
Zaklina Kovacevic, Sherin Chikhani, David B. Lovejoy, and Des R. Richardson

Iron Metabolism and Chelation Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, New South Wales, Australia

Received May 18, 2011; accepted June 30, 2011

ABSTRACT
Pancreatic cancer is an aggressive neoplasm, with a mortality rate close to 100%. The most successful agent for pancreatic cancer treatment is gemcitabine, although the overall effect in terms of patient survival remains very poor. This study was initiated to evaluate a novel class of anticancer agents against pancreatic cancer. This group of compounds belongs to the dipyridyl thiosemicarbazone class that have been shown to have potent and selective activity against a range of different neoplasms in vitro and in vivo. We demonstrate for the first time in pancreatic cancer that these agents increase the expression of the growth and metastasis suppressor N-myc downstream-regulated gene 1 and its phosphorylation at Ser330 and Thr346 that is important for its activity against this tumor. In addition, these agents increased expression of the cyclin-dependent kinase inhibitor p21CIP1/WAF1, whereas decreasing cyclin D1 in pancreatic cancer cells. Together, these molecular alterations account, in part, for the pronounced antitumor activity observed. Indeed, these agents had significantly higher antiproliferative activity in vitro than the established treatments for pancreatic cancer, namely gemcitabine and 5-fluorouracil. Studies in vivo demonstrated that a novel thiosemicarbazone, namely di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone hydrochloride, completely inhibited the growth of pancreatic cancer xenografts with no evidence of marked alterations in normal tissue histology. Together, our studies have identified molecular effectors of a novel and potent antitumor agent that could be useful for pancreatic cancer treatment.

Introduction
Pancreatic cancer is a devastating disease that is fatal in 98 to 100% of cases, with the survival from this disease being the same today as it was 20 years ago (Jemal et al., 2009). Although there have been increasing efforts to better understand the pathogenesis and improve treatment options for pancreatic cancer (Custodio et al., 2009; Furukawa, 2009), the prognosis for those with this illness remains poor. The standard treatment for pancreatic cancer is the anticancer agent gemcitabine (Fig. 1A), often given in combination with other chemotherapeutics such as 5-fluorouracil (Fig. 1B) (Custodio et al., 2009). Gemcitabine is an analog of the nucleoside deoxycytidine, which functions to inhibit ribonucleotide reductase and also initiates DNA strand termination and apoptosis (Wong et al., 2009). However, the success of gemcitabine and its combinations with other agents for pancreatic cancer treatment has been limited, with an average increase in patient lifespan of only 3 months (Custodio et al., 2009).

Considering the highly aggressive nature of this disease and the limited progress in the development of effective therapeutic strategies, we sought to examine a new approach to pancreatic cancer treatment that involves targeting the product of the growth and metastasis suppressor N-myc down-streamregulated gene 1, NDRG1.
stream-regulated gene 1 (NDRG1) (Kovacevic and Richardson, 2006; Ellen et al., 2008). This latter protein inhibits both growth and metastasis and angiogenesis of pancreatic cancer in vivo, leading to reduced tumor progression (Maruyama et al., 2004). Moreover, NDRG1 expression has also been correlated with increased differentiation of pancreatic cancers (Angst et al., 2006). Therefore, NDRG1 may be a promising therapeutic target for the treatment of this disease.

One potential strategy for targeting NDRG1 in pancreatic cancer is through the use of novel thiosemicarbazones, which have been demonstrated previously to up-regulate NDRG1 in vitro and in vivo via their ability to increase the hypoxia-inducible factor-1 (HIF-1) (Le and Richardson, 2004; Whitnall et al., 2006; Kovacevic et al., 2008). The mechanism involved in this effect is mediated through the binding of intracellular iron by thiosemicarbazones and other iron chelators, which inhibits HIF-1α degradation (Le and Richardson, 2004).

Iron is an essential element necessary for a variety of crucial metabolic processes, including ribonucleotide reductase, which catalyzes the rate-limiting step in DNA synthesis (Kalinowski and Richardson, 2005). The suitability of iron chelators as anticancer agents was first discovered when the iron chelator desferrioxamine (DFO; Fig. 1C), which is primarily used for iron-overload diseases such as α-thalassemia (Aouad et al., 2002), was successfully used in clinical trials for neuroblastoma (Buss et al., 2003). Since then, iron chelators designed specifically for the treatment of cancer have been developed with the thiosemicarbazone 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP; Fig. 1D), entering a wide variety of phase I and II clinical trials (Landry et al., 2010). However, the latter agent has shown considerable problems, including low efficacy and serious side effects, including methemoglobinemia and hypoxia (Kalinowski and Richardson, 2005).

Thiosemicarbazones can bind both iron and copper, leading to the formation of redox-active complexes that produce reactive oxygen species (ROS) that induce cancer cell cytotoxicity (Yuan et al., 2004; Kalinowski and Richardson, 2005; Jansson et al., 2010). One of the most active thiosemicarbazones developed to date is di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1E) (Yuan et al., 2004; Kalinowski and Richardson, 2005; Whitnall et al., 2006).

Dp44mT has been demonstrated to markedly reduce the growth of multiple tumors in vitro and in vivo, being more potent and less toxic than 3-AP (Whitnall et al., 2006). However, studies using high, nonoptimal doses of Dp44mT found that it induced cardiotoxicity in nude mice (Whitnall et al., 2006). Hence, in an effort to develop highly potent yet less toxic thiosemicarbazones, Dp44mT was modified to generate a novel second-generation thiosemicarbazone, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone hydrochloride (DpC; Fig. 1F).

The aim of this study was to examine the mechanism of action and the activity of Dp44mT and its novel analog, DpC, in vitro and in vivo. We demonstrate that these thiosemicarbazones affect a variety of molecular targets including NDRG1, p21WAF1/CIP1, and cyclin D1 and are significantly more effective at inhibiting proliferation and inducing apoptosis in vitro compared with the current agent of choice, gemcitabine, in three of four pancreatic cancer cell types. Furthermore, in vivo studies showed that DpC completely inhibits pancreatic tumor growth, being significantly more effective and less toxic than Dp44mT. Hence, DpC may be an effective new treatment strategy against pancreatic cancer.

Materials and Methods

Cell Culture. The pancreatic cancer cell lines MIAPaCa-2, PANC-1, CAPAN-2, and CFPAC-1 were from the American Type Culture Collection (Manassas, VA). MIAPaCa-2 and PANC-1 cells are both epithelial cells that were derived from pancreatic carcinomas. CAPAN-2 cells are polygonal cells derived from a pancreatic adenocarcinoma, whereas CFPAC-1 cells are epithelial cells derived from a liver metastasis that originated from a pancreatic adenocarcinoma.

The MIAPaCa-2, PANC-1, and CFPAC-1 cell types were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Sydney, Australia), whereas CAPAN-2 cells were grown in McCoy’s medium (Invitrogen). All media were supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (all from Invitrogen). Cells were grown in an incubator (Thermo Fisher Scientific, Waltham, MA) at 37°C in a humidified atmosphere of 5% CO2 and 95% air and subcultured by standard methods, as described previously (Le and Richardson, 2004).

Reagents. Gemcitabine (Gemzar) was purchased from Eli Lilly & Co. (Indianapolis, IN). 5-Fluorouracil was obtained from Sigma-
Aldrich (St. Louis, MO). DFO was from Novartis (Basel, Switzerland). The iron chelator Dp44mT was synthesized and characterized as described previously (Richardson et al., 2006).

The novel iron chelator DpC was synthesized using a combination of established methods (Scovill, 1990; Richardson et al., 2006). In brief, carbon disulfide (0.2 mol) was added drop-wise to N-methylcyclohexylamine (0.2 mol) in NaOH solution (250 ml, 0.8 M) and allowed to react until the organic layer almost disappeared. Next, sodium chloroacetate (0.2 mol) was added to the aqueous extract and allowed to react overnight at room temperature. The addition of concentrated HCl (25 ml) produced the solid carboxymethyl thiocarbanilate intermediate. Then, 0.08 mol of the latter compound was dissolved in 20 ml of hydrzone hydrate plus 10 ml of water. This was followed by five cycles of gentle heating until fuming) and cooling. The solution was then allowed to stand until fine white crystals of thiosemicarbazide formed. A solution of the thiosemicarbazide (10 mmol) in water (15 ml) was added to di-2-pyridyl ketone (10 mmol) dissolved in EtOH (15 ml). Next, five drops of glacial acetic acid was added, and the mixture was refluxed for 2 h and cooled to 5°C to give the yellow Dp4cyH4mT precipitate. Finally, Dp4cyH4mT was dissolved in a minimum volume of ice-cold hexane, and equimolar HCl was added to create the HCl salt Dp4cych4mT-HCl (DpC). The purity of the compound was characterized using a combination of elemental analysis (calculated: C, 47.52%; H, 6.82%; N, 14.58%; Found: C, 47.04%; H, 6.54%; N, 15.02%; Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia), infrared spectroscopy, mass spectroscopy, and 1H NMR spectroscopy (data not shown).

Western Blot Analysis. Protein isolation was performed as described previously (Dunn et al., 2006), and Western analysis was achieved via established protocols (Gao and Richardson, 2001). The primary antibodies used were goat anti-human NDRG1 (Abcam Inc., Cambridge, MA); rabbit anti-human p21(WAF1/CIP1), rabbit anti-human pNDRG1 (Ser330), rabbit anti-human pNDRG1 (Thr346), rabbit anti-human cleaved PARP, rabbit anti-human Bax, and rabbit anti-human Bcl-2 (all from Cell Signaling Technology, Danvers, MA); and mouse anti-human cyclin D1 and β-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA).

Flow Cytometry. Flow cytometry using Annexin V and propidium iodide (PI) labeling was used to examine apoptosis in response to the thiosemicarbazones and gemcitabine using standard methods (Yuan et al., 2004). In brief, cells were seeded in T25 flasks and allowed to adhere overnight. The cells were then treated with either 10 or 20 μM gemcitabine, Dp44mT, or DpC and incubated for 48 h at 37°C. The cells were harvested and prepared using the Annexin V apoptosis kit (BD Biosciences, San Jose, CA) following the manufacturer’s instructions and examined using a FACScalibur flow cytometer (BD Biosciences). Results were analyzed using CellQuest software (BD Biosciences).

MTT Cellular Proliferation Assay. Cell proliferation was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT; Sigma-Aldrich) assay after a 72-h/37°C incubation (data not shown). Western Blot Analysis. Protein isolation was performed as described previously (Dunn et al., 2006), and Western analysis was achieved via established protocols (Gao and Richardson, 2001). The primary antibodies used were goat anti-human NDRG1 (Abcam Inc., Cambridge, MA); rabbit anti-human p21(WAF1/CIP1), rabbit anti-human pNDRG1 (Ser330), rabbit anti-human pNDRG1 (Thr346), rabbit anti-human cleaved PARP, rabbit anti-human Bax, and rabbit anti-human Bcl-2 (all from Cell Signaling Technology, Danvers, MA); and mouse anti-human cyclin D1 and β-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA).

Flow Cytometry. Flow cytometry using Annexin V and propidium iodide (PI) labeling was used to examine apoptosis in response to the thiosemicarbazones and gemcitabine using standard methods (Yuan et al., 2004). In brief, cells were seeded in T25 flasks and allowed to adhere overnight. The cells were then treated with either 10 or 20 μM gemcitabine, Dp44mT, or DpC and incubated for 48 h at 37°C. The cells were harvested and prepared using the Annexin V apoptosis kit (BD Biosciences, San Jose, CA) following the manufacturer’s instructions and examined using a FACScalibur flow cytometer (BD Biosciences). Results were analyzed using CellQuest software (BD Biosciences).

MTT Cellular Proliferation Assay. Cell proliferation was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT; Sigma-Aldrich) assay after a 72-h/37°C incubation using standard methods (Richardson et al., 1995). As shown previously, MTT color formation was directly proportional to the number of viable cells (Richardson et al., 1995), validating its use in these experiments. Maximum Tolerated Dose Studies in Nude Mice. In vivo experiments were approved by the Animal Ethics Committee (University of Sydney). Before studies assessing antitumor activity of the novel thiosemicarbazone DpC were initiated, maximum tolerated dose (MTD) experiments were performed, as described previously (Yuan et al., 2004; Whitnall et al., 2006) using BALBc nu/nu nude mice (Animal Resources Facility, Perth, Western Australia). The MTD was defined as the dose at which 30% of the cohort was killed because of markedly deteriorating health or lost body weight in excess of 10% (Yuan et al., 2004; Whitnall et al., 2006).

Tumor Xenografts in Nude Mice. In these studies, 8-week-old female nude mice (BALBc nu/nu) were used and tumor xenografts established by standard techniques (Whitnall et al., 2006). In brief, each mouse was injected subcutaneously with 2 × 106 PANC-1 cells suspended in Matrigel (BD Biosciences). Tumor size was measured by Vernier calipers, and tumor volume was calculated as described previously (Balsari et al., 2004). Once the tumors reached an average of 90 mm3, the treatment began (day 0; Fig. 7A). The chelators, Dp44mT and DpC, were dissolved in 30% propylene glycol in 0.9% saline and injected intravenously (via the tail vein) 5 days/week (Monday to Friday) (Whitnall et al., 2006). Gemcitabine was dissolved in 15% propylene glycol/0.9% saline and injected intraperitoneally every third day as per an established protocol (Laquente et al., 2008). Each group of mice (n = 8) received either gemcitabine (5 mg/kg), Dp44mT (0.4 mg/kg), DpC (5 mg/kg), or the vehicle control. This treatment regimen was implemented based on the MTD studies performed in our laboratory and previous studies using these agents (Whitnall et al., 2006; Laquente et al., 2008). The vehicle control group was subdivided into two groups (n = 4) with the first group receiving an intravenous injection of 30% propylene glycol/0.9% saline, 5 days/week, which acted as a control for the iron chelator treatment group. The second control group received 15% propylene glycol/0.9% saline intraperitoneally every third day and was the appropriate control for the gemcitabine treatment. Once control tumors reached 1000 mm3, the animals were euthanized because of ethical requirements.

Hematology and Histology. Upon completion of the in vivo experiment, blood was collected by cardiac puncture and hematological indices assayed by standard methods (Dunn et al., 2006). Tissues, including organs and tumors, were embedded in paraffin blocks and sectioned. Three different stains were used, namely hematoxylin and eosin, Perls, or Gomori-Trichrome. The histological analysis and quantification of pathological features was performed by an independent veterinary pathologist, Dr. Terrence Rathwell (Rathwell Consulting, Avalon Beach, NSW, Australia).

Statistical Analysis. Data were compared by using the Student’s t test. Results were expressed as mean ± S.D. unless otherwise indicated. Data were considered statistically significant when p < 0.05.

Results

In Vitro Analysis of Novel Thiosemicarbazones for the Treatment of Pancreatic Cancer. To assess the efficacy of novel thiosemicarbazones against pancreatic cancer and to compare their activity to gemcitabine, we first performed in vitro studies examining crucial molecular targets. These included the growth and metastasis suppressor, NDRG1 (Kovacevic and Richardson, 2006), the cyclin-dependent kinase inhibitor, p21(WAF1/CIP1), the cyclin-depent transcription factor, WAF1/CIP1, and the growth and metastasis suppressor, P300 (Kovacevic et al., 2009). These included the growth and metastasis suppressor, NDRG1 (Kovacevic and Richardson, 2006), the cyclin-dependent kinase inhibitor, p21(WAF1/CIP1) (Yu et al., 2007), and cyclin D1 that is necessary for cell cycle progression (Yu et al., 2007). Moreover, a number of apoptosis markers, as well as the ability of these agents to induce apoptosis, was also investigated. We also examined the anti-proliferative activity of DFO, Dp44mT and DpC against four different pancreatic cancer cell types in vitro compared with the standard chemotherapeutics for this disease, namely gemcitabine and 5-fluorouracil (Custodio et al., 2009).

Thiosemicarbazones Up-Regulate the Growth and Metastasis Suppressor NDRG1 in Pancreatic Cancer Cells. In the current study, we examined the effect of the novel thiosemicarbazones, Dp44mT and DpC, and gemcitabine on total and phosphorylated NDRG1 (Ser330 and Thr346) expression in four pancreatic cancer cell types (Fig. 2, A–D). This was crucial to assess considering the widely reported antitumor function of NDRG1 (Kovacevic and Richardson, 2006; Ellen et al., 2008) and its potential as a prom...
ising therapeutic target against pancreatic cancer (Angst et al., 2006; Maruyama et al., 2006). Four pancreatic tumor cell types, namely MIAPaCa-2, PANC-1, CAPAN-2, and CFPAC-1 were incubated with 5 or 10 μM Dp44mT, DpC or gemcitabine for 24 h/37°C, and NDRG1 protein expression was then examined. Our results demonstrate that both Dp44mT and DpC significantly (p < 0.05) up-regulated total and phosphorylated (Ser330 and Thr346) NDRG1 protein levels in all cell types examined (Fig. 2, A–D). On the other hand, gemcitabine did not significantly (p > 0.05) alter total or phosphorylated NDRG1 expression in any of the cell types assessed compared with the untreated controls (Fig. 2).

As demonstrated previously (Murray et al., 2004; Kovacevic et al., 2011), we observed two bands for total NDRG1 in each cell type examined (migrating at 43 and 44 kDa; Fig. 2), and these may represent the different phosphorylation states of this protein, as has been described previously (Murray et al., 2004). Considering that both Dp44mT and DpC significantly (p < 0.05) up-regulated the top band of NDRG1, as well as the two different phosphorylated forms of this protein (Ser330 and Thr346; at 44 kDa) in each cell-type examined (Fig. 2), the top band may correspond to its phosphorylated form. It is important to note that we also observed a very faint lower band when probing with the antibody for Thr346 phosphorylated NDRG1, which may indicate another NDRG1 isoform. Although the biological relevance of the different phosphorylation states of NDRG1 is yet to be conclusively determined, a recent study has demonstrated that phosphorylation of NDRG1 is important for its antitumor function in pancreatic cancer (Murakami et al., 2010). Thus, our current results are important for understanding the antitumor activity of these thiosemicarbazones.

**Novel Thiosemicarbazones Modulate Other Key Proteins Involved in Cell Cycle Progression, Namely p21\(^{CIP1/WAF1}\) and Cyclin D1.** We recently discovered that NDRG1 can up-regulate the expression of the cyclin-dependent kinase inhibitor p21\(^{CIP1/WAF1}\) in a variety of cancer cell types (Kovacevic et al., 2011). Considering this, together with the fact that p21\(^{CIP1/WAF1}\) is regulated by cellular iron levels (Fu and Richardson, 2007) and that it plays a crucial role in preventing G1/S progression (Yu et al., 2007), we further examined the effect of our novel thiosemicarbazones and gemcitabine on p21\(^{CIP1/WAF1}\) expression. Moreover, we also investigated the expression of another crucial protein involved in cell cycle progression, namely cyclin D1, which has been demonstrated to be markedly decreased by iron chelators in cancer cells (Nurtjahja-Tjendraputra et al., 2007) and is another potential molecular target of thiosemicarbazones.

MIAPaCa-2, PANC-1, CFPAC-1, and CAPAN-2 cells were incubated with either gemcitabine, Dp44mT, or DpC at a concentration of 5 or 10 μM for 24 h/37°C, and protein levels of p21\(^{CIP1/WAF1}\) and cyclin D1 were examined. Both Dp44mT
and DpC significantly \((p < 0.05)\) increased \(p21^{\text{CIP1/WAF1}}\) expression, whereas significantly \((p < 0.05)\) reducing cyclin D1 levels in each of the four cell types examined (Fig. 3, A–D). It is noteworthy that gemcitabine was also able to markedly reduce cyclin D1 levels in the MIAPaCa-2, PANC-1, and CFPAC-1 cells, whereas having no significant effect in CAPAN-2 cells (Fig. 3C). On the other hand, gemcitabine reduced \(p21^{\text{CIP1/WAF1}}\) expression in the PANC-1, CFPAC-1, and CAPAN-2 cells, with no effect being observed in MIAPaCa-2 cells relative to the control (Fig. 3). Hence, the molecular effects of gemcitabine seem to be cell type-dependent.

Together, the results above demonstrate that NDRG1 and \(p21^{\text{CIP1/WAF1}}\) are markedly up-regulated, whereas cyclin D1 is reduced in the pancreatic cancer cell types by Dp44mT and DpC. Considering the antitumor function of NDRG1 in pancreatic cancer (Maruyama et al., 2006), these results suggest that thiosemicarbazones may be a beneficial treatment strategy against this disease. Hence, further in vitro studies examining the anti-proliferative efficacy of these agents were performed.

**Novel Thiosemicarbazones Are Significantly More Effective at Inhibiting Proliferation of Pancreatic Cancer Cells In Vitro Compared with Gemcitabine and 5-Fluorouracil.** To examine the antiproliferative activity of Dp44mT and DpC against pancreatic cancer in vitro, we performed MTT proliferation assays with each of the four pancreatic cancer cell types studied above compared with currently used treatments for this disease, namely gemcitabine and 5-fluorouracil (Custodio et al., 2009). Moreover, as a further control, we also examined the well characterized iron chelator DFO (Kalinowski and Richardson, 2005).

Examining the MIAPaCa-2, PANC-1, and CAPAN-2 cell types, the highest antiproliferative activity was observed with Dp44mT and DpC (Fig. 4, A–C) with their IC\(_{50}\) values being significantly \((p < 0.01)\) lower compared with gemcitabine and 5-fluorouracil (Table 1). In fact, the IC\(_{50}\) values for Dp44mT and DpC were at least 4- and 2000-fold lower in three of the four cell types compared with gemcitabine and 5-fluorouracil, respectively (Table 1). On the other hand, DFO had relatively low antiproliferative activity, being significantly \((p < 0.001)\) less effective than the thiosemicarbazones probably because of its low membrane permeability (Kalinowski and Richardson, 2005).

In contrast to the other cell types in which the thiosemicarbazones had the highest antiproliferative activity, CFPAC-1 cells were more sensitive to gemcitabine than either Dp44mT or DpC (Fig. 4D). In fact, the IC\(_{50}\) value for gemcitabine was significantly \((p < 0.05)\) lower than that of Dp44mT or DpC (Fig. 4D; Table 1). However, it is notable that Dp44mT and DpC had lower IC\(_{90}\) values than gemcitabine in CFPAC-1 cells (Fig. 4D; Supplemental Table 1), suggesting that the thiosemicarbazones are effective at inhibiting proliferation of this cell type in vitro when used at higher concentrations (Fig. 4D). These results demonstrate the heterogeneity of pancreatic cancer and may reflect the
different molecular alterations that determine sensitivity to anticancer agents (Furukawa, 2009).

Both Gemcitabine and the Novel Thiosemicarbazones Dp44mT and DpC Induce Apoptosis in Pancreatic Cancer Cells. Considering that Dp44mT and DpC significantly altered the expression of a number of proteins that play key roles in growth and metastasis, namely NDRG1, p21\(^{\text{CIP1/WAF1}}\), and cyclin D1, we further examined the effects of these agents by assessing apoptosis in the four cell types compared with gemcitabine.

Each cell type was incubated with 5 or 10 \(\mu\)M concentrations of either gemcitabine, Dp44mT, or DpC for 24 h and a number of apoptosis markers assessed, including cleaved PARP, Bax, and Bcl-2 (Tang and Porter, 1996). We observed that cleaved PARP was most effectively up-regulated by gemcitabine in the MIAPaCa-2 and CFPAC-1 cell types compared with the other pancreatic cancer cells examined (Fig. 5), and this corresponds to the higher sensitivity of these cells to gemcitabine (Fig. 4 and Table 1). However, PARP was also cleaved by the thiosemicarbazones in these cells types, but to a lesser degree than gemcitabine (Fig. 5, A and D). It is noteworthy that PANC-1 and CAPAN-2 cells were more or similarly sensitive to the thiosemicarbazones than gemcitabine, with Dp44mT (10 \(\mu\)M) being the most efficient at inducing cleaved PARP (Fig. 5, B and C).

Expression of proapoptotic Bax (Tang and Porter, 1996) was up-regulated by each of the compounds examined in all four cell types with the exception being Dp44mT in MIAPaCa-2 cells, in which no significant effect was observed (Fig. 5A). Moreover, the expression of the antiapoptotic protein Bcl-2 (Tang and Porter, 1996) was significantly \((p < 0.01)\) reduced by the thiosemicarbazones in each of the cell types assessed, whereas gemcitabine only markedly reduced Bcl-2 in CFPAC-1 cells (Fig. 5). Further studies examining the expression of these molecules after a 48-h incubation with these agents revealed generally similar results to that observed after 24 h (Supplemental Fig. 1). The results above highlight the different sensitivities of the pancreatic cancer cells to the agents examined, demonstrating various molecular responses that indicate the induction of apoptosis. To clarify whether apoptosis was occurring, and to what degree, we further examined pancreatic cancer cells by flow cytometry after incubation with these agents.

Using Annexin V and PI labeling, we examined apoptosis in each cell type via flow cytometry after treatment with either 10 or 20 \(\mu\)M concentration of gemcitabine, Dp44mT, or DpC for 48 h/37°C. These concentrations were used because our molecular studies suggested that the thiosemicarbazones induced markers of apoptosis at 10 \(\mu\)M more efficiently than at 5 \(\mu\)M (Fig. 5). In addition, we examined the higher con-
centration of 20 μM to better distinguish the apoptotic effects of the different agents. In these studies, DpC was consistently the most effective agent at inducing late apoptosis (Annexin V- and PI-positive) in each of the four cell types examined at a concentration of 20 μM compared with both gemcitabine and Dp44mT at the same concentration (Fig. 6). The effects of DpC were most pronounced in the MIAPaCa-2, PANC-1, and CAPAN-2 cell types, in which it was significantly ($p < 0.05$) more effective than gemcitabine at the same concentration (Fig. 6, A–C). However, in CFPAC-1 cells,

Fig. 5. Dp44mT, DpC, and gemcitabine modulate molecular markers of apoptosis in pancreatic cancer cells. To examine the ability of Dp44mT, DpC, or gemcitabine to induce apoptosis, the following pancreatic cancer cell types were assessed: MIAPaCa-2 (A), PANC-1 (B), CAPAN-2 (C), and CFPAC-1 (D). Cells were incubated for 24 h/37°C with gemcitabine (Gem), Dp44mT, or DpC (5 or 10 μM), and then cleaved PARP, Bax, and Bcl-2 were examined by Western analysis. The gel photographs are representative of three experiments, whereas the densitometric analysis is the mean ± S.D. (three experiments). For statistical analysis, each treatment was compared with the untreated control; *, $p < 0.05$ versus control; **, $p < 0.01$ versus control.

Fig. 6. DpC was most efficient at inducing late apoptosis in each of the four pancreatic cancer cell types examined as determined by flow cytometry. MIAPaCa-2 (A), PANC-1 (B), CAPAN-2 (C), and CFPAC-1 (D) were incubated with 10 or 20 μM concentration of either gemcitabine (Gem), Dp44mT, or DpC for 48 h/37°C, and apoptosis was examined by flow cytometry using PI and Annexin V (AV) staining. The amount of cells in early apoptosis was defined as cells positive for AV only, whereas late apoptosis was defined as cells positive for both AV and PI. The amount of cells in late apoptosis was most pronounced in cells treated with DpC (20 μM). The data presented are representative of three separate experiments performed and are presented as mean ± S.D. *, $p < 0.05$ versus control; **, $p < 0.01$ versus control; ***, $p < 0.001$ versus control. DpC treatments were also compared with Gem treatments at the same concentration, and statistical significance is depicted using #, $p < 0.05$, and ##, $p < 0.01$. 604 Kovacevic et al.
there was no significant difference between DpC and gemcitabine at both concentrations, with both agents being equally effective at inducing apoptosis in this cell type (Fig. 6D). Again, these results confirm our earlier findings that CFPAC-1 cells are more sensitive to gemcitabine compared with the other cell types examined (Fig. 4D). It is noteworthy that PANC-1 cells were only sensitive to DpC at a concentration of 20 μM, with the other treatments having no significant effect on apoptosis in this cell type (Fig. 6B).

Overall, our results demonstrate that each agent examined was able to modulate markers of apoptosis in the pancreatic cancer cells. However, DpC was the only agent that induced apoptosis in all cell types, being significantly more effective than gemcitabine in three of the four cell types.

In Vivo Analysis of Novel Thiosemicarbazones versus Gemcitabine in Pancreatic Cancer. To further characterize the efficacy of thiosemicarbazones against pancreatic cancer and their potential as a novel therapeutic strategy, further studies examining these agents were performed in vivo. In these experiments, PANC-1 cells were used because they have been demonstrated to be suitable for generating xenografts that are more resistant to gemcitabine compared with other pancreatic tumors in vivo (Régibá et al., 2009). Once established in nude mice, the tumors were allowed to grow to 90 mm³, and the treatment was then initiated with either the vehicle alone, gemcitabine (5 mg/kg i.p. every third day), Dp44mT (0.4 mg/kg i.v. 5 days/week), or DpC (5 mg/kg i.v. 5 days/week). This dosing schedule and route of administration for Dp44mT was used because it showed good tolerability and high antitumor efficacy against other tumor types in previous studies (Whitnall et al., 2006), whereas for DpC and gemcitabine, preliminary maximum tolerated dose studies (data not shown) demonstrated that this administration schedule was also well tolerated and demonstrated substantial efficacy against tumors.

After 44 days of treatment, the vehicle control mice had reached an average volume of 675 ± 138 mm³ (Fig. 7A). It should be noted that there were two sets of vehicle controls administered either intraperitoneally or intravenously as the active agents were administered via these routes (see Materials and Methods). However, both controls led to the same response, and thus, these data have been combined and presented as one group throughout. Treatment of mice with gemcitabine, Dp44mT, or DpC decreased tumor volumes to 202 ± 70, 230 ± 52, and 86 ± 20 mm³, respectively. In fact, gemcitabine (p < 0.01), Dp44mT (p < 0.05), and DpC (p < 0.001) all significantly reduced tumor volumes to 30 ± 10, 34 ± 8, and 13 ± 3% of the control, respectively (Fig. 7A). Furthermore, the final tumor weights after 44 days of treatment reflected the tumor volumes. In fact, control tumors weighed 292 ± 65 mg, whereas tumors treated with gemcitabine, Dp44mT, and DpC were significantly smaller and weighed 67 ± 25 (p < 0.01), 122 ± 33 (p < 0.05), and 40 ± 12 mg (p < 0.001), respectively (Fig. 7B). It is noteworthy that DpC was significantly (p < 0.05) more effective than Dp44mT at reducing tumor weight. Hence, each treatment was able to markedly inhibit the growth and progression of the pancreatic tumor xenografts in vivo, with DpC showing the greatest antitumor efficacy (Fig. 7, A–C).

Although the difference between DpC and gemcitabine was not significant (p > 0.05) at all time points, these data indicate that after day 32, both gemcitabine and Dp44mT treatments were increasingly less effective at inhibiting tumor growth compared with DpC (Fig. 7A). Considering that the tumor size of the vehicle control group was the limiting factor in the length of this experiment because of ethical reasons, it was not possible to continue further treatment after 44 days. This was due to the tumor volume in some control animals reaching the maximum limit prescribed by the local animal ethics committee. However, future studies examining the longer-term effects of gemcitabine and DpC are warranted.

**Fig. 7.** Dp44mT, DpC, and gemcitabine inhibit pancreatic cancer growth in vivo. PANC-1 tumor xenografts were allowed to grow to 90 mm³ subcutaneously, and the treatment was then initiated with either the vehicle alone (Control), gemcitabine (5 mg/kg i.p. every third day), Dp44mT (0.4 mg/kg/day i.v. 5 days/week), or DpC (5 mg/kg i.v. 5 days/week). A, each agent examined effectively inhibited the growth of PANC-1 pancreatic cancer xenografts in vivo with DpC completely inhibiting tumor growth. B, average tumor weights were lowest in the DpC and gemcitabine (Gem)-treated animals. DpC was significantly (p < 0.05) more effective at reducing tumor weight compared with Dp44mT after 44 days of treatment. C, photograph of a representative tumor from the control, gemcitabine, Dp44mT, and DpC groups at euthanasia after 44 days of therapy. D, the average weight of animals in each treatment group during the course of the study. Data presented in A, B, and D are shown as the average ± S.E.M. (n = 8). For statistical analysis, each treatment was compared with the untreated control; *, p < 0.05 versus control; **, p < 0.01 versus control; ***, p < 0.001 versus control. Dp44mT was also compared with DpC as indicated on the graph.
and will further distinguish the efficacy of these two anticancer agents against pancreatic cancer.

Examing Weight, Hematological Indices, and Histology to Determine Toxicity. To determine whether the different agents used in the in vivo studies above were associated with any toxicity, the hematological indices and the body and organ weights of the mice were analyzed after euthanasia. The body weight of the animals after 44 days of treatment remained close to 100% of the pretreatment weight for each group with the exception of DpC (Fig. 7D; Table 2). These animals showed a significant (p < 0.001) weight loss of 12% compared with their pretreatment weight (Table 2). Although we found no significant differences in most organ weights (Table 2) between the different treatment groups, we did observe that the DpC group also had a significantly (p < 0.01) smaller spleen compared with the vehicle control group (i.e., 0.08 versus 0.12 g; Table 2). Histological analysis of the spleen found that the splenic red pulp of mice in all groups contained a normal population of hematopoietic cells (Fig. 8).

Another crucial parameter examined in the animals treated with these agents was the hematological indices in relation to the potential side effect of anemia considering that iron-chelating agents (Dp44mT and DpC) were used. We found no significant difference in the red blood cell, white blood cell, or platelet counts between the control and different treatment groups (Table 3). However, we did observe that the Dp44mT and DpC groups had significantly (p < 0.01) lower hemoglobin levels and a slight but significant (p < 0.05) increase in reticulocyte counts compared with the control group (Table 3). This may be an indicator of a slight anemia in these animals.

To further investigate the potential toxic effects of the different treatments on the organs, a histological analysis of the spleen, kidney, liver, heart, lungs, brain, and bone marrow was performed by staining with 1) hematoxylin and eosin (to detect general ultrastructural pathological conditions), 2) Perls (for presence of iron), and 3) Gomori-Trichrome (for fibrosis). An independent veterinary pathologist performed the histological analysis, and these findings

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>Dp44mT (0.4 mg/kg/day i.v.)</th>
<th>DpC (5 mg/kg/day i.v.)</th>
<th>Gemcitabine (5 mg/kg/3 days i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight loss, % of total weight</td>
<td>104.9 ± 4.6</td>
<td>99.4 ± 5.7</td>
<td>88.0 ± 6.5***</td>
<td>104.5 ± 2.2</td>
</tr>
<tr>
<td>Liver, g</td>
<td>0.96 ± 0.11</td>
<td>0.88 ± 0.03</td>
<td>0.80 ± 0.03</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>Spleen, g</td>
<td>0.12 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.08 ± 0.01**</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Kidney, g</td>
<td>0.15 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Heart, g</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Brain, g</td>
<td>0.32 ± 0.02</td>
<td>0.31 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Tumor, g</td>
<td>0.29 ± 0.07</td>
<td>0.12 ± 0.03*</td>
<td>0.04 ± 0.01***</td>
<td>0.07 ± 0.02**</td>
</tr>
</tbody>
</table>

*p < 0.05.
**p < 0.01.
***p < 0.001.

Fig. 8. Histological analysis of the heart, spleen, and liver after euthanasia after 44 days treatment of nude mice bearing a PANC-1 pancreatic tumor xenograft with either the vehicle alone (control), gemcitabine, Dp44mT, or DpC. The study was performed as described in the legend to Fig. 7. Black arrows indicate myocardial fibrosis in the Dp44mT group only. Scale bar, 200 μm; original magnification, 100×. Histological assessment was performed as described under Materials and Methods. The images shown are representative of the results obtained for each group. Further analysis of the histological data are provided in Supplemental Table 2.
are presented in Supplemental Table 2. Two of the eight Dp44mT-treated mice showed some evidence of hematopoietic cells in the liver. In approximately half of the Dp44mT- and DpC-treated mice, there was also some evidence of mild histopathological conditions in the liver. In addition, iron deposits were identified in the kidneys of four of the eight control-treated mice and all of the Dp44mT- and DpC-treated animals (Supplemental Table 2). These observations could be related to iron in the diet and the excretion of the chelator-iron complex in the urine, respectively. On the other hand, the gemicabine-treated group had no evidence of iron deposits in the kidney (Supplemental Table 2). Moreover, the myocardium of each mouse in the Dp44mT group displayed myocardial lesions that were characterized by myocardial fiber degeneration and necrosis, with replacement by fibrous tissue (Fig. 8 and Supplemental Table 2). The pathological changes observed were most pronounced in the wall of the right ventricle and in the myocardium beneath the endocardium of the left ventricle (Fig. 8). This is in agreement with an earlier study that also detected cardiofibrosis in Dp44mT-treated nude mice (Whitnall et al., 2006). It is noteworthy that there was no evidence of fibrotic lesions in the heart of the DpC-treated group, demonstrating that this compound exhibits potent antitumor activity at the dose used and is far less toxic than Dp44mT in vivo. Significantly, these results represent a substantial improvement in the selective antitumor activity of this class of compounds. There was no evidence of marked pathological tissue in any of the other organs examined (Supplemental Table 2), suggesting that neither DpC nor gemicabine induced significant tissue damage compared with the vehicle control-treated group.

Discussion

Pancreatic cancer is an aggressive disease, with a poor response to the currently available treatments, including the standard gemicabine (Jemal et al., 2009). To this end, we examined a new class of thiosemicarbazones that are designed to target the crucial nutrient iron (Richardson et al., 2009). Thiosemicarbazones have been found to have potent and selective activity against a range of different tumors (Yuan et al., 2004; Kalinowski and Richardson, 2005; Whitnall et al., 2006). In fact, these agents were also demonstrated to overcome chemoresistance (Whitnall et al., 2006), which is an appreciable problem in the treatment of pancreatic cancer (Custodio et al., 2009). However, the efficacy of these novel thiosemicarbazones against pancreatic cancer has not been assessed previously.

One of the first indicators that thiosemicarbazones and other iron chelators may be a suitable strategy for the treatment of pancreatic cancer was the finding that they up-regulate the growth and metastasis suppressor NDRG1 in a range of cancer cell types (Le and Richardson, 2004; Whitnall et al., 2006; Kovacevic et al., 2008). In fact, earlier studies have demonstrated that iron-depletion is responsible for increased NDRG1 levels, which occurs in part, through HIF-1 (Le and Richardson, 2004). In agreement with these studies, we found that both Dp44mT and DpC markedly increased the expression and phosphorylation of NDRG1 in each of the four pancreatic cancer cell types examined, whereas gemicabine did not significantly modulate its expression. The increase in phosphorylated NDRG1 in response to the thiosemicarbazones is of significance in terms of the mechanism of action of these agents, because NDRG1 phosphorylation at Ser330 and Thr346 is necessary for its antitumor activity in pancreatic cancer (Murakami et al., 2010).

We have demonstrated that NDRG1 is also able to up-regulate the cyclin-dependent kinase inhibitor p21<sup>CIP1/WAF1</sup> in a number of cancer cell types (Kovacevic et al., 2011). Here, we further demonstrated that both Dp44mT and DpC also increased p21<sup>CIP1/WAF1</sup> expression in pancreatic cancer cells, which was correlated with increased NDRG1 levels. It is noteworthy that an earlier study examining MCF-7 breast cancer cells demonstrated that other iron chelators, [DFO and 2-hydroxy-1-naphthaldehyde isonicotinoyl (311)], reduced p21<sup>CIP1/WAF1</sup> protein levels (Fu and Richardson, 2007). This may indicate that the response of p21<sup>CIP1/WAF1</sup> to chelators may be cell type-specific or dependent on the type of ligand used, because chelators demonstrate different effects depending on their structure. In fact, thiosemicarbazones induce ROS generation upon binding iron, whereas DFO and 311 bind iron without inducing ROS (Kalinowski and Richardson, 2005). In contrast to the thiosemicarbazones, gemicabine reduced p21<sup>CIP1/WAF1</sup> expression in three of the four pancreatic cancer cell types tested.

It is notable that the function of p21<sup>CIP1/WAF1</sup> in cell cycle regulation is complex, with its overexpression leading to G<sub>1</sub>/S arrest due to its ability to act as a cyclin-dependent kinase inhibitor, whereas a reduction of p21<sup>CIP1/WAF1</sup> expression induces apoptosis (Cheng et al., 1999). In fact, basal expression of p21<sup>CIP1/WAF1</sup> is required to stabilize the cyclin D1/cdk complex, which is necessary for cell cycle progression (Cheng et al., 1999). Hence, the decreased p21<sup>CIP1/WAF1</sup> expression after incubation with gemicabine in some cells may contribute to its antitumor activity via the induction of apoptosis, whereas increased p21<sup>CIP1/WAF1</sup> levels in response to the
thiosemicarbazones could inhibit cell cycle progression and proliferation, as demonstrated in this study.

We also examined the expression of another important cell cycle regulatory molecule, cyclin D1, which is involved in proliferation (Yu et al., 2007). Both Dp44mT and DpC reduced cyclin D1 levels in each cell type examined, which was in agreement with an earlier study that also demonstrated the ability of iron chelators to reduce cyclin D1 (Nurtjahja-Tjendraputra et al., 2007). Gemcitabine was also able to markedly decrease cyclin D1 levels in three of the four cell types, as demonstrated previously using pancreatic cancer cells (Kunnumakkara et al., 2007), but had no effect on its expression in CAPAN-2 cells. Significantly, cyclin D1 functions as an oncogene in pancreatic cancer, often being overexpressed in these tumors, and is correlated with poor patient survival (Kornmann et al., 1998). Hence, anticancer agents that are able to effectively reduce cyclin D1 levels are likely to be beneficial for pancreatic cancer treatment.

Considering the marked effect of the thiosemicarbazones on the three key molecular targets described above, it was of interest that Dp44mT and DpC were >4-fold more effective at inhibiting proliferation of three of the four pancreatic cancer cell types compared with gemcitabine and >2000-fold more effective than 5-fluorouracil in all cell types. Overall, our in vitro analysis demonstrated that the novel thiosemicarbazones, Dp44mT and DpC, were more effective at inhibiting the proliferation of pancreatic cancer cells compared with gemcitabine and 5-fluorouracil. Because these agents up-regulate both NDRG1 and p21\(^{CIP1/WAF1}\) expression and considering the role of these molecules in inducing apoptosis (Stein et al., 2004; Yu et al., 2007), we further examined their effect on apoptosis compared with gemcitabine. Each agent modulated markers of apoptosis including cleaved PARP, Bax, and Bcl-2. However, the extent to which these were affected was dependent on the cell type and drug concentration. Using flow cytometry, we demonstrated that DpC was the most efficient agent at inducing late apoptosis, being significantly more effective than gemcitabine in three of the four pancreatic cancer cell-types.

It is notable that CFPAC-1 cells were consistently less sensitive to the thiosemicarbazones than the other three cell types but were more vulnerable to the antiproliferative effects of gemcitabine. These results demonstrate that CFPAC-1 cells have other molecular attributes that render them more resistant to these agents. In fact, recent studies have demonstrated that CFPAC-1 and MIAPaCa-2 cells have higher endogenous levels of ROS compared with PANC-1 cells, which makes them more sensitive to gemcitabine (Donadelli et al., 2007). This is in agreement with our results showing that among the pancreatic tumor cell types tested, CFPAC-1 and MIAPaCa-2 cells were most sensitive to gemcitabine. It is unclear how endogenous ROS levels may lead to greater resistance of CFPAC-1 cells to thiosemicarbazones. However, higher ROS could lead to these cells having bolstered antioxidant defense mechanisms (e.g., increased catalase, etc.) and because the cytotoxic effector mechanisms of these thiosemicarbazones is due to their ability to generate ROS (Yuan et al., 2004; Richardson et al., 2006), this may potentially explain the greater resistance of CFPAC-1 cells to these agents.

Studies in vivo examining the efficacy of these agents against PANC-1 pancreatic cancer xenografts found that the most effective treatment was DpC, which seemed to completely inhibit tumor growth. Considering the high efficacy of DpC against pancreatic cancer, it was important to examine any potential toxic side effects of this therapeutic regimen. In contrast to the other treatment groups, we noted weight loss (12%) by the last day of treatment with DpC. In addition, both DpC- and Dp44mT-treated groups exhibited a slight but significant increase in reticulocyte counts and decreased hemoglobin levels. This may be indicative of mild anemia and highlights the importance of establishing an effective treatment regimen that will overcome these side effects while maintaining antitumor activity.

Another important outcome of the current study was the comparison between the two thiosemicarbazones Dp44mT and DpC. Earlier studies examining Dp44mT against melanoma xenografts in vivo in nude mice noted some cardiac fibrosis at higher, nonoptimal doses of this chelator (Whitnall et al., 2006). It is noteworthy that we also observed limited cardiac fibrosis in mice treated with Dp44mT (0.4 mg/kg), whereas there was no cardiotoxicity after treatment with DpC (5 mg/kg). Therefore, DpC was able to overcome the major toxicity observed with Dp44mT while maintaining potent anti-cancer activity. These results clearly establish DpC as the most effective and selective iron chelator developed in our laboratories and warrants further studies into its potential for pancreatic cancer treatment.

It is notable that the thiosemicarbazone 3-AP has already been through clinical trials for the treatment of a range of tumors including pancreatic cancer (Attia et al., 2008). In fact, phase II clinical trials were performed using 3-AP in combination with gemcitabine, because these drugs were observed to have synergistic effects (Mackenzie et al., 2007). However, these studies found that 3-AP induced significant toxicity with little therapeutic benefit (Mackenzie et al., 2007; Attia et al., 2008). Considering that Dp44mT is far more potent and less toxic than 3-AP (Yuan et al., 2004; Whitnall et al., 2006), both Dp44mT and DpC are new and more effective alternatives to this agent. Hence, studies examining potential synergy between gemcitabine and DpC are warranted and may result in a more effective therapeutic regimen.

In conclusion, the current study is the first to examine the anticancer activity of novel thiosemicarbazones against pancreatic cancer. We demonstrated that Dp44mT and DpC up-regulate NDRG1 and p21\(^{CIP1/WAF1}\) and down-regulate cyclin D1, which are key molecular targets that lead to inhibition of proliferation. Furthermore, DpC was generally more effective than the current standard treatments, namely gemcitabine and 5-fluorouracil. Additional studies demonstrated that DpC completely inhibited pancreatic tumor xenograft growth and, unlike Dp44mT, did not lead to cardiac fibrosis. These data clearly highlight the potential of DpC as an effective treatment strategy against pancreatic cancer.

**Acknowledgments**

We thank Dr. Christopher Austin, Dr. Katie Dixon, Dr. Patric Jansson, Dr. Yu Yu, Dr. Darius Lane, Angelica Merlot, and Dr. Daohai Zhang of the Iron Metabolism and Chelation Program (University of Sydney) for their critical appraisal of the manuscript before submission.
Authorship Contributions

Participated in research design: Kovacevic, Lovejoy, and Richardson.
Conducted experiments: Kovacevic and Chikhani.
Contributed new reagents or analytic tools: Lovejoy.
Performed data analysis: Kovacevic and Richardson.
Wrote or contributed to the writing of the manuscript: Kovacevic and Richardson.

References


Kovacevic, Lovejoy, and Richardson.


Address correspondence to: Dr. D. R. Richardson, Iron Metabolism and Chemotherapy Program, Department of Pathology and Bosoh Institute, University of Sydney, Sydney, New South Wales, 2006 Australia. E-mail: d.richardson@med.usyd.edu.au