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RESEARCH ARTICLE

Cladribine inhibits a diltiazem-induced increase in red blood cell purine nucleotide concentrations in a zebrafish model

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Abstract

Minimizing drug interactions is paramount to improving the efficacy and tolerability of cancer therapy. The zebrafish represents an innovative cancer model due to highly conserved genetics and inherent capacity for high-throughput chemical screening. This pilot study extends the utility of the zebrafish to a preclinical model for pharmacodynamics by examining the interaction of the nucleoside analogue, cladribine with the calcium channel blocker, diltiazem. Cladribine (0.7–3.5 mM) and/or diltiazem (2.4 mM), was injected intraperitoneally into adult zebrafish and red blood cell (RBC) lysates were assayed by HPLC for levels of purine nucleotides (e.g. ATP), potential biomarkers of cardiovascular health. Diltiazem increased RBC ATP concentrations, which were inhibited by co-injection of cladribine. These results suggest a novel drug interaction and highlight the feasibility of the zebrafish as an *in vivo* model for pharmacodynamic studies.

Keywords: *Zebrafish; pharmacodynamics; cladribine; diltiazem; HPLC; ATP*

Introduction

Tremendous advances have been made in treating both adult and childhood cancer through the use of novel anticancer agents and combination chemotherapy. However, these drugs are associated with numerous potential side-effects and they frequently interact with other concomitantly administered medications. Cladribine (2-CdA) is one of the prototypic nucleoside antineoplastic agents that has been shown to be effective in the treatment of hairy cell leukaemia (Belani & Saven 2006, Else et al. 2009) and systemic mastocytosis (Pardanani et al. 2004, Quintas-Cardama et al. 2006, Tefferi et al. 2001), and has a role as salvage chemotherapy in relapsed or refractory acute myeloid leukaemia (AML) (Rubnitz et al. 2009, Wierzbowska et al. 2008). It may also have efficacy in the treatment of a variety of solid tumours (Armitage et al. 2004) and autoimmune diseases, such as multiple sclerosis (Brousil et al. 2006, Robak et al. 2009). Cladribine is transported across the plasma membrane by passive and/or facilitated diffusion. The prodrug is phosphorylated intracellularly to cladribine monophosphate (2-CdAMP) by the nuclear/cytosolic enzyme deoxycytidine kinase (dCK) and the mitochondrial enzyme deoxyguanosine kinase. 2-CdAMP resists deamination by adenosine deaminase (ADA), and accumulates intracellularly. Subsequent phosphorylation events yield 2-chloro-2-deoxy-β-D-adenosine triphosphate (2-CdATP), the active form, which inhibits the ribonucleotide reductase-catalysed conversion of deoxyribonucleotides to ribonucleotides (Elledge et al. 1992). 2-CdATP is incorporated into replicating DNA and competitively inhibits DNA polymerases resulting in termination of DNA chain elongation and inhibition of repair, ultimately leading to cellular apoptosis (Beutler 1992, Carson et al. 1980, Lotfi et al. 2003). Cladribine and other nucleoside analogues, like fludarabine are

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associated with a number of well-described toxicities including myelosuppression, nausea and vomiting, and less commonly cardiac toxicity (Morandi et al. 2005, Tallman et al. 1995). More frequently, adults undergoing treatment with cladribine may have pre-existing cardiac disease or hypertension and may receive antiarrhythmic or antihypertensive medications. An underlying cardiac condition has the potential to exacerbate the cardiac toxicity associated with cladribine, which may precipitate clinically important drug interactions. Preclinical pharmacodynamic studies that reliably predict drug delivery and interaction are necessary for developing effective chemotherapy regimens and anticipating complications. These studies are best achieved using reproducible validated *in vitro* assays in conjunction with relevant animal model systems.

We have previously posited that erythrocyte (red blood cell, RBC) purine nucleotide concentrations could serve as biomarkers for cardiovascular health (Yeung et al. 2008a, 2009). These nucleotides such as adenosine 5'-triphosphate (ATP), have been shown to play critical roles in regulating coronary blood flow (Jeremias et al. 2000, Oxhorn et al. 2000, Tune et al. 2002), ischaemic preconditioning (de Jonge et al. 2001, Donato & Gelpi 2003, Funahashi 2003), and protection of the myocardium (Ashraf et al. 1994, Kitakaze et al. 1994). ATP produced through cellular respiration and released into the bloodstream is quickly taken up by myocardial cells and RBCs. Within both of these cell types, ATP is dephosphorylated to ADP and AMP, and subsequently metabolized into oxypurine metabolites (Berne 1980, Rounds et al. 1994, Yeung et al. 1997). While some differences may exist regarding the precise mechanisms underlying ATP metabolism in myocardial cells and erythrocytes, this response occurring in the RBC may reflect similar metabolic effects occurring in cardiac myocytes. Thus, ATP levels in the RBC may provide a surrogate measure of cardiac function.

We have recently developed a rapid ion-paired high-performance liquid chromatography (HPLC) assay that reproducibly measures purine nucleotide concentrations in RBCs (Yeung et al. 2008a). We demonstrated that this assay could accurately quantify purine nucleotide levels in rodent RBCs with resultant increases in ATP levels following exercise and/or administration of the calcium channel blocking agent, diltiazem. This HPLC assay was the first to be used successfully for *in vivo* pharmacodynamic monitoring studies of RBC purine nucleotide concentrations.

The fresh water fish, *Danio rerio* (zebrafish) has emerged as a robust vertebrate model for studying blood cell development (Berman et al. 2003, Carradice & Lieschke 2008) and tumorigenesis (Grabher & Look 2006, Langenau et al. 2003). Zebrafish demonstrate highly conserved genetics with mammals both in maintaining normal developmental programmes (Berman et al. 2005) and in possessing orthologous classic proto-oncogenes and tumour suppressor genes (Grabher & Look 2006). Transgenic technology has been well developed in the zebrafish, which has enabled the generation of a number of transgenic models of human cancers, including acute lymphoblastic leukaemia (Langenau et al. 2003), melanoma (Patton et al. 2005) and rhabdomyosarcoma (Langenau et al. 2007). Studies in the zebrafish complement those in mammalian models and cell culture, due to the inherent capacity for high-throughput screening of small-molecule inhibitors in the fish. We wanted to extend the utility of the zebrafish from recapitulating human malignancy to serving as an *in vivo* tool for pharmacodynamic studies of chemotherapeutic drug delivery and interaction. As we have generated and are currently characterizing transgenic zebrafish models of systemic mastocytosis and AML, cladribine was a logical agent for this pilot study. In this investigation, we have pioneered an approach to deliver cladribine and/or diltiazem repeatedly to wild-type adult zebrafish by intraperitoneal (IP) injection and adopted the HPLC assay to quantify RBC purine nucleotide levels in the zebrafish from whole blood obtained by cardiac puncture. We have found that this approach is feasible and demonstrated that cladribine inhibited the effect of diltiazem to increase RBC ATP levels in the zebrafish model.

Materials and methods

Fish maintenance

Adult wild-type (AB) zebrafish (*Danio rerio*) were maintained in the Aquatics Laboratory at IWK Health Centre (Halifax, NS) as described by Westerfield (Westerfield 1995). This study was approved by the Dalhousie University Animal Care Committee.

Optimization of intraperitoneal injection and sample preparation

To determine the number of IP injections and dosing frequency for the study, wild-type adult zebrafish were anaesthetized in pairs with 100 μg ml^{-1} tricaine (MS-222) in a 'treatment tank' and injected IP with 4 μl of saline in combination with 1 μl of 0.07% bromophenol blue, a marker dye used to ensure correct anatomical location, at 09:00 h and 15:00 h daily to a maximum of five doses. The zebrafish were allowed to recover and monitored for 12 h postinjection (hpi) after the final injection, before being euthanized in 2 mg ml^{-1} tricaine. We found that

three injections per fish over a 2-day period (09:00 h, 15:00 h and 09:00 h the following day) were well tolerated by most fish.

Heparin is required for proper RBC processing to prevent sample clotting, but may also result in decreased ATP levels (Sun & Chai 2002). A pilot study was conducted to determine the minimum amount of heparin necessary to preserve RBC lysate samples. Six wild-type adult zebrafish were injected with saline and bromophenol blue at the optimal frequency described above, and sacrificed 1 hpi by tricaine overdose as described above. Immediately postmortem, each fish was laid on a fitted platform, and a small ventral slit was made using microdissection scissors to reveal the heart. A fine-tipped pipette filled with heparin was used to rupture the heart and blood was collected and dispensed into a 1.5 ml Eppendorf tube containing heparin. Five sets of the experiment were performed using different concentrations and volumes of heparin (Table 1). Heparin stock solutions were diluted with an equal volume of 1x phosphate-buffered saline (PBS). Samples were then centrifuged (855*g*, 5 min at 4°C) and the plasma removed. The pellet was resuspended with 2 µl of PBS and lysed with the addition of 2 µl of ice-cold 10% trichloroacetic acid (TCA) followed by centrifugation (855*g* for 10 min at 4°C). The lysate was collected in PCR tubes, and stored at -80°C. A heparin volume of 10 µl in the pipette tip for cardiac puncture and 20 µl in the Eppendorf tube for whole blood collection (both 1000 USP units ml^{-1} stock concentration) was found to be optimal.

Table 1. Optimization of heparin concentration and volume.

Trial	Volume in pipette tip (µl)	Concentration in pipette tube (USP units ml^{-1})	Volume in Eppendorf tube (µl)	Concentration in Eppendorf tube (USP units ml^{-1})
1	20	1000	20	1000
2	10	1000	20	1000
3	10	1000	20	500
4	10	1000	20	333
5	10	1000	20	200

Table 2. Zebrafish intraperitoneal injection drug combinations.

Trial	Treatment 1	Treatment 2
1	Saline	N/A
2	Cladribine (0.7 mM)	Saline
3	Cladribine (1.4 mM)	Saline
4	Cladribine (2.1 mM)	Saline
5	Cladribine (3.5 mM)	Saline
6	Saline	Diltiazem (2.4 mM)
7	Cladribine (0.7 mM)	Diltiazem (2.4 mM)
8	Cladribine (1.4 mM)	Diltiazem (2.4 mM)
9	Cladribine (3.5 mM)	Diltiazem (2.4 mM)

Drug trials

Six wild-type adult zebrafish were injected per trial with the applicable drug combination (Table 2) together with two control fish, which were injected with saline. For each trial, fish were anaesthetized and temporarily paralysed in 100 µg ml^{-1} tricaine and injected IP for a total of three doses administered at 09:00 h, 15:00 h and 09:00 h the following day, as described above. A total volume of 5 µl (4 µl of drug and/or saline with 1 µl of 0.07% bromophenol blue) was injected per dose. Cladribine solutions were prepared from either 1 mg ml^{-1} or 2 mg ml^{-1} stock solutions. Concentrations of cladribine solutions injected ranged from 0.7 mM to 3.5 mM. Diltiazem was prepared from a 5 mg ml^{-1} stock solution and a 2.4 mM solution was generated for injection. Injection concentrations correspond to doses of 1–5 mg kg^{-1} of cladribine and 5 mg kg^{-1} of diltiazem, respectively, based on positive results observed for equivalent doses in the Sprague–Dawley rat model (Yeung et al. 2008a, b). Cardiac puncture was performed and a median of 6 µl (mode 5 µl, range 2–16 µl, n = 70) samples were obtained 1 h after the third injection. Collected blood samples were centrifuged at 855g for 10 min at 4°C and processed as described above.

HPLC analysis

Details of the HPLC method for measuring purine nucleotide concentrations have been reported (Yeung et al. 2008a). Briefly, the system consisted of a Shimadzu LC-10AT *VP* solvent delivery module (Man-Tech Associate Inc., Guelph, ON, Canada), a Rheodyne syringe loading injector (model 9725), with a 100 µl PEEK injection loop (Scientific Products & Equipment, Concord, ON, Canada) and a Spectra-Physics variable wavelength ultraviolet detector (Spectra 100; Spectra-Physics Inc., San Jose, CA, USA). Chromatographic separation was achieved by ion-paired chromatography on a 250 × 3.0 mm ID Supelcosil LC-18-T column bonded to a 5 µm spherical silica packing, pore size 120 Å (Supelico Inc., Bellefonte, PA, USA) preceded with a 5 µm 4.0 × 4.0 mm ID C_{18} reversed-phase cartridge guard column (LiChrocart®; E.M. Merck, Darmstadt, Germany). Separation was achieved using a mobile phase of a 0.0005 M tetrabutyl ammonium hydrogen sulfate (TBAS) solution in 0.1 M KH_2PO_4:acetonitrile:methanol (9.6:0.3:0.1) with a final pH of 6.3. The system was operated at room temperature with a flow rate of 0.5 ml min^{-1} and an operating pressure of 11.5 mPa (ca. 1.7 kpsi). The wavelength was set at 254 nm for detection and quantification. Detector output was recorded by an HP3395 Integrator (Hewlett-Packard, Palo Alto, CA, USA), and digitalized using the

Peak Simple® software (Chromatographic Specialties Inc., Brockville, ON, Canada).

Statistical analysis

Data between treated groups of fish were compared by ANOVA and a difference considered significant when $p < 0.05$.

Results

IP injection of cladribine into wild-type adult zebrafish was made at concentrations of 0.7–3.5 mM. Red cell lysates were prepared and assayed by HPLC for levels of purine nucleotides after correction for dilution as described previously (Yeung et al. 2008a). RBC ATP and other purine nucleotide levels following cladribine treatment were not significantly different following increasing cladribine dose (0.7–3.5 mM) (Figure 1). However, diltiazem at a dose of 2.4 mM increased RBC concentrations of ATP, ADP, AMP, GTP and GDP (Figure 2), although only the increase of the adenine nucleotides reached statistical significance compared with control samples (ATP, 0.75 ± 0.098 vs 0.48 ± 0.22 mM; ADP, 0.32 ± 0.042 vs 0.14 ± 0.072 mM; AMP, 0.093 ± 0.019 vs 0.043 ± 0.019 mM) ($p < 0.05$). Co-injection of cladribine even at the 0.7 mM dose significantly inhibited the cardiovascular effects of diltiazem on increasing adenine nucleotide concentrations ($p < 0.05$) (Figure 2). However, a further increase of cladribine dose to 1.4 mM or 3.5 mM did not result in a further decrease in nucleotide levels (data not shown).

Discussion

Chemotherapeutic approaches to treating cancer have become more complex incorporating classical cytotoxic agents in combination with molecularly targeted and immunologically based therapies. These multifaceted approaches of polypharmacy significantly increase the potential for drug interactions and unforeseen toxicities particularly for patients with a background of other comorbidities and associated therapeutic interventions. While *in vitro* studies are beneficial for demonstrating efficacy, animal modelling is a necessary preclinical segue before a drug or drug combination can proceed to a clinical trial. Small size, high reproductive capacity and ease of genetic manipulation have catapulted the zebrafish into its current position for studying human cancers. High-throughput chemical screening with zebrafish has begun to identify promising new anticancer agents (Kalen et al. 2009, Lally et al. 2007, Murphey et al. 2006). While these studies are tremendously exciting in their capacity to identify new possible therapeutic strategies, they limit the zebrafish as a screening tool for drug discovery. We aimed to expand further the potential of this model system for study of pharmacodynamic drug interactions.

We had previously investigated the utility of the zebrafish as a pharmacokinetic model. Following a similar approach to that described here, we performed IP injection of a single dose of cladribine into adult zebrafish with subsequent measurement of serum levels of the drug. Our initial studies appeared promising, with

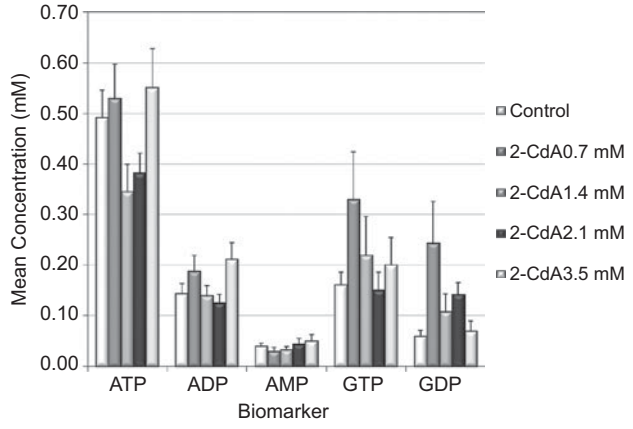

Figure 1. High-performance liquid chromatography (HPLC) on zebrafish red blood cell (RBC) lysates demonstrates no significant increase in nucleotide levels following intraperitoneal injection of cladribine (2-CdA) at different doses. Data expressed as mean concentration in mM with standard error of the mean (SEM). (Graph was generated using Microsoft Excel 2007 and formatted using Adobe Photoshop CS3 Extended, Version 10.0.)

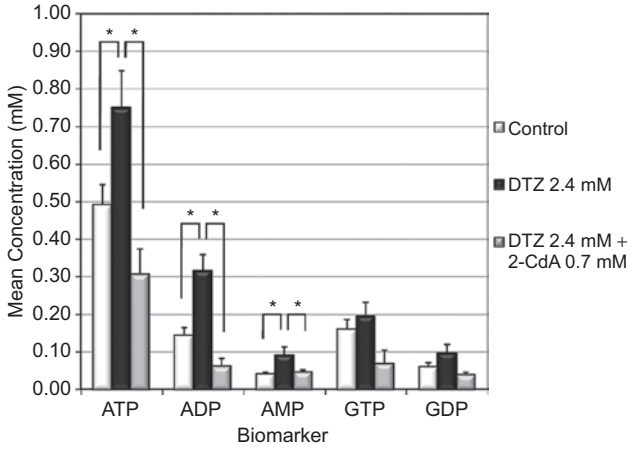

Figure 2. High-performance liquid chromatography (HPLC) on zebrafish red blood cell (RBC) lysates demonstrates a significant increase in adenine nucleotide levels ($p < 0.05$) following intraperitoneal injection of 2.4 mM diltiazem (DTZ) and a significant decrease in adenine nucleotide levels ($p < 0.05$) following intraperitoneal injection of 2.4 mM diltiazem together with 0.7 mM cladribine (2-CdA). Data expressed as mean concentration in mM with standard error of the mean (SEM). (Graph was generated using Microsoft Excel 2007 and formatted using Adobe Photoshop CS3 Extended, Version 10.0.)

drug clearance paralleling an established rodent model. However, concerns regarding inherent features of the zebrafish as a pharmacokinetic system were apparent, such as the potential for drug diffusion (i.e. excretion and re-absorption) through the integument of the fish (Yeung et al. 2008b). Thus, we embarked upon the current pharmacodynamic study, in which we incorporated a previously validated and reproducible assay measuring rodent RBC nucleotide levels and effectively adopted it for use in the zebrafish model. Some technical restrictions remain with these zebrafish assays when compared with similar mammalian models, such as the inability to perform sequential blood sampling from the same fish due to the small volume of blood available and the need for invasive intracardiac sampling. However, these limitations are more than compensated for by the opportunities afforded by the zebrafish to expose a large study population to numerous drug doses and experimental conditions in a rapid cost-effective manner, particularly when supply of drug is a limiting factor.

Injection of cladribine at several different dose levels resulted in no demonstrable change in zebrafish RBC nucleotide levels (Figure 1). While a decrease in nucleotide levels may have been anticipated, particularly as the doses administered were up to ten times the human dose, these findings are not out of keeping with clinical data suggesting that cardiovascular effects are an uncommon toxicity of cladribine, when given as a single agent (Morandi et al. 2005, Tallman et al. 1995). However, diltiazem at a dose of 2.4 mM resulted in an increase of nucleotide levels and ATP levels, in particular (Figure 2). This finding mirrors those observed in the exercise rodent model (Yeung et al. 2008a) and suggests conservation of physiological and biochemical responses to calcium channel blocking agents. Most significant is the novel observation of the interaction between cladribine and diltiazem. We observed that this combination resulted in a reduction in the levels of RBC ATP and other nucleotides even below baseline levels (Figure 2). These results suggest that cladribine inhibits one of the key cardiovascular effects of diltiazem, and demonstrate the feasibility of the zebrafish as an *in vivo* readout for these types of pharmacodynamic studies. While the clinical relevance of measuring the levels of purine nucleotides as biomarkers for cardiovascular health still needs to be validated (Yeung et al. 2009), the evidence we have shown for antihypertensive and anti-ischaemic agents earlier in the rodent model (Yeung et al. 2008a), and now in the zebrafish model, warrants investigation in a defined clinical setting.

Cladribine has been frequently used as a salvage therapy for systemic mastocytosis (Pardanani et al. 2004, Quintas-Cardama et al. 2006, Tefferi et al. 2001) and refractory AML (Rubnitz et al. 2009, Wierzbowska et al. 2008). Its efficacy has often been tempered by high toxicity. Transgenic zebrafish models of human mastocytosis and high-risk AML have been established in our laboratory and are currently being characterized (L.C. Klein and J.N. Berman, unpublished observations). In addition to subjecting these zebrafish cancer models to high-throughput small-molecule inhibitor screens, with the approach piloted in this study, we can further exploit this system as an *in vivo* platform for evaluating the interactions of novel targeted therapies identified with current chemotherapeutic agents. Moreover, this study also hearkens at the potential to adapt additional pharmacodynamic assays to the zebrafish model.

In summary, this study contributes to the expanding portfolio of the zebrafish in cancer research. With the addition of capacity for high-throughput pharmacodynamic studies, we have suggested that the zebrafish can move beyond serving as a screening tool to a more complete preclinical animal model for developing novel cancer therapies.

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