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NOVEL THIOSEMICARBAZONE IRON CHELATORS INDUCE UP-REGULATION AND PHOSPHORYLATION OF THE METASTASIS SUPPRESSOR, NDRG1: A NEW STRATEGY FOR THE TREATMENT OF PANCREATIC CANCER.

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Running Title: Novel Iron Chelators for Pancreatic Cancer Treatment.

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Abbreviations:

Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2; DFO, desferrioxamine; DpC, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone hydrochloride; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; HIF-1, hypoxia-inducible factor-1; i.v., intravenous; i.p., intraperitoneal; MTD, Maximum tolerated dose; NDRG1, N-myc down-stream regulated gene 1; PARP, Poly (ADP-ribose) polymerase; 3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 311, 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone.

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Abstract

Pancreatic cancer is an aggressive neoplasm, with a mortality rate close to 100%. Currently, 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 anti-cancer agents against pancreatic cancer. This group of compounds belongs to the dipyridyl thiosemicarbazone (DpT) class that have been shown to have potent and selective activity against a range of different neoplasms *in vitro* and *in vivo* (Whitnall M., Howard J., Ponka P. and Richardson D.R. 2006, *PNAS* 40:14901-6). We demonstrate for the first time in pancreatic cancer that these agents increase the expression of the growth and metastasis suppressor, *N-myc down-stream regulated gene 1* (NDRG1), and also its phosphorylation at Ser-330 and Thr-346 that is important for its activity against this tumor. In addition, these agents increased expression of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, while decreasing cyclin D1 in pancreatic cancer cells. Together, these molecular alterations account, in part, for the pronounced anti-tumor activity observed. Indeed, these agents had significantly higher anti-proliferative 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 (DpC), completely inhibited the growth of pancreatic cancer xenografts with no evidence of marked alterations in normal tissue histology. Collectively, our studies have identified molecular effectors of a novel and potent anti-tumor agent that could be useful for pancreatic cancer treatment.

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Introduction

Pancreatic cancer is a devastating disease being fatal in 98-100% 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 suffering this illness remains poor. The "gold-standard" treatment for pancreatic cancer is the anti-cancer agent, gemcitabine (Figure 1A), often given in combination with other chemotherapeutics such as 5-fluorouracil (Figure 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 life-span 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 downstream regulated gene-1* (NDRG1) (Ellen et al., 2007; Kovacevic and Richardson, 2006). This latter protein inhibits both growth and metastasis as well as angiogenesis of pancreatic cancer *in vivo*, leading to reduced tumor progression (Maruyama et al., 2006). 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 previously demonstrated to up-regulate NDRG1 *in vitro* and *in vivo via* their ability to increase the hypoxia-inducible factor-1 (HIF-1) (Kovacevic et al., 2008; Le and Richardson, 2004; Whitnall et al., 2006). The mechanism involved in this effect is mediated

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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 anti-cancer agents was first discovered when the iron chelator, desferrioxamine (DFO; Figure 1C), which is primarily used for iron-overload diseases such as β-thalassemia (Aouad et al., 2002), was successfully utilized 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; Figure 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 which produce reactive oxygen species (ROS) that induce cancer cell cytotoxicity (Jansson et al., 2011; Kalinowski and Richardson, 2005; Yuan et al., 2004). One of the most active thiosemicarbazones developed to date is di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Figure 1E) (Kalinowski and Richardson, 2005; Whitnall et al., 2006; Yuan et al., 2004). 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, non-optimal 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; Figure 1F).

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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, $p21^{CIP1/WAF1}$ and cyclin D1, and are significantly more effective at inhibiting proliferation and inducing apoptosis *in vitro* when compared to the current agent of choice, gemcitabine, in 3 of 4 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.

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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 (ATCC; 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, while 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 DMEM medium (Invitrogen, Sydney, Australia), while CAPAN-2 cells were grown in McCoy's medium (Invitrogen). All media was supplemented with 10% (v/v) fetal calf serum (Invitrogen), 1% (v/v) non-essential amino acids (Invitrogen), 1% (v/v) sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 μg/mL of streptomycin (Invitrogen) and 100 U/mL penicillin (Invitrogen). Cells were grown in an incubator (Forma Scientific, Ohio, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and sub-cultured by standard methods, as described previously (Le and Richardson, 2004).

Reagents

Gemcitabine (Gemzar®) was purchased from Lilly (IN, USA). 5-fluorouracil was obtained from Sigma-Aldrich (Sigma-Aldrich; St. Louis, MO). Desferrioxamine (DFO) was from Novartis (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 (Richardson et al., 2006; Scovill, 1990). Briefly, carbon disulphide (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

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aqueous extract and allowed to react over-night at room temperature. The addition of concentrated HCl (25 mL) produced the solid carboxymethyl thiocarbamate intermediate. Then, 0.08 mol of the latter compound was dissolved in 20 mL of hydrazine 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, 5 drops of glacial acetic acid were added and the mixture was refluxed for 2 h and cooled to 5°C to give the yellow Dp4cycH4mT precipitate. Finally, Dp4cycH4mT was dissolved in a minimum volume of 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, New South Wales), infrared spectroscopy, mass spectroscopy and ^{1}H-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 (Cat. #: ab37897; Abcam Inc, MA, USA), rabbit anti-human p21$^{CIP1/WAF1}$ (Cat. #: 2947; Cell Signalling, MA, USA), rabbit anti-human pNDRG1 (Ser-330; Cat. #: 3506, Cell Signalling), rabbit anti-human pNDRG1 (Thr 346; Cat. #: 3217, Cell Signalling), rabbit anti-human cleaved PARP (Cat. #: 9541S, Cell Signalling), rabbit anti-human Bax (Cat. #: 2772S, Cell Signalling), rabbit anti-human Bcl-2 (Cat. #: 2870S; Cell Signalling), mouse anti-human cyclin D1 (Cat. #: SC-8396; Cruz, CA, USA) and β-actin (Cat. #: SC-130301; Santa Cruz).

Flow cytometry

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Flow cytometry using Annexin V and propidium iodide (PI) labeling was utilized to examine apoptosis in response to the thiosemicarbazones and gemcitabine using standard methods (Yuan et al., 2004). Briefly, cells were seeded in T25 flasks and allowed to adhere overnight. The cells were then treated with either 10 or 20 μM of gemcitabine, Dp44mT or DpC and incubated for 48 h/37^{o}C. The cells were harvested and prepared using the Annexin V apoptosis kit (BD Biosciences, NJ, USA) following the manufacturer's instructions and examined using a FACS Calibur flow cytometer (BD Biosciences). Results were analysed using CellQuest software (BD Biosciences).

MTT Cellular Proliferation Assay

Cellular proliferation was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; Sigma-Aldrich) assay after a 72 h/37^{o}C incubation, using standard methods (Richardson et al., 1995). As shown previously, MTT colour formation was directly proportional to the number of viable cells (Richardson et al., 1995), validating its use in these studies.

Maximum Tolerated Dose (MTD) Studies in Nude Mice

In vivo experiments were approved by the Animal Ethics Committee (University of Sydney). Before studies assessing anti-tumour activity of the novel thiosemicarbazone, DpC, were initiated, MTD experiments were performed, as described previously (Whitnall et al., 2006; Yuan et al., 2004) 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% (Whitnall et al., 2006; Yuan et al., 2004).

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). Briefly, each mouse was injected subcutaneously with 2 x 10^6 PANC-1 cells suspended in Matrigel (BD Biosciences). Tumor size

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was measured by Vernier calipers and tumor volume calculated as described previously (Balsari et al., 2004). Once the tumors reached an average of 90 mm^3, the treatment began (Day 0; Figure 7A). The chelators, Dp44mT and DpC, were dissolved in 30% propylene glycol in 0.9% saline and injected intravenously (i.v.; *via* the tail vein) 5 days/week (Monday-Friday) (Whitnall et al., 2006). Gemcitabine was dissolved in 15% propylene glycol/0.9% saline and injected intra-peritoneally (i.p.) every 3rd 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 (Laquente et al., 2008; Whitnall et al., 2006). The vehicle control group was sub-divided 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 intra-peritoneally every 3rd day and was the appropriate control for the gemcitabine treatment. Once control tumors reached 1,000 mm^3, the animals were euthanized due to 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 utilized, namely hematoxylin and eosin (H&E), Pearl's or Gomori-Trichrome. The histological analysis and quantification of pathological features was performed by an independent veterinary pathologist, Dr. Terrence Rothwell (Rothwell Consulting, Avalon Beach, NSW, Australia).

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Statistical Analysis

Data were compared by using the Student's *t*-test. Results were expressed as mean ± SD unless otherwise indicated. Data were considered statistically significant when $p < 0.05$.

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Results

In Vitro Analysis of Novel Thiosemicarbazones for the Treatment of Pancreatic Cancer.

In order 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, $p21^{CIP1/WAF1}$ (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 4 different pancreatic cancer cell-types *in vitro* in comparison to 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, as well as gemcitabine on total and phosphorylated NDRG1 (Ser-330 and Thr-346) expression in four pancreatic cancer cell-types (Figure 2A-D). This was crucial to assess considering the widely reported anti-tumor function of NDRG1 (Ellen et al., 2007; Kovacevic and Richardson, 2006) and its potential as a promising 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^{o}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 (Ser-330 and Thr-346) NDRG1 protein levels in all cell-types examined (Figure 2A-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 when compared to the untreated controls (Figure 2).

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As demonstrated previously (Kovacevic et al., 2011; Murray et al., 2004), we observed two bands for total NDRG1 in each cell-type examined (migrating at 43- and 44-kDa; Figure 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 (Ser-330 and Thr-346; at 44 kDa) in each cell-type examined (Figure 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 Thr-346 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 anti-tumor function in pancreatic cancer (Murakami et al., 2010). Thus, our current results are important for understanding the anti-tumor 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 G_1/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.

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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^{o}C and protein levels of p21$^{CIP1/WAF1}$ and cyclin D1 examined. Both Dp44mT and DpC significantly (p<0.05) increased p21$^{CIP1/WAF1}$ expression, while significantly (p<0.05) reducing cyclin D1 levels in each of the four cell-types examined (Figure 3A-D). Interestingly, gemcitabine was also able to markedly reduce cyclin D1 levels in the MIAPaCa-2, PANC-1 and CFPAC-1 cells, while having no significant effect in CAPAN-2 cells (Figure 3C). On the other hand, gemcitabine reduced p21$^{CIP1/WAF1}$ expression in the PANC-1, CFPAC-1 and CAPAN-2 cells, with no effect being observed in MIAPaCa-2 cells relative to the control (Figure 3). Hence, the molecular effects of gemcitabine appear to be cell-type-dependent.

Collectively, the results above demonstrate that NDRG1 and p21$^{CIP1/WAF1}$ are markedly up-regulated, while cyclin D1 is reduced in the pancreatic cancer cell-types by Dp44mT and DpC. Considering the anti-tumor 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 when compared to gemcitabine and 5-fluorouracil.

To examine the anti-proliferative activity of Dp44mT and DpC against pancreatic cancer *in vitro*, we performed MTT proliferation assays with each of the 4 pancreatic cancer cells types studied above in comparison to 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).

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Examining the MIAPaCa-2, PANC-1 and CAPAN-2 cell-types, the highest anti-proliferative activity was observed with Dp44mT and DpC (Figure 4A, B and C) with their IC_{50} values being significantly ($p<0.01$) lower when compared to gemcitabine and 5-fluorouracil (Table 1). In fact, the IC_{50} values for Dp44mT and DpC were at least 4-fold and 2000-fold lower in 3 out of the 4 cell-types when compared to gemcitabine and 5-fluorouracil, respectively (Table 1). On the other hand, DFO had relatively low anti-proliferative activity, being significantly ($p<0.001$) less effective than the thiosemicarbazones probably due to its low membrane permeability (Kalinowski and Richardson, 2005).

In contrast to the other cell-types where the thiosemicarbazones had the highest anti-proliferative activity, CFPAC-1 cells were more sensitive to gemcitabine than either Dp44mT or DpC (Figure 4D). In fact, the IC_{50} value for gemcitabine was significantly ($p<0.05$) lower than that of Dp44mT or DpC (Figure 4D and Table 1). However, it is notable that Dp44mT and DpC had lower IC_{90} values than gemcitabine in CFPAC-1 cells (Figure 4D, Supplemental Table 1), suggesting that the thiosemicarbazones are effective at inhibiting proliferation of this cell-type *in vitro* when used at higher concentrations (Figure 4D). These results demonstrate the heterogeneity of pancreatic cancer and may reflect the different molecular alterations that determine sensitivity to anti-cancer 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, $p21^{CIP1/WAF1}$ and cyclin D1, we further examined the effects of these agents by assessing apoptosis in the 4 cell-types when compared to gemcitabine.

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Each cell-type was incubated with 5 or 10 μM 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 when compared to the other pancreatic cancer cells examined (Figure 5) and this corresponds to the higher sensitivity of these cells to gemcitabine (Figure 4, Table 1). However, PARP was also cleaved by the thiosemicarbazones in these cells types, but to a lesser degree than gemcitabine (Figure 5A, D). Interestingly, PANC-1 and CAPAN-2 cells were more or similarly sensitive to the thiosemicarbazones than gemcitabine, with Dp44mT (10 μM) being the most efficient at inducing cleaved PARP (Figure 5B and C).

Expression of pro-apoptotic 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, where no significant effect was observed (Figure 5A). Moreover, the expression of the anti-apoptotic protein Bcl-2 (Tang and Porter, 1996) was significantly (p<0.01) reduced by the thiosemicarbazones in each of the cell-types assessed, while gemcitabine only markedly reduced Bcl-2 in CFPAC-1 cells (Figure 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 Figure 1). The results above highlight the different sensitivities of the pancreatic cancer cells to the agents examined, demonstrating various molecular responses which indicate the induction of apoptosis. To clarify whether apoptosis was occurring, and to what degree, we further examined pancreatic cancer cells by flow cytometry following 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 μM of gemcitabine, Dp44mT or DpC for 48 h/37^{o}C. These concentrations were used as our molecular studies suggested that the thiosemicarbazones induced

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markers of apoptosis at 10 μM more efficiently than at 5 μM (Figure 5). In addition, we examined the higher concentration 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 when compared to both gemcitabine and Dp44mT at the same concentration (Figure 6). The effects of DpC were most pronounced in the MIAPaCa-2, PANC-1 and CAPAN-2 cell-types where it was significantly (p<0.05) more effective than gemcitabine at the same concentration (Figure 6A, B and C). However, in CFPAC-1 cells, 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 (Figure 6D). Again, these results confirm our earlier findings that CFPAC-1 cells are more sensitive to gemcitabine when compared to the other cell-types examined (Figure 4D). Interestingly, 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 (Figure 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 3 of the 4 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 as they have been demonstrated to be suitable for generating xenografts that are more resistant to gemcitabine when compared to other pancreatic tumors *in vivo* (Rejiba et al., 2009). Once established in nude mice, the tumors were allowed to grow to 90 mm^3 and the treatment was then initiated with either the vehicle alone, gemcitabine (5 mg/kg i.p.; every 3rd day), Dp44mT (0.4 mg/kg i.v.; 5 days/week) or DpC (5 mg/kg i.v.; 5

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days/week). This dosing schedule and route of administration for Dp44mT was used as it showed good tolerability and high anti-tumor efficacy against other tumor types in previous studies (Whitnall et al., 2006), while 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 mm^3 (Figure 7A). It should be noted that there were 2 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 mm^3, 230 ± 52 mm^3 and 86 ± 20 mm^3, 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 (Figure 7A). Furthermore, the final tumor weights after 44 days of treatment reflected the tumor volumes. In fact, control tumors weighed 292 ± 65 mg, while tumors treated with gemcitabine, Dp44mT and DpC were significantly smaller and weighed 67 ± 25 mg (p<0.01), 122 ± 33 mg (p<0.05) and 40 ± 12 mg (p<0.001), respectively (Figure 7B). Notably, 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 anti-tumor efficacy (Figure 7A,B and 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 when compared to DpC (Figure 7A). Considering that the tumor size of the vehicle control group was the limiting factor in the length of

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this experiment due to 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 and will further distinguish the efficacy of these two anti-cancer agents against pancreatic cancer.

Examining 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 as well as the body and organ weights of the mice were analyzed following euthanasia. The body weight of the animals after 44 days of treatment remained close to 100% of the pre-treatment weight for each group with the exception of DpC (Figure 7D, Table 2). These animals showed a significant ($p<0.001$) weight loss of 12% when compared to their pre-treatment 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 ($p<0.001$) smaller spleen when compared to the vehicle control group (*i.e.*, 0.08 g 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 (Figure 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 (RBC), white blood cell (WBC) 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 (Hb) levels and a slight, but significant ($p<0.05$) increase in reticulocyte counts when compared to the control group (Table 3). This may be an indicator of a slight anemia in these animals.

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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)** H&E (to detect general ultra-structural pathology); **(2)** Perls' (for presence of iron) and **(3)** Gomori-Trichrome (for fibrosis). The histological analysis was performed by an independent veterinary pathologist and these findings 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 histopathology in the liver. In addition, iron deposits were identified in the kidneys of 4 of the 8 control-treated mice and all 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 gemcitabine-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 fibre degeneration and necrosis, with replacement by fibrous tissue (Figure 8, Supplemental Table 2). The pathological changes observed were most pronounced in the wall of the right ventricle and also in the myocardium beneath the endocardium of the left ventricle (Figure 8). This is in agreement with an earlier study that also detected cardio-fibrosis in Dp44mT-treated nude mice (Whitnall et al., 2006). Importantly, there was no evidence of fibrotic lesions in the heart of the DpC-treated group demonstrating that this compound exhibits potent anti-tumor activity at the dose used and is far less toxic than Dp44mT *in vivo*. Significantly, these results represent a substantial improvement in the selective anti-tumor activity of this class of compounds.

There was no evidence of marked pathology in any of the other organs examined (Supplemental Table 2), suggesting that both DpC and gemcitabine did not induce significant tissue damage when compared to the vehicle control treated group.

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Discussion

Pancreatic cancer is an aggressive disease, with a poor response to the currently available treatments, including the "gold standard" gemcitabine (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 (Kalinowski and Richardson, 2005; Whitnall et al., 2006; Yuan et al., 2004). 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 previously assessed.

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 (Kovacevic et al., 2008; Le and Richardson, 2004; Whitnall et al., 2006). 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 also phosphorylation of NDRG1 in each of the four pancreatic cancer cell-types examined, while gemcitabine 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, since NDRG1 phosphorylation at Ser-330 and Thr-346 is necessary for its anti-tumor activity in pancreatic cancer (Murakami et al., 2010).

We recently demonstrated that NDRG1 is also able to up-regulate the cyclin-dependent kinase inhibitor, $p21^{CIP1/WAF1}$, in a number of cancer cell-types (Kovacevic et al., 2011). Here, we further

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demonstrated that both Dp44mT and DpC also increased $p21^{CIP1/WAF1}$ expression in pancreatic cancer cells, which was correlated with increased NDRG1 levels. Interestingly, an earlier study examining MCF-7 breast cancer cells demonstrated that other iron chelators, (DFO and 2-hydroxy-1-naphthaldehyde isonicotinoyl (311)), reduced $p21^{CIP1/WAF1}$ protein levels (Fu and Richardson, 2007). This may indicate that the response of $p21^{CIP1/WAF1}$ to chelators may be cell-type specific or dependent on the type of ligand utilized, as chelators demonstrate different effects depending on their structure. In fact, thiosemicarbazones induce ROS generation upon binding iron, while DFO and 311 bind iron without inducing ROS (Kalinowski and Richardson, 2005). In contrast to the thiosemicarbazones, gemcitabine reduced $p21^{CIP1/WAF1}$ expression in 3 of the 4 pancreatic cancer cell-types tested.

It is notable that the function of $p21^{CIP1/WAF1}$ in cell cycle regulation is complex, with its over-expression leading to G_1/S arrest due to its ability to act as a cyclin-dependent kinase inhibitor, while a reduction of $p21^{CIP1/WAF1}$ expression induces apoptosis (Cheng et al., 1999). In fact, basal expression of $p21^{CIP1/WAF1}$ is required to stabilize the cyclin D1/cdk complex which is necessary for cell cycle progression (Cheng et al., 1999). Hence, the decreased $p21^{CIP1/WAF1}$ expression after incubation with gemcitabine in some cells may contribute to its anti-tumor activity *via* the induction of apoptosis, while increased $p21^{CIP1/WAF1}$ 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 3 of the 4 cell-types, as demonstrated previously using pancreatic cancer cells (Kunnumakkara et al., 2007), but had no

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effect on its expression in CAPAN-2 cells. Significantly, cyclin D1 functions as an oncogene in pancreatic cancer, often being over-expressed in these tumors and is correlated with poor patient survival (Kornmann et al., 1998). Hence, anti-cancer 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 3 key molecular targets described above, it was of interest that Dp44mT and DpC were >4-fold more effective at inhibiting proliferation of 3 of the 4 pancreatic cancer cell-types when compared to gemcitabine and >2,000-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 when compared to gemcitabine and 5-fluorouracil. Since 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 in comparison to 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 3 of the 4 pancreatic cancer cell-types.

It is notable that CFPAC-1 cells were consistently less sensitive to the thiosemicarbazones than the other 3 cell-types, but were more vulnerable to the anti-proliferative effects of gemcitabine. These results demonstrate that CFPAC-1 cells have other molecular attributes which 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 reactive oxygen species (ROS) when compared to 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

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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 anti-oxidant defence mechanisms (*e.g.*, increased catalase *etc*) and since the cytotoxic effector mechanisms of these thiosemicarbazones is due to their ability to generate ROS (Richardson et al., 2006; Yuan et al., 2004), 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 appeared 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 Hb 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 anti-tumor 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, non-optimal doses of this chelator (Whitnall et al., 2006). Interestingly, we also observed limited cardiac fibrosis in mice treated with Dp44mT (0.4 mg/kg), while 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.

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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, as 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 (Attia et al., 2008; Mackenzie et al., 2007). Considering that Dp44mT is far more potent and less toxic than 3-AP (Whitnall et al., 2006; Yuan et al., 2004), 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 anti-cancer activity of novel thiosemicarbazones against pancreatic cancer. We demonstrated that Dp44mT and DpC up-regulate NDRG1 and $p21^{CIP1/WAF1}$ and down-regulate cyclin D1which are key molecular targets that lead to inhibition of proliferation. Further, DpC was generally more effective than the current "gold-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.

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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.

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Footnotes:

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Figure Legends

Figure 1: Chemical structures of: (A) gemcitabine, (B) 5-fluorouracil, (C) DFO, (D) 3-AP, (E) Dp44mT and (F) DpC.

Figure 2: The thiosemicarbazones, Dp44mT and DpC, significantly up-regulate NDRG1 and its phosphorylation at Ser-330 and Thr-346, while gemcitabine (Gem) has no effect. (**A**) MIAPaCa-2, (**B**) PANC-1, (**C**) CAPAN-2 and (**D**) CFPAC-1 cells were incubated with either control medium or this medium containing Gem (5 or 10 μM), Dp44mT (5 or 10 μM) or DpC (5 or 10 μM) for 24 h/37^{o}C and NDRG1 expression examined by western blotting. Two bands were detected for NDRG1 at approximately 43 and 44 kDa and both were quantitated using densitometry. Antibodies specific for NDRG1 phosphorylated at Ser-330 and Thr-346 were also utilized to assess the effect of the agents on its phosphorylation. The gel photographs in (**A**), (**B**), (**C**) and (**D**) are representative of 3 experiments performed, while the densitometric analysis is mean ± SD (3 experiments). For statistical analysis, each treatment was compared to the untreated control; * *vs*. control, $p<0.05$; ** *vs*. control, $p<0.01$; *** *vs*. control, $p<0.001$.

Figure 3: Effects of gemcitabine (Gem), Dp44mT and DpC on p21$^{CIP1/WAF1}$ and cyclin D1 expression in pancreatic cancer cells. The thiosemicarbazones, Dp44mT and DpC, markedly and significantly up-regulate p21$^{CIP1/WAF1}$ expression, while significantly reducing cyclin D1 levels. (**A**) MIAPaCa-2, (**B**) PANC-1, (**C**) CAPAN-2 and (**D**) CFPAC-1 cells. Cells were incubated with either control medium or this medium containing either: Gem (5 or 10 μM), Dp44mT (5 or 10 μM) or DpC (5 or 10 μM) for 24 h/37^{o}C and western blotting then performed. The gel photographs are representative of 3 experiments performed, while the densitometric analysis is mean ± SD (3 experiments). For statistical analysis, each treatment was compared to the untreated control; * *vs*. control, $p<0.05$; ** *vs*. control, $p<0.01$.

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Figure 4: The thiosemicarbazones, Dp44mT and DpC, are more effective at inhibiting proliferation of pancreatic cancer cells when compared to gemcitabine and 5-fluorouracil. (**A**) MIAPaCa-2, (**B**) PANC-1 and (**C**) CAPAN-2 cells were significantly more susceptible to the anti-proliferative effects of Dp44mT and DpC compared to gemcitabine and 5-fluorouracil after an incubation for 72 h/37^{o}C. (**D**) CFPAC-1 cells were most sensitive to gemcitabine at lower doses (< 0.16 μM) and to Dp44mT at higher doses (> 0.63 μM). MTT analysis was performed as described in the *Materials and Methods*. The data presented are mean of 3-5 experiments and the calculated IC_{50} and IC_{90} values from these studies are presented in Table 1 and Supplemental Table 1, respectively.

Figure 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: (**A**) MIAPaCa-2, (**B**) PANC-1, (**C**) CAPAN-2 and (**D**) CFPAC-1. Cells were incubated for 24 h/37^{o}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 3 experiments, while the densitometric analysis is mean ± SD (3 experiments). For statistical analysis, each treatment was compared to the untreated control; * *vs*. control, $p<0.05$; ** *vs*. control, $p<0.01$.

Figure 6: DpC was most efficient at inducing late apoptosis in each of the four pancreatic cancer cell-types examined as determined by flow cytometry. (**A**) MIAPaCa-2, (**B**) PANC-1, (**C**) CAPAN-2 and (**D**) CFPAC-1 were incubated with 10 or 20 μM of either gemcitabine (Gem), Dp44mT or DpC for 48 h/37^{o}C and apoptosis was examined by flow cytometry using propidium iodide (PI) and Annexin V (AV) staining. The amount of cells in early apoptosis was defined as cells positive for AV only, while 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

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data presented is representative of 3 separate experiments performed and is presented as mean ± SD. * *vs*. control, $p<0.05$; ** *vs*. control, $p<0.01$; *** *vs*. control, $p<0.001$. DpC treatments were also compared to Gem treatments at the same concentration and statistical significance is depicted using $^{\#}$ ($p<0.05$) and $^{\#\#}$ ($p<0.01$).

Figure 7: Dp44mT, DpC and gemcitabine inhibit pancreatic cancer growth *in vivo*. PANC-1 tumor xenografts were allowed to grow to 90 mm^3 subcutaneously and the treatment was then initiated with either the vehicle alone (Control), gemcitabine (5 mg/kg i.p.; every 3rd day), Dp44mT (0.4 mg/kg/day i.v.; 5 days/week) or DpC (5 mg/kg/day 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 when compared to 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 average ± SEM (n = 8). For statistical analysis, each treatment was compared to the untreated control; * *vs*. control, $p<0.05$; ** *vs*. control, $p<0.01$; *** *vs*. control, $p<0.001$. Dp44mT was also compared to DpC as indicated on the graph.

Figure 8: Histological analysis of the heart, spleen and liver following 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 for Figure 7. Black arrows indicate myocardial fibrosis in the Dp44mT group only. Scale bar represents 200 μm in the images (Magnification: 100 x). Histological assessment was performed as described in the *Materials and Methods*. The images shown are representative of the results

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obtained for each group. Further analysis of the histological data is provided in Supplemental Table 2.

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Table 1: IC_{50} values (μM) of DFO, Dp44mT, DpC and gemcitabine in 4 different pancreatic cancer cell lines after a 72 h incubation. Data is presented as IC_{50} values ± SD (3-5 experiments).

	IC_{50} (μM)				
	DFO	**Dp44mT**	**DpC**	**Gemcitabine**	**5-fluorouracil**
MIAPaCa-2	38.703 ± 6.205	0.001 ± 0.001	0.005 ± 0.001	0.016 ± 0.005	24.267 ± 6.345
PANC-1	9.463 ± 1.415	0.004 ± 0.001	0.030 ± 0.002	10.988 ± 0.799	62.303 ± 6.536
CAPAN-2	6.954 ± 5.427	0.001 ± 0.001	0.020 ± 0.008	40.791 ± 4.723	54.247 ± 17.129
CFPAC-1	14.742 ± 3.059	0.200 ± 0.054	0.203 ± 0.155	0.022 ± 0.020	41.221 ± 1.069

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Table 2: Body weight loss (% of total weight) and organ and tumor weights (g) in mice treated with vehicle control, Dp44mT (0.4 mg/kg i.v.; 5 days/week), DpC (5 mg/kg i.v.; 5 days/week) and gemcitabine (5 mg/kg i.p.; every 3rd day) following 44 days of treatment. Values are mean ± SEM (n = 8 mice/group).

Organ	Experimental Groups (n=8)			
	Control	**Dp44mT (0.4 mg/kg/day)**	**DpC (5 mg/kg/day)**	**Gemcitabine (5 mg/kg/3 days)**
Body weight loss (% of total weight)	104.9 ± 4.6	99.4 ± 5.7	88.0 ± 6.5***	104.5 ± 2.2
Liver (g)	0.96 ± 0.11	0.88 ± 0.03	0.80 ± 0.03	1.04 ± 0.03
Spleen (g)	0.12 ± 0.01	0.13 ± 0.01	0.08 ± 0.01 **	0.13 ± 0.01
Kidney (g)	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.19 ± 0.03
Heart (g)	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01
Brain (g)	0.32 ± 0.02	0.31 ± 0.01	0.30 ± 0.01	0.32 ± 0.01
Tumor (g)	0.29 ± 0.07	0.12 ± 0.03*	0.04 ± 0.01***	0.07 ± 0.02**

* p<0.05, ** p<0.01, *** p<0.001

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Table 3: Hematological indices of mice treated with vehicle control, Dp44mT (0.4 mg/kg i.v.; 5 days/week), DpC (5 mg/kg i.v.; 5 days/week) and gemcitabine (5 mg/kg i.p.; every 3rd day) after 44 days of treatment. Values are displayed as mean ± SEM (*n* = 8 mice/group).

	Experimental Groups (*n*=8)			
	Control	**Dp44mT (0.4 mg/kg/day)**	**DpC (5 mg/kg/day)**	**Gemcitabine (5 mg/kg/3 days)**
RBC x 10^{12}/L	10.17 ± 0.15	9.99 ± 0.12	9.46 ± 0.31	9.65 ± 0.08
Hb g/L	146.44 ± 1.68	137 ± 1.77**	131.88 ± 4.31**	147.88 ± 1.19
Hct	0.44 ± 0.01	0.43 ± 0.01	0.41 ± 0.01	0.45 ± 0.01
Platelets x 10^9/L	1042.22 ± 146.57	1418.63 ± 164.11	1281.88 ± 159.97	1060.13 ± 204.75
WBC x 10^9/L	4.53 ± 0.44	3.15 ± 0.51	3.5 ± 0.34	5.51 ± 0.56
Reticulocytes x 10^{12}/L	0.56 ± 0.06	0.87 ± 0.11*	0.77 ± 0.08*	0.35 ± 0.12

* $p<0.05$, ** $p<0.01$

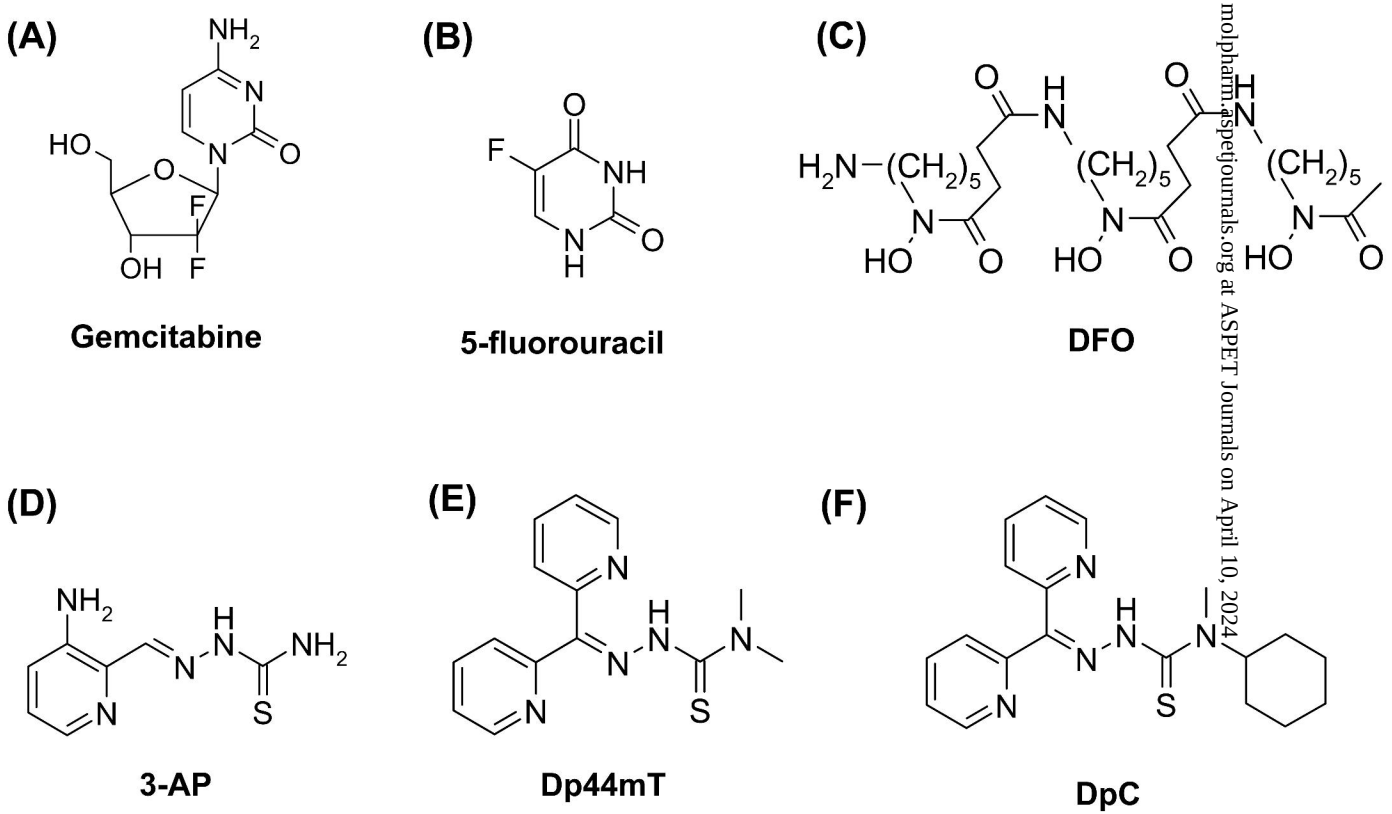
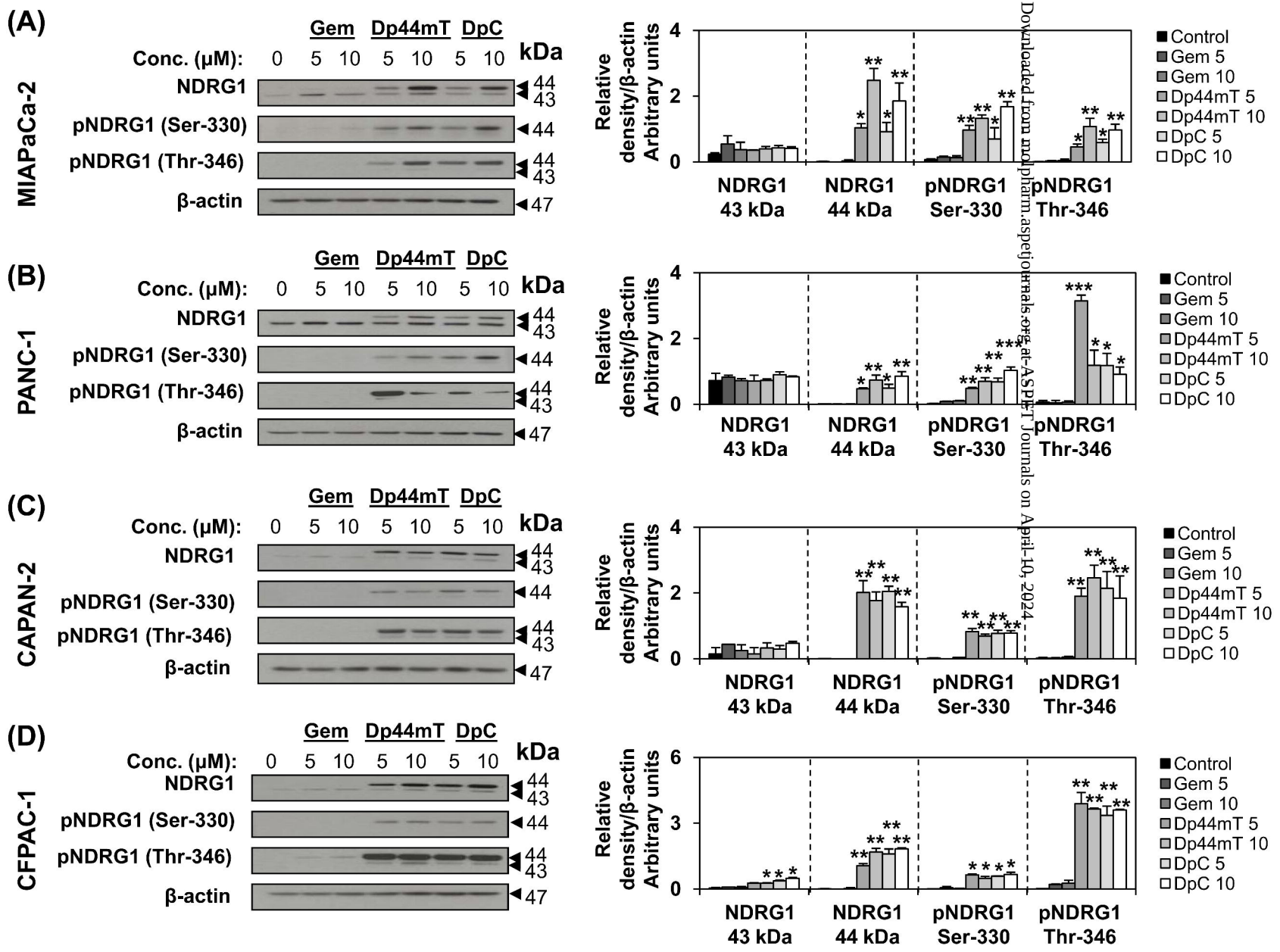


Figure 1



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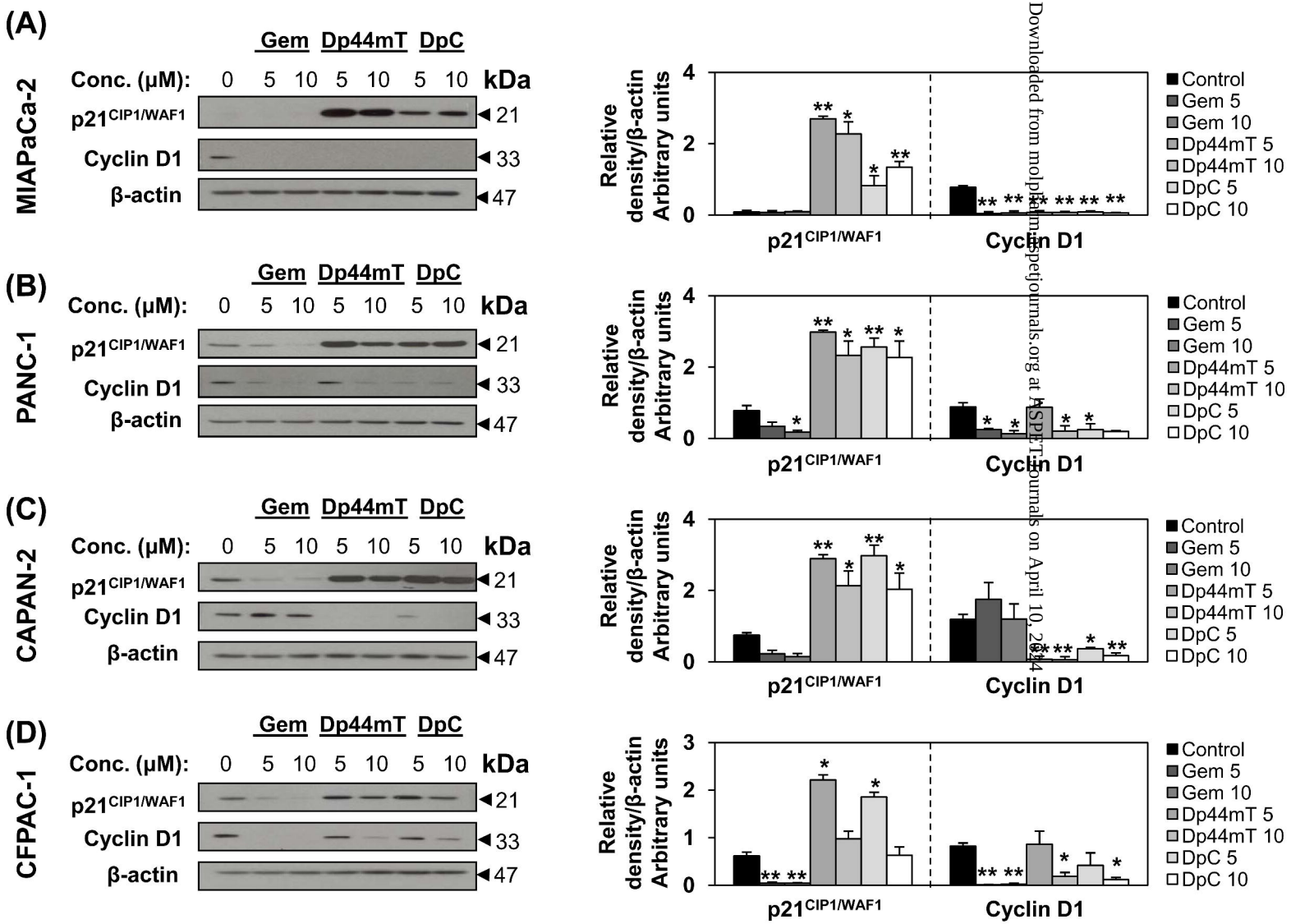

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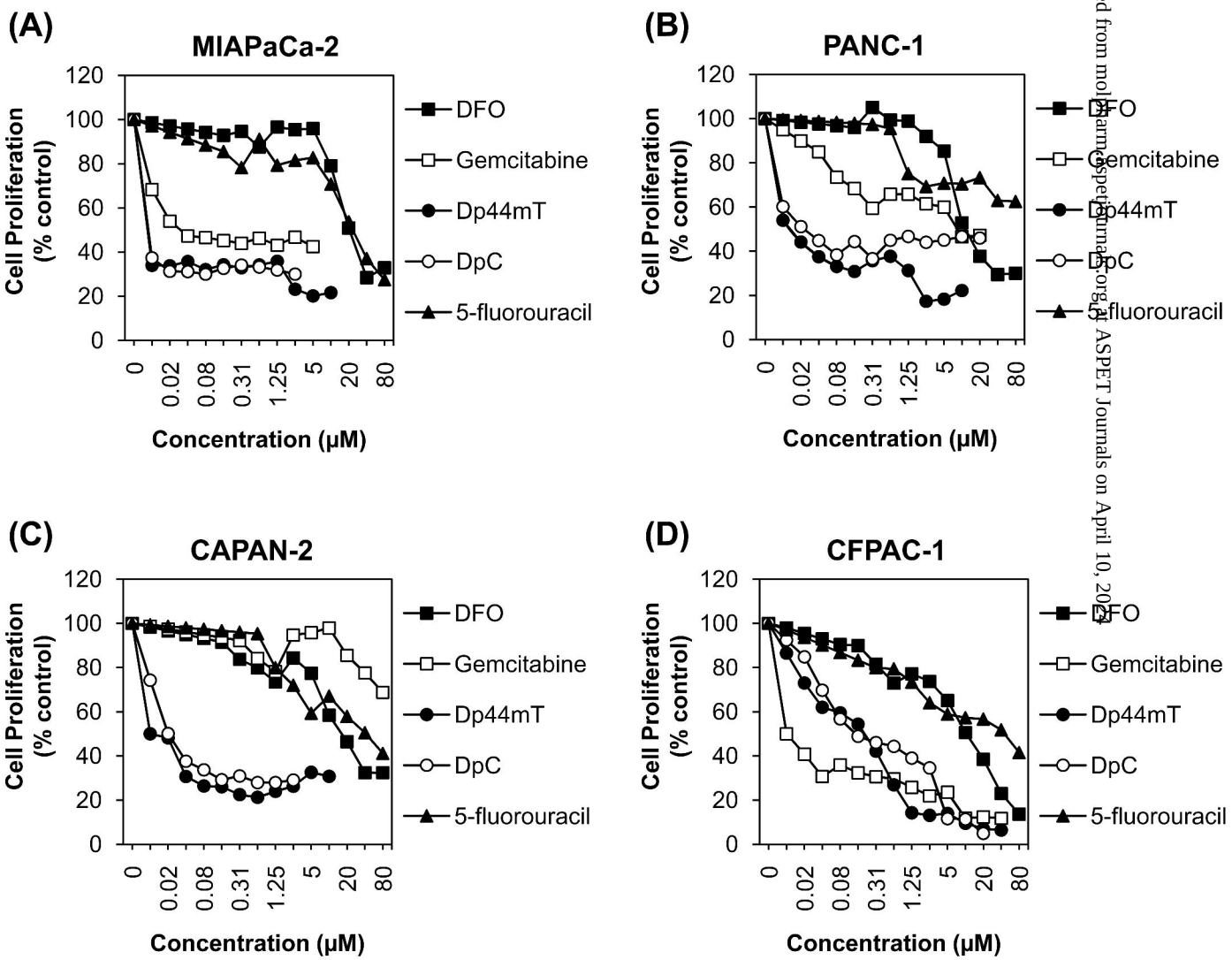


Figure 4

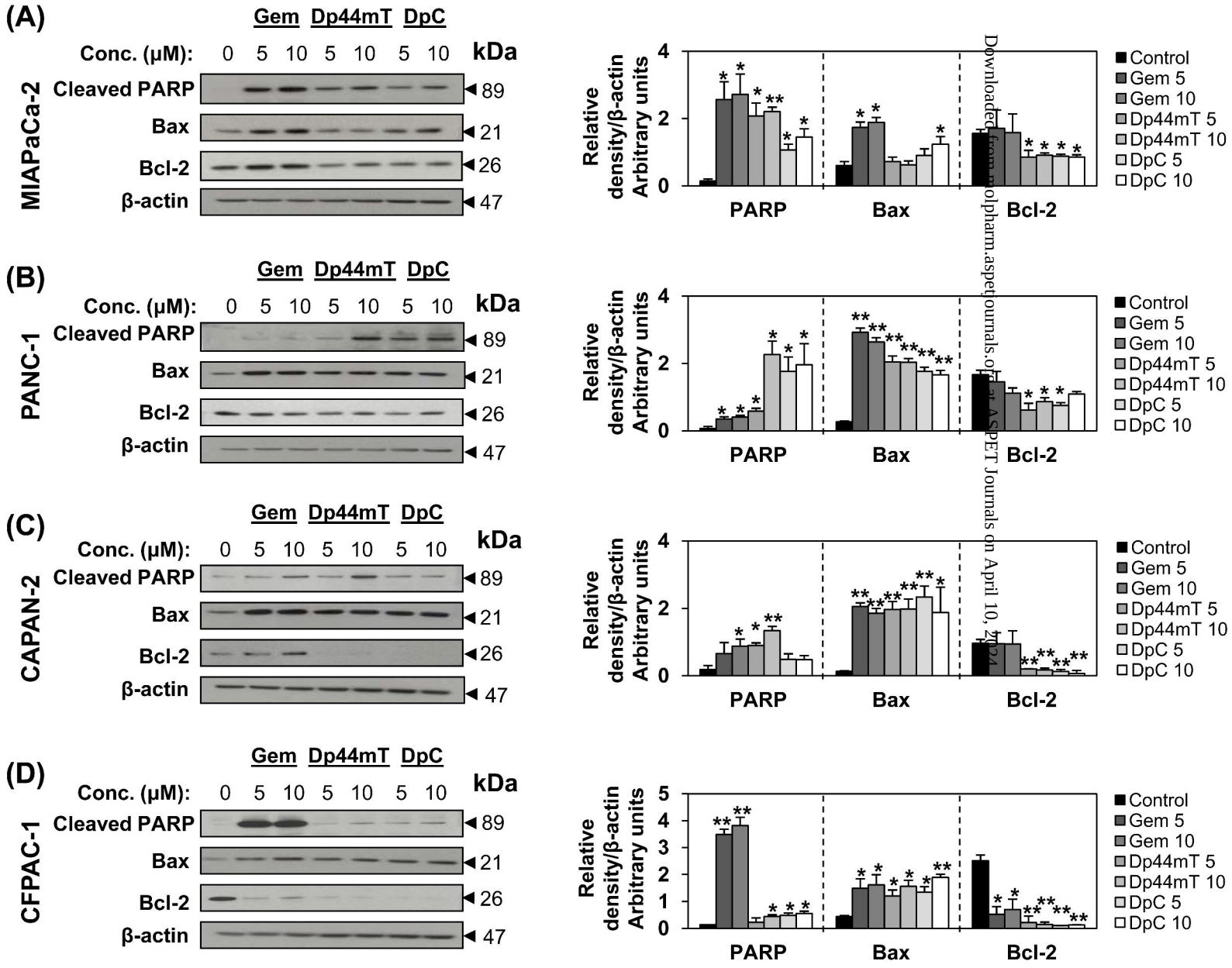
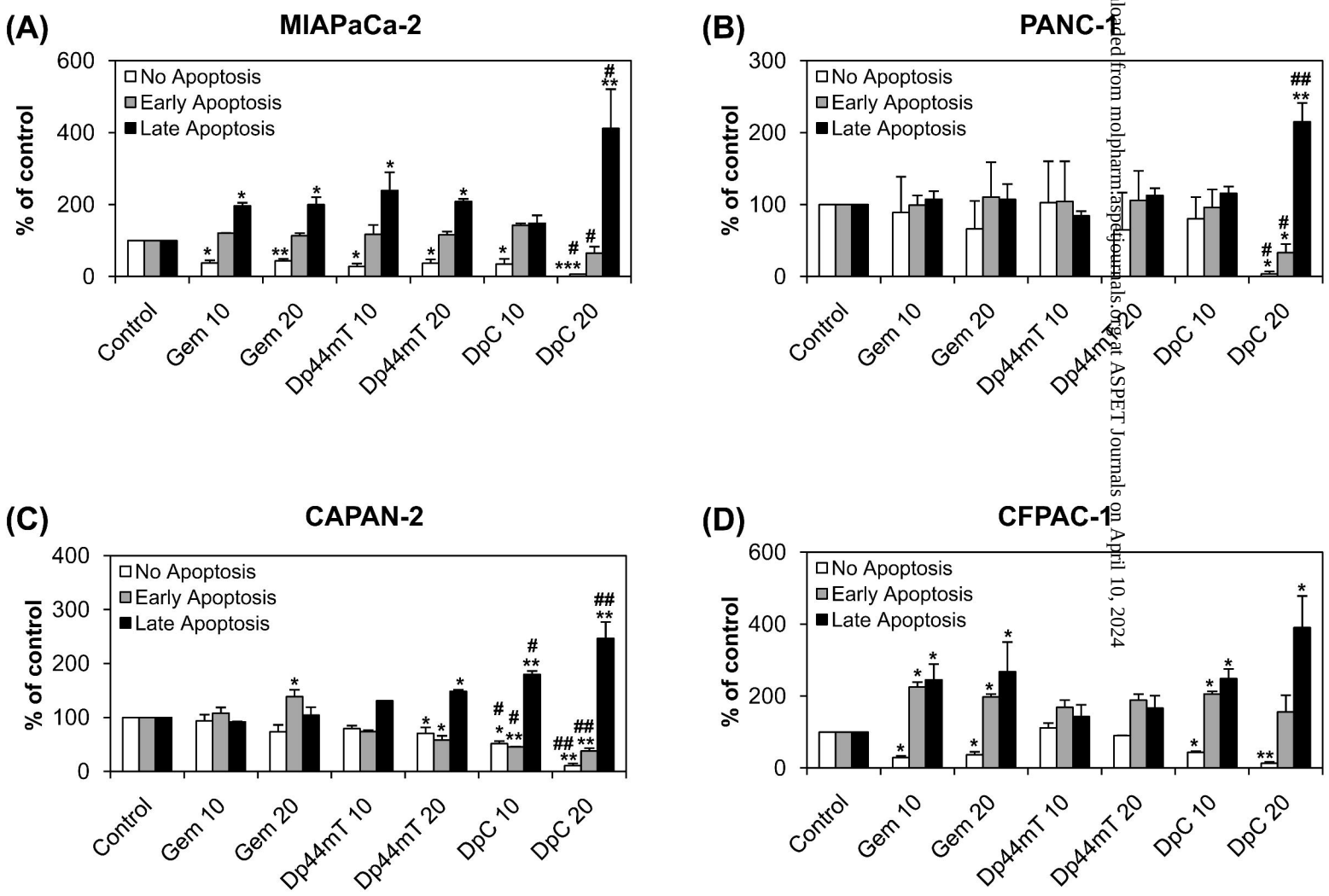


Figure 5



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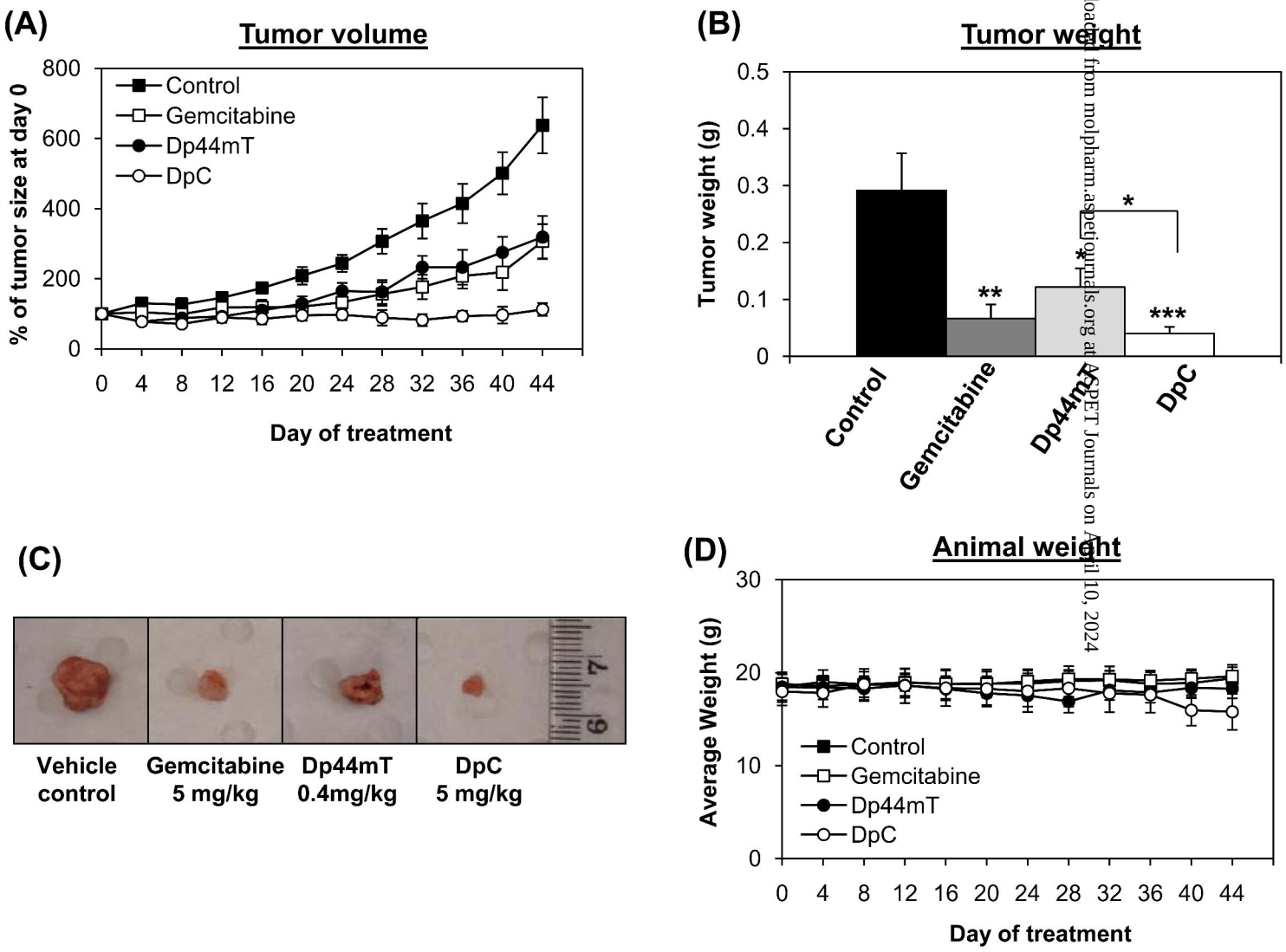

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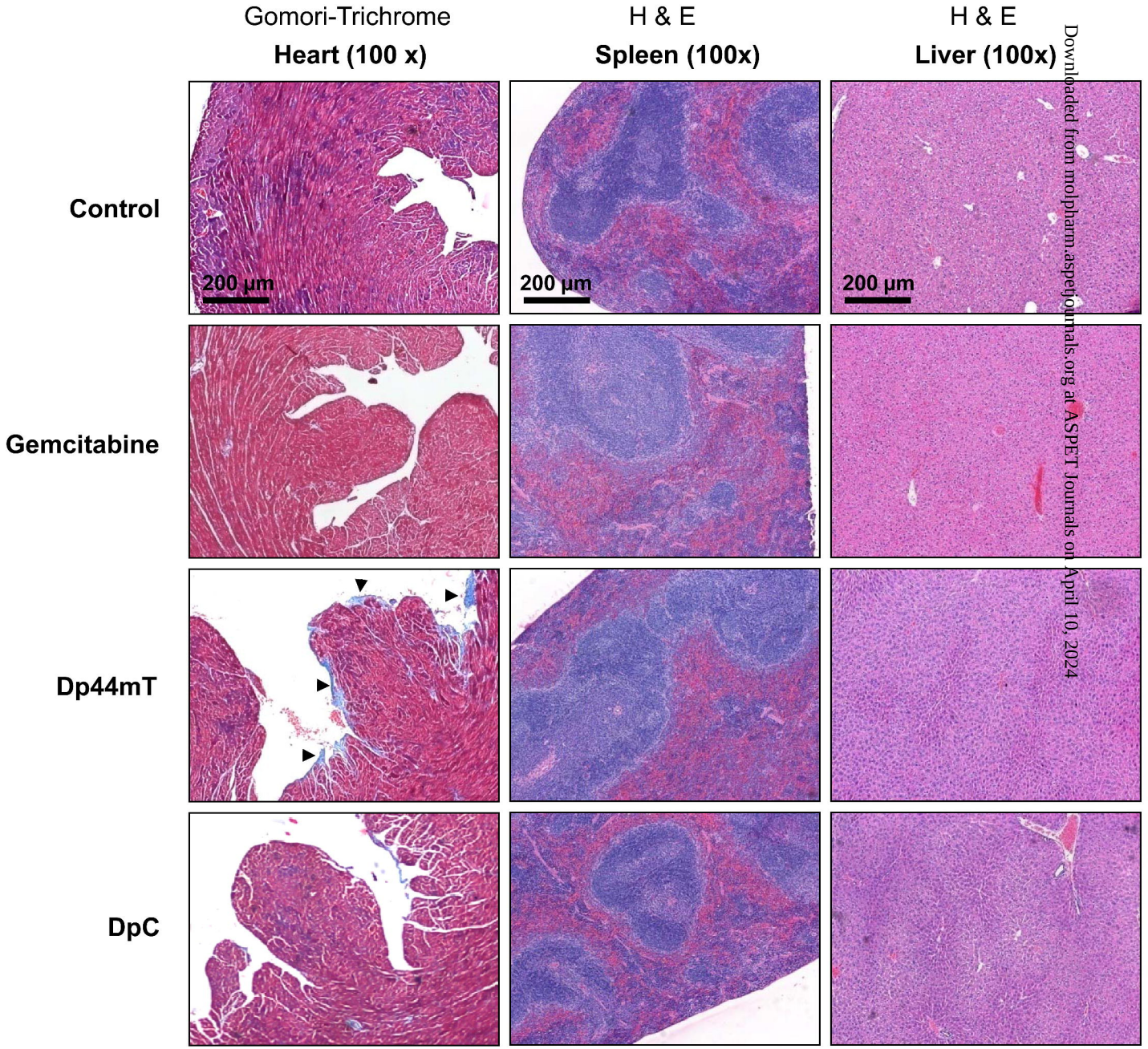

Figure 8