Biphasic Effects of Ingenol 3, 20-Dibenzoate on the Erythropoietin Receptor: Synergism at Low Doses and Antagonism at High Doses

Jin-Gyo Oh, Young-Won Chin, Sung-Jo Kim, Jong Min Choi, Sang Kyum Kim, Hee Eun Kang, Tae-Hwe Heo

Laboratory of Pharmacoimmunology, Integrated Research Institute of Pharmaceutical Sciences, College of Pharmacy, The Catholic University of Korea, Bucheon, Republic of Korea (J.G.O., H.E.K., T.H.H.), College of Pharmacy, Dongguk University-Seoul, Seoul, Republic of Korea (Y.W.C.), Department of Biotechnology, Hoseo University, Baebang, Asan, Chungnam, Republic of Korea (S.J.K.), College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea (J.M.C., S.K.K.)

1

MOL #97436

Running title
IDB Targets both PKC and EPO Receptor

Correspondence to: Dr. Tae-Hwe Heo
NP512, Hall of Cardinal Jin-Suk Cheong, The Catholic University of Korea, 43 Jibong-ro, Wonmi-gu, Bucheon-si, Gyeonggi-do, 420-743, Republic of Korea
Tel: +82-2-2164-4053
Fax: +82-2-2164-4059
E-mail: thhur92@catholic.ac.kr

The number of text pages: 19
Number of tables: 0
Number of figures: 4
Number of references: 39
Number of words in the *Abstract*: 200
Number of words in the *Introduction*: 677
Number of words in the *Discussion*: 858

Abbreviations
IDB, 3, 20-dibenzoate; EPO, erythropoietin; PKC, protein kinase C; EPOR, erythropoietin receptor; JAK-2; Janus tyrosine kinase-2; STAT-5, signal transducer and activator of transcription-5; IGN, ingenol; SPR, surface plasmon resonance

Abstract

Although ingenol 3,20-dibenzoate (IDB) is known as a selective novel protein kinase C (PKC) agonist, its biological actions and underlying mechanisms remain incompletely understood. In this study, we identified IDB as a proliferative agent for an erythropoietin (EPO) dependent cell line, UT-7/EPO, through the screening of a natural compound library. To clarify the underlying mechanism of IDB's EPO-like activities, we thoroughly analyzed the mutual relation between EPO and IDB in terms of *in vitro* and *in vivo* activities, signaling molecules, and a cellular receptor. IDB substantially induced the proliferation of UT-7/EPO cells, but not as much as EPO. IDB also lessened the anemia induced by 5-fluorouracil in an *in vivo* mouse model. Interestingly, IDB showed a synergistic effect on EPO at low concentration, but an antagonistic effect at higher concentration. Physical interaction and activation of PKCs by IDB and EPO-competitive binding of IDB to EPO receptor (EPOR) explains these synergistic and antagonistic activities, respectively. Importantly, we addressed IDB's mechanism of action by demonstrating the direct binding of IDB to PKCs, and by identifying EPOR as a novel molecular target of IDB. Based on these dual targeting properties, IDB holds promise as a new small molecule modulator of EPO-related pathologic conditions.

Introduction

The ingenol diterpenoid derivatives from natural products have been reported to possess important biomedical activities, including anticancer (Challacombe et al., 2006; Jørgensen et al., 2013), antiviral (Hong et al., 2011), and thrombopoietic (Racke et al., 2012). In particular, ingenol mebutate (Picato®, PEP005, ingenol 3-angelate) was recently US approved by the FDA for actinic keratosis and was effective against human melanoma (Challacombe et al., 2006) and basal cell carcinomas (Siller et al., 2010). Ingenol protects human T cells from HIV infection (Hong et al., 2011) and ingenol triacetate inhibited the replication of HIV strain (Fujiwara et al., 1996). Ingenol 3,20-dibenzoate (IDB) induces megakaryocytic differentiation (Racke et al., 2001; 2012). Nonetheless, the underlying mechanisms of the pleiotropic actions of ingenol derivatives are not well understood.

Ingenol derivatives mimic endogenous diacylglycerol (DAG) by activation and translocation of protein kinase C (PKC) that is responsible for a number of cellular signal transduction pathways and functions (Newton, 1995). PKC isozymes are classified into three groups: conventional PKC (cPKC) which includes α, β, and γ isoforms, novel PKC (nPKC) which includes δ, ε, η, θ, and μ isoforms, and atypical PKC (aPKC) which includes ζ and λ isoforms (Newton, 1995). Ingenol binds and activates partially purified PKC (Hasler et al., 1992) and ingenol mebutate binds cPKC and nPKC (Kedei et al, 2004). However, the interactions between ingenol derivatives and PKCs were only analyzed by the indirect method of [³H]PDBu binding (Hasler et al., 1992; Kedei et al, 2004) or the docking simulation model (Grue-Sørensen et al., 2014). IDB can selectively activate nPKC (Asada et al, 1998), but direct binding kinetics have never been measured. Instead of PKC as a molecular target, evidence for the existence of cell membrane receptors for phorbol or ingenol esters was

suggested over 30 years ago (Shoyab and Todaro, 1980; Driedger and Blumberg, 1980; Dunphy et al., 1980; Sando et al., 1981), but they have not been identified to date.

Human erythropoietin (EPO) is the main cytokine that stimulates proliferation and differentiation of erythroid progenitor cells. EPO receptor (EPOR) mediates the activities of EPO. EPO binding to EPOR induces a conformational change, which results in phosphorylation of Janus tyrosine kinase-2 (JAK-2), EPOR itself, and other signal-transduction proteins, including the signal transducer and activator of transcription-5 (STAT-5) (Constantinescu et al., 2001). PKC appears to play an important role in EPOR signaling especially for the activation of RAF-1 and ERK1/2 (Lindern et al, 2000; Szenajch et al., 2010).

Recombinant EPO protein and its modified forms have been successfully used in the clinic to treat anemia. Because of some of EPO's limitations, including the existence of EPO-resistant anemia, pure red cell aplasia (PRCA), tumor-promoting effects, high cost, and invasive route of administration, researchers are trying to develop alternative EPOR activating molecules (Kim et al., 2013). Small molecules and peptide EPO mimetics that activate EPOR have been reported (Qureshi et al., 1999; Goldberg et al., 2002). However, until now, small molecule mimetics were not effective enough to be used as a therapeutic agent and the development of peptide mimetics are underway (Kessler et al., 2012).

UT-7/EPO is a human erythroleukemia cell line that is absolutely dependent on EPO for long term maintenance (Komatsu et al., 1993). We and other researchers have used UT-7/EPO cell line for the EPO bioassay, EPO signaling study, and EPO mimetic drug test (Fan et al., 2006;

Liu et al., 2007; Shin et al., 2010; 2012; Kessler et al., 2012). Little information is available regarding non-peptide small molecules with UT-7/EPO proliferative activities.

Although several herbal extracts promote erythropoiesis, most of their direct molecular targets are unidentified due to the presence of a large number of active compounds in the extracts. Using synthetic chemical libraries, researchers have tried to discover the single active compounds, but have been unsuccessful up to this point (Qureshi et al., 1999; Goldberg et al., 2002). Considering the diverse and complex conformations of ingredients of natural products, we believe that identification of EPO mimetic small molecules from the natural world is promising.

In the present study, we discover IDB possesses EPO-modulatory properties, and investigate its underlying mechanism of actions by identifying direct molecular targets.

Materials and Methods

EPO bioassay system for screening natural product library

A commercial library with 502 purified natural products of known structure (BML-2865, Enzo Life Sciences, NY, USA) was screened using UT-7/EPO cells (Shin et al., 2010). UT-7/EPO cells were maintained in alpha-MEM (Hyclone, UT, USA), supplemented with 10% FBS (Gibco BRL, NY, USA) and 1 IU/ml of erythropoietin (EPO, LG life science, Korea) in a humidified CO_2 incubator. UT-7/EPO cells were washed 3 times with alpha-MEM and 10^4 cells in EPO free culture medium were added in triplicate to wells and starved for 1 day. After starvation, 0.2 IU/ml EPO or 20 µg/ml of herbal compound was added to the cells and incubated for 3 days at 37°C in a CO_2 incubator. After incubation, EZ-Cytox cell viability WST assay kits (Daeil lab service, Korea) were used to measure cell proliferation. Fifteen microliters of kit reagent was added to each well and incubated for 1 to 6 hours in a CO_2 incubator. After incubation, optical density was measured at an absorbance of 450 nm (reference wavelength: 690 nm).

Bioassay for analyzing the dose-dependent activities of IDB and EPO

A proliferative response of UT-7/EPO cells to EPO, IDB (Santa Cruz Biotechnology, CA, USA, Enzo Life Sciences), or ingenol (IGN, Santa Cruz Biotechnology) were measured. EPO (4-fold dilutions from 5 IU/ml), IDB (4-fold dilutions from 5 µg/ml), or IGN (4-fold dilutions from 5 µg/ml) was added to the cells and incubated for 3 days. Viability assay was performed as described above.

Analysis of the activity of IDB or EPO on human bone marrow CD34+ progenitor cells

Human bone marrow CD34+ progenitor cells (1×10^4 cells) (Lonza, Maryland, USA) were incubated with EPO (4 IU/ml), IDB (1 µg/ml), or IGN (1 µg/ml) in StemSpan SFEM supplemented with cytokine cocktail CC100 (StemCell Technologies, Vancouver, Canada) in a 12-well tissue culture dish. After incubation at 37°C, 5% CO2 for 10 d, viability assays were performed as described above.

In vivo model of 5-fluorouracil (5-FU)-induced anemia for analyzing the activities of IDB and EPO

Male C57BL/6 mice (8 to 12 weeks old) were used (5 mice per treatment group). On day 0, anemia was induced in mice via a single intraperitoneal (IP) injection of 3mg of 5-FU (Sigma-Aldrich, MO, USA). On days 3, 7, and 11, mice were administered EPO (100 IU), IDB (20 µg), or vehicle in 0.2 ml saline. Blood samples were collected via the retro orbital plexus at the indicated time points (days 0, 4, 8, 12, 16, and 20) using a heparinized capillary. Hematological parameters were measured using a Procyte DX hematology analyzer (IDEXX Laboratories, CA, USA). Two independent experiments were performed. Animal studies were approved by the department of laboratory animal, institutional animal care and use committee at Sungsim campus of The Catholic University of Korea (Bucheon, Korea).

Bioassay for analyzing the activity of an EPO and IDB mixture

UT-7/EPO cell proliferative activity of an EPO and IDB mixture was measured. IGN (0.078 µg/ml or 1.25 µg/ml), IDB (0.078 µg/ml or 1.25 µg/ml), EPO (0.078 IU/ml or 1.25 IU/ml), or

mixture of EPO (0.078 IU/ml) and IDB (0.078 μg/ml) or EPO (1.25 IU/ml) and IDB (1.25 μg/ml) were applied to UT-7/EPO cell bioassay as described above.

Erythroid (BFU-E) colony assay

MethoCult assay media for human colony-forming cell assay were prepared by adding 50 ng/ml hSCF, 20 ng/ml of hGM-CSF, 20 ng/ml of hIL-3, 20 ng/ml of hIL-6, and 20 ng/ml of hG-CSF (PeproTech, NJ, USA) into MethoCult media (Stemcell Technologies, Vancouver, Canada). Human bone marrow CD34+ progenitor cells (1×10^4 cells) (Lonza, Maryland, USA) were incubated with 0.3 ml of EPO (30 IU/ml), IDB (0.03 or 1 μg/ml), or a mixture in MethoCult assay media in 35 mm dishes. After incubation at 37°C in 5% CO_2 for 12~14 d, BFU-E colonies were counted using inverted microscope (Zeiss, Oberkochen, Germany).

Western blot (WB) for analyzing the PKC activation signaling by IDB

EPO or IDB-induced translocation of PKC-α, PKC-δ, or PKC-ε to cellular membrane was analyzed by WB. EPO-starved UT-7/EPO cells (2×10^6 cells) were prepared on 100 mm plates and treated with EPO (3 IU/ml), IDB (3 μM), IGN (3 μM), or vehicle for 24 hrs. Membrane proteins and cytosolic proteins were prepared with MEM-per plus membrane protein extraction kit (Thermo Scientific, IL, USA) according to the manufacturer's instructions. From each sample, 30 μg of protein was electrophoresed on a SDS-PAGE gel, and transferred to a PVDF membrane (Millipore, MA, USA). The protein containing membrane was blocked using superblock T20 blocking buffer (Thermo Scientific) and then probed with anti-PKC-α Abs (Santa Cruz Biotechnology), anti-PKC-δ (Cell signaling, MA, USA), or anti-PKC-ε (Cell signaling). Primary antibodies were diluted 1:500 or 1:1000 in

TBS with 5% skim milk (BD science) and 0.1% Tween 20 (Sigma-Aldrich). Anti-mouse IgG

HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and Super Signal West

Pico Luminal/Enhancer solution (Pierce, IL, USA) were used for chemiluminescence. All

bands were detected using chemidoc XRS+ system (Bio-rad, CA, USA).

Bioassay for analyzing the inhibition of EPO or IBD activity by PKC inhibitors

Inhibition of UT-7/EPO cell proliferative activity of EPO or IDB by PKC inhibitors was

measured. Gö6976 (Calbiochem, NJ, USA) or bisindolylmaleimides (BIM, Merck, Germany)

was serially diluted 2-fold from 10 μM stock and added to cells. After a 30 min incubation in

a CO_2 incubator, EPO (0.25 IU/ml) or IDB (0.8 μg/ml) was applied to the UT-7/EPO cell

bioassay as described above.

Surface plasmon resonance (SPR) for analyzing the IDB interaction with PKC-α and PKC-ε

To monitor the binding kinetics between IDB and human PKC-α (Abcam, MA, USA) or

PKC-ε (Abcam), surface plasmon resonance (SPR) analysis was performed using Biacore

T200 model (GE healthcare, NJ, USA) at 25°C with buffer HBS-ET$^+$ (10 mM HEPES, pH

7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% tween-20) containing 5% DMSO. The pH

scouting to immobilize PKC isoforms was performed in 10 mM acetate buffer at a pH 4.0,

4.5, 5.0, and 5.5. PKC-α was immobilized on a CM5 sensor chip up to 5012 response unit

(RU) and PKC-ε was up to 3356 RU with the standard amine coupling method at pH 4.5.

IDB was injected into the PKC isoforms-immobilized flow cell at concentrations of 6.25,

3.13, 1.56, 0.78, and 0.39 μM with a flow rate of 20 μl/min for 45 s, and allowed to

dissociated for 180 s. T-200 BIAevaluation software was used to subtract references and determine a steady state affinity constant (K_D). Between sample series, a solvent correction cycle was run to adjust for referencing errors due to refractive index mismatches between running buffer and samples.

Bioassay for analyzing the inhibition of EPO activity by increasing amount of IDB

The inhibition of EPO-induced UT-7/EPO cell proliferation by increasing concentrations of IDB was measured. EPO (1 IU/ml) was co-incubated with IDB or IGN (4-fold serial dilutions from 5 µg/ml stock), and applied to the UT-7/EPO cell bioassay as described above.

Bioassay for analyzing the inhibition of IDB activity by sEPOR

The inhibition of EPO- and IDB-induced cell proliferation by sEPOR was monitored. UT-7/EPO cells were incubated with IDB (0.8 µg/ml) or EPO (0.25 IU/ml) in the presence of sEPOR (2-fold serial dilutions from 2.5 µg/ml stock), and applied to the UT-7/EPO cell bioassay as described above.

Enzyme-linked immunosorbent assay (ELISA) for analyzing the inhibition of EPO binding to sEPOR with IDB

sEPOR was diluted to 1 µg/ml in PBS and coated on a 96-well Maxisorp Nunc Immunoplate (Nunc, Denmark) at 4°C overnight. After washing with PBST (0.05% Tween-20 in phosphate buffered saline) using an automated washer (Bio-rad), the plates were blocked with PBSA (PBS containing 1% BSA (Sigma-Aldrich)) for 1 h at room temperature (RT).

11

After washing, 5 μg/ml EPO in PBSAT (1% BSA and 0.05% Tween-20 in PBS) or mixtures

of 5 μg/ml EPO plus IDB (25, 50, 100, or 200 μg/ml) were added to each well and incubated

for 1 h at RT. After washing, anti-EPO Ab (#ab20473, Abcam) diluted in PBSAT (1:200)

was added to each well and incubated for 1 h. After washing, rabbit anti-mouse HRP Ab

(Santa Cruz Biotechnology) diluted in PBSAT (1:2500) was added to each well and

incubated for 1 h. After washing, ABTS solution (Roche, Germany) was added to each well

and optical density was measured at an absorbance of 405 nm.

Western blot for analyzing the inhibition of IDB or EPO signaling by sEPOR

Inhibition of IDB or EPO-induced ERK1/2 phosphorylation by sEPOR co-incubation was

analyzed by Western blot (WB). Starved UT-7/EPO cells (2×10^6 cells) were treated with

EPO (0.25 IU/ml), IDB (0.8 μg/ml), or vehicle in the presence or absence of sEPOR (5

μg/ml) for 5 min. Total proteins were extracted using RIPA buffer (Sigma-Aldrich) with a

protease inhibitor cocktail (Fisher, MA, USA) and applied to the WB assay as described

above. Anti-ERK1/2 (Thermo Scientific) and phospho (p)-ERK1/2 (Thermo Scientific) were

used for primary Abs.

WB for analyzing IDB-induced phosphorylation of EPOR and ERK1/2 in the presence or
absence of EPO

Starved UT-7/EPO cells (2×10^6 cells) were treated with various concentrations of IDB (1.6,

0.8, 0.4, 0.2, and 0.1 μg/ml) with or without EPO (0.25 IU/ml) for 5 min. Total proteins were

extracted using RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Fisher) and

applied to the WB assay as described above. Anti-EPOR (Santa Cruz Biotechnology), p-EPOR (Santa Cruz Biotechnology), ERK1/2, and p-ERK1/2 were used for primary Abs.

WB for analyzing the IDB-induced phosphorylation of JAK-2

Starved UT-7/EPO cells (2×10^6 cells) were treated with EPO (1, 0.25, or 0.0625 IU/ml), IDB (1.6, 0.4, or 0.1 μg/ml), or IGN (1.6, 0.4, or 0.1 μg/ml) for 5 min. Total proteins were extracted using RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Fisher) and applied to the WB assay as described above. Anti-JAK-2 (Cell signaling) and p-JAK-2 (Cell signaling) were used for primary Abs.

SPR for analyzing the IDB binding to sEPOR

To analyze the interaction between IDB and human sEPOR, the pH scouting for immobilization of recombinant sEPOR was performed in 10 mM acetate buffer at pHs 4.0, 4.5, 5.0, and 5.5. sEPOR was immobilized on a CM5 sensor chip up to 1612 RU with a standard amine coupling method at pH 5.0. IDB was injected into the sEPOR-immobilized flow cell at concentrations of 6.25, 3.13, 1.56, 0.78, and 0.39 μM with a flow rate of 20 μl/min for 45 s and allowed to dissociate for 180 s.

Data analysis

Results are expressed or plotted as the mean ± standard deviation (SD) or standard error of the mean (SEM), and data were compared for statistical significance with the Student's t-test

MOL #97436

or one-way ANOVA, and $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered

significant.

Results

EPO mimetic properties of IDB in cell and mouse models

Through the screening of natural compound library, ingenol 3,20-dibenzoate (IDB) was identified as an active compound that induced proliferation of the EPO-dependent cell line, UT-7/EPO (Fig. 1A). IDB (Fig. 1B) and ingenol (IGN) (Fig. 1C) are diterpenoids related to phorbol, and IDB is a known PKC-ε activator and IGN is a weak activator of PKC-ε and PKC-δ. Despite the same core structure between IDB and IGN, IGN did not induce UT-7/EPO proliferation (Fig. 1D). IDB dose-dependently promoted UT-7/EPO cell proliferation with an EC_{50} of 0.27 μg/ml (485 nM) (Fig. 1D). The maximum effect of IDB was about 70% that of EPO. Additionally, we assessed the IDB-driven expansion of human bone marrow CD34+ progenitor cells. Like EPO, IDB also positively affects the proliferation of primary CD34+ cells (Fig. 1E).

Given that IDB was shown to have EPO like capacity in promoting the proliferation of a solely EPO-dependent cell line, its performance was assessed in a 5-fluorouracil (5-FU)-induced anemia model (Fig. 2). Overall, severity of 5-FU-induced anemia was lessened by IDB treatment on days 12 and 20 (Figs. 2A, 2C, and 2E), which was shown by the significant elevation of red blood cells (RBC), hematocrit (HCT), and hemoglobin (Hb) levels compared with vehicle treated group (Figs. 2B, 2D, and 2F). IDB also elevated reticulocytes (RET) on day 16 (Figs. 2G and 2H).

Both synergistic and antagonistic effects of IDB against EPO

In order to check if the activities of EPO and IDB are either cooperative or competitive, a bioassay was performed with the subsaturated concentration mixture of 0.08 IU/ml EPO and 0.08 μg/ml IDB (Fig. 3A) or with the saturated one of 1.25 IU/ml EPO and 1.25 μg/ml IDB (Fig. 4A). While IDB and EPO synergistically stimulated UT-7/EPO cell proliferation at a subsaturated concentration (Fig. 3A), IDB suppressed EPO activity in cells similarly to those treated with IDB alone at saturated concentrations (Fig. 4A). We demonstrated this outcome in more detail with mixtures of concentration-fixed EPO (1 IU/ml) and serially diluted IDB (Fig. 4B). EPO activity was completely downregulated by IDB in a dose-dependent manner, but IDB's own activity was maintained (Fig. 4B). Similarly, synergistic (Fig. 3B) or antagonistic activity (Fig. 4C) between EPO and IDB was examined in an *ex vivo* assay. Although 0.003 μg/ml of IDB alone was unable to stimulate the differentiation of human bone marrow CD34+ cells into erythroid colonies (BFU-E), but it significantly synergized EPO activity (Fig. 3B). In contrast, 0.1 μg/ml of IDB suppressed EPO-induced BFU-E colony formation (Fig. 4C). To understand the differential actions of IDB on EPO, we began to investigate the underlying mechanisms.

Synergistic effects of IDB at subsaturated concentration with EPO via direct binding to PKCs

At first, the effects of IDB on the activation and membrane translocation of PKC isoforms were studied in UT-7/EPO cells (Fig. 3C). PKC-α, PKC-δ, and PKC-ε were translocated to the membrane fraction in IDB-treated cells and EPO slightly activated PKC-δ and PKC-ε. No PKCs tested by us were affected by IGN treatment. Next, the effect of a classical PKC inhibitor on the IDB or EPO-mediated UT-7/EPO cell proliferation was assessed (Fig. 3D and 3E). While EPO-induced cell proliferation was almost completely blocked by Gö6976, a

16

PKC-α and PKC-β inhibitor, in a dose-dependent manner, IDB-induced proliferation was partially blocked (Fig. 3D). Bisindolylmaleimides (BIM), a general PKC inhibitor, was added to EPO or IDB-treated cells (Fig. 3E). Although both activities of EPO and IDB were almost completely inhibited by BIM treatment in a dose-dependent manner, IDB activity was much more sensitive (Fig. 3E). Further evidence of IDB's action on PKCs was provided by surface plasmon resonance (SPR) analysis by BIAcore (Figs. 3F, 3G, 3H, and 3I). Purified PKC-α or PKC-ε was covalently cross-linked to the dextran matrix of a CM5 sensor chip and various concentrations of IDB were passed over this surface as well as the reference surface. Representative reference-subtracted overlaid sensorgrams are displayed. IDB bound directly to PKC-α (Fig. 3F) and PKC-ε (Fig. 3H) with similar binding affinities (K_D), but IGN did not (Figs. 3G and 3I).

IDB competes and interferes with EPO via direct binding to EPO receptor (EPOR).

It was observed that a relatively high amount of IDB down-regulated EPO activity nearly to the level of the group treated with IDB alone (Fig. 4A and 4B). We postulated that most activities of EPO (1 IU/ml) plus IDB (high concentrations) seems to be attributed by remaining IDB that targets PKCs but not by IDB that participates in the EPO blockade via direct competition for the EPOR.

To prove this hypothesis, we investigated the impact of soluble EPOR on IDB related activity. IBD's cell proliferation activity was partially hampered by sEPOR co-incubation, whereas the activity of EPO was almost completely suppressed by sEPOR (Fig. 4D). Partial inhibition of IDB activity by sEPOR co-incubation led to the idea that it may be possible for IDB to target at least two molecules, sEPOR and PKCs. To determine if IDB competes with

17

EPO in binding to sEPOR at a molecular level, ELISA was used to analyze the interaction between EPO and sEPOR. Protein-protein interaction of EPO and sEPOR was substantially disrupted by the addition of IDB to the EPO sample in a dose-dependent manner (Fig. 4E). In addition, it was demonstrated that sEPOR was able to block EPO and IDB-induced phosphorylation of ERK1/2, likely through the direct binding to EPO or IDB (Fig. 4F).

Since preceding data have shown that IDB possibly binds directly to EPOR, we tested whether IDB is able to stimulate the internal EPOR-related signaling. IDB stimulated the phosphorylation of ERK1/2, but not the phosphorylation of EPOR and JAK-2 (Figs. 4G and 4H). By demonstrating the blockade of EPO-induced EPOR phosphorylation by IDB (Fig. 4G) and the specific binding kinetics between IDB and EPOR with a K_D of 4.02×10^{-6} M (Figs. 4I and 4J), we collectively concluded that IDB does bind to EPOR in a competitive fashion with EPO, but does not activate the EPOR-initiated signaling pathway.

MOL #97436

Discussion

To find an EPO mimetic agent from natural sources, an herbal compound library was screened using an EPO-dependent cell line, and it is from this work that IDB was discovered. To our knowledge, the data firstly showed the dual functions of IDB, EPO-supportive activity via PKC and EPO-suppressive activity via the EPOR. It was previously reported that EPO mimetic synthetic compounds from chemical collections exhibited much less potency than EPO (under 20% that of EPO) with no *in vivo* data (Qureshi et al., 1999; Goldberg et al., 2002), but we demonstrated the substantial *in vitro* efficacy of IDB (about 70% that of EPO) with an *in vivo* hematopoietic activity (Figs. 1 and 2).

Although very recent research regarding ingenol derivatives shed a new light on the chemical synthesis (Jørgensen et al., 2013; McKerrall et al., 2014) and clinical application (Challacombe et al., 2006; Siller et al., 2010), their mode of action is still obscure. Moreover, the data about IDB are much more limited in terms of activities and their mechanisms. Unlike the known agonistic action of IDB on the novel PKCs (Racke et al., 2001; 2012), IDB also seems to activate classical PKCs, which was demonstrated by the partial inhibition of IDB activity by a PKC-α inhibitor (Fig. 3D), complete inhibition by a broad PKC inhibitor (Fig. 3E), and the physical interactions of IDB with PKC-α and PKC-ε (Fig. 3F and 3H). IDB possesses erythropoietic activities *in vivo* (Fig. 2), and this effect is likely attributable to the EPO-mimic activation of PKC (Lindern et al, 2000). Usually, binding affinity of chemical PKC agonists were measured by the inhibition of [^{3}H]PDBu binding (Hasler et al., 1992; Kedei et al, 2004), but we firstly adopted surface plasmon resonance analysis to demonstrate the direct binding of IDB to PKC-α and PKC-ε (Figs. 3F and 3H). IGN did not bind to PKC-α and PKC-ε and did not show any biological activities.

19

While the synergistic effect of IDB and EPO on UT-7/EPO proliferation appears to be implicated in the targeting of PKC and EPOR, respectively, EPO antagonistic effect (Fig. 4A, 4B, and 4C) of IDB could not be explained with PKC targeting alone. Considering the EPO mimicking, but competitive effects of IDB, we placed the EPOR on the list of novel target suspects. IDB does directly bind to EPOR (Fig. 4I), which could interfere with the EPO binding to EPOR (Fig. 4E) and a subsequent blockade of EPO signaling (Fig. 4G). In addition, sEPOR was able to bind to IDB, blocking its cellular proliferation (Fig. 4D) and downstream signaling activity (Fig. 4F). IDB is not regarded as an EPOR agonist but as an antagonist, because it failed to stimulate JAK-2 and EPOR phosphorylation (Fig. 4G and 4H). Although some synthetic small molecules as a multimeric form stimulated EPO-responder cells, direct evidence regarding the triggering of internal signaling was missing (Qureshi et al., 1999; Goldberg et al., 2002). This is the first report that ingenol ester, a PKC agonist, could target and antagonize EPOR.

The dual role of IDB as an EPO-synergizing (Fig. 3) or -suppressing molecule (Fig. 4) is due to the dual targeting of PKC and EPOR in a concentration-dependent manner by IDB and EPO. At limiting concentrations of IDB and EPO, IDB tends to strengthen the intracellular PKC/ERK signaling of EPO (Fig. 4G) by direct activation of PKC (Fig. 3C, 3F, and 3H), rather than compete with EPO for EPOR binding due to the possible availability of EPO-unbound EPOR on the cell surface, resulting in enhanced stimulation of cell proliferation by the mixture of IDB and EPO (Fig. 3A). At saturating concentrations of IDB and EPO, IDB appears to inhibit the cell surface binding of EPO to EPOR by competitively binding to EPOR (Fig. 4D, 4E, and 4I). IDB also appears to sustain the PKC/ERK activation signal (Fig. 4G) via excess IDB that does not participate in EPOR binding, but instead penetrates

the membrane and binds to PKC, resulting in suppressed stimulation of cell proliferation by the mixture of IDB and EPO (Fig. 4A and 4B).

The antagonistic activity of IDB against EPO provides a speculation that IDB may counteract the potential deleterious effects of EPO, such as the growth of some EPO-dependent tumors (Pérès et al., 2011; Wu et al., 2012), increased mortality rate of cancer patients treated with recombinant EPO for cancer-associated anemia (Henke et al., 2003; Leyland-Jones et al, 2003; Wright et al., 2007; Bennett et al., 2008), and high endogenous EPO levels related to certain diseases, such as myelodysplastic syndrome (Wallvik et al., 2002; Moyo et al., 2008). IDB and IDB derivatives induce apoptosis in Jurkat leukemia cells (Blanco-Molina et al., 2001), and ingenol mebutate is effective against actinic keratosis, human melanoma (Challacombe et al., 2006), and basal cell carcinomas (Siller et al., 2010). Considering the intrinsic hematopoietic, anti-cancer, and EPOR antagonistic activities of IDB, researchers believe that utilizing IDB to treat cancer-related anemia is a promising approach.

In conclusion, we have identified a new biological effect and IDB target. These findings could provide novel insights into the underlying mechanisms of biologically active ingenol derivatives and could be basis for the development of novel therapeutic strategies for EPO-related disorders.

MOL #97436

Acknowledgements

MOL #97436

Authorship contribution statement

Participated in research design: Oh, S.K. Kim, S.J. Kim, Kang, and Heo.

Conducted experiments: Oh and Choi

Contributed new reagents or analytic tools: Chin.

Performed data analysis: Oh, Kang, and Heo.

Wrote or contributed to the writing of the manuscript: Oh, S.K. Kim, and Heo

References

Asada A, Zhao Y, Kondo S, and Iwata M (1998) Induction of thymocyte apoptosis by Ca2+-independent protein kinase C (nPKC) activation and its regulation by calcineurin activation. *J Biol Chem* **273**:28392–28398.

Bennett CL, Silver SM, Djulbegovic B, Samaras AT, Blau CA, Gleason KJ, Barnato SE, Elverman KM, Courtney DM, McKoy JM, Edwards BJ, Tigue CC, Raisch DW, Yarnold PR, Dorr DA, Kuzel TM, Tallman MS, Trifilio SM, West DP, Lai SY, and Henke M (2008) Venous thromboembolism and mortality associated with recombinant erythropoietin and darbepoetin administration for the treatment of cancer-associated anemia. *JAMA* **299**:914–924.

Blanco-Molina M, Tron GC, Macho A, Lucena C, Calzado MA, Muñoz E, and Appendino G (2001) Ingenol esters induce apoptosis in Jurkat cells through an AP-1 and NF-kappaB independent pathway. *Chem Biol* **8**:767–778.

Challacombe JM, Suhrbier A, Parsons PG, Jones B, Hampson P, Kavanagh D, Rainger GE, Morris M, Lord JM, Le TT, Hoang-Le D, and Ogbourne SM (2006) Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. *J Immunol* **177**:8123–8132.

Constantinescu SN, Keren T, Socolovsky M, Nam H, Henis YI, and Lodish HF (2001) Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *Proc Natl Acad Sci USA* **98**:4379–4384.

Driedger PE and Blumberg PM (1980) Specific binding of phorbol ester tumor promoters. *Proc Natl Acad Sci USA* **77**:567–571.

Dunphy WG, Delclos KB, and Blumberg PM (1980) Characterization of specific binding of [3H] phorbol 12, 13-dibutyrate and [3H] phorbol 12-myristate 13-acetate to mouse brain. *Cancer Res* **40**:3635–3641.

Fan Q, Leuther KK, Holmes CP, Fong KL, Zhang J, Velkovska S, Chen MJ, Mortensen RB, Leu K, Green JM, Schatz PJ, and Woodburn KW (2006) Preclinical evaluation of Hematide, a novel erythropoiesis stimulating agent, for the treatment of anemia. *Exp Hematol* **34**:1303–1311.

Fujiwara M, Ijichi K, Tokuhisa K, Katsuura K, Shigeta S, Konno K, Wang GY, Uemura D, Yokota T, and Baba M (1996) Mechanism of selective inhibition of human immunodeficiency virus by ingenol triacetate. *Antimicrob Agents Chemother* **40**:271–273.

Goldberg J, Jin Q, Ambroise Y, Satoh S, Desharnais J, Capps K, and Boger DL (2002) Erythropoietin mimetics derived from solution phase combinatorial libraries. *J Am Chem Soc* **124**:544–555.

Grue-Sørensen G, Liang X, Månsson K, Vedsø P, Dahl Sørensen M, Soor A, Stahlhut M, Bertelsen M, Engell KM, and Högberg T (2014) Synthesis, biological evaluation and SAR of 3-benzoates of ingenol for treatment of actinic keratosis and non-melanoma skin cancer. *Bioorg & Med Chem Lett* **24**:54–60.

Hasler CM, Acs G, and Blumberg PM (1992) Specific binding to protein kinase C by ingenol and its induction of biological responses. *Cancer Res* **52**:202–208.

Henke M, Laszig R, Rübe C, Schäfer U, Haase KD, Schilcher B, Mose S, Beer KT, Burger U, Dougherty C, and Frommhold H (2003) Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* **362**:1255–1260.

Hong KJ, Lee HS, Kim YS, and Kim SS (2011) Ingenol Protects Human T Cells From HIV-1 Infection. *Osong Public Health Res Perspect* **2**:109–114.

Jørgensen L, McKerrall SJ, Kuttruff CA, Ungeheuer F, Felding J, and Baran PS (2013) 14-step synthesis of (+)-ingenol from (+)-3-carene. *Science* **341**:878–882.

Kedei N, Lundberg DJ, Toth A, Welbum P, Garfield SH, and Blumberg PM (2004) Characterization of the interaction of ingenol 3-angelate with protein kinase C. *Cancer Res* **64**:3243–3255.

25

Kessler C, Greindl A, Breuer B, Haberl U, Rybka A, Emgenbroich M, Frank HG, and Pötgens AJ (2012) Erythropoietin mimetic compound AGEM400(HES) binds to the same receptor as erythropoietin but displays a different spectrum of activities. *Cytokine* **57**:226–237.

Kim SJ, Chin YW, and Heo TH (2013) Erythropoietic agents from natural sources. *Altern Ther Health Med* **19**:54–60.

Komatsu N, Yamamoto M, Fujita H, Miwa A, Hatake K, Endo T, Okano H, Katsube T, Fukumaki Y, and Sassa S (1993) Establishment and characterization of an erythropoietin-dependent subline, UT-7/Epo, derived from human leukemia cell line, UT-7. *Blood* **82**:456–464.

Leyland-Jones B, BEST Investigators and Study Group (2003) Breast cancer trial with erythropoietin terminated unexpectedly. *Lancet Oncol* **4**:459–460.

von Lindern M, Parren-van Amelsvoort M, van Dijk T, Deiner E, van den Akker E, van Emst-de Vries S, Willems P, Beug H, and Löwenberg B (2000) Protein kinase C alpha controls erythropoietin receptor signaling. *J Biol Chem* **275**:34719–34727.

Liu Z, Stoll VS, Devries PJ, Jakob CG, Xie N, Simmer RL, Lacy SE, Egan DA, Harlan JE, Lesniewski RR, and Reilly EB (2007) A potent erythropoietin-mimicking human antibody interacts through a novel binding site. *Blood* **110**:2408–2413.

McKerrall SJ, Jørgensen L, Kuttruff CA, Ungeheuer F, and Baran PS (2014) Development of a Concise Synthesis of (+)-Ingenol. *J Am Chem Soc* **136**:5799–5810.

Moyo V, Lefebvre P, Duh MS, Yektashenas B, and Mundle S (2008) Erythropoiesis-stimulating agents in the treatment of anemia in myelodysplastic syndromes: a meta-analysis. *Ann Hematol* **87**:527–536.

Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* **270**:28495–28498.

Pérès EA, Valable S, Guillamo JS, Marteau L, Bernaudin JF, Roussel S, Lechapt-Zalcman E, Bernaudin M, and Petit E (2011) Targeting the erythropoietin receptor on glioma cells reduces tumour growth. *Exp Cell Res* **317**:2321–2332.

Qureshi SA, Kim RM, Konteatis Z, Biazzo DE, Motamedi H, Rodrigues R, Boice JA, Calaycay JR, Bednarek MA, Griffin P, Gao YD, Chapman K, and Mark DF (1999) Mimicry of erythropoietin by a nonpeptide molecule. *Proc Natl Acad Sci USA* **96**:12156–12161.

Racke FK, Baird M, Barth RF, Huo T, Yang W, Gupta N, Weldon M, and Rutledge H (2012) Unique in vitro and In vivo thrombopoietic activities of ingenol 3,20 dibenzoate, A Ca++-independent protein kinase C isoform agonist. *PLoS ONE* **7**:e51059.

Racke FK, Wang D, Zaidi Z, Kelley J, Visvader J, Soh JW, and Goldfarb AN (2001) A potential role for protein kinase C-epsilon in regulating megakaryocytic lineage commitment. *J Biol Chem* **276**:522–528.

Sando JJ, Hilfiker ML, Salomon DS, and Farrar JJ (1981) Specific receptors for phorbol esters in lymphoid cell populations: role in enhanced production of T-cell growth factor. *Proc Natl Acad Sci USA* **78**:1189–1193.

Shin SK, Ha SK, Lee KW, Yoo TH, Yun SR, Yoon SH, Kim SJ, Lee SK, and Heo TH (2010) Application of a bridging ELISA for detection of anti-erythropoietin binding antibodies and a cell-based bioassay for neutralizing antibodies in human sera. *J Pharm Biomed Anal* **52**:289–293.

Shin SK, Moon SJ, Ha SK, Jo YI, Lee TW, Lee YS, Kim YW, Kim DJ, Kim JK, Yoo TH, Lee KB, Choi SO, Kang EW, Lee KW, Kim SJ, Kim SK, and Heo TH (2012) Immunogenicity of recombinant human erythropoietin in Korea: A two-year cross-sectional study. *Biologicals* **40**:254-261.

Shiroshita N, Musashi M, Sakurada K, Kimura K, Tsuda Y, Ota S, Iwasaki H, Miyazaki T, Kato T, Miyazaki H, Shimosaka A, and Asaka M (2001) Involvement of protein kinase C-epsilon in

27

signal transduction of thrombopoietin in enhancement of interleukin-3-dependent proliferation of primitive hematopoietic progenitors. *J Pharmacol Exp Ther* **297**:868–875.

Shoyab M and Todaro GJ (1980) Specific high affinity cell membrane receptors for biologically active phorbol and ingenol esters. *Nature* **288**:451–455.

Siller G, Rosen R, Freeman M, Welburn P, Katsamas J, and Ogbourne SM (2010) PEP005 (ingenol mebutate) gel for the topical treatment of superficial basal cell carcinoma: results of a randomized phase IIa trial. *Australas J Dermatol* **51**:99–105.

Szenajch J, Wcislo G, Jeong JY, Szczylik C, and Feldman L (2010) The role of erythropoietin and its receptor in growth, survival and therapeutic response of human tumor cells From clinic to bench - a critical review. *Biochim Biophys Acta* **1806**:82–95.

Wallvik J, Stenke L, Bernell P, Nordahl G, Hippe E, and Hast R (2002) Serum erythropoietin (EPO) levels correlate with survival and independently predict response to EPO treatment in patients with myelodysplastic syndromes. *Eur J Haematol* **68**:180–185.

Wright JR, Ung YC, Julian JA, Pritchard KI, Whelan TJ, Smith C, Szechtman B, Roa W, Mulroy L, Rudinskas L, Gagnon B, Okawara GS, and Levine MN (2007) Randomized, double-blind, placebo-controlled trial of erythropoietin in non-small-cell lung cancer with disease-related anemia. *J Clin Oncol* **25**:1027–1032.

Wu P, Zhang N, Wang X, Zhang C, Li T, Ning X, and Gong K (2012) The Erythropoietin/Erythropoietin Receptor Signaling Pathway Promotes Growth and Invasion Abilities in Human Renal Carcinoma Cells. *PLoS ONE* **7**:e45122.

MOL #97436

Footnotes

Reprint request to **Dr. Tae-Hwe Heo**

NP512, Hall of Cardinal Jin-Suk Cheong, The Catholic University of Korea, 43 Jibong-ro, Wonmi-gu, Bucheon-si, Gyeonggi-do, 420-743, Republic of Korea

E-mail: thhur92@catholic.ac.kr

MOL #97436

Figure legends

Fig. 1. Ingenol 3,20-dibenzoate (IDB) stimulates the proliferation of UT-7/EPO cell line with absolute dependence on erythropoietin (EPO) as well as human bone marrow CD34+ progenitor cells. **(A)** Compound library from natural products were screened using UT-7/EPO cells to identify a compound with proliferative activity. After EPO starvation for 24 h, UT-7/EPO cells were incubated with EPO (0.2 IU/ml) or one of the library compounds (20 μg/ml), and cell viability was measured by WST assay. IDB was identified as a hit out of about 400 compounds and representative data are shown. **(B)** Chemical structure of IDB. **(C)** Chemical structure of ingenol (IGN) used as a control. **(D)** Dose-dependent enhancement of UT-7/EPO proliferation by IDB. EPO (5 IU/ml), IDB (5 μg/ml), or IGN (5 μg/ml) was serially diluted and applied to UT-7/EPO cell proliferation assay. Mean OD values of triplicate samples ± standard deviation (SD) are shown. **(E)** Stimulation of proliferation of human bone marrow CD34+ progenitor cells by IDB. Human bone marrow CD34+ progenitor cells were incubated with EPO (4 IU/ml), IDB (1 μg/ml), or IGN (1 μg/ml) in StemSpan SFEM supplemented with cytokine cocktail CC100. After a 10-day incubation, viability assays were performed. Mean OD values of triplicate samples ± standard error of the mean (SEM) are shown. * $p < 0.05$, *** $p < 0.001$, with respect to the vehicle-treated group.

Fig. 2. In vivo activity of IDB. Anemia was induced by an IP injection of 5-FU (3 mg/mouse) into C57BL/6 mice on day 0. EPO (100 IU/mouse), IDB (20 μg/mouse), or vehicle was administered on days 3, 7, and 11. Hematological parameters, including RBC (red blood cells, 10^6 cells/μl) **(A, B)**, HCT (hematocrit, %) **(C, D)**, Hb (hemoglobin, g/dl) **(E, F)**, RET (reticulocytes, 10^3 cells/μl) **(G, H)**, and PLT (platelets, 10^3 cells/μl) **(I, J)** were measured using a Procyte DX hematology analyzer. Representative data from two

30

independent experiments are shown as the mean ± standard error of the mean (SEM) from 5

mice for vehicle, EPO, and IDB treated groups. * $p < 0.05$, ** $p < 0.01$, compared with

values from vehicle-treated mice.

Fig. 3. EPO synergistic activity of IDB is attributable to the direct targeting of PKC. (A)
Promotion of EPO activity with IDB in a bioassay. IGN (0.08 µg/ml), IDB (0.08 µg/ml), EPO
(0.08 IU/ml), or a mixture of EPO (0.08 IU/ml) and IDB (0.08 µg/ml) was applied to the UT-
7/EPO cell proliferation assay. Mean OD values of triplicate samples ± SD are shown. **p <
0.01, *** < 0.001 in comparison with EPO alone group. **(B)** Stimulation of the differentiation
of human CD34+ bone marrow cells into BFU-E colonies by EPO or EPO plus IDB. CD34+
cells were incubated for 12~14 d in MethoCult media in the presence of IDB (0.003 µg/ml),
EPO (3 IU/ml), or IDB (0.003 µg/ml) plus EPO (3 IU/ml). BFU-E colonies were counted
using an inverted microscope (*left*). Representative images were taken at $100 \times$ magnification
(*right*). **(C)** Translocations of PKCs to the cell membrane were analyzed by Western blot
(WB) after treatment with EPO (3 IU/ml), IDB (3 µM), or IGN (3 µM) for 24 h. WB bands
were quantified by densitometric analysis. **(D) (E)** Inhibition of cell proliferative activity of
EPO and IDB by PKC inhibitors. Gö6976 **(D)** or bisindolylmaleimides (BIM) **(E)** was
serially diluted 2-fold from 10 µM stock and added to the cells. After 30 m incubation in a
CO_2 incubator, EPO (0.25 IU/ml) or IDB (0.8 µg/ml) was added to each well and incubated
for another 72 h. Cell proliferation was measured by WST assay. **(F), (G), (H), (I)** Surface
plasmon resonance (SPR) analysis was performed using a BIAcore instrument. PKC-α or
PKC-ε was immobilized on a CM5 sensor chip with the standard amine coupling method and
IDB or IGN was injected to the PKC-coated flow cell at indicated concentrations. T-200
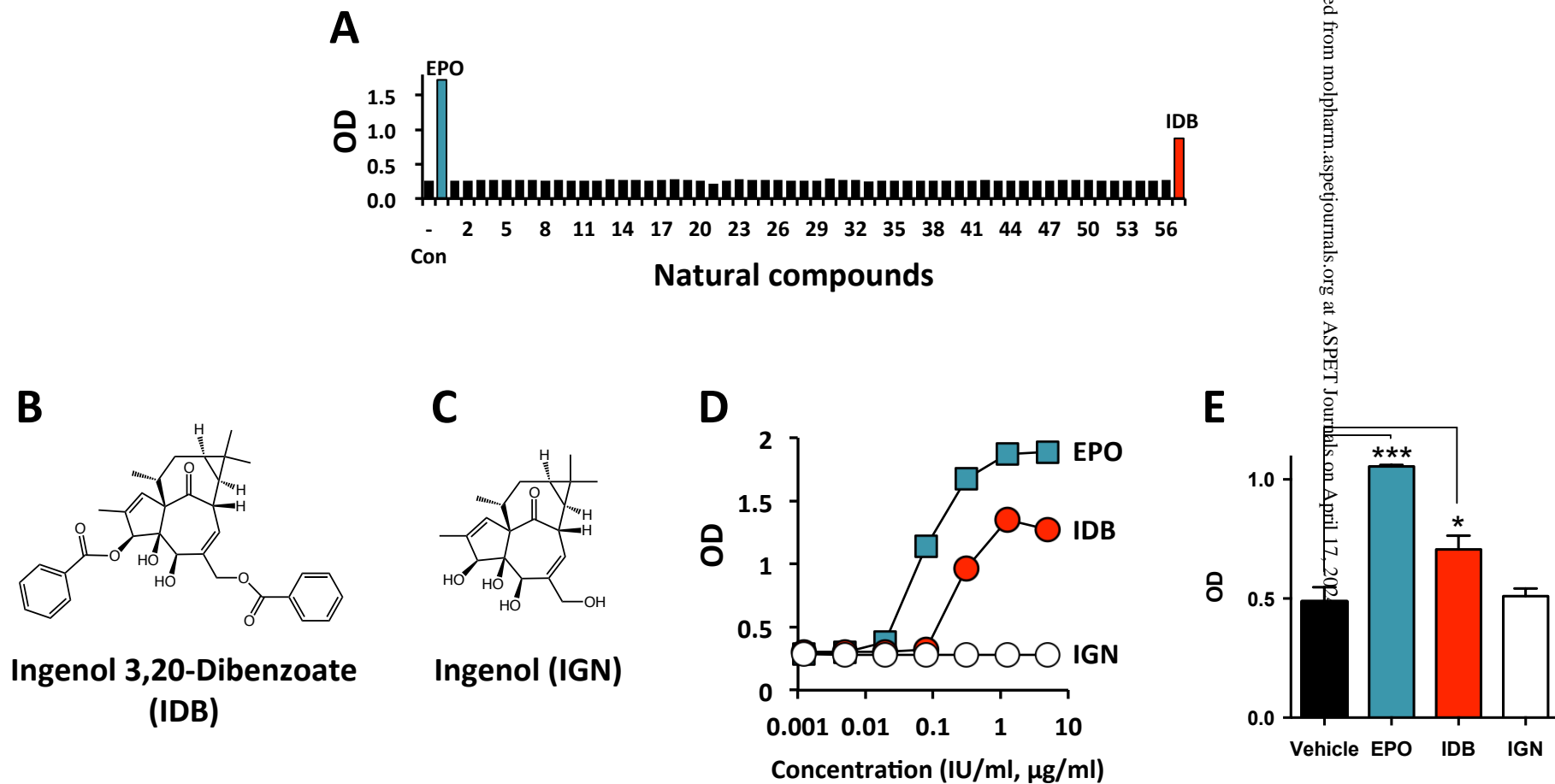BIAevaluation software was used to subtract references and determine the affinity constant
(K_D).

31

MOL #97436

Fig. 4. EPO inhibitory activity of IDB is attributable to the competitive binding of IDB and EPO towards the EPO receptor (EPOR). (A) Inhibition of EPO activity with a high concentration of IDB in a bioassay. IGN (1.25 μg/ml), IDB (1.25 μg/ml), EPO (1.25 IU/ml), or a mixture of EPO (1.25 IU/ml) and IDB (1.25 μg/ml) was applied to the UT-7/EPO cell proliferation assay. Mean OD values of triplicate samples ± SD are shown. **p < 0.01, *** < 0.001 in comparison with EPO alone group. **(B)** Inhibition of activity using a fixed concentration of EPO with increasing concentrations of IDB or IGN. 1 IU/ml of EPO was co-incubated with increasing amounts of IDB or IGN up to 5 μg/ml and applied to the UT-7/EPO cell proliferation assay. For comparison, serial dilutions of IDB or IGN were applied to cells in the absence of EPO. Mean OD values of triplicate samples ± SD are shown. **(C)** Suppression of EPO-induced differentiation in human CD34+ bone marrow cells into BFU-E colonies by a high concentration of IDB. CD34+ cells were incubated for 12~14 d in MethoCult media in the presence of IDB (0.1 μg/ml), EPO (3 IU/ml), or IDB (0.1 μg/ml) plus EPO (3 U/ml). **(D)** Inhibition of cell proliferative activity by IDB or EPO with soluble EPOR (sEPOR). UT-7/EPO cells were incubated with IDB (0.8 μg/ml) or EPO (0.25 IU/ml) in the presence of sEPOR (2-fold dilutions from 2.5 μg/ml) and the WST assay was performed. Mean OD values of triplicate samples ± SD are shown. **(E)** Inhibition of EPO binding to plate-coated sEPOR by IDB. ELISA plate was coated with sEPOR (1 μg/ml) and added by EPO (5 μg/ml) in the presence of serially diluted IDB (200, 100, 50, 25 μg/ml). After washing, bound EPO was detected by anti-EPO primary Abs. Mean OD values of triplicate samples ± SD are shown. *p < 0.05, **p < 0.01, and *** < 0.001 indicate statistical significance in comparison with cells treated with EPO alone. **(F)** Inhibition of IDB- or EPO-induced ERK1/2 phosphorylation by co-incubation with sEPOR. ERK1/2 signaling in UT-7/EPO cells stimulated by IDB (0.8 μg/ml) or EPO (0.25 IU/ml) in the presence of sEPOR (5
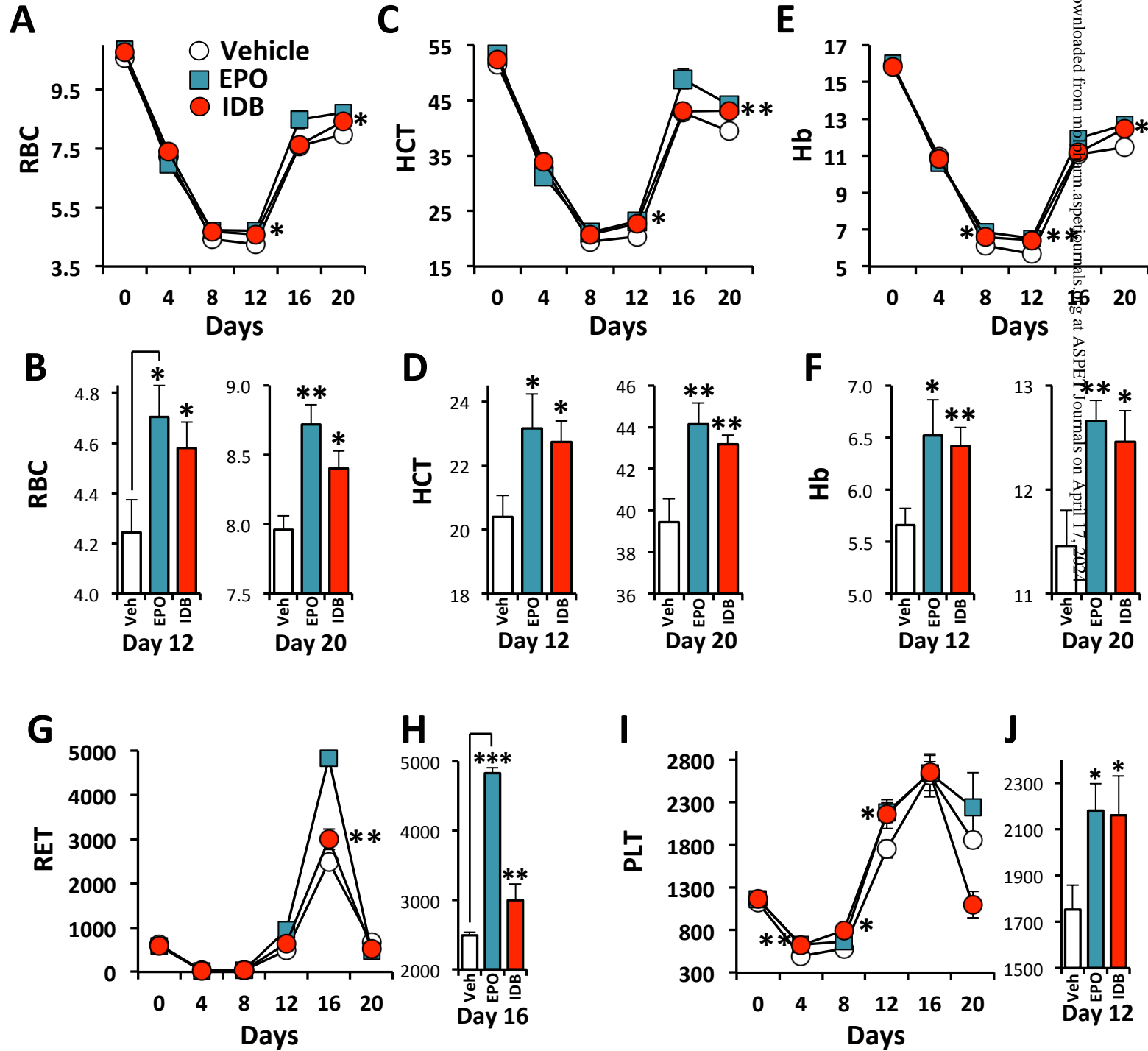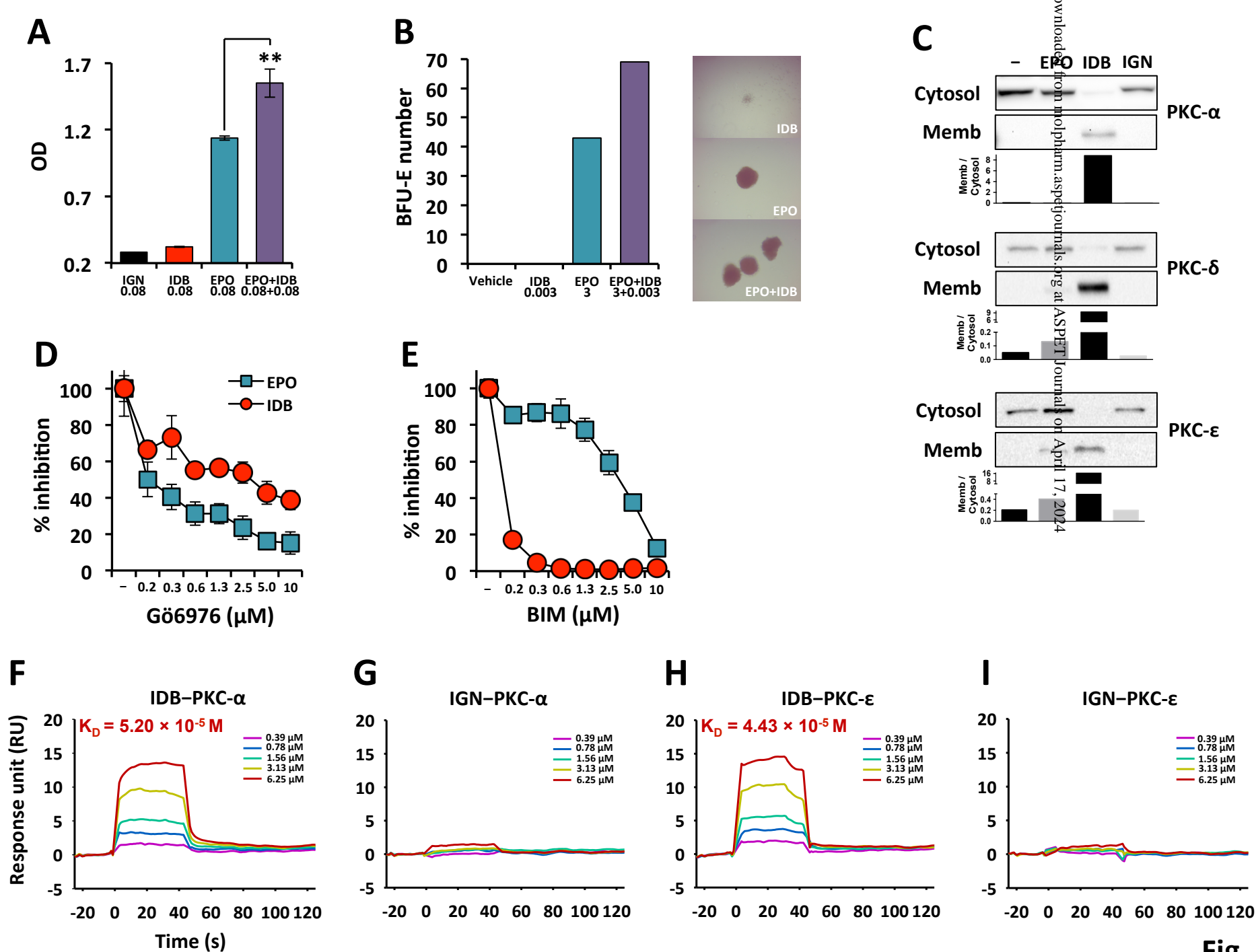
μg/ml) for 5 m was analyzed by Western blot (WB). WB bands were quantified by densitometric analysis, and the data are presented as the mean ± SD of three experiments. Data were analyzed by Student t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(G)** Dose-dependent inhibition of EPO-induced EPOR phosphorylation by IDB. Effect of IDB (1.6, 0.8, 0.4, 0.2, or 0.1 μg/ml) co-incubation in the EPO (0.25 IU/ml)-induced phosphorylation of EPOR and ERK1/2 were analyzed by WB. WB bands were quantified by densitometric analysis, and the data are presented as the mean ± SD of three experiments. Data were analyzed by one-way ANOVA with Dunnett post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(H)** Inefficiency of JAK-2 phosphorylation by IDB. WB analysis of JAK-2 phosphorylation by EPO (1, 0.25, or 0.0625 IU/ml), IDB (1.6, 0.4, or 0.1 μg/ml), or IGN (1.6, 0.4, or 0.1 μg/ml) was performed. WB bands were quantified by densitometric analysis, and the data are presented as the mean ± SD of three experiments. Data were analyzed by one-way ANOVA with Dunnett post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(I)**, **(J)** SPR analysis was performed using BIAcore. sEPOR was immobilized on a CM5 sensor chip with a standard amine coupling method and IDB or IGN was injected into the sEPOR-coated flow cell at indicated concentrations. T-200 BIAevaluation software was used to subtract references and determine the affinity constant (K_D).
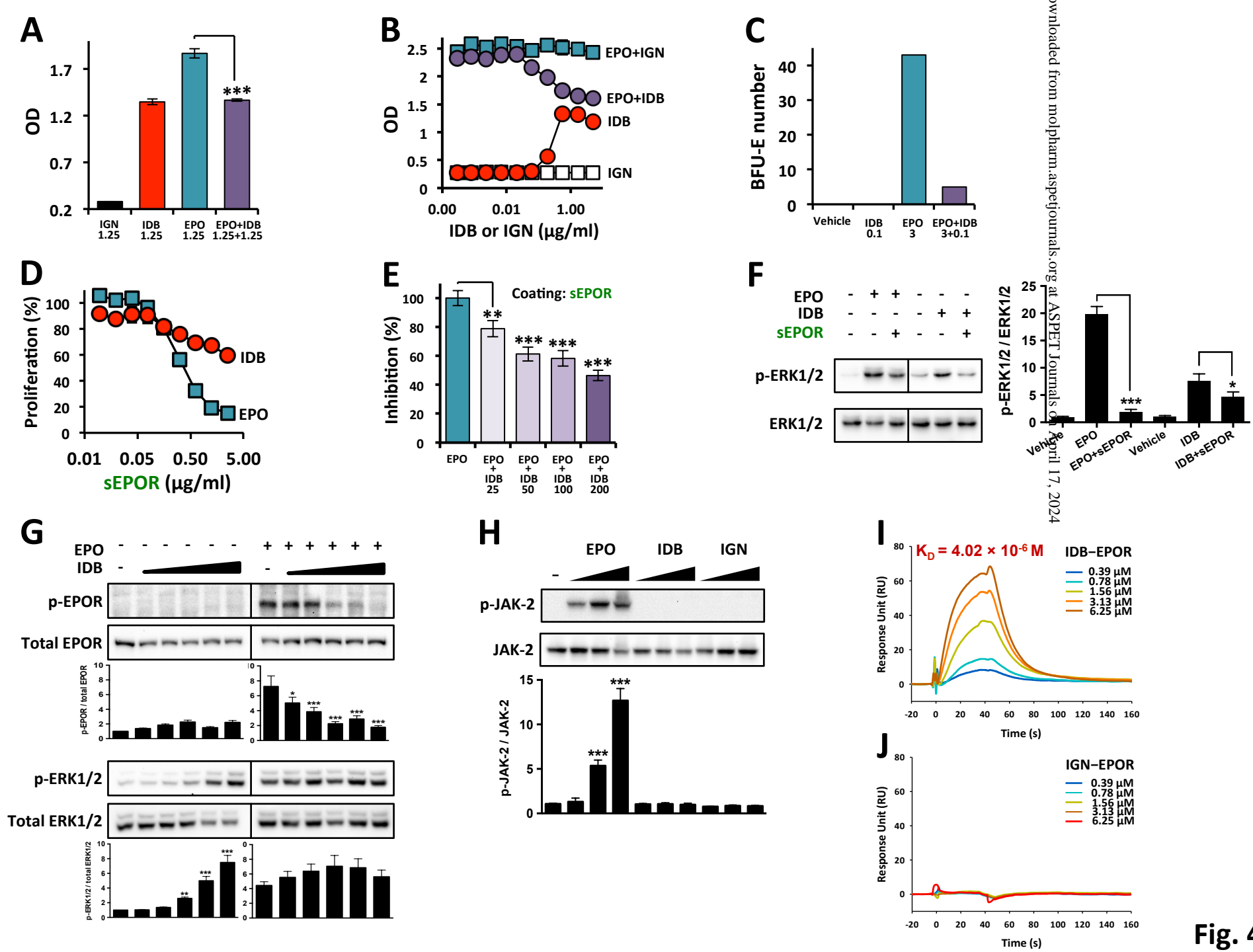
A

B

Ingenol 3,20-Dibenzoate
(IDB)

C

Ingenol (IGN)

D

E

Fig. 1

Fig. 2

Fig. 3

Fig. 4