Diindolylmethane Inhibits the Activity of P-glycoprotein in 17-71 Canine B-Cell Lymphoid Tumor Cells

Ala Mansour, Kodye Abbott, Patrick Flannery, Elaine Coleman, Amit Tiwari, and Satyanarayana Pondugula

ABSTRACT

B-cell lymphoma is the most common hematopoietic tumor in dogs and is usually treated with multiple chemotherapy drugs. However, relapses are often seen and chemoresistance is a significant concern in cases of relapses. Chemoresistance in B-cell lymphoma was shown to be associated with upregulation of the expression/activity of multidrug transporters, particularly P-glycoprotein (P-gp). The existing P-gp inhibitors have limited success mainly due to undesired toxicities. Novel and safer approaches are therefore crucial for overcoming chemoresistance by downregulating P-gp. Here, we studied whether 3,3’-diindolylmethane (DIM), a natural dietary supplement, affects the function of P-gp in the chemoresistant 17-71 canine B-cell lymphoid tumor cells. Cell viability, P-gp function, and P-gp gene expression were studied using ATP-based CellTiter-Glo luminescent cell viability assays, intracellular rhodamine 123 accumulation assays, and RT-PCR, respectively. DIM, at its physiologically relevant concentrations, did not significantly affect the viability of either the chemoresistant 17-71 and chemosensitive GL-1 canine lymphoid tumor cells, suggesting that DIM is non-cytotoxic at physiologically relevant concentrations. P-gp specific inhibitor PSC-833 significantly increased the intracellular accumulation of P-gp substrate rhodamine 123 in 17-71 but not GL-1 cells, suggesting that 17-71, but not GL-1, expresses functional P-gp. Similar to PSC-833, DIM at its non-cytotoxic concentrations significantly increased the intracellular accumulation of rhodamine 123 in 17-71 cells, indicating that DIM inhibits the activity of P-gp in 17-71 cells. These results are consistent with our conclusion that DIM inhibits the function of P-gp in the chemoresistant canine 17-71 B-cell lymphoid tumor cells.

INTRODUCTION

Lymphoma is one of the most common cancers in dogs, accounting for up to 25% of all canine cancers (Meuten, 2002). Notably, B-cell lymphoma accounts for about 85% of lymphomas (Ruslander, Gebhard, Tompkins, Grindem, & Page, 1997). Most untreated dogs diagnosed with B-cell lymphoma will only survive for 4-6 weeks (Meuten, 2002). B-cell lymphoma is usually treated with aggressive chemotherapy protocols involving a combination of chemotherapeutics, including vincristine (VCR) and doxorubicin (DOX). While these multi-agent chemotherapy regimens (Dervisis et al., 2007; Fahey et al., 2011; Flory et al., 2008; Griessmayr, Payne, Winter, Barber, & Shofer, 2009; Lori, Stein, & Thamm, 2010; Moore et al., 1999; Northrup et al., 2009; Rassnick et al., 2002; Rebhun et al., 2011) improve the survival time between 6 and 18 months (Lori et al., 2010), relapses are frequently seen. Most importantly, relapsed lymphoma often displays resistance to chemotherapy drugs, resulting in a poor prognosis. Due to high risk of cardiotoxicity, the lifetime cumulative dose of DOX must be limited (Banco, Grieco, Servida, & Giudice, 2011; MacDonald, 2009). Likewise, to
avoid potential gastrointestinal abnormalities, myelosuppression, and extravasation injury, the lifetime cumulative dose of VCR must also be limited (Banco et al., 2011; MacDonald, 2009). As such, novel therapeutic approaches are sought to combat chemoresistance as well as avoid the limiting threshold of the chemotherapy drugs.

Chemoresistance in a variety of cancers is associated with upregulation of expression/function of multidrug transporters (drug efflux pumps), such as P-glycoprotein (P-gp) (Borst, Evers, Kool, & Wijnholds, 2000; Chen et al., 2012; Marzag et al., 2011; Pondugula and Mani, 2013). Chemoresistance in canine B-cell lymphoma is also associated with P-gp upregulation (Fan, 2003; Lee, Hughes, Fine, & Page, 1996; Moore, Leveille, Reimann, Shu, & Arias, 1995; Steingold et al., 1998; Uozurmi, Nakaichi, Yamamoto, Une, & Taura 2005). Functionally, P-gp prevents the retention of cytotoxic drugs within tumor cells resulting in little or no cytotoxic effect. For example, P-gp can preclude the cytotoxic effect of VCR and DOX by exporting them from tumor cells (Borst et al., 2000; Okamura, Sakaeda, & Okumura, 2004; Sharom, 2008; Sodani, Patel, Kathawala, & Chen, 2012).

A common approach to avoiding chemoresistance and drug cytotoxicity involves the use of rationally designed combinatorial treatments. The dual employment of one of several pharmaceutical P-gp inhibitors, such as verapamil, valsodar, cyclosporine A, PSC-833, or Tariquidar (Baer et al., 2002; Daenen et al., 2004; Fox and Bates, 2007; Kolitz et al., 2004), along with anticancer agents is one conventional method of reversing chemoresistance (Dano, 1973; Tsuruo, Iida, Tsukagoshi, & Sakurai, 1981). However, such P-gp inhibitors have had limited success because of their undesired dose-dependent toxicity and unpredictable pharmacokinetic side effects (Thomas and Coley, 2003). Due to these restraints, the use of naturally occurring compounds as alternatives to these pharmaceutical P-gp inhibitors has been a topic of massive interest in cancer research.

3,3′-Diindolylmethane (DIM) is a natural health supplement and is the major active metabolite of indole-3-carbinol (I3C) (Anderton et al., 2004b), which is also a natural health supplement as well as a naturally occurring compound in cruciferous vegetables (Bonnesen, Eggleston, & Hayes, 2001). DIM is used to treat recurrent respiratory papillomatosis (Auborn, 2002; Wiatrak, 2003). The emerging evidence from several studies indicates that DIM could be used for both the treatment and prevention of a variety of human cancers, including prostate and breast cancers (Azmi et al., 2008; Biersack and Schobert, 2012; Chen et al., 2012). The aim of this study was to investigate whether DIM inhibits the function of P-gp in the chemoresistant canine B-cell lymphoid tumor cells.

MATERIALS AND METHODS

Cell Culture

17-71 and GL-1 canine B-cell lymphoid tumor cell lines were provided by Dr. Steven Suter, North Carolina State University. These lymphoid tumor cell lines are well characterized to reflect in vivo properties and widely used for B-cell lymphoma studies (Jamadar-Shroff, Papich, & Suter, 2009; Kojima, Fujino, Goto-Koshino, Ohno, & Tsujimoto, 2013; Matsuda et al., 2010; Uozurmi et al., 2005; Zandvliet, Teske, Chapuis, Fink-Gremmels, & Schrickx, 2013). 17-71 and GL-1 cell lines were grown in RPMI-1640 medium (Lonza, Walkersville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA), 2 mM L-glutamine (Cellgro), and 1 mM sodium pyruvate (Cellgro). Both cell lines were cultured in a humidified incubator with an atmosphere of 5% CO₂ and 95% air at 37°C.

Chemicals

3,3′-Diindolylmethane (DIM), Dimethyl sulfoxide, Valspodar (PSC-833), and Rhodamine 123 (R123) were purchased from Sigma-Aldrich (St Louis, MO). DIM, PSC-833, and R123 were reconstituted in DMSO to obtain stock solutions.

Cell Viability Assays

17-71 and GL-1 cells were plated into 96-well culture plates
(PerkinElmer, Waltham, MA) at a density of 10,000 cells per well in a final volume of 100 µl medium. The cells were then either untreated or treated with DMSO or DIM (25 to 100 µM) for 24 h. The CellTiter-Glo luminescent cell viability assays (Promega, Madison, WI) were used to determine the number of viable cells by quantifying the ATP present, which indicates the presence of metabolically active cells. Luminescence was measured with a FLUOStar Optima plate reader (BMG Labtech, Cary, NC).

### Intracellular Rhodamine 123 Accumulation Assays

The efflux activity of P-gp in the lymphoid tumor cells was determined by measuring the intracellular accumulation of the fluorescent P-gp probe rhodamine 123 (Harmsen et al., 2010; Ishikawa et al., 2010). The tumor cells were washed with Hank’s Balanced Salt Solution (HBBS, without Ca²⁺, Mg²⁺ and phenol red) and incubated at 37°C for 15 min with or without DMSO, DIM, or PSC-833 (P-gp specific inhibitor) (Keller et al., 1992; Zandvliet et al., 2013) in HBBS. Rhodamine 123 (5 µM) was added later to the cells in the presence or absence of DMSO, DIM, or PSC-833 and incubated for another 45 min. The cells were washed with ice-cold-HBBS and solubilized in Triton-HBBS. To determine the intracellular concentration of rhodamine 123, the fluorescence was measured using the Infinite microplate reader (TECAN, San Jose, CA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

### RT-PCR analysis

Total RNA was extracted from 17-71 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). The quality and quantity of the total RNA was assessed using NanoVue Plus Spectrophotometer (GE Healthcare, Pittsburg, PA). Dog liver RNA was provided by Dr. Bruce Smith, Auburn University. Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen) to synthesize

### Table 1. Forward (F) and Reverse (R) Primers Used for Quantitative RT-PCR of 18S rRNA and P-gp

<table>
<thead>
<tr>
<th>Gene/Primer</th>
<th>Amplified Segment (bp)</th>
<th>Gene Bank Accession No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F: 5’- GAGGTTCAAGACGACGAGA-3’</td>
<td>315</td>
<td>BK000964</td>
<td>Pondugula, Tong, Wu, Cui, &amp; Chen (2010)</td>
</tr>
<tr>
<td>R: 5’- TCGCTCCACCACTAAGAAC-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F: 5’- CCATCTGGAGGAGAAATGA-3’</td>
<td>227</td>
<td>AF045016</td>
<td>Greger, Gropp, Morel, Sauter, &amp; Blum (2006); Gropp, Gregor, Morel, Sauter, &amp; Blum (2006)</td>
</tr>
<tr>
<td>R: 5’- TGGAGACATCGTCTGAGC-3’</td>
<td></td>
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[Figure 1. Detection of P-gp mRNA by RT-PCR in 17-71 cells. A single band was visualized for P-gp mRNA at the expected size in 17-71 cells as well as in positive control tissue dog liver. No signal was observed when RNA was used as negative control template. The identity of the band was verified by sequence analysis. M, 100-bp ladder.]
complementary DNA (cDNA) from total RNA. This cDNA was subsequently used as a template for polymerase chain reaction (PCR). PCR was performed by using Taq PCR Master Mix Kit (Qiagen) and PCR Detection System (Bio-Rad; Hercules, CA) according to the manufacturer’s protocol. Transcripts of the 18S small subunit ribosomal RNA (18S rRNA) housekeeping gene and P-gp were amplified using gene-specific primers (Table 1). Each of the 40 PCR cycles was conducted at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were electrophoresed on 2% agarose gel and detected by ethidium bromide to check the amplicon size. The specific bands were excised from agarose gels and the PCR products were purified using the gel extraction kit (Qiagen). Purified PCR product was sequenced by Auburn University gene sequencing facility. The sequences were then verified for their identity using the National Center for Biotechnology Information’s (NCBI’s) Basic Local Alignment Search Tool (BLAST) program.

Data Presentation and Statistical Analysis

Cell viability data are expressed as percentage of DMSO treated cells (control), where control was set as 100% viability. The vehicle DMSO treatment did not significantly affect cell viability when compared to untreated cells. Data are shown as mean values from at least three independent experiments with bars indicating the standard deviation. The Student’s t-test was used to determine statistical significance (*p < 0.05) of unpaired samples by comparing the viability of cells treated with DMSO to DIM. In accumulation assays, fluorescence intensity of the samples without R123 was considered as the background. Fluorescence intensity of all the samples with R123 was subtracted with the background fluorescence before data normalization. The efflux activity of P-gp is presented as relative R123 accumulation by normalizing the fluorescence intensity of the samples with vehicle DMSO, DIM, or PSC-833 to the samples without the vehicle, DIM, or PSC-833. Statistical significance (*p < 0.05 vs control) was determined using unpaired Student’s t-test.

RESULTS

P-gp Gene Expression in 17-71 Canine B-cell Lymphoid Tumor Cells

Other studies in our lab have shown that only 17-71, but not GL-1, cells are resistant to traditional chemotherapeutic drugs such as VCR and DOX, which are commonly used to treat canine B-cell lymphoma (Dervisis et al., 2007; Fahey et al., 2011; Flory et al., 2008; Griessmayr et al., 2009; Lori et al., 2010; Lymphoma, n.d.; Moore et al., 1999; Northrup et al., 2009; Rassnick et al., 2002; Rebhun et al., 2011). These observations led us to ask whether the chemoresistant 17-71 cells express P-gp, which is known to be associated with chemoresistance in canine B-cell lymphoma (Fan, 2003; Lee et al., 1996; Moore et al., 1995; Steingold et al., 1998; Uozurmi et al., 2005). Indeed, the transcript for P-gp was detected in 17-71 cells (Figure 1). Dog liver was used as a positive control tissue for P-gp gene expression.

Effect of DIM on Viability of the Lymphoid Tumor Cells

Some dietary supplements are known to exert antitumorigenic properties by inducing selective

![Figure 2. Effect of DIM on viability of canine B-cell lymphoid tumor cells. Cell viability was measured after treating 17-71 (A) and GL-1 (B) cells with vehicle DMSO or increasing concentrations of DIM as indicated for 24 or 48 h. Data are presented as mean ± SD of three separate experiments (*P < 0.05 determined by Student’s unpaired t-test).](image-url)
cytotoxicity in tumor cells. Recently, DIM has been shown to exert antitumor actions in a variety of human cancer cells (Azmi et al., 2008; Biersack and Schobert, 2012; Chen et al., 2012). We studied the effect of DIM on viability of the lymphoid tumor cells. DIM was found to be non-cytotoxic to 17-71 cells at 25 µM treatment for 24 or 48 h, although it was cytotoxic at higher concentrations (Figure 2A), suggesting that DIM is nontoxic at physiologically relevant concentrations (up to 20 µM) (Anderton et al., 2004a; Anderton et al., 2004b; Fan, Meng, Saha, Sarkar, & Rosen, 2009; Moiseeva, Almeida, Jones, & Manson, 2007; Reed et al., 2006; Reed et al., 2008), but can inhibit the growth of the lymphoid tumor cells at supraphysiological concentrations. Similarly, DIM was found to be non-cytotoxic to GL-1 cells at its physiologically relevant concentrations (Figure 2B).

**DIM is Not a Fluorescent Substrate**

DIM, similar to DMSO, did not exhibit a noticeable intracellular fluorescence in 17-71 cells in intracellular substrate accumulation assays (Figure 3). In contrast, treatment with fluorescent dye R123, a substrate of P-gp, resulted in a significant increase in intracellular fluorescence (Figure 3). Together, these results suggest that only R123, but not DIM, behaves as a fluorescent substrate in our experimental conditions.

**Effect of DIM on the Efflux Activity of P-gp**

Chemoresistance in a variety of cancers is associated with upregulation of expression/function of multidrug transporters, particularly P-gp (Borst et al., 2000; Chen, 2010; Marzac et al., 2011; Pondugula and Mani, 2013). It is known that some dietary supplements suppress chemoresistance by downregulating the expression/activity of multidrug transporters (Borst et al., 2000; Gelsomino et al., 2013; Kuan, Walker, Luo, & Chen, 2011; Okamura et al., 2004; Rahman, Veigas, Williams, & Fernandes, 2013; Sharom, 2008; Sodani et al., 2012). Chemoresistance in canine lymphoma was shown to be associated with P-glycoprotein (P-gp) (Fan, 2003; Lee et al., 1996; Moore et al., 1995; Steingold et al., 1998; Uozurmi et al., 2005). Recently, it was shown in DOX-resistant canine lymphoid tumor cells that Masitinib, a tyrosine kinase inhibitor, reversed DOX resistance by inhibiting the function of P-gp (Zandvliet et al., 2013).

We tested whether DIM can inhibit the function of P-gp in the chemoresistant 17-71 lymphoid tumor cells. Intracellular accumulation of the fluorescent dye R123, a substrate of P-gp, was used to study the efflux activity of P-gp. Treatment of 17-71 cells with PSC-833, a prototypical inhibitor of P-gp, resulted in a significant increase in intracellular accumulation of P-gp substrate R123 (Figure 4A). This result suggests that the chemoresistant 17-71 cells functionally express P-gp. On the other hand, in the chemosensitive GL-1 cells, there was no change in R123 accumulation after PSC-833 treatment (Figure 4B), suggesting that GL-1 cells lack functional P-gp expression. The absence of functional P-gp system in GL-1 cells is in agreement with a previous report (Zandvliet et al., 2013) and is also consistent with lack of resistance towards VCR.
and DOX induced cytotoxicity in the other studies in our lab. Similar to PSC-833, DIM at its non-cytotoxic physiologically relevant concentrations, 10 and 25 µM, considerably increased the intracellular accumulation of R123 in 17-71 cells, suggesting that DIM inhibits the efflux activity of P-gp in 17-71 cells.

**DISCUSSION**

To our knowledge, our study is the first to report DIM at its physiologically relevant concentrations could inhibit the activity of P-gp in any chemoresistant cancer cell line. This study identifies a natural dietary supplement that inhibits the efflux activity of P-gp in the chemoresistant canine lymphoid tumor cells.

DIM is not only a health supplement, but also the major active metabolite of I3C (Anderton et al., 2004b), which is also a health supplement and naturally occurring compound enriched in cruciferous vegetables such as broccoli, cabbage, and cauliflower (Bonnesen et al., 2001). It is noteworthy that the acidic environment of the stomach fosters the non-enzymatic self-condensation of about 20% I3C to form DIM (Spande, 1979; Stresser, Williams, Griffin, & Bailey, 1995). Therefore, DIM could be taken as a direct supplement, indirectly in the form of I3C as a supplement, or through I3C-rich cruciferous vegetables.

While its therapeutic potential has been well emphasized, the plasma concentration of DIM is not fully evaluated. While DIM was being tested in several in vitro studies at concentrations ranging from 1-100 µM, up to 20 µM has been reported as physiological concentration of DIM as these concentrations would be comparable with serum or tissue concentrations achievable in vivo in humans/rodents (Anderton et al., 2004a; Anderton et al., 2004b; Fan et al., 2009; Moiseeva et al., 2007; Reed et al., 2006; Reed et al., 2008). Together, based on published in vitro and in vivo studies, our results show that DIM can inhibit P-gp activity at its physiologically relevant concentrations.

The mechanism of DIM inhibition of P-gp is unknown, although it is possible that it can directly inhibit the activity of P-gp by serving as a substrate of P-gp, like other P-gp inhibitors such as PSC-833. The results of our research suggest that DIM can be administered in combinatorial chemotherapeutic treatment as a means to overcoming chemoresistance by blocking P-gp activity in chemoresistant lymphoid tumor cells. However, it remains to be determined whether DIM sensitizes the chemoresistant lymphoid tumor cells, including 17-71 cells, to chemotherapy drugs such as VCR and/or DOX. Our future studies will address these questions.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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