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Trombinol, a bioactive fraction of *Psidium guajava*, stimulates thrombopoietin expression in HepG2 cells

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ABSTRACT

Objective: To study the regulation of trombinol on thrombopoietin, an essential regulator of thrombocyte production.
Methods: Effect of trombinol on thrombopoietin regulation was evaluated at the mRNA and protein levels in human hepatoma HepG2 cells. The mRNA expressions were revealed by PCR and real-time PCR, while the protein expressions were analyzed using western blotting and human ELISA kit. Statistical differences between the test were determined by student's *t*-test with $P < 0.05$ was considered statistically significant.
Results: Trombinol significantly increased the expression of thrombopoietin at the level of mRNA and protein secretion in HepG2 cell lines. Trombinol with the concentration of 15 μg/mL, positively induces 2.5-fold of thrombopoietin expression. Up-regulation of GABP, a transcription factor of thrombopoietin, is suggested to be involved in cellular regulatory mechanisms of trombinol. Here, our result shows convincing evidence that trombinol affects the thrombopoietin productions *in vitro*. This molecular explanation of thrombopoietin's stimulating function is in line with the traditional use of *Psidium guajava* for treatment of diseases involving thrombocytopenia.
Conclusions: Thrombopoietin stimulating function of trombinol could be potentially considered as one of alternative treatment for thrombocytopenia-related cases, including post chemotherapy shock, dengue fever and liver failure.

1. Introduction

Thrombopoietin (TPO) is a glycoprotein hormone produced mainly by the liver and the kidney which regulates the production of thrombocytes by the bone marrow [1,2]. It stimulates the production and differentiation of megakaryocytes, the bone marrow cells that fragment into large numbers of thrombocytes [3,4]. Binding of TPO and its receptor, c-Mpl, activates a wide variety of signal transduction molecules [5–7]. Both *in vitro* and *in vivo* studies have demonstrated that TPO induces a series of signaling events including activation of JAK/STAT, Shc/Ras/MAPK and PI3K/AKT/FOXO3a pathways [8–10]; these pathways overlap with those induced by other cytokines and differently lead to the unique biological effect [11]. In the upstream, the expression of TPO is known to be regulated by other proteins, *i.e.* GABP that acts as its transcription factor [12,13] and IL-6, another cytokine that known has a relation with thrombocytopoiesis [14–16]. Such pathways determine the role of TPO on the production of thrombocytes.

Psidium guajava (*P. guajava*) has been widely used as a folk medicine to increase thrombocyte count in dengue viral infection [17,18]. However, preclinical research about *P. guajava* is still limited and the mechanism is still not yet clearly understood. Many pharmacological studies have demonstrated the ability of *P. guajava* to exhibit antioxidant, hepatoprotection, anti-allergy, antimicrobial, antigenotoxic, antiplasmodial, and antiinflammatory activities, supporting its traditional uses [19,20]. Several studies indicated a strong correlation between *P. guajava* with the increase of thrombocyte in dengue viral infection [17,18,21]. Trombinol is a bioactive fraction (BAF) which was fractionated with ethanol derived from *P. guajava* leave, with DMSO as BAF solvent.

In this study, we evaluated the effects of trombinol on TPO expression as well as other genes and proteins related to TPO. Mechanism of action of trombinol in HepG2 cells was elucidated by measuring the expression of TPO. The safety dosage of trombinol

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and its ranges were determined, and subsequently were used to evaluate the effects of trombinol on TPO at mRNA and protein levels. Taken together, these results showed molecular evidences of trombinol as a potential drug candidate to treat diseases related to thrombocytopenia condition, including dengue fever, liver failure, coagulation disorder, and detrimental side effect of chemotherapy.

2. Materials and methods

2.1. Preparation of trombinol

Trombinol was bioactive fraction extracted from *P. guajava* leaf. The *P. guajava* leaf was originated from Cianjur, West Java, Indonesia. This plant has been identified by the Herbarium Bogoriense, Research Center of Biology, Indonesian Institute of Sciences with certificate No. 155/IPH.1.02/If.8/II/2013. The dried milled of *P. guajava* leaf was macerated in 70% ethanol with ratio 1:10 w/v at 50 °C for 2 h, then followed by filtration. The filtrate was concentrated under low pressure at 60 °C using rotary evaporator machine. The concentrate was dried in conventional oven at 70 °C for 24 h and then stored in a well closed container at 25–30 °C.

The identification of Trombinol was done using thin layer chromatography plate Silica Gel 60 F254 with toluene–acetone–formic acid (6:6:1, v/v) as mobile phase. The eluent was allowed to move along the TLC plate for a distance of 8 cm. The chromatogram was then observed under UV λ366 nm with R_f ± 0.3 as black band before derivatization and yellow band after derivatization by 1% $AlCl_3$ in ethanol. The marker compound in trombinol was quantified using high-pressure liquid chromatography Waters® under UV λ352 nm with Symmetry C18, 4.6 × 150 mm 5 μm as a column and methanol–acetonitrile–water–acetic acid (45:15:38:2, v/v) as mobile phase. All of analytic reagent was purchased from Merk, Germany. Content of the marker compound was not less than 4% in trombinol.

2.2. Cell culture and sample treatment

The human hepatoma cell line, HepG2 (ATCC No. HB-8065), was purchased from ATCC (Rockville, MD, USA) and seeded in culture plates. HepG2 cells were cultured in Modified Eagle's Medium (MEM) (Gibco, Grand Island, USA) basal medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, USA), 100 μg/mL penicillin-streptomycin (Gibco, Grand Island, USA), and 1 mmol/L sodium bicarbonate (Gibco, Grand Island, USA). The culture was incubated at 37 °C in a 5% CO_2 atmosphere. The medium was replaced every 2–3 days until the cells reached 80% confluence. Prior to the treatment, the medium was aspirated and replenished with serum-free media for 4 h, then treated with trombinol at various concentrations in the range of 0–60 μg/mL and incubated for 24 h.

2.3. RNA analysis

Total cellular RNA was extracted using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The RNA concentration was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, MA, USA). A quantitative real-time polymerase chain reaction (RT-PCR) was developed using i-Cycler (BioRad Laboratories). cDNA was synthesized from 2 μg total RNA using GoScript Reverse Transcription System (Promega, Madison, USA). The reaction was performed at 42 °C for 1 h and followed by heat inactivation at 70 °C for 15 min. Synthesized cDNA was amplified with GoTaq Green Master Mix (Promega, Madison, USA). The primers used in this study were: TPO forward, 5′-CGTTTCCTG ATGCTTGTAGG-3′; TPO reverse, 5′-GAAGGAGAATATCC AGGCTG-3′; GABPα forward, 5′-AAAGAGCGCCGAGGAT TTCAG-3′; GABPα reverse, 5′-CCAAGAAATGCAGTCTC GAG-3′; IL-6 forward, 5′-TACCCCCAGGAGAAGATTCC-3′; and IL-6 reverse, 5′-GCCATCTTTGGAAGGTTCAG-3′. All primers were synthesized by First Base Laboratories (Singapore). The relative mRNA level was measured by relative quantification of gene expression with GAPDH or actin as internal control.

2.4. Preparation of protein isolation and western blotting

Extracellular and intracellular proteins were concentrated separately from the medium and the cells. Medium was first collected into a 15 mL Falcon tube and then centrifuged at 5 000 r/min for 15 min using a 50-kDa Amicon filter (Milipore, Carrightwohil, Ireland) to obtain more extracellular protein concentration in medium. Meanwhile, to obtain the intracellular protein, cells were washed with ice-cold phosphate-buffer saline (PBS) and lysed in a hypotonic buffer (150 mmol/L NaCl, 20 mmol/L Tris, 10% glycerol, 0.1% SDS, 1 mmol/L EDTA) containing a protease inhibitor cocktail set I Calbiochem (Milipore, Carrightwohill, Ireland). Total of 35 μg proteins were boiled for 10 min in SDS buffer (1 mol/L Tris pH 6.8, 87% glycerol, 10% SDS, 2-merkaptoetanol, Bromphenol Blue) and were size fractionated by sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto polyvinyledene difluoride (PVDF) membranes. The blots were incubated with primary and secondary antibodies (ratio of dilution 1:500 and 1:10 000 respectively), visualized by the Amersham chemiluminescence kit (GE Life Sciences, Buckinghamshire, UK) and quantified using a ChemiDoc MP Imaging System (BioRad).

2.5. Quantitative measurement of TPO production

The production of TPO of the cells was measured using a TPO human ELISA kit (Abcam, Cambridge, USA). Standard solutions and medium are filled into a well-plate coated by immobilized TPO specific antibody. Biotinylated anti-human TPO antibody was added into the wells. After washing unbound biotinylated antibody, the HRP conjugated streptavidin was added. A 3.3′, 5.5′-tetramethylbenzidine (TMB) substrate solution was added and color developed in proportion to the amount of TPO bound. Intensity of the color was measured at 450 nm.

2.6. Statistical analysis

The statistical differences between the test and control samples were determined by Student's *t*-test using the StatView software package (Abacus Concepts, Piscataway, NJ, USA). Values were expressed as means ± SD for at least three independent experiments ($P < 0.05$).

3. Results

3.1. Trombinol induces TPO expression in HepG2 cells

The human hepatoma cell line, HepG2 cell, has been reported to be an *in vitro* model for studying physiological

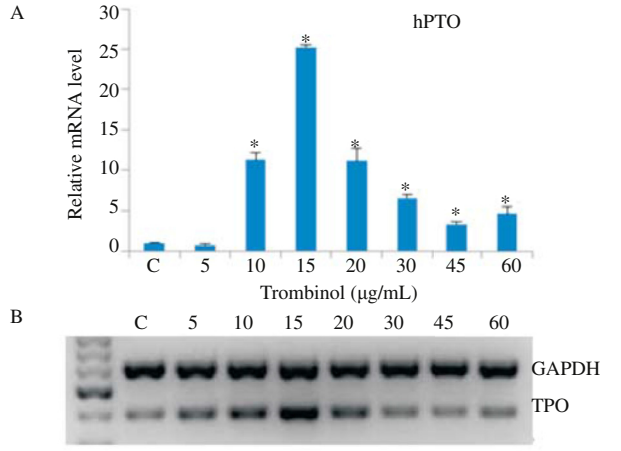

Figure 1. Dose responses study of trombinol on the TPO mRNA expression.
In vitro effect of trombinol on TPO mRNA was evaluated using (A) real-time PCR and (B) conventional PCR in HepG2 cells. The figure represents the average ± standard deviation of three independent experiments. $^{*}P < 0.05$ *vs*. control.

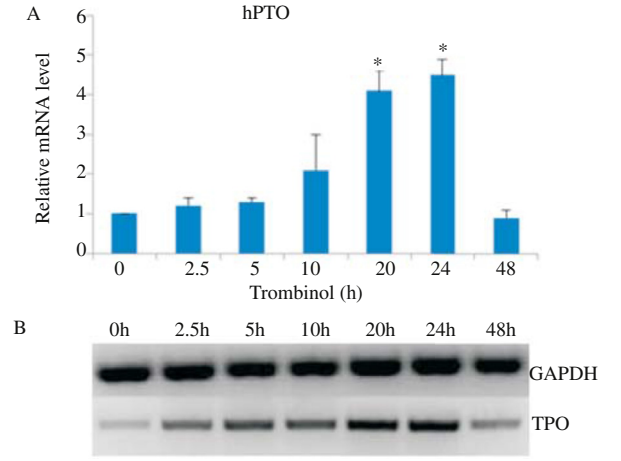

Figure 2. Time-course studies of trombinol on the TPO mRNA expression.
In vitro effect of 15 μg/mL trombinol on TPO mRNA were evaluated using (A) real-time PCR and (B) conventional PCR in HepG2. The figure represents the average ± SD of three independent experiments. $^{*}P < 0.05$ *vs*. control.

regulation of TPO expression in liver [6,12]. To reveal the effects of trombinol on the expression of TPO, different doses of Trombinol at 5, 10, 15, 20, 30, 45, and 60 μg/mL were applied overnight in HepG2 cells. The dose ranges were used based on the limit of safe doses of trombinol in HepG2 cells, which is done previously in different experiment (data not shown). The results of conventional and real-time PCR showed that trombinol-induced TPO expression at dose-dependent manner, with a peak response of Trombinol was seen at 15 μg/mL (Figure 1). Time-course study of Trombinol towards the increasing of TPO expression was also conducted to obtain the optimum hours of treatment. A gradual increase was found at 10–24 h and decreased after 48 h of treatment. The peak of the up-regulation was found to be maximum at 24 h after treatment (Figure 2).

Meanwhile, western blotting and human TPO ELISA kit were also conducted to evaluate the response of treatment on TPO at protein level. As seen on the graph, there is a correlation between mRNA (Figure 3A, B) and protein levels (Figure 3C, D), which indicated that trombinol increased TPO expression of both molecules.

3.2. Effect of trombinol on GABP expression level

GABP, an Ets family transcription factor, was known to be very essential for the expression of TPO in the liver [12,13]. To confirm this notion, we also evaluated the effects of trombinol on GABP expression. Trombinol induced the expression of GABP, a transcription activation domain (TAD) of GABP complex, not only at the gene but also at the protein level, in a dose-dependent manner (Figure 4). This data suggested that the mechanism of trombinol induced TPO expression is done through up-regulation of its transcription factor, GABP.

3.3. Trombinol induces the expression of hematopoietic cytokine IL-6

Hematopoiesis is regulated by various hematopoietic cytokines [22,23]. The expression of IL-6, a cytokine that known to be

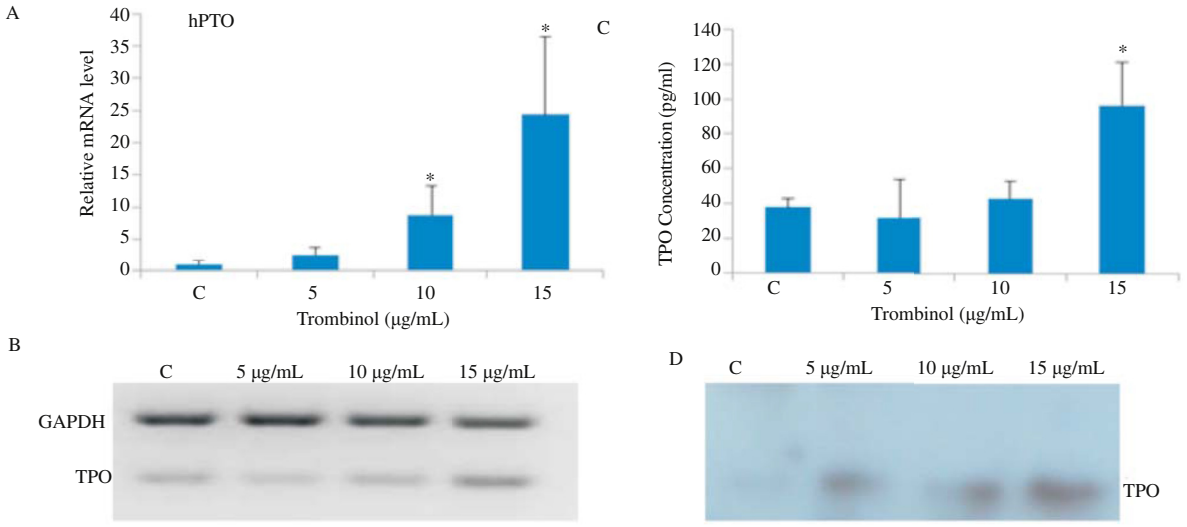

Figure 3. Effect of trombinol on thrombopoietin expression at mRNA and protein levels.
Gene expression of TPO was analyzed using (A) real-time PCR and (B) conventional PCR with GAPDH as the internal control, while the protein level was measured using (C) Western blotting and (D) human TPO ELISA kit. Figures represent the average ± SD of three independent experiments. $^{*}P < 0.05$ *vs.* control.

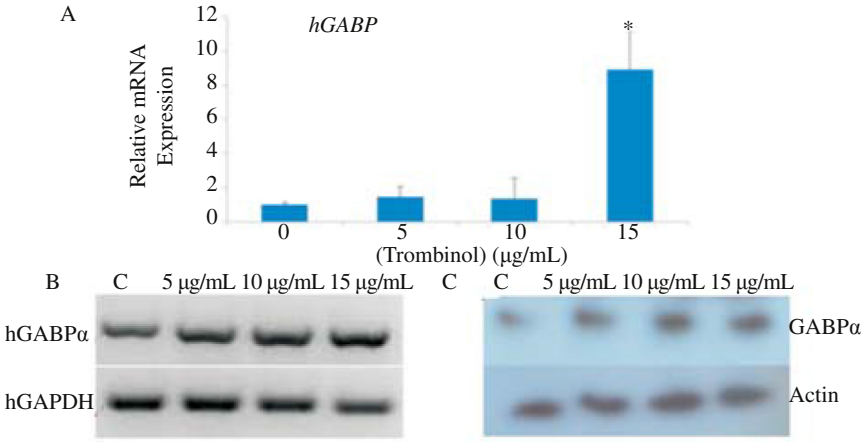

Figure 4. Effect of trombinol on GABPα expression at mRNA and protein levels.
Gene expression of GABPα was analyzed using (A) real-time PCR and (B) conventional PCR. (C) Semi quantitative analysis of protein expression of GABPα by western blot. The figure is representative of three independent experiments. $^{*}P < 0.05$ *vs*. control.

associated with thrombocytosis, was also evaluated (Figure 5). Trombinol increased mRNA levels of IL-6 (Figure 5A, B) in a dose dependent manner. The effect of trombinol on protein level of this cytokine was also determined by western blot in cells treated with trombinol. Trombinol increased the cytokine expression insignificantly (Figure 5C).

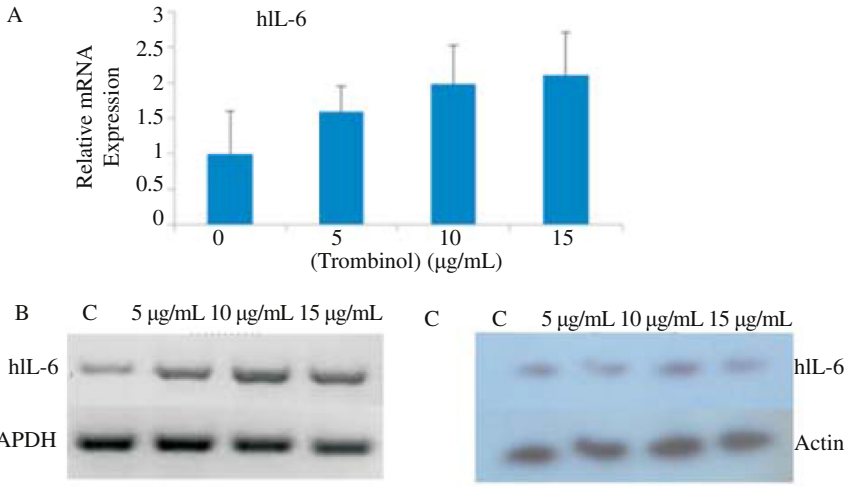

Figure 5. Effect of trombinol on IL-6 expression at mRNA and protein levels.
Gene expression of TPO was analyzed using (A) real-time PCR and (B) conventional PCR. (C) Semi quantitative analysis of protein expression of GABPα by western blot. The figure is representative of three independent experiments. $P < 0.05$ *vs.* control.

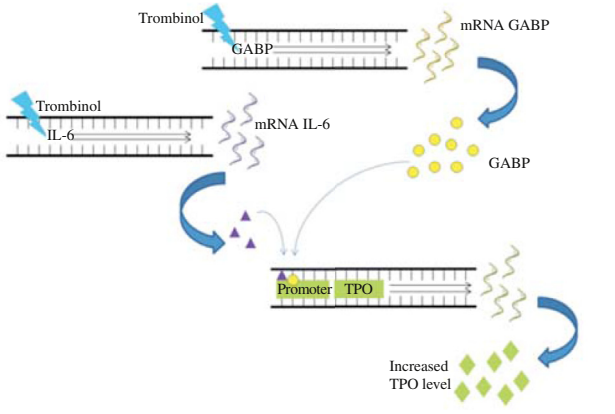

Figure 6. Simplified model of MoA of trombinol-increasing TPO expression in HepG2 cells.
Trombinol up-regulates the expression of GABP transcription factor and IL-6. IL-6 increases the DNA binding activity between GABP and TPO promoter to promote TPO expression.

4. Discussion

TPO is the primary regulator in physiological regulation of thrombocyte production. Lack of TPO causes significant reduction of thrombocyte level in the blood, which leads further into thrombocytopenia condition [2]. Cancer patients also frequently experience side effects such as hematoxicity, leukopenia and thrombocytopenia, from both chemotherapy and radiation. Recently, stimulation of TPO has become the most promising medical treatment for thrombocytopenia-related diseases [24,25].

In this study, we have shown that the application of trombinol could regulate the TPO mRNA expression, as well as the secretion of TPO protein in cultured HepG2 cells. Dose response and time course studies showed that the most optimum dose and incubation time of trombinol on increasing TPO expression were 15 μg/mL and 24 h treatment, respectively. These treatments were further used to analyze other mRNA and protein that literally involved in TPO expression. The increasing of TPO production in liver will in turn binding c-Mpl receptor in

megakaryocytes, activates its downstream signaling pathway to determine the differentiation, fragmentation and maturation of thrombocytes cell mass in bone marrow [7]. As a consequence, increasing number of thrombocyte may alleviate the problem of thrombocytopenia. Additional evidence to explain how trombinol induce TPO expression in liver cells was also provided. In this study, the cellular regulatory mechanisms of trombinol in activating TPO expression via GABP and IL-6 have been hypothesized. These two proteins have been known to be playing important roles in regulating the expression of TPO, both in *in vivo* and *in vitro* studies [12,26,27].

GABP, an Ets family transcription factor of TPO, binds to TPO promoter and, subsequently, enhanced the TPO promoter activity [12]. More specifically, Kosone *et al.* reported that binding of GABP to the sequence of 5′-ACTTCCG-3′ in human and transgenic mice's TPO promoter significantly increased the TPO expression [12]. The regulation activity of GABP depends mostly upon GABPα subunit, regarding to its function as a transcriptional activation domain [28,29]. Together with its β subunit, GABP transcriptionally regulates several cytokines that are critical for cellular differentiation, maturation, and activation. Thus, GABP also regulates the TPO gene that organizes the differentiation and maturation of megakaryocytes [12,29]. In line with the *in vitro* study, the effect of GABP in thrombopoiesis acceleration has been reported to play a key role in the gene activation of TPO in transgenic mice [12,28]. In addition, IL-6 also reported to play a role in hepatic protein synthesis through influencing the transcription of several genes in liver [30–32]. Few studies have been reported that the expression of IL-6 might be associated with thrombocytosis through TPO regulation by inflammatory mediator pathways [15,16]. IL-6 has been shown to rapidly induce a DNA-binding activity of Ets transcription factor with the promoter [13], which suggested that an Ets family transcription factor, GABP, might be involved in the enhancement of TPO gene by the cytokine [16]. Another experiment also reported that there is a multitude putative of IL-6 response element found in the promoter region of TPO that strongly suggests a transcriptional activation of TPO gene might be regulated by IL-6 [26].

Our study showed that the regulation of GABP signaling cascade by Trombinol could be regulated at two distinct levels: (i) at the transcriptional level as a result of an increase of GABPα mRNA expression and (ii) at the translational level as a result of an increase of GABPα protein. This is in contrast to the increase of IL-6 expression which happened at the transcriptional level only. We exhibited that the increase of TPO could be more affected by up-regulating its transcription factor, GABP, instead of regulating the DNA-binding activity of IL-6. Since other cytokines, such as IL-11 and TNFα, could also be involved in TPO stimulation [14,23,26], we infer that, in a case of trombinol, the DNA-binding activity might also be regulated by other cytokines.

Our findings show convincing evidence that trombinol affects the TPO productions via up-regulation of GABP and IL-6 in human hepatoma cells (Figure 6). This molecular explanation of TPO's stimulating function is in line with the ancient of medicinal theory for the functional usage of *P. guajava* in thrombocytopenia-dengue fever cases. Although clinical studies are still required, TPO stimulating function of trombinol could be considered as one of alternative treatment for thrombocytopenia-related cases, including post chemotherapy shock, dengue fever and liver failure.

Conflict of interest statement

We declare that we have no conflict of interest.

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