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Research Article
Dabrafenib, as a Novel Insight into Drug Repositioning Against Secretory Group IIa Phospholipase A2

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Abstract

The screening of bioactive compound libraries can be an effective approach for repositioning FDA-approved drugs or discovering new treatments for human diseases. The expression of secretory group IIa phospholipase A2 (sPLA2-IIa) is enhanced by development of inflammatory disorders. In this study, sPLA2-IIa expression was induced in the lipopolysaccharide (LPS)-stimulated Human Umbilical Vein Endothelial Cells (HUVECs) and mice to evaluate the effect of dabrafenib. This study illustrates drug repositioning with dabrafenib (DAB) for the modulation of sPLA2-IIa expression and activity. Dabrafenib is a B-Raf inhibitor and initially used for the treatment of metastatic melanoma therapy. Results showed that dabrafenib remarkably suppressed the LPS-mediated protein expression and activity of sPLA2-IIa via inhibition of phosphorylation of cytosolic phospholipase A2 (cPLA2) and extracellular signal-regulated kinase (ERK) 1/2. These results demonstrated that dabrafenib might play an important role in the modulation of sPLA2-IIa expression and activity in response to the inflammatory diseases.

Key words: Dabrafenib, HUVECs, sPLA2-IIa, inflammation, mice, drug repositioning

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The classic approach to drug discovery involves *de novo* identification and validation of new molecules, which is a time-consuming and very expensive process (Padhy and Gupta, 2011; Medina-Franco *et al*., 2013; Baek *et al*., 2015). Despite huge investment, the number of new drugs identified by this traditional approach has not increased significantly during past decades (Baek *et al*., 2015). An interesting alternative is drug repositioning (also known as drug reprofiling, drug repurposing or drug retasking) that consists of identifying and developing new uses for existing drugs (Ashburn and Thor, 2004). More and more companies are scanning the existing pharmacopoeia for repositioning candidates. This can be achieved by two approaches: *In silico* drug repositioning which utilizes public databases and bioinformatics tools to systematically identify interaction networks between drugs and protein targets whereas activity-based drug repositioning refers to the use of existing drugs for screening. Several governments worldwide are investing in drug repositioning and related activities (Langedijk *et al*., 2015). Drug repositioning has gained particular attention from the scientific community engaged in anticancer research due to the combination of great demand for new anticancer drugs and the availability of a wide variety of cell and target-based screening assays (Shim and Liu, 2014). With the successful clinical introduction of a number of non-cancer drugs for cancer treatment, drug repositioning now becomes a powerful alternative strategy to discover and develop novel anticancer drug candidates from the existing drug space (Padhy and Gupta, 2011; Medina-Franco *et al*., 2013; Baek *et al*., 2015).

A superfamily of enzymes phospholipases A2 (PLA2) hydrolyzes the ester bond at sn-2 position of phosphoglycerides to release a free fatty acid and lysophospholipids (Six and Dennis, 2000; Kudo and Murakami, 2002). The PLA2 is consisted with four individual groups according to the molecular weight and Ca^{2+}-dependence such as secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca^{2+}-dependent PLA2 and lipoprotein-associated PLA2 (Six and Dennis, 2000; Kudo and Murakami, 2002). Among them, sPLA2 is triggered with the various inflammatory diseases containing rheumatoid arthritis, sepsis, bowel disease, respiratory distress syndrome and so on (Dennis, 1997). Especially, the patients suffered from sepsis, septic shock and polytrauma showed the high levels of sPLA2-IIa (Waydhas *et al*., 1989; Nakano *et al*., 1990; Crowl *et al*., 1991; Oka and Arita, 1991; Pruzanki and Vadas, 1991; Menschikowski *et al*., 2006). In the previous reports, the secretory group IIa phospholipase A2 (sPLA2 IIa) has been thought as a regulator for variety of biological mechanisms in mammalian cells involved to coagulation, signal transduction, apoptosis, remodeling of cellular membranes and host defense (Kuwata *et al*., 1998; Mounier *et al*., 1998; Murakami *et al*., 1998).

In this study for repositioning FDA-approved drugs (total 1,163), 327 drugs were selected which are related to vascular inflammation and infection. Among selected drugs, high contents screening system (PerkinElmer Operetta, Waltham, MA) was used to select the compounds which modulate the expression and activity of sPLA2-IIa and found that dabrafenib (DAB) had the inhibitory effects on the expression and activity of sPLA2-IIa. Dabrafenib (brand name; Tafinlar™) is a kinase inhibitor indicated as a single agent for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E mutation as detected by an FDA-approved test (Li *et al*., 2014). Therefore, investigation on the effect of dabrafenib on sPLA-IIa using *in vitro* and *in vivo* system was done.

MATERIALS AND METHODS

Reagents: Dabrafenib was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The LPS, an ERK 1/2 inhibitor U1026 and a cPLA2α inhibitor arachidonyl trifluoromethyl ketone (AACO) were obtained from Sigma (St. Louis, MO, USA). The sPLA2-IIa from GenWay Biotech Inc. (San Diego, CA, USA) was also used.

Cell culture: Primary HUVECs obtained from Cambrex Bio Science (Charles City, IA, USA) were maintained as previously described (Bae *et al*., 2014; Ku and Bae, 2014; Ku *et al*., 2014; Lee *et al*., 2015). For all experiments, HUVECs were used at passages from 3-5.

Animals and husbandry: Six to seven weeks old male ICR mice (the average of body weight, 27 g) were obtained from Orient Bio Co. (Sungnam, Korea) and used after acclimatization period for 12 days. The five mice per a polycarbonate cage were cared with 12:12 h light/dark cycle and controlled humidity (RH, 40-45%) at 20-25°C. Mice received a normal pellet diet and water *ad libitum*. All animal experiments were performed according to the

'Guidelines for the Care and Use of Laboratory Animals' by Kyungpook National University (KNU 2012-13).

Cecal ligation and puncture (CLP): Before sepsis induction, mice were anesthetized with 2% isoflurane (JW Pharmaceutical, Seoul, Korea) in oxygen using a small rodent gas anesthesia machine (RC2, Vetequip, Pleasanton, CA, USA). The CLP-induced sepsis model was induced as previous study (Bae *et al*., 2014). To expose the cecum and adjoining intestine, a 2 cm midline incision was made. Then, the cecum was tightly ligated with 3-0 silk suture at 5 mm from the cecal tip and punctured using a 22 gauge needle (Rittirsch *et al*., 2008). It was gently squeezed to extrude a small amount of feces from the perforation site and returned to peritoneal cavity followed by the 4-0 silk suture for laparotomy site. For sham control mice, the cecum was exposed without ligation and puncture, then returned to the abdominal cavity. For this experiment, the protocol was authorized by the Animal Care Committee at Kyungpook National University (IRB No. KNU 2012-13).

Measurement of sPLA2-IIa protein expression: The primary HUVECs were pre-incubated with dabrafenib for 6 h and 5 μM U1026 or 20 μM AACO for 2 h, respectively. Then, the serum-free media for a control or 100 ng mL^{-1} LPS were treated to the cells for 24 h. On the other hand, 15 mg kg^{-1} LPS-treated mice by intraperitoneal (i.p.) injection or CLP-operated mice were administered with dabrafenib 20.8 or 31.1 μg/mouse via intravenous (i.v.) injection for *in vivo* study. The plasma was prepared after 2 days.

For detection of sPLA2-IIa in the cell culture supernatants, the enzyme-linked immunosorbent assay (ELISA) was performed using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction. Briefly, the diluted media or mouse plasma was applied to each well of a plate. After plate washing, the acetylcholinesterase-sPLA2-Fab' conjugate was treated. Then, Ellman's reagent was added and the color development resulted by the acetylcholinesterase-catalyzed reaction was measured at 412 nm using a microplate reader (Tecan, Männedorf, Switzerland). The sPLA2-IIa concentration was estimated based on a standard curve of recombinant sPLA2-IIa.

sPLA2-IIa activity assay: To measure the sPLA2-IIa activity, 1-palmitoyl-2-[12-[7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-sn-glycero-3-phospho-ethanolamine (NBD-PE; Avanti Polar Lipids, Alabaster, AL, USA) was used as a substrate (Shimoyama *et al*., 2001). The reaction mixture (100 μL) which is consisted with 50 mM Tris-HCl (pH 8.0), 123 μM NBD-PE, 2 mM Ca^{2+} and the indicated amounts of sPLA2-IIa was incubated at 30°C for 30 min with or without dabrafenib.

Western blotting: The collected protein was quantified using Bovine Serum Albumin (BSA) protein assay kit and equilibrated. The protein was applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked at Room Temperature (RT) for 1 h with 0.1% Tween 20 in tris-buffered saline (TBST, pH 7.4) supplemented with 5% non-fat milk followed by the reaction with primary antibodies against ERK 1/2, phospho-ERK 1/2, cPLA2a and phospho-cPLA2 at 4°C overnight. After membrane washing with TBST, the secondary antibodies were treated to membranes at RT for 1 h. The blots were detected by enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) and the densities of protein blots were analyzed using ImageJ Gel Analysis software.

Statistical analysis: All the experiments were performed three times at least. Data were shown as Means±SEM and the statistical differences were determined with $p<0.05$ calculated by Student's t-test.

RESULTS

Effects of dabrafenib on the expression and activity of sPLA2-IIa: When the various concentrations of LPS were treated to HUVECs for 24 h, the sPLA2-IIa levels in the cell culture media were peaked from 100 ng mL^{-1} LPS (data not shown). In addition, this trend was also shown in the serum-free culture media supplemented with 0.2% BSA, therefore, the possibility of which the effect of LPS on sPLA2-II2 was resulted by the serum content was excluded. Thus, 100 ng mL^{-1} LPS was used for the subsequent experiments to stimulate the endothelial cells.

As shown in Fig. 1a, dabrafenib significantly suppressed the LPS-induced sPLA2-IIa expression of HUVECs in a concentration-dependent manner. In addition, the enhanced activity of sPLA2-IIa was also inhibited by dabrafenib treatment (IC_{50} = 24.7 μM, Fig. 1b). From these data, it was observed that dabrafenib had a remarkable effect on sPLA2-IIa-related inflammatory mechanisms stimulated by LPS.

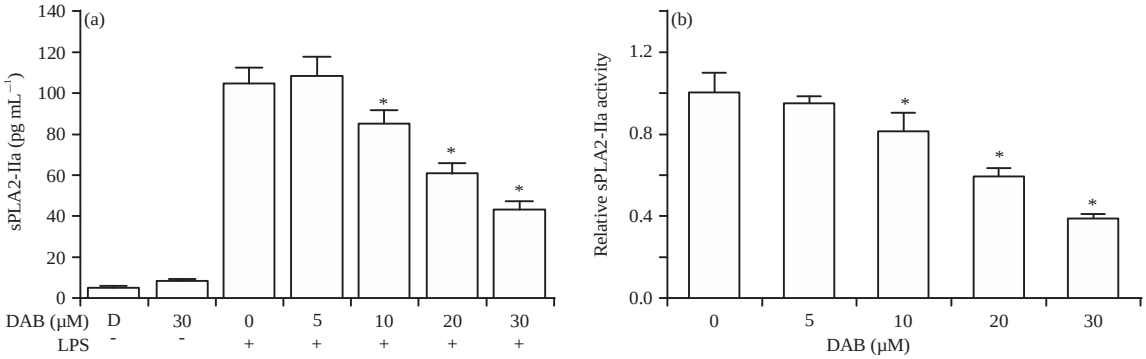

Fig. 1(a-b): Effects of dabrafenib on the expression and activity of the sPLA2-IIa in HUVECs, (a) The primary HUVECs were treated with dabrafenib for 6 h before 100 ng mL^{-1} LPS stimulation. After 24 h, the sPLA2-IIa in the cell culture media was quantified by ELISA and (b) To assess the sPLA2-IIa activity, 100 μL of reaction mixture consisted with 20 μM tris-HCl (pH 8.0), 123 μM NBD-PE (a substrate), 2 mM Ca^{2+} and sPLA2-IIa were reacted at 30°C for 30 min in the presence or absence of dabrafenib, D: A vehicle control treated with 0.5% dimethyl sulfoxide (DMSO), *p<0.05 as compared to (a) LPS only or (b) Zero

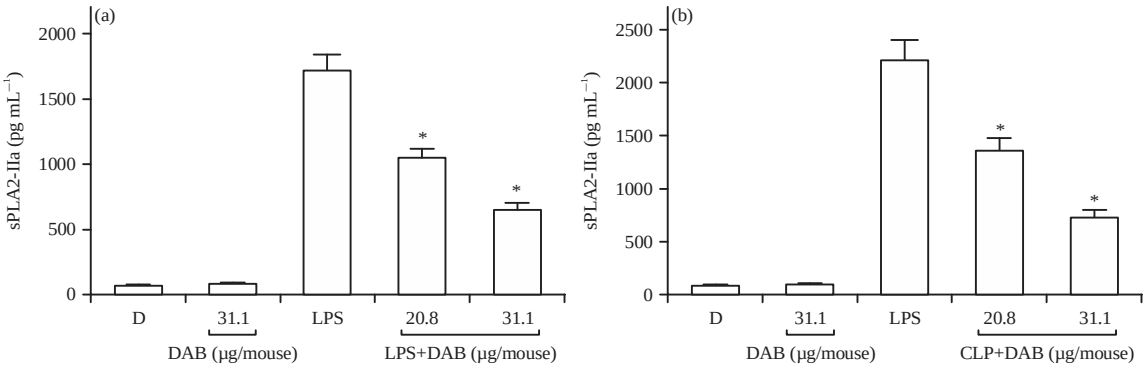

Fig. 2(a-b): Effects of dabrafenib on LPS or CLP-induced sPLA2-IIa expression *in vivo*. The male ICR mice were administered with 20.8 and 31.1 μg dabrafenib/mouse before the injection of (a) 15 mg kg^{-1} LPS or (b) CLP surgery. Two days after, the mouse serum was collected and sPLA2-IIa expression was analyzed by ELISA, *p<0.05 as compared to (a) LPS only or (b) CLP

Effect of dabrafenib on sPLA2-IIa expression in LPS-induced endotoxemia and CLP-induced sepsis: To prove the effect of dabrafenib *in vivo*, mice were induced endotoxemia and sepsis via LPS injection and CLP, respectively. After induction of sepsis, mice showed the clinical signs such as shivering, weakness and bristled hair. When the mice were administered with 20.8 or 31.1 μg/mouse, the sPLA2-IIa expression mediated by LPS and CLP were significantly decreased in concentration-dependent manner (Fig. 2a and b). Since, the average circulating blood volume for mice is 72 mL kg^{-1} (Diehl *et al*., 2001) and the average weight of used mouse is 27 g and the average blood volume is 2 mL, the amounts of 20.8 and 31.1 μg dabrafenib in peripheral blood were estimated to 20 and 30 M, respectively.

Inhibitory effects of dabrafenib on LPS-induced activation of ERK 1/2 and cPLA2α: The LPS-induced phosphorylation of ERK 1/2 and cPLA2α in HUVECs were attenuated by 20 and 30 μM of dabrafenib (Fig. 3a). Thus, the roles of ERK 1/2 and cPLA2α were tested by activation in 100 ng mL^{-1} LPS-mediated sPLA2-IIa expression in HUVECs. When HUVECs were pre-treated with 5 μM U0126 or 20 μM AACO before LPS stimulation, sPLA2-IIa expression were significantly reduced compared to the cells treated with LPS only (Fig. 3b). These data indicated that 100 ng mL^{-1} LPS-induced the activation of ERK 1/2 and cPLA2α can regulate the expression of sPLA-IIa and the inhibitory effects of dabrafenib on LPS-mediated sPLA-IIa production were originated from the suppressed phosphorylation of ERK 1/2 and cPLA2α.

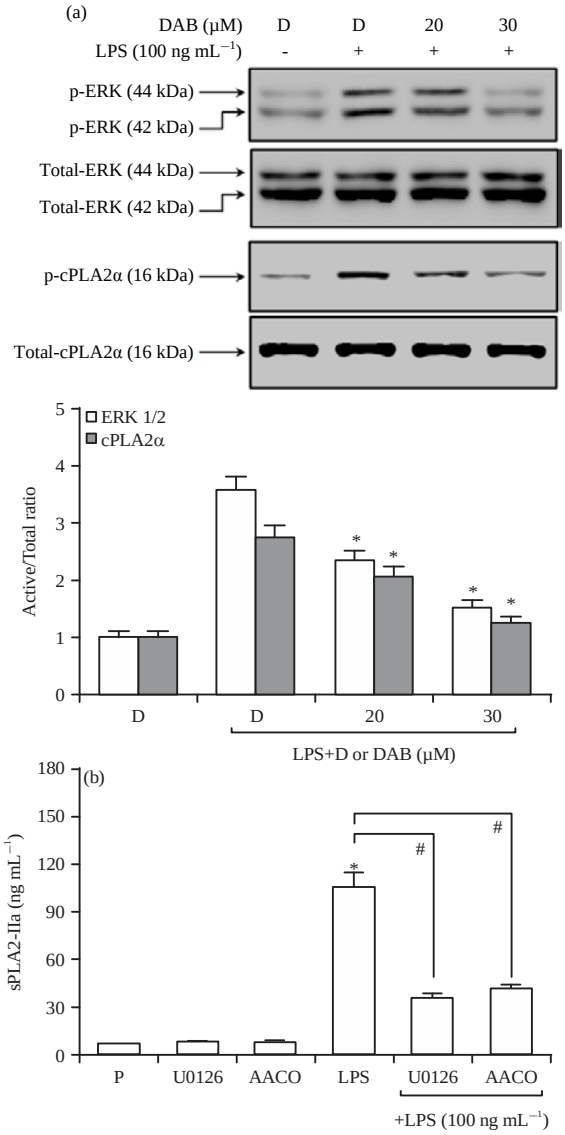

Fig. 3(a-b): The inhibitory effects of dabrafenib on LPS-mediated phosphorylation of ERK 1/2 and cPLA2α in HUVECs, (a) Dabrafenib was treated to HUVECs for 6 h before 100 ng mL^{-1} LPS stimulation for 24 h. The levels of phosphorylated ERK 1/2 and cPLA2α were assessed by Western blotting. The graphs show the densities of protein blots compared to total proteins such as ERK 1/2 and cPLA2α and (b) HUVECs were pre-treated with 5 μM U0126 (an ERK 1/2 inhibitor) or 20 μM AACO (a cPLA2α inhibitor) for 2 h prior to LPS stimulation, *: (a) and $^{\#}$: (b), $p<0.05$ as compared to LPS only

DISCUSSION

The involvement of sPLA2-IIa in inflammatory diseases in humans is well documented such as sepsis, septic shock and polytrauma and it is well correlated with the severity of inflammation diseases (Waydhas *et al*., 1989; Nakano *et al*., 1990; Crowl *et al*., 1991; Oka and Arita, 1991; Pruzanki and Vadas, 1991; Menschikowski *et al*., 2006). The expression level of sPLA2-IIa is markedly induced by pro-inflammatory mediators and downregulated by anti-inflammatory cytokines in a variety of cells and tissues in mammalians (Nakano *et al*., 1990; Crowl *et al*., 1991; Oka and Arita, 1991). Therefore, the sPLA2-IIa is thought to associate with the initiation and multiplication of inflammatory reactions. In supporting this, the inflammatory diseases are attenuated by sPLA2-IIa inhibitors (Tanaka *et al*., 1993; Balsinde *et al*., 1999; Bradley *et al*., 2005) and in turn, purified sPLA2-IIa aggravates these responses when injected into inflamed tissues (Vadas *et al*., 1989). Thus, sPLA2-IIa seems to be pertinent to in the pathophysiology of various inflammatory diseases. In this study, it was demonstrated that the expression level and activity of sPLA2-IIa were significantly inhibited by dabrafenib *in vitro* and *in vivo* (Fig. 1 and 2), indicating the possible use of dabrafenib as anti-inflammatory reagent candidates against sepsis, septic shock and polytrauma.

Despite specific inhibitors were used to oppose the abnormal production of sPLA2-IIa, it was ineffective to improve the clinical outcome for the patients with severe sepsis or rheumatoid arthritis (Bradley *et al*., 2005; Zeiher *et al*., 2005). Therefore, improved approach needed for the cure of severe inflammatory diseases. Noting that the abnormal production or secretion of sPLA2-IIa was suppressed by dabrafenib, it may be one of the candidates for the inhibition of sPLA2-IIa expression. This concept is supported by the finding that sPLA2-IIa transgenic mice develop hyper permeability (Grass *et al*., 1996) and sPLA2-IIa itself directly induces the expression of chemokines and cell adhesion molecules in vascular endothelium (Beck *et al*., 2003). In this perspective dabrafenib could be of special interest since in this study dabrafenib showed the inhibitory effect of sPLA2-IIa expression.

Recently, it was reported that the protective effects of dabrafenib in mouse septic model, which showed that dabrafenib inhibited sepsis mediator induced production of high mobility group box 1 protein (HMGB1), hyperpermeability and expressions of HMGB1 receptors such as TLR2, TLR4 and RAGE (Jung *et al*., 2015). In addition, dabrafenib suppressed the adhesion and migration of immune cells by inhibiting the expressions of cell adhesion molecules (Jung *et al*., 2015) such as vascular cell-adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) and E-selectin, which promote inflammation via the recruitment of leukocytes (Bae and Rezaie, 2011). Furthermore, dabrafenib had protective effect on CLP-induced septic lethality

(Jung *et al*., 2015). Mechanical study also showed the inhibitory effects of dabrafenib on the activation of nuclear factor (NF)-κB/ERK and production of interleukin (IL)-6 and Tumor Necrosis Factor (TNF) which was approved by this study (Fig. 3).

This study clearly illustrates the aspects of drug repositioning of dabrafenib. Indeed, it was shown that dabrafenib could be reprofiled to evolve from a drug that was used for the treatment of metastatic melanoma, towards an anti-inflammatory agent. This is very interesting since the existence of unresponsive severe inflammation and the appearance of resistant inflammatory diseases during the course of treatments both justify that increase urgently the panel of pharmacological molecules able to fight vascular inflammatory diseases. Dabrafenib has been used for other diseases in the clinic, indicating its acceptable human safety.

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