ($P < 0.05$) increase in the heart/body weight ratio of Dp44mT-treated (0.75 mg·kg⁻¹ i.v.) mice, which was not observed in any i.v. or oral Bp44mT-treatment groups. The organ/body weight ratios of the other organs, including kidney, brain and lung, were not significantly affected by either Bp44mT or Dp44mT. This indicated that Bp44mT treatment led to potential liver and splenic effects but had no effect on heart weight.

Haematology and serum biochemistry. Haematological indices were examined at the end of the i.v. treatment and showed that Bp44mT at 9 and 12 mg·kg⁻¹ caused a significant ($P < 0.01$) decrease in red blood cell (RBC) count, compared with that after the vehicle (Table 2). In contrast, only the highest dose of oral Bp44mT (50 mg·kg⁻¹) resulted in a slight, but significant ($P < 0.05$) decrease in RBC count as compared with the vehicle (Table 2). A small decrease in haemoglobin concentration ($P < 0.05$) was also observed in this latter group. These results were not unexpected, since Bp44mT has potent iron chelation efficacy as demonstrated previously *in vitro* (Kalinowski *et al.*, 2007). Interestingly, oral Bp44mT at 10 and 50 mg·kg⁻¹ significantly ($P < 0.05$) increased the white blood cell (WBC) count, compared with control ($6.50 \pm 0.70 \times 10^9$ L⁻¹). There was no significant change in haematocrit or platelets in any of the thiosemicarbazone-treated groups (Table 2).

Serum biochemical indices of the treated mice were also assessed. There was a significant ($P < 0.05$) decrease in serum iron levels in the 9 mg·kg⁻¹ (i.v.) and 50 mg·kg⁻¹ (oral) Bp44mT-treated mice, while there was no effect in the other treatment groups (Table 2). A significant increase ($P < 0.05$) in the serum levels of alkaline phosphatase (ALP) (up to 2.1-fold) in all i.v. and oral Bp44mT treatment groups was observed (Table 2). In all cases, serum alanine aminotransferase (ALT) was also significantly ($P < 0.05$) elevated, with the oral group (up to a 6.3-fold increase) being more markedly affected than the i.v. group (up to a 3.1-fold increase). Furthermore, there was also a slight, but significant ($P < 0.05$) decrease in the serum albumin levels in the i.v. (9 and 12 mg·kg⁻¹) and oral (25 and 50 mg·kg⁻¹) Bp44mT groups. Dp44mT (i.v. 0.75 mg·kg⁻¹) also significantly ($P < 0.05$) elevated serum ALP and ALT but did not significantly affect albumin levels after 4 weeks of treatment (Table 2). The oral, but not i.v., treatment also affected cholesterol levels, which were elevated in the 25 and 50 mg·kg⁻¹ Bp44mT-treated mice. The triglyceride level, total iron-binding capacity (TIBC) and unsaturated iron-binding capacity (UIBC) were unaffected. Collectively, Bp44mT treatment resulted in an increase in the level of serum liver enzymes (ALP and ALT) in the i.v. and oral studies. Other indices such as RBC count, serum iron and albumin were also affected, while alterations in WBC count or cholesterol level were only observed in groups given Bp44mT orally.

Total tissue iron, copper and zinc levels. Due to the ability of BpT chelators to bind metals (Kalinowski *et al.*, 2007), the total tissue iron, copper and zinc were determined for the Bp44mT-treated groups that had received the highest doses via the two administration routes (i.e. 12 mg·kg⁻¹ i.v. and 50 mg·kg⁻¹ oral). These experiments showed that Bp44mT at these doses significantly ($P < 0.05$) decreased the total iron

Figure 4

Bp44mT does not induce significant body weight loss but increases the liver/body weight ratio. (A) Bp44mT treatment did not cause body weight loss in BALBc *nu/nu* mice, bearing a DMS-53 lung carcinoma xenograft when administered i.v. (once per day, five times per week for 28 days) at 9 or 12 mg·kg⁻¹. Dp44mT (0.75 mg·kg⁻¹) once per day, five times per week for 28 days was included as relative control and resulted in significant weight loss. (B) No significant body weight loss was observed when Bp44mT was administered via oral gavage once per day, three times per week at 10, 25 or 50 mg·kg⁻¹ for 23 days. Body weight is expressed as a percentage of the initial body weight of the mice before treatment. (C) Organ weight expressed as percentage of total body weight after the treatment in (A) using Bp44mT and Dp44mT (i.v.). (D) Organ weight expressed as percentage of total body weight after the treatment with oral Bp44mT shown in (B). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

content in liver and spleen, with a greater reduction occurring in the mice given oral Bp44mT (Table 3). This is probably due to the high concentrations of chelator used for the oral treatment. Total iron content in the liver after oral and i.v.

Bp44mT were decreased by approximately 1.8- and 1.3-fold respectively. In the spleen, total iron content was reduced by 2.1- and 1.5-fold for oral and i.v. treated groups respectively. In addition, the total iron level in the brain was also slightly

Table 2Haematological and serum indices from mice treated with i.v. Bp44mT (9 and 12 mg·kg⁻¹), Dp44mT (0.75 mg·kg⁻¹) or oral Bp44mT (10, 25 and 50 mg·kg⁻¹)

Indices	Units	Treatment group								
		i.v. for 28 days (5 days per week)		Oral gavage for 23 days (3 days per week)						
		Vehicle control (n = 10)	Bp44mT 9 mg·kg ⁻¹ (n = 11)	Bp44mT 12 mg·kg ⁻¹ (n = 11)	Dp44mT 0.75 mg·kg ⁻¹ (n = 7)	Vehicle control (n = 10)	Bp44mT 10 mg·kg ⁻¹ (n = 8)	Bp44mT 25 mg·kg ⁻¹ (n = 9)	Bp44mT 50 mg·kg ⁻¹ (n = 7)	
Haematological indices										
RBC	10 ¹² L ⁻¹	9.80 ± 0.21	9.10 ± 0.09**	9.11 ± 0.10**	9.93 ± 0.24	9.48 ± 0.13	9.40 ± 0.21	9.57 ± 0.20	9.07 ± 0.11*	
WBC	10 ⁹ L ⁻¹	8.43 ± 0.92	8.58 ± 1.20	8.26 ± 0.38	9.70 ± 1.15	6.50 ± 0.70	9.68 ± 0.84*	7.98 ± 1.19	12.93 ± 2.10*	
Haemoglobin	g·L ⁻¹	160 ± 4	152 ± 1	148 ± 2	156 ± 4	154 ± 2	152 ± 3	154 ± 3	147 ± 2*	
Haematocrit	%	0.48 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	0.50 ± 0.02	0.47 ± 0.01	0.47 ± 0.01	0.48 ± 0.01	0.46 ± 0.02	
Platelets	10 ⁹ L ⁻¹	883 ± 43	886 ± 48	849 ± 41	835 ± 80	752 ± 11	753 ± 61	978 ± 87	1032 ± 42	
Serum biochemical indices										
Serum iron	μmol·L ⁻¹	26.4 ± 2.6	15.5 ± 1.9***	21.0 ± 1.6	32.7 ± 2.7	23.7 ± 1.6	23.2 ± 1.4	19.9 ± 2.4	19.7 ± 1.1*	
TIBC	μmol·L ⁻¹	64.0 ± 4.3	58.3 ± 2.4	64.8 ± 3.8	67.1 ± 4.9	46.1 ± 1.1	45.6 ± 1.8	47.8 ± 1.5	43.2 ± 3.0	
UIBC	μmol·L ⁻¹	51.4 ± 5.6	46.3 ± 1.8	45.2 ± 3.8	54.6 ± 4.8	66.6 ± 1.7	67.1 ± 2.1	61.1 ± 1.9	65.5 ± 3.3	
ALP	U·L ⁻¹	75.1 ± 7.8	111.2 ± 3.5***	136.2 ± 9.1***	124.3 ± 12.0*	75.2 ± 2.0	120.2 ± 7.4***	140.3 ± 9.8***	156.9 ± 10.5***	
ALT	U·L ⁻¹	36.0 ± 4.7	113.9 ± 15.9***	61.8 ± 7.8*	64.7 ± 11.3*	32.9 ± 1.5	60.0 ± 10.3*	150.0 ± 17.9***	206.9 ± 46.5***	
Albumin	g·L ⁻¹	37.1 ± 0.7	34.2 ± 0.65*	34.8 ± 0.60*	35.7 ± 1.0	33.5 ± 0.3	34.0 ± 1.1	29.6 ± 1.0***	30.2 ± 0.5***	
Cholesterol	mmol·L ⁻¹	2.79 ± 0.16	2.52 ± 0.16	3.22 ± 0.25	2.80 ± 0.15	2.11 ± 0.07	2.43 ± 0.11	2.46 ± 0.11*	2.73 ± 0.09***	
Triglyceride	mmol·L ⁻¹	1.13 ± 0.10	1.01 ± 0.09	1.02 ± 0.10	1.35 ± 0.17	0.82 ± 0.05	0.95 ± 0.09	0.90 ± 0.17	1.11 ± 0.11	

Values expressed as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared with their respective vehicle control, as determined using Student's t-test.

Table 3

Tissue iron, copper or zinc of mice after treatment with either Bp44mT i.v. (12 mg·kg⁻¹) or oral (50 mg·kg⁻¹)

Parameters	Units	Treatment group			
		i.v. for 28 days (5 days per week)		Oral gavage for 23 days (3 days per week)	
		Vehicle control (n = 10)	Bp44mT 12 mg·kg ⁻¹ (n = 11)	Vehicle control (n = 7)	Bp44mT 50 mg·kg ⁻¹ (n = 7)
Iron					
Heart	µg·g ⁻¹	505 ± 29	540 ± 97	448 ± 13	424 ± 48
Liver	µg·g ⁻¹	573 ± 29	432 ± 25*	495 ± 27	268 ± 18*
Kidney	µg·g ⁻¹	480 ± 39	519 ± 45	263 ± 5	218 ± 7*
Spleen	µg·g ⁻¹	2266 ± 348	1492 ± 105*	3039 ± 296	1412 ± 164*
Brain	µg·g ⁻¹	100 ± 3	83 ± 3*	98 ± 3	86 ± 2*
Tumour	µg·g ⁻¹	122 ± 8	104 ± 9	140 ± 11	222 ± 43
Copper					
Heart	µg·g ⁻¹	45 ± 3	44 ± 2	47 ± 4	34 ± 1
Liver	µg·g ⁻¹	17 ± 1	15 ± 0.2	15 ± 0.5	14 ± 0.4
Kidney	µg·g ⁻¹	19 ± 1	19 ± 0.3	19 ± 0.7	18 ± 0.1
Spleen	µg·g ⁻¹	14 ± 1	10 ± 0.4	13 ± 2	9 ± 0.5
Brain	µg·g ⁻¹	18 ± 0.2	18 ± 0.2	18 ± 0.4	18 ± 0.3
Tumour	µg·g ⁻¹	6 ± 0.3	6 ± 0.6	5 ± 0.3	6 ± 0.6
Zinc					
Heart	µg·g ⁻¹	98 ± 6	103 ± 4	111 ± 6	100 ± 6
Liver	µg·g ⁻¹	132 ± 13	124 ± 3	111 ± 3	126 ± 2
Kidney	µg·g ⁻¹	86 ± 3	87 ± 1	84 ± 1	88 ± 2
Spleen	µg·g ⁻¹	111 ± 5	103 ± 3	116 ± 7	112 ± 3
Brain	µg·g ⁻¹	75 ± 1	75 ± 1	79 ± 3	76 ± 2
Tumour	µg·g ⁻¹	94 ± 1	96 ± 1	95 ± 1	90 ± 2

Values expressed as mean ± SEM. **P* < 0.05 compared with their respective vehicle control, as determined using Student's *t*-test.

reduced by a similar extent (1.1- to 1.2-fold). The total iron level in the kidneys was also slightly reduced, but only in mice administered oral Bp44mT. The heart iron levels in both groups were not affected.

Despite indications of some iron depletion after Bp44mT administration as shown by several haematological indices (Table 2) and total iron levels in the liver and spleen (Table 3), we observed no significant difference in the total iron content in the tumours of treated versus non-treated mice (Table 3). In fact, after oral gavage, total tumour iron in the Bp44mT (50 mg·kg⁻¹)-treated group increased by 1.6-fold, but this was not significant. This is consistent with previous studies using Dp44mT, which also found no reduction in tumour iron level (Whitnall *et al.*, 2006). These observations indicate that these thiosemicarbazones may not deplete, but rather sequester, iron in the tumours. Total copper and zinc levels in major organs and the tumour (Table 3) were not significantly affected, indicating that Bp44mT did not deplete these metals.

Histology of major organs. Major organs of mice treated with 12 mg·kg⁻¹ (i.v.) and 50 mg·kg⁻¹ (oral) Bp44mT were taken for histopathological examination (Figure 5A,B). As Bp44mT increased liver weight (Figure 4C–D), cytoplasmic vacuola-

tion of the liver was assessed by H&E staining. The vacuolation was more pronounced after oral administration of Bp44mT (50 mg·kg⁻¹), compared with the respective vehicle control (Figure 5Bii, C). Mice treated with i.v. Bp44mT (12 mg·kg⁻¹) also showed hepatic vacuolation when compared with its control (Figure 5Aii, C). Comparing administration of the control vehicle (30% propylene glycol/saline) via the i.v. and oral routes, it was notable that the latter induced more hepatic vacuolation. This is probably due to the hepatic portal circulation which would directly deliver the vehicle to the liver for first pass metabolism. No increase in hepatocellular necrosis or fibrosis was observed in any of the livers from Bp44mT-treated mice. Therefore, the increased liver weights could be due to increased vacuolation.

The majority of the H&E sections of the kidney appeared normal (Figure 5Aiv, Biv). However, there were very small focal abnormalities detected infrequently in kidney sections of oral Bp44mT-treated mice (see arrow Figure 5Biv), while none were identified in the kidneys of control or i.v. Bp44mT-treated mice (Figure 5Aiv, Biv). The pathogenesis of these lesions is unclear, especially as the majority of renal tissue was normal. There was no significant alteration in the number of haematopoietic cells in the spleen between control and Bp44mT-treated mice (data not shown). This is in contrast to

Figure 5

Bp44mT does not induce cardiac fibrosis, but increases liver vacuolation and reduces splenic haemosiderin levels. Histology of the major organs (heart, liver, spleen or kidney) from (A) mice after i.v. vehicle control or Bp44mT (12 mg·kg⁻¹) administration, once per day, 5 days per week for 28 days; (B) Oral Bp44mT (50 mg·kg⁻¹, once per day, 3 days per week for 23 days) and the respective vehicle control given as described in Figure 3. (i) Gomori trichrome-stained cardiac tissue showed no fibrotic lesions (magnification: ×40). (ii) Examination of H&E-stained liver sections demonstrate an increase in vacuolation in liver from treated mice (magnification: ×400). (iii) Perl's-stained spleen showed reduction in iron level consistent with reduction in haemosiderin (magnification: ×100). (iv) H&E-stained kidney tissue illustrates mild and small focal abnormalities (see arrow) in the treated groups (magnification: ×400). (C, D) Histological quantification of liver vacuolation and Prussian blue staining in the spleen was performed using ImageJ software (48 sections per group; mean ± SEM). ****P* < 0.001.

our previous study examining Triapine® treatment (i.v. 12 mg·kg⁻¹ for 2 weeks), where an increased number of haematopoietic cells in the splenic red pulp of mice was reported (Whitnall *et al.*, 2006). Perl's stain was also used for staining macrophages for iron-containing haemosiderin, which is generally present in the splenic red pulp of normal mice (Suttie, 2006). There was significantly ($P < 0.001$) less splenic iron deposits in i.v and oral Bp44mT-treated mice, compared with their respective controls, especially after the oral treatment (Figure 5Aiii, Biii, D). This is consistent with the reduction of total iron content in the spleen of the treated mice (Table 3).

There was no evidence to suggest myocardial fibre degeneration, necrosis or fibrosis, as judged from Gomori trichrome staining of cardiac sections (Figure 5Ai, Bi). In contrast, administration of Dp44mT i.v. at 0.7 mg·kg⁻¹ for 2 weeks resulted in evidence of some myocardial fibrosis, as described previously (Whitnall *et al.*, 2006). Moreover, there were no abnormalities observed in any of the brain or lung sections (data not shown).

The effect of Bp44mT withdrawal on its toxicological profile

In order to determine if Bp44mT caused permanent pathology, further investigations were performed using 50 mg·kg⁻¹ oral Bp44mT (once per day, 3 days per week for 21 days), as most alterations occurred at this dose (Table 2, Figures 4D and 5B). A group of mice (seven control and nine treated

animals) were randomly culled after 21 days of treatment, and these studies confirmed the pathological changes reported in the previous sections above. The remaining mice were then allowed to recover from day 22–38 without any further treatment. The vehicle control-treated mice were also included in the recovery study to ensure an appropriate age comparison.

As an additional haematological indicator, the percentage of reticulocytes in peripheral blood was also determined to assess anaemia and to ensure adequate bone marrow function after Bp44mT treatment (Finch, 1982). Orally administered Bp44mT (50 mg·kg⁻¹) for 3 weeks resulted in a slight, but significant ($P < 0.01$), increase in reticulocytes as compared with the control (5.46 ± 0.41 vs. $3.32 \pm 0.54\%$; $n = 7$; Table 4). These results suggest that Bp44mT-treated mice developed a slight anaemia with an appropriate bone marrow response and are in agreement with the reduced RBC and haemoglobin levels observed.

During treatment with oral Bp44mT (50 mg·kg⁻¹), tumour growth was inhibited, such that by the end of treatment, the tumour volume was $32 \pm 5\%$ of the vehicle control (Figure 6A). After the recovery period, the tumour volume in the treated mice was still significantly ($P < 0.001$) smaller than the vehicle control ($35 \pm 6\%$ of that found in the control). Throughout the treatment and recovery period, no significant alteration to body weight was observed at any point (data not shown). It should be noted that the recovery period was limited to 17 days as the tumour volume of the control

Table 4

Haematological and serum indices from mice treated with oral Bp44mT (50 mg·kg⁻¹; three times per week) for 21 days and then after a 17-day recovery period without drug treatment

Parameter	Units	Treatment group			
		Oral gavage 3 days per week for 21 days Bp44mT 50 mg·kg ⁻¹		After recovery period (17 days) Bp44mT 50 mg·kg ⁻¹	
		Control (n = 7)	(n = 9)	Control (n = 11)	(n = 12)
Haematological indices					
RBC	10 ¹² L ⁻¹	10.63 ± 0.16	10.05 ± 0.21*	10.58 ± 0.19	10.57 ± 0.19
WBC	10 ⁹ L ⁻¹	3.00 ± 0.40	5.09 ± 0.68*	2.82 ± 0.48	3.27 ± 0.44
Haemoglobin	g·L ⁻¹	156 ± 2	146 ± 3*	154 ± 3	152 ± 3
Haematocrit	%	0.46 ± 0.004	0.44 ± 0.006	0.46 ± 0.006	0.46 ± 0.007
Platelets	10 ⁹ L ⁻¹	1229 ± 96	1129 ± 184	1452 ± 75	1299 ± 66
Reticulocytes	%	3.32 ± 0.54	5.46 ± 0.41**	4.23 ± 0.40	5.02 ± 0.41
Clinical chemistry indices					
Serum iron	μmol·L ⁻¹	24.7 ± 1.4	18.4 ± 1.2*	25.0 ± 1.6	30.2 ± 1.7
TIBC	μmol·L ⁻¹	50.7 ± 4.1	59.0 ± 2.8	60.0 ± 1.8	60.3 ± 1.6
UIBC	μmol·L ⁻¹	63.5 ± 4.8	50.2 ± 3.3	51.6 ± 4.1	59.5 ± 1.5
ALP	U·L ⁻¹	102.3 ± 14.6	226.1 ± 15.3***	74.8 ± 5.5	100.7 ± 6.5**
ALT	U·L ⁻¹	38.6 ± 1.4	141.4 ± 4.2***	62.3 ± 8.3	135.2 ± 14.2**
Albumin	g·L ⁻¹	30.5 ± 0.5	25.0 ± 0.6***	28.9 ± 0.3	28.3 ± 0.4
Cholesterol	mmol·L ⁻¹	2.90 ± 0.12	3.73 ± 0.12*	2.87 ± 0.22	3.08 ± 0.08
Triglyceride	mmol·L ⁻¹	0.76 ± 0.11	1.23 ± 0.07	0.87 ± 0.10	0.84 ± 0.09

Values expressed as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with their respective vehicle control, as determined using Student's *t*-test.

Figure 6

Oral administration of Bp44mT ($50 \text{ mg} \cdot \text{kg}^{-1}$) does not cause irreversible toxicity in nude mice bearing DMS-53 lung carcinoma xenografts. (A) The net tumour volume of mice treated with oral Bp44mT ($50 \text{ mg} \cdot \text{kg}^{-1}$; once per day, three times per week) during treatment from days 0–21 and throughout the recovery period without treatment (days 22–38). (B) Organ weight expressed as a percentage of body weight at the end of the treatment or recovery period. (C) Photographs of liver (H&E) and spleen (Perl's) sections from mice at the end of treatment or recovery periods. (D, E) Liver vacuolation and splenic iron deposits were quantified as described in Figure 5 $**P < 0.01$; $***P < 0.001$.

animals had reached the maximum ethical limit prescribed by the local animal ethics committee and had to be culled.

After the recovery period, the majority of the adverse effects previously observed either totally recovered or improved. The pathology that completely recovered included the increased spleen/body weight (Figure 6B), all haematological indices (RBC, haemoglobin, WBC and reticulocyte count; Table 4) and a number of biochemical indices, including serum iron, albumin and cholesterol levels (Table 4). The liver/body weight and serum ALP and ALT levels of mice after the recovery period were still significantly ($P < 0.01$) increased as compared with their relevant vehicle control (Figure 6B and Table 4). However, a less pronounced effect on the liver/body weight (1.2- vs. 1.7-fold), as well as the ALP (1.3- vs. 2.2-fold) and ALT (2.2- vs. 3.7-fold) levels were observed after the recovery period relative to after the treatment period.

The liver H&E stain showed an improvement in the degree of vacuolation in the Bp44mT-treated group after the recovery period (Figure 6Ciii vs. 6Ci, D). This is consistent with the improved liver/body weight observed. The histological assessment of the spleen using Prussian blue staining demonstrated that the Bp44mT-treated group after recovery showed a significant ($P < 0.001$) increase in iron deposits (Figure 6Civ vs. 6Cii, E). Hence, together with the observed improvement in the haematological indices (Table 4), these results suggested that the Bp44mT-treated mice were able to recover to normal levels of iron stores. The histology also did not show any other additional abnormalities in the treated mice. Collectively, these data indicated that orally administered Bp44mT (50 mg·kg⁻¹) did not cause irreversible toxicity.

Effects of Bp44mT on the expression of iron-regulated proteins in the liver and tumour

To further characterize whether the mechanism of Bp44mT anti-tumour activity *in vivo* was a result of depletion of iron, we evaluated the expression of proteins known to be regulated by intracellular iron levels, namely TfR1 and FTH in tumours excised from mice treated with oral Bp44mT (50 mg·kg⁻¹) or the vehicle. During cellular iron depletion, TfR1 expression is up-regulated for compensatory iron uptake, while FTH is down-regulated to prevent iron storage (Hentze and Kuhn, 1996; Kalinowski and Richardson, 2005). As a relevant comparison, the expression of these molecules in the liver was also determined. This study was important because oral Bp44mT (50 mg·kg⁻¹) did not deplete total iron content in the tumour, while it significantly ($P < 0.05$) reduced the iron levels in liver, kidney, spleen and brain (Table 3). The cell cycle control molecules, cyclin D1 and the cyclin-dependent kinase inhibitor p21^{CIP1/WAF1} were also tested, as they have been previously shown to be regulated by intracellular iron levels (Gao and Richardson, 2001; Le and Richardson, 2003; Fu and Richardson, 2007; Nurtjahja-Tjendraputra *et al.*, 2007).

In the liver, there was significant ($P < 0.001$) up-regulation of TfR1, accompanied by a significant ($P < 0.001$) reduction in FTH levels (Figure 7A). This was consistent with the significant ($P < 0.05$) reduction in the total iron level in the liver after treatment with Bp44mT (Table 3). Cyclin D1 and p21^{CIP1/WAF1} levels in the liver were not significantly affected.

In contrast to these results in the liver, within the tumour, TfR1 was significantly ($P < 0.001$) decreased, while FTH was up-regulated ($P < 0.05$) (Figure 7B). These results suggest a condition of excess iron in the tumour rather than a lack of iron and were consistent with the increased tumour iron level (Table 3). Cyclin D1 and p21^{CIP1/WAF1} was also significantly ($P < 0.05$) down-regulated in the tumour (Figure 7B).

The expression of hypoxia inducible factor-1 α (HIF-1 α) in the tumour was also determined as this molecule is generally induced after iron chelation *in vitro* as a result of low iron levels (An *et al.*, 1998). This is observed because iron is necessary for the function of prolyl hydroxylase, which assists in proteasomal degradation of HIF-1 α (Semenza, 1999). There was no significant alteration in HIF-1 α protein expression in the xenografts after treatment with oral Bp44mT as compared with the control (Figure 7B), which is consistent with the observation that Bp44mT did not induce tumour iron depletion (Table 3).

In addition, the expression of downstream target of HIF-1 α , namely NDRG1 (Le and Richardson, 2004), in the tumour was not significantly altered after treatment with Bp44mT (Figure 7B), and despite repeated attempts, we were unable to identify Ndrgr1 expression in the liver. Previously, *NDRG1* mRNA expression was found to be up-regulated in tumour xenografts after i.v. Dp44mT (0.75 mg·kg⁻¹ per day, 5 days per week for 2 weeks) (Whitnall *et al.*, 2006). This does not agree with the protein expression observed in this study using oral Bp44mT (50 mg·kg⁻¹, 3 times per week for 3 weeks) and potentially could be due to the differences in dose and administration route and/or the different molecular targets between these two thiosemicarbazones.

Discussion

The current study examined the anti-tumour activity and toxicological effects of the next generation BpT iron chelator, Bp44mT (Kalinowski *et al.*, 2007). This compound demonstrated marked anti-tumour efficacy when given by either the i.v. or oral routes. Evaluation of the toxicity profile of Bp44mT demonstrated an appreciable difference when compared with Dp44mT. The major toxicological issue with Dp44mT was its effect at inducing cardiac fibrosis at non-optimal doses in a nude mouse model (Whitnall *et al.*, 2006). However, Bp44mT did not induce such pathology when administered via either the i.v. or oral routes even when given at much higher doses than Dp44mT.

Evaluation of Bp44mT revealed its influence on the liver, as suggested by the consistent dose-dependent increase in liver weight after i.v. and oral studies. Invariably, there was also an increase in the serum liver biochemical enzymes, ALP and ALT. Collectively, these results suggested some hepatic toxicity associated with Bp44mT therapy, although it must be noted that these serum enzyme activities are not direct measures of either the severity of liver injury or its function (Field *et al.*, 2008a). Moreover, these standard biochemical tests of liver function are often altered during chemotherapy, as well as after a variety of common treatments in current clinical use (Field and Michael, 2008). Liver tissue histology established that the increased liver weight in the treated mice was likely to be related to increased hepatocyte vacuolation, while sig-

A Mouse liver **B Human tumour xenograft** **Figure 7**

Oral Bp44mT differentially regulates the expression of transferrin receptor-1 (TfR1), ferritin heavy chain (FTH), cyclin D1 and the cyclin-dependent kinase inhibitor, p21^{CIP1/WAF1}, in the tumour xenograft as compared with liver in nude mice. (A) Oral administration of Bp44mT (50 mg·kg⁻¹, once per day, three times per week for 3 weeks) up-regulates the protein expression of the iron-regulated molecule, TfR1, while down-regulating FTH in the liver. Cyclin D1 and p21^{CIP1/WAF1} expression were not significantly altered in the liver when comparing the vehicle control and Bp44mT-treatment groups. (B) In contrast, the same treatment with oral Bp44mT as in (A) down-regulates the expression of TfR1, cyclin D1 and p21^{CIP1/WAF1}, while up-regulating FTH in human DMS-53 tumour xenografts. HIF-1α and NDRG1 expression were not significantly altered. Protein expression was determined by Western blotting from at least three to five different mice. The densitometric results are mean ± SEM from three experiments. **P* < 0.05; ****P* < 0.001.

nificantly there was no necrosis or fibrosis. Moreover, these hepatic changes related to Bp44mT treatment were largely reversible on stopping treatment.

Mechanistically, it was unclear as to the exact cause of Bp44mT-induced hepatic toxicity and little is known about the relationship between iron chelation therapy and liver injury. The clinically used, orally active, iron chelator, deferasiprone, which has been marketed for thalassemia treatment

in Europe, was suggested to cause hepatic fibrosis in patients (Olivieri *et al.*, 1998), although this remains controversial (Wanless *et al.*, 2002). The orally effective aroylhydrazone, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH), when administered to mice also increased liver weight when given at 200 mg·kg⁻¹ twice daily for 8 days, although histopathological examination showed no atypical characteristics (Wong *et al.*, 2004). Deferasirox is another

orally effective, clinically used iron chelator that can also elevate liver enzyme levels in patients and can be managed by discontinuation of treatment (Vichinsky, 2008). Collectively, these results indicate a general association between oral iron chelation therapy and mild hepatic toxicity, resulting in elevated serum liver enzymes.

In relation to the *in vivo* anti-tumour mechanism of Bp44mT, it remains to be investigated as to why iron was retained in the xenografts and was not markedly depleted when assessing tissue iron levels (Table 3). Examination of the iron-regulated molecules, TfR1 and FTH, also confirmed that iron depletion did not occur in tumour xenografts as opposed to the liver. This was an interesting observation, as classically it would be expected that a chelator could deplete the tumour of iron. Furthermore, our studies *in vitro* have shown that this ligand shows marked activity at mobilizing iron from cells in culture (Kalinowski *et al.*, 2007). Despite these seemingly paradoxical observations, it is notable that the current lack of tumour iron depletion *in vivo* was consistent with the observations made for the related chelator, Dp44mT, in a previous study from our laboratory (Whitnall *et al.*, 2006). The ability of thiosemicarbazones to chelate iron has been shown to be important for their anti-cancer activity (Yuan *et al.*, 2004). This was demonstrated from studies examining an analogue of the DpT series of ligands, namely di-2-pyridylketone-2-methyl-3-thiosemicarbazone (Dp2mT), which cannot bind metals due to a methyl substituent in the ligation site. This structural alteration blocks both its iron chelation efficacy and anti-proliferative activity (Yuan *et al.*, 2004). Hence, the iron-binding property of this series of compounds is crucial for their biological activity. However, from the current investigation and our previous study (Whitnall *et al.*, 2006), it is clear that overall depletion of iron in the tumour is not required for the anti-cancer activity observed.

The fact that iron depletion is not correlated to anti-tumour activity probably reflects the different mechanism of action of these thiosemicarbazone ligands, relative to that of DFO or deferiprone, compounds that have low anti-tumour efficacy and act simply to bind iron (Selig *et al.*, 1998; Simonart *et al.*, 2002). Indeed, in contrast to these latter chelators, which bind iron through hard oxygen donor atoms (Figure 1A), the thiosemicarbazones chelate iron through soft donors, namely nitrogen and sulphur (Richardson *et al.*, 2006; Kalinowski *et al.*, 2007). This leads to a marked alteration in biological activity, enabling the ligand to not only bind intracellular iron but also to form a cytotoxic redox-active chelator-iron complex within tumour cells (Richardson *et al.*, 2006; Kalinowski *et al.*, 2007; Jansson *et al.*, 2010). In fact, from previous structure-activity relationships studies, the redox activity of thiosemicarbazone-iron complexes has been shown to be a strong determinant of its anti-cancer activity (Richardson *et al.*, 2006; Kalinowski *et al.*, 2007). Hence, we suggest that the iron accumulation within the tumour could occur by the ligand entering the cell, binding iron and forming a cytotoxic iron complex that is then sequestered within the cancer cell. The iron complex of these ligands are highly lipophilic, and such a mechanism involving sequestration of the metal complex could explain the observations *in vivo* in this study and our previous investigation (Whitnall *et al.*, 2006), where significant tumour iron depletion was not observed.

Considering the tumour iron accumulation observed using Bp44mT, it is of interest to note that other studies in cell culture examining the related thiosemicarbazone, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT), showed that this agent has high membrane permeability (Merlot *et al.*, 2010). Moreover, Bp4eT evades rapid efflux leading to its cellular retention when compared to the iron chelator, pyridoxal isonicotinoyl hydrazone (PIH), which does not possess potent anti-tumour activity. In addition, it was shown that the intracellular level of Bp4eT in MCF-7 breast cancer and SK-N-MC neuroepithelioma cells were increased, compared with non-immortalized MRC-5 fibroblasts under confluent conditions (Merlot *et al.*, 2010). These observations highlight the intracellular retention of this class of ligands in neoplastic cells and may explain the accumulation of their iron complexes in tumours *in vivo*.

At the molecular level in the tumour, orally administered Bp44mT down-regulated the expression of several molecules, including TfR1, cyclin D1 and p21^{CIP1/WAF1}, but increased FTH expression (Figure 7B). The down-regulation of TfR1 and up-regulation of FTH in tumours could possibly be due to the iron sequestration within tumour cells (due potentially to accumulation of the Bp44mT-iron complex) and the sensing of this increased iron content via the well-known iron-regulatory protein-iron responsive element (IRP-IRE) mechanism (Hentze and Kuhn, 1996). An opposite response to Bp44mT was observed in the liver, where TfR1 and FTH expression were up- and down-regulated respectively (Figure 7A). These alterations in expression were in good agreement with the liver iron levels, which were significantly decreased after Bp44mT treatment (Table 3). Hence, in contrast to the tumour, the effect of the ligand on the liver was markedly different and could reflect the fundamental differences in metabolism between these tissues. It is also notable that the response of cyclin D1 and p21 expression to Bp44mT was also clearly different between the liver and tumour. Indeed, while there was no alteration in the expression of cyclin D1 and p21 in the liver, there was a significant decrease in expression of these molecules in the tumour. These cell cycle molecules play crucial functions in tumour growth (Schrump *et al.*, 1996; Arber *et al.*, 1997; Yang *et al.*, 2001), and the alterations in expression could, in part, explain the anti-cancer activity of Bp44mT. In fact, the down-regulation of cyclin D1 and p21^{CIP1/WAF1} in the tumour were consistent with previous observations examining the effect of iron chelators in cancer cells *in vitro* (Fu and Richardson, 2007; Nurtjahja-Tjendraputra *et al.*, 2007).

The function of p21^{CIP1/WAF1} in cell cycle regulation is complex, as the molecule has several effects. In addition to the well-known effect of p21^{CIP1/WAF1} over-expression on inhibiting cyclin-dependent kinase (CDK) activity and inducing a G₁ arrest (Xiong *et al.*, 1993), it also has a positive influence on the cell cycle (Sherr, 1994). Indeed, the assembly and activation of cyclin-CDK complexes which are necessary for cell cycle progression require a basal level of p21^{CIP1/WAF1} (Cheng *et al.*, 1999). Considering our results showing that the expression of cyclin D1 and p21^{CIP1/WAF1} were decreased after Bp44mT treatment, it is likely that this effect will promote G₁/S arrest since p21^{CIP1/WAF1} can stabilise cyclin D1-CDK complexes (Cheng *et al.*, 1999). Of relevance, it is well known that the closely related thiosemicarbazone, Dp44mT, induces a

G₁/S arrest and a similar response has also been observed after incubation of cells with a range of iron chelators, including DFO and 311 (Lalande, 1990; Brodie *et al.*, 1993; Gharagozloo *et al.*, 2008; Noulisri *et al.*, 2009). Furthermore, p21^{CIP1/WAF1} has also been shown to have anti-apoptotic activity (Janicke *et al.*, 2007), and high p21^{CIP1/WAF1} expression in some cancers can provide a growth advantage by subverting apoptosis induced by DNA-damaging chemotherapeutic agents (Weiss, 2003). In fact, a reduction of p21^{CIP1/WAF1} expression using anti-sense oligonucleotides can induce cancer cell apoptosis (Fan *et al.*, 2003). Hence, the decrease of p21^{CIP1/WAF1} expression after Bp44mT treatment may facilitate apoptosis in addition to its effect of preventing the assembly and activation of cyclin-cdk complexes.

Considering the mechanism of action of Bp44mT, it is notable that there was no alteration in HIF-1 α expression in the tumour, which was consistent with the observation that treatment with Bp44mT did not induce tumour iron depletion (Table 3), which is known to increase HIF-1 α expression (An *et al.*, 1998). Our previous studies on the BpT class of chelators demonstrated that Bp44mT forms a redox-active iron complex (Kalinowski *et al.*, 2007), which we believe is important for its anti-tumour activity. Moreover, as described above, we demonstrate after treatment of mice with Bp44mT that there was a marked decrease in cyclin D1 and p21^{CIP1/WAF1} expression in the tumours, which should prevent cell cycle progression and induce apoptosis (Fu and Richardson, 2007; Nurtjahja-Tjendraputra *et al.*, 2007). This effect on cyclin D1 and p21^{CIP1/WAF1} was not observed in the liver (Figure 7), suggesting selective anti-tumour activity. Together, these multiple cytotoxic mechanisms are probably important for the marked anti-tumour efficacy of Bp44mT.

In conclusion, Bp44mT can be administered orally with potent anti-tumour efficacy *in vivo*, and this agent is well tolerated in mice. Moreover, in contrast to Dp44mT, Bp44mT has the marked advantage of not causing cardiac toxicity or weight loss even when given at much higher doses. However, Bp44mT treatment was associated with mild reversible anaemia and hepatotoxicity. Collectively, these results demonstrate the substantial promise of this novel, orally active thiosemicarbazone for cancer therapy.

Acknowledgements

The authors acknowledge the critical comments on the manuscript prior to submission by Dr Katie Dixon, Dr Patric Jansson, Dr Danuta Kalinowski, Dr Zaklina Kovacevic, Dr Darius Lane, Dr David Lovejoy, Dr Helena Mangs and Ms. Angelica Merlot of the Iron Metabolism and Chelation Program, University of Sydney.

YY was supported by PhD scholarships from the University of Sydney (University Postgraduate Award) and Cancer Institute New South Wales. YSR was supported by a Cancer Institute New South Wales Early Career Development Fellowship. This research was supported by a Senior Principal Research Fellowship and Project Grants from the National Health and Medical Research Council of Australia (NHMRC) and a Discovery Grant from the Australian Research Council (ARC) to D.R.R.

Conflict of interest

The authors declare no competing financial interests.

References

- Abramoff MD, Magelhaes PJ, Ram SJ (2004). Image processing with ImageJ. *Biophoton Int* 11: 36–42.
- An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1 α . *Nature* 392: 405–408.
- Arber N, Doki Y, Han EK, Sgambato A, Zhou P, Kim NH *et al.* (1997). Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. *Cancer Res* 57: 1569–1574.
- Attia S, Kolesar J, Mahoney MR, Pitot HC, Laheru D, Heun J *et al.* (2008). A phase 2 consortium (P2C) trial of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) for advanced adenocarcinoma of the pancreas. *Invest New Drugs* 26: 369–379.
- Balsari A, Tortoreto M, Besusso D, Petrangolini G, Sfondrini L, Maggi R *et al.* (2004). Combination of a CpG-oligodeoxynucleotide and a topoisomerase I inhibitor in the therapy of human tumour xenografts. *Eur J Cancer* 40: 1275–1281.
- Blatt J (1994). Deferoxamine in children with recurrent neuroblastoma. *Anticancer Res* 14: 2109–2112.
- Brodie C, Siriwardana G, Lucas J, Schleicher R, Terada N, Szepesi A *et al.* (1993). Neuroblastoma sensitivity to growth inhibition by deferrioxamine: evidence for a block in G1 phase of the cell cycle. *Cancer Res* 53: 3968–3975.
- Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM *et al.* (1999). The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin d-dependent kinases in murine fibroblasts. *EMBO J* 18: 1571–1583.
- Donfrancesco A, Deb G, Dominici C, Pileggi D, Castello MA, Helson L (1990). Effects of a single course of deferrioxamine in neuroblastoma patients. *Cancer Res* 50: 4929–4930.
- Donfrancesco A, Deb G, Angioni A, Maurizio C, Cozza R, Jenkner A *et al.* (1993). D-CECaT: a breakthrough for patients with neuroblastoma. *Anticancer Drugs* 4: 317–321.
- Fan Y, Borowsky AD, Weiss RH (2003). An antisense oligodeoxynucleotide to p21(Waf1/Cip1) causes apoptosis in human breast cancer cells. *Mol Cancer Ther* 2: 773–782.
- Field KM, Michael M (2008b). Part II: liver function in oncology: towards safer chemotherapy use. *Lancet Oncol* 9: 1181–1190.
- Field KM, Dow C, Michael M (2008a). Part I: liver function in oncology: biochemistry and beyond. *Lancet Oncol* 9: 1092–1101.
- Finch CA (1982). Erythropoiesis, erythropoietin, and iron. *Blood* 60: 1241–1246.
- Finch RA, Liu MC, Cory AH, Cory JG, Sartorelli AC (1999). Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-AP): an inhibitor of ribonucleotide reductase with antineoplastic activity. *Adv Enzyme Regul* 39: 3–12.
- Fu D, Richardson DR (2007). Iron chelation and regulation of the cell cycle: 2 mechanisms of posttranscriptional regulation of the universal cyclin-dependent kinase inhibitor p21CIP1/WAF1 by iron depletion. *Blood* 110: 752–761.

- Gao J, Richardson DR (2001). The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents, IV: the mechanisms involved in inhibiting cell-cycle progression. *Blood* 98: 842–850.
- Gharagozloo M, Khoshdel Z, Amirghofran Z (2008). The effect of an iron (III) chelator, silybin, on the proliferation and cell cycle of Jurkat cells: a comparison with desferrioxamine. *Eur J Pharmacol* 589: 1–7.
- Hentze MW, Kuhn LC (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc Natl Acad Sci USA* 93: 8175–8182.
- Hershko C (1994). Control of disease by selective iron depletion: a novel therapeutic strategy utilizing iron chelators. *Baillieres Clin Haematol* 7: 965–1000.
- Janicke RU, Sohn D, Essmann F, Schulze-Osthoff K (2007). The multiple battles fought by anti-apoptotic p21. *Cell Cycle* 6: 407–413.
- Jansson PJ, Hawkins CL, Lovejoy DB, Richardson DR (2010). The iron complex of Dp44mT is redox-active and induces hydroxyl radical formation: an EPR study. *J Inorg Biochem* 104: 1224–1228.
- Kalinowski DS, Richardson DR (2005). The evolution of iron chelators for the treatment of iron overload disease and cancer. *Pharmacol Rev* 57: 547–583.
- Kalinowski DS, Yu Y, Sharpe PC, Islam M, Liao YT, Lovejoy DB *et al.* (2007). Design, synthesis, and characterization of novel iron chelators: structure-activity relationships of the 2-benzoylpyridine thiosemicarbazone series and their 3-nitrobenzoyl analogues as potent antitumor agents. *J Med Chem* 50: 3716–3729.
- Karp JE, Giles FJ, Gojo I, Morris L, Greer J, Johnson B *et al.* (2008). A phase I study of the novel ribonucleotide reductase inhibitor 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine) in combination with the nucleoside analog fludarabine for patients with refractory acute leukemias and aggressive myeloproliferative disorders. *Leuk Res* 32: 71–77.
- Kingston HM, Jassie LB (1988). Introduction to Microwave Sample Preparation – Theory and Practice. American Chemical Society Professional Reference Book: Washington D.C.
- Lalande M (1990). A reversible arrest point in the late G1 phase of the mammalian cell cycle. *Exp Cell Res* 186: 332–339.
- Le NT, Richardson DR (2003). Potent iron chelators increase the mRNA levels of the universal cyclin-dependent kinase inhibitor p21(CIP1/WAF1), but paradoxically inhibit its translation: a potential mechanism of cell cycle dysregulation. *Carcinogenesis* 24: 1045–1058.
- Le NT, Richardson DR (2004). Iron chelators with high antiproliferative activity up-regulate the expression of a growth inhibitory and metastasis suppressor gene: a link between iron metabolism and proliferation. *Blood* 104: 2967–2975.
- Liu DY, Liu ZD, Hider RC (2002). Oral iron chelators – development and application. *Best Pract Res Clin Haematol* 15: 369–384.
- Lovejoy DB, Richardson DR (2002). Novel ‘hybrid’ iron chelators derived from aroylhydrazones and thiosemicarbazones demonstrate selective antiproliferative activity against tumor cells. *Blood* 100: 666–676.
- Ma B, Goh BC, Tan EH, Lam KC, Soo R, Leong SS *et al.* (2008). A multicenter phase II trial of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine) and gemcitabine in advanced non-small-cell lung cancer with pharmacokinetic evaluation using peripheral blood mononuclear cells. *Invest New Drugs* 26: 169–173.
- Mackenzie MJ, Saltman D, Hirte H, Low J, Johnson C, Pond G *et al.* (2007). A Phase II study of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) and gemcitabine in advanced pancreatic carcinoma. A trial of the Princess Margaret hospital Phase II consortium. *Invest New Drugs* 25: 553–558.
- Merlot AM, Pantarat N, Lovejoy DB, Kalinowski DS, Richardson DR (2010). Membrane transport and intracellular sequestration of novel thiosemicarbazone chelators for the treatment of cancer. *Mol Pharmacol* 78: 675–684.
- Noulsri E, Richardson DR, Lerdwana S, Fucharoen S, Yamagishi T, Kalinowski DS *et al.* (2009). Antitumor activity and mechanism of action of the iron chelator, Dp44mT, against leukemic cells. *Am J Hematol* 84: 170–176.
- Nurtjahja-Tjendraputra E, Fu D, Phang JM, Richardson DR (2007). Iron chelation regulates cyclin D1 expression via the proteasome: a link to iron deficiency-mediated growth suppression. *Blood* 109: 4045–4054.
- Olivieri NF, Brittenham GM (1997). Iron-chelating therapy and the treatment of thalassemia. *Blood* 89: 739–761.
- Olivieri NF, Brittenham GM, McLaren CE, Templeton DM, Cameron RG, McClelland RA *et al.* (1998). Long-term safety and effectiveness of iron-chelation therapy with deferiprone for thalassemia major. *N Engl J Med* 339: 417–423.
- Rahmanto YS, Richardson DR (2009). Generation and characterization of transgenic mice hyper-expressing melanoma tumour antigen p97 (Melanotransferin): no overt alteration in phenotype. *Biochim Biophys Acta* 1793: 1210–1217.
- Richardson DR, Baker E (1990). The uptake of iron and transferrin by the human malignant melanoma cell. *Biochim Biophys Acta* 1053: 1–12.
- Richardson DR, Milnes K (1997). The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents II: the mechanism of action of ligands derived from salicylaldehyde benzoyl hydrazone and 2-hydroxy-1-naphthylaldehyde benzoyl hydrazone. *Blood* 89: 3025–3038.
- Richardson DR, Tran EH, Ponka P (1995). The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood* 86: 4295–4306.
- Richardson DR, Sharpe PC, Lovejoy DB, Senaratne D, Kalinowski DS, Islam M *et al.* (2006). Dipyriddy thiosemicarbazone chelators with potent and selective antitumor activity form iron complexes with redox activity. *J Med Chem* 49: 6510–6521.
- Sanceau J, Poupon MF, Delattre O, Sastre-Garau X, Wietzerbin J (2002). Strong inhibition of Ewing tumor xenograft growth by combination of human interferon-alpha or interferon-beta with ifosfamide. *Oncogene* 21: 7700–7709.
- Schrump DS, Chen A, Consoli U (1996). Inhibition of lung cancer proliferation by antisense cyclin D. *Cancer Gene Ther* 3: 131–135.
- Selig RA, White L, Gramacho C, Sterling-Levis K, Fraser IW, Naidoo D (1998). Failure of iron chelators to reduce tumor growth in human neuroblastoma xenografts. *Cancer Res* 58: 473–478.
- Semenza GL (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 15: 551–578.
- Shen DW, Fojo A, Roninson IB, Chin JE, Soffir R, Pastan I *et al.* (1986). Multidrug resistance of DNA-mediated transformants is linked to transfer of the human mdr1 gene. *Mol Cell Biol* 6: 4039–4045.

- Sherr CJ (1994). G1 phase progression: cycling on cue. *Cell* 79: 551–555.
- Simonart T, Boelaert JR, Andrei G, van den Oord JJ, Degraef C, Hermans P *et al.* (2002). Desferrioxamine enhances AIDS-associated Kaposi's sarcoma tumor development in a xenograft model. *Int J Cancer* 100: 140–143.
- Sparreboom A, de Jonge MJ, Verweij J (2002). The use of oral cytotoxic and cytostatic drugs in cancer treatment. *Eur J Cancer* 38: 18–22.
- Summers MR, Jacobs A, Tudway D, Perera P, Ricketts C (1979). Studies in desferrioxamine and ferrioxamine metabolism in normal and iron-loaded subjects. *Br J Haematol* 42: 547–555.
- Suttie AW (2006). Histopathology of the spleen. *Toxicol Pathol* 34: 466–503.
- Vichinsky E (2008). Clinical application of deferasirox: practical patient management. *Am J Hematol* 83: 398–402.
- Wanless IR, Sweeney G, Dhillon AP, Guido M, Piga A, Galanello R *et al.* (2002). Lack of progressive hepatic fibrosis during long-term therapy with deferiprone in subjects with transfusion-dependent beta-thalassemia. *Blood* 100: 1566–1569.
- Weiss RH (2003). p21Waf1/Cip1 as a therapeutic target in breast and other cancers. *Cancer Cell* 4: 425–429.
- Whitnall M, Howard J, Ponka P, Richardson DR (2006). A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. *Proc Natl Acad Sci USA* 103: 14901–14906.
- Wong CS, Kwok JC, Richardson DR (2004). PCTH: a novel orally active chelator of the aroylhydrazone class that induces iron excretion from mice. *Biochim Biophys Acta* 1739: 70–80.
- Wu CP, Shukla S, Calcagno AM, Hall MD, Gottesman MM, Ambudkar SV (2007). Evidence for dual mode of action of a thiosemicarbazone, NSC73306: a potent substrate of the multidrug resistance linked ABCG2 transporter. *Mol Cancer Ther* 6: 3287–3296.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* 366: 701–704.
- Yang DC, Jiang XP, Elliott RL, Head JF (2001). Inhibition of growth of human breast carcinoma cells by an antisense oligonucleotide targeted to the transferrin receptor gene. *Anticancer Res* 21: 1777–1787.
- Yu Y, Kalinowski DS, Kovacevic Z, Siafakas AR, Jansson PJ, Stefani C *et al.* (2009). Thiosemicarbazones from the old to new: iron chelators that are more than just ribonucleotide reductase inhibitors. *J Med Chem* 52: 5271–5294.
- Yuan J, Lovejoy DB, Richardson DR (2004). Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: in vitro and in vivo assessment. *Blood* 104: 1450–1458.