22 มี.ค. 2543

เอกสารปกปิด
กรุณาส่งคืนศูนย์ฯ

รายงานการวิจัย พัฒนาและวิศวกรรม ฉบับสมบูรณ์

โครงการการคัดหาสารแก้การอักเสบจากสารสกัดจากธรรมชาติโดยใช้ dermal fibroblasts ของคน และเซลล์ของหนูที่ไม่มี cyclooxygenase-1 หรือ –2

Screening for anti-inflammatory compounds from natural extracts using human dermal fibroblasts and murine cyclooxygenase-1 or –2 null cells

รหัสโครงการ BRT641007

โดย กัญญวิมว์ กีรติกร

ได้รับทุนสนับสนุนจากโครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย
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(1 มกราคม – 31 ธันวาคม 2542)

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Abstract

Nonsteroidal antiinflammatory drugs (NSAIDs) reduce inflammation, pain, and fever by decreasing prostaglandin biosynthesis via the inhibition of prostaglandin G/H synthase (PGHS). Two isozymes of PGHS have been reported, PGHS-1 and PGHS-2. Recently developed NSAIDs that are more selective for PGHS-2 maintain their antiinflammatory properties but exhibit fewer unfavorable gastrointestinal side effects. Here, we report on a whole cell assay system for testing the efficacy of PGHS isozyme-specific inhibitors using murine PGHS-1 or PGHS-2 null cell lines. This system, using cells that express either PGHS-1 or PGHS-2, offers a convenient and reliable method to determine IC_{50} and IC_{80} values of the two PGHS isoforms independent of each another in the same cell type. To evaluate the usefulness of the PGHS null cell system we tested three widely used NSAIDs, aspirin, ibuprofen and indomethacin, using both external arachidonic acid and endogenous, calcium ionophore A23187-elicited arachidonic acid in these cells.

This assay system was later used to screen Thai medicinal plants, fungal growth extracts, and pure chemical compounds. Two plant samples showed a preferential inhibition of PGHS-2 over PGHS-1. These are methanol fractions of *Zingiber offcinale* and *Artemisia scoparia*.

บทคัดย่อ

ยาแก้ปวดประเภทที่ไม่มีสารสเตียรอยด์หรือที่เรียกว่ายาประเภท NSAIDs (nonsteroidal anti-inflammatory drugs) บรรเทาอาการปวด บวม และอาการไข้โดยยับยั้งการทำงานของเอนไซม์ prostaglandin G/H synthase (PGHS, cyclooxygenase) ซึ่งนำไปสู่การสร้างพรอสตาแกลนดินให้น้อยลง ในเซลล์มี PGHS อยู่ 2 isoforms คือ PGHS-1 และ PGHS-2 ยาประเภท NSAIDs ที่สามารถยับยั้ง PGHS-2 มากกว่า PGHS-1 จะให้ผลข้างเคียง เช่น การเกิดแผลในกระเพาะอาหาร น้อยกว่ายาที่ยับยั้งทั้ง 2 isoforms

ในการคัดหาสารบรรเทาอาการปวดที่มีผลข้างเคียงน้อยที่สุด เราได้เสนอวิธีการใช้ cell line 2 ชนิดจากหนูที่ได้รับการเปลี่ยนแปลงทางพันธุกรรมให้ไม่มี PGHS-1 หรือ PGHS-2 การใช้ cell line จากหนูในการทดสอบสารที่มีคุณสมบัติยับยั้ง PGHS-2 มากกว่า PGHS-1 นี้ เป็นการเสนอระบบทดสอบที่มาจากเซลล์ชนิดเดียวกันซึ่งปราศจาก PGHS isoform ที่ไม่ต้องการเป็นครั้งแรก จาก cell line 2 ชนิดนี้เราสามารถหาค่า IC_{50} และ IC_{80} ของสารแต่ละชนิดได้ เมื่อเปรียบเทียบเทียบค่า IC_{50} และ IC_{50} ratio ของ PGHS-2/PGHS-1 ของยาแก้ปวด 3 ชนิด (แอสไพริน ไอบิวโพรฟีน และ อินโดเมทาซิน) กับข้อมูลทางคลีนิค พบว่าสามารถใช้ค่า IC_{50} ratio ทำนายความแรงของผลข้างเคียงได้ โดยเฉพาะเมื่อเซลล์ใช้ arachidonic acid จากแหล่งภายในเซลล์

เมื่อใช้วิธีทดสอบนี้ในการคัดหาสารบรรเทาอาการปวดจากสารสกัดจากธรรมชาติทั้งจากพืช และเชื้อรา เราพบว่า ส่วนสกัด methanol จาก *Zingiber offcinale* และ *Artemisia scoparia* มีคุณสมบัติในการเลือกยับยั้ง PGHS-2 มากกว่า PGHS-1

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Summary of the first 6 months of the project (1 January - 30 June 1999)

1) Fibroblasts primary cell lines were established routinely from the foreskins supplied by the hospital. These cell lines were used in developing the standard assay of prostaglandin E_2 (PGE_2) determination.

2) The radioimmuno assay (RIA) has been established as previously reported and further scaled down by 50% in volume to reduce cost. Comparison has been made between results using the original volume and the scaled down volume to demonstrate that there is no difference in sensitivity of PGE_2 detection level.

3) *PGHS* null cells, $PGHS\text{-}1^{-/-}$ and $PGHS\text{-}2^{-/-}$, were grown in the lab and their characteristics in PGE_2 production were confirmed with interleukin-1 (IL-1) induction. $PGHS\text{-}1^{-/-}$ cells generate more IL-1 induced PGE_2 than in $PGHS\text{-}2^{-/-}$ cells.

4) Aspirin was tested for its ability to inhibit PGE_2 production in both human fibroblast and mouse cells.

5) Fifty three plant samples were screened for ability to inhibit PGE_2 production in IL-1 induced human fibroblast cells and their IC_{50} values were determined for 11 samples.

Introduction

Prostaglandins act as mediators of inflammation, fever, and pain during disease. However, they also play a significant role in maintaining cellular homeostasis such as controlling the mucous secretion in the stomach and balancing water resorption in the kidney (1-3). The production of prostaglandins is regulated by the conversion of membrane phospholipid to arachidonic acid, by phospholipase enzymes, and the successive conversion of arachidonic acid to prostaglandin H_2 (PGH_2), by prostaglandin H synthase/cyclooxygenase (PGHS-1 and PGHS-2) (4). Subsequently, PGH_2 can be converted to a number of different prostaglandins by the non-rate limiting actions of isomerases expressed by different types of cells. PGHS-1 and PGHS-2 are encoded by two different genes and each exhibits a different pattern of expression (5). PGHS-1 is constitutively expressed in most types of cells and is associated with the maintenance of cellular homeostasis (6). On the other hand, PGHS-2 is highly inducible by many stimuli and believed to be responsible for the increased prostaglandin levels associated with inflammation (2, 4, 7). PGHS is the primary target of a group of anti-inflammatory drugs with diverse structures, nonsteroidal anti-inflammatory drugs (NSAIDs), which include aspirin (8, 9). It is widely recognized that the untoward side effects of most NSAIDs such as stomach ulcers and kidney failure result from the indiscriminate inhibition of both PGHS-1 and PGHS-2 by NSAIDs (10, 11).

Since the discovery of PGHS-2 and its direct association with inflammation and pain, several new compounds have been developed in an effort to selectively inhibit PGHS-2 activity without affecting PGHS-1 activity. Many methods for testing PGHS-1 and PGHS-2 specific inhibitors were developed using pure enzymes, cell fractions or whole cell assays (12-17). In cell fraction or whole cell systems, different types of cells(platelets for PGHS-1 and synovial cells or mononuclear cells for -2) were used as sources of PGHS-1 and -2 (15, 18) making direct comparisons difficult. In some whole cell systems, PGHS-1 or PGHS-2 gene transfectants have been produced in order to study

each isozyme independently (19, 20) in an attempt to make comparisons of the IC_{50} values for each potential inhibitor more meaningful.

Here we examine the pharmacological profiles of NSAIDs using the whole cell assay system employing murine fibroblast cell lines derived from lung tissues from PGHS-1 or PGHS-2 deficient mice. These immortalized, PGHS-null cells have been characterized previously and shown to be devoid of the alternate PGHS isozyme so that each enzyme can be studied independently (21). Perhaps more importantly, these cells constitutively overexpress PGHS-1 or PGHS-2, respectively thus eliminating the need for treatment of PGHS-2 expressing cells with agonists to induce PGHS-2 expression that could potentially affect enyzme/inhibitor interactions (21). These characteristics make this PGHS-null cell system particularly convenient and useful for the direct comparison of PGHS selective inhibitors in the same cell type, without the need for the induction of PGHS-2. To demonstrate the usefulness of this cell system we simply compared the pharmacological profiles of NSAIDs in PGHS-null cells utilizing either external or internal arachidonic acid as sources of substrate. The PGHS-2/PGHS-1 IC_{50} and IC_{80} ratios for each NSAID tested were then determined and compared with previously published data.

In addition, we have employed this assay system to test 1) Thai medicinal plants used traditionally as antipyretic and anti-inflammatory agents, 2) a representative of fungi collected in Thailand, and 3) pure chemical compounds isolated by the Bioresource Unit of BIOTEC. We identified two plants with potential for preferential inhibition of PGHS-2 over PGHS-1.

Materials and methods

Materials

All tissue culture components were purchased from Gibco BRL (Gaithersburg, MD). Aspirin, ibuprofen, indomethacin, calcium ionophore A23187 and arachidonic acid were purchased from

Sigma (St. Louis, MO). ^{3}H-PGE_2 was from NEN Life Science (Boston, MA) and anti-PGE_2 antibody was from Upstate Biotechnology (Upstate, NY).

Extraction of plant materials

Plant materials were prepared by the Bioresource Unit at BIOTEC-Yothi or the Plant cell Culture Laboratory. They were first air dried and ground with mortar and pestle. The powder were then sequentially extracted with hexane and methanol. The hexane and methanol fractions were evaporated. The dried extracts were weighed and dissolved in DMSO at a concentration of 10 mg/ml.

Preparation of fungus extract

Fungi were grown in different medium in either static or shaking condition in the Fermentation Laboratory at BIOTEC-Yothi. Both fungus and growth medium were lyophilized and extracted with dichloromethane and ethanol (1:1). The dried powder was weighted and dissolved in DMSO at a concentration of 10 mg/ml.

Cell culture and treatment

Immortalized mouse *PGHS-1* null cells (*PGHS-1*$^{-/-}$) and *PGHS-2* null cells (*PGHS-2*$^{-/-}$) were seeded at 1×10^5 cells/ml in complete Dubelcco's Modified Eagle Medium (DMEM) supplemented with PenStrep at a concentration of 100,000 U/liter penicillin G and 100 mg/liter streptomycin sulfate, non essential amino acids (0.1 mM), glutamine (292 mg/liter), ascorbic acid (50 mg/liter), and 10% FCS in 96-well (83 µl/well) flat-bottomed tissue culture plates. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 72 hours. Cells were then washed with DMEM medium without FCS and preincubated for 30 minutes with 83 µl serum-free DMEM medium containing vehicle or drug. Aspirin, indomethacin and ibuprofen were dissolved and serially diluted in ethanol before they were added to the medium. Final ethanol concentration was 1%. Following a preincubation period, the medium was removed and cells were immediately treated with serum-free medium containing

vehicle or drug and 20 μM arachidonic acid or 2 μM A23187 for 30 minutes. Medium samples were then collected from wells and analyzed for PGE_2 concentration as previously described (21) except that the reaction volume was reduced to one forth of the original. Inhibition was calculated as percent PGHS activity of drug treated cells relative to vehicle treated cells. IC_{50}s for each NSAID were determined using SOFTmax software (Molecular Devices, Sunnyvale, CA).

Screening of plant/fungal extracts and pure compounds for PGHS-2 selective inhibitors

Immortalized mouse *PGHS-1* null cells (*PGHS-1*$^{-/-}$) were seeded as above. Samples were serially diluted in 10% DMSO and the final DMSO concentration in medium was 0.1%. Samples were preincubated with cells for 30 minutes and aspirin was included in separate wells as a positive control. Following a preincubation period, the medium was removed and cells were immediately treated with serum-free medium containing vehicle or samples and 2 μM A23187 for 30 minutes. Medium samples were then collected from wells and analyzed for PGE_2 concentration. Samples that inhibit PGE_2 synthesis at 10^{-5} g/ml were further tested for the ability to inhibit PGE_2 synthesis in PGHS-2 null cells (*PGHS-1*$^{-/-}$). PGHS-2/PGHS-1 IC_{50} ratios were determined for samples that inhibit PGHS-2 more than PGHS-1.

Results

PGE_2 production in PGHS-null cells from external and internal sources of arachidonic acid

We first examined the activity of PGHS-1 or PGHS-2 in each respective cell type in the presence of exogenously added arachidonic acid (20 μM) during a 30 minute incubation. Under these conditions we found that PGHS-1 activity, as represented by the level of PGE_2 produced by PGHS-2$^{-/-}$ cells, was 3 to 4-fold higher than the activity of PGHS-2 in PGHS-1$^{-/-}$ cells (Table 1). Alternatively, when cells were treated with the calcium ionophore A23187 in order to mobilize arachidonic acid from the intracellular sources, PGHS-1 and PGHS-2 activities were essentially

equivalent (Table 1). These data suggest that PGHS-1 is able to utilize exogenous AA better than PGHS-2 and that both enzymes use endogenous substrate equally well.

Inhibition of PGHS-1 and PGHS-2 enzyme activity by NSAIDs

Next, we tested PGHS-null cell lines in an inhibition study of three clinically employed NSAIDs; aspirin, ibuprofen and indomethacin. Cells were preincubated for 30 minutes with drug or vehicle to allow for "slow-binding" PGHS inhibitors to interact before changing to fresh media containing 20 μM arachidonic acid or 2 μM A23187. Cells were incubated for an additional 30 minutes after which time PGE_2 levels in the media were analyzed by RIA. Aspirin concentration response curves using either exogenous AA or A23187-mobilized endogenous AA as sources of substrate are shown in Fig. 1. Using exogenous AA, aspirin was a stronger inhibitor of PGHS-1 than PGHS-2. However, aspirin was almost equally effective against both PGHS-1 and PGHS-2 when cells used endogenous AA as indicated by the nearly identical response curves for PGHS-1 and PGHS-2 (Fig. 1B). When the IC_{50} values of PGHS-1 and PGHS-2 were determined in the presence of exogenously added AA, aspirin preferentially inhibited PGHS-1 (4-fold) more than PGHS-2. The IC_{50} ratio of PGHS-2/PGHS-1 was 1.4 in cells utilizing endogenous (A23187-derived) AA as substrate (Table 2). We also found that the IC_{50} values for each isozyme, either using external or internal sources of AA, were in a similar concentration range.

Ibuprofen concentration response curves are shown in Fig. 2. In the presence of exogenous AA, ibuprofen was a more potent inhibitor of PGHS-2 than PGHS-1 with an IC_{50} value for PGHS-2 18 times lower than the PGHS-1 IC_{50} value. However, with A23187-derived endogenous AA, there was a slight shift of the PGHS-1 curve resulting in a decrease in the PGHS-1 IC_{50} value. Consequently, there was no difference between PGHS-1 and PGHS-2 IC_{50} values when using endogenous AA. The IC_{50} values of each isozyme, using either external or internal sources of AA, fall within the concentration ranges of 10^{-7}-10^{-6} M (Table 2).

In the presence of exogenous AA, the indomethacin response curves showed a preferential inhibition of PGHS-1 over PGHS-2 (Fig. 3); the difference in IC_{50} values was 2.5 fold (Table 2). Similar to the effect seen in the experiment with ibuprofen, when A23187-mobilized endogenous AA was used as substrate, there was a decrease in the IC_{50} value of PGHS-1. In addition, this decrease was also accompanied by a greater reduction in the IC_{50} value of PGHS-2 resulting in a PGHS-2/PGHS-1 ratio of approximately 1.6. The IC_{50} values of each isozyme, either using external or internal sources of AA, fall within the concentration ranges of 10^{-9}-10^{-8}M.

When IC_{80} values for each NSAID were determined, the values obtained from experiments using exogenously added AA were very similar to their IC_{50} ratios (Table 2). In the experiments using A23187 to mobilize endogenous AA the IC_{80} ratios of aspirin and ibuprofen were similar to their respective IC_{50} ratios while the IC_{80} ratios of indomethacin increased from 1.6 to 19.2.

PGHS-2/PGHS-1 IC_{50} and IC_{80} ratio ranking

The three NSAIDs tested are widely used as anti-inflammatory, antipyretic, and analgesic drugs. The potency of PGHS-2 selective inhibition is determined by their ability to preferentially inhibit PGHS-2 over PGHS-1. Based on the PGHS-2/PGHS-1 IC_{50} ratios, each drug is ranked from more selective (low PGHS-2/PGHS-1 ratio) to less selective (high PGHS-2/PGHS-1 ratio) for PGHS-2 (Table 2). In the presence of exogenously added AA, ibuprofen ranked the highest for its selective inhibition for PGHS-2 followed by indomethacin and then aspirin. When cells utilized A23187-derived endogenous AA, ibuprofen still ranked the highest for its selectivity with respect to PGHS-2 but aspirin ranked higher than indomethacin. Since IC_{50} and IC_{80} ratios of the three NSAIDs with external arachidonic acid did not change significantly, there was no change in the order of PGHS-2 selectivity ranking based on IC80 values. Although the indomethacin IC_{80} value increased 12-fold in cells utilizing A23187 mobilized AA, the PGHS-2 selectivity ranking remained the same as those based on the IC_{50} values.

Screening of Thai plants for PGHS-2 selective inhibition

We selected a number of plants based on their traditional use in Thai medicinal protocols to test with our murine cell lines (Table 3). Plants were first tested for their ability to inhibit PGE_2 synthesis in PGHS-1 null cells which contains only PGHS-2. Each sample was assayed at two different concentrations at 10^{-7} and 10^{-5} g/ml. Aspirin was used as a standard. At 10^{-7} g/ml and 10^{-5} g/ml, aspirin inhibits PGE_2 production of PGHS-2 80% and 60%, respectively. To classify a plant sample as active, the sample must inhibit at least 50% PGE_2 synthesis at 10^{-5} g/ml concentration. Relying on this criterion, two out of 184 plant samples are considered active (Table 2). After further test in PGHS-2 null cells which contain only PGHS-1 to determined the IC_{50} ratios of PGHS-2/PGHS-1, data showed that these two samples preferentially inhibit PGHS-2 over PGHS-1. Cytotoxicity test was conducted on Vero cells (ATCC) according to the method. At the concentration of $20x10^{-6}$ g/ml *Zingiber officinale* methanol fraction is not toxic to Vero cells while *Artemisia scoparia* methanol fraction at $2x10^{-6}$ g/ml is toxic to Vero cells (Table 3).

Screening of Thai fungi for PGHS-2 specific inhibition

We selected a group of fungi representing different species of fungi collected in Thailand (Table 4). The extracts were tested on *PGHS-1*$^{-/-}$ cells for PGE_2 inhibition. From thirty samples tested, none could reduce PGE_2 synthesis at 10^{-5} g/ml.

Testing pure chemical compounds for PGHS-2 specific inhibition

Chemical compounds (AP-A, CP-B, CP-A, SAEW05, SAEW06, SAEW07, and SAEW10) were tested for the ability to inhibit PGE_2 synthesis in *PGHS-1*$^{-/-}$ cells. SAEW05 and SAEW10 showed weak PGE_2 inhibiting effect and were subsequently tested in *PGHS-2*$^{-/-}$ cells. SAEW05 exhibited PGHS-2 IC_{50} value of $1x10^{-5}$ g/ml and PGHS-1 IC_{50} value $>1x10^{-5}$ g/ml.

Discussion

Using mouse PGHS null cells for primary screening instead of the human dermal fibroblasts

We originally proposed to use primary culture of human dermal fibroblasts in screening the anti-inflammatory compounds from plant and fungal extracts. In this procedure, cells of human dermal fibroblasts induced with IL-1 to express higher levels of PGHS-2 will be incubated with the crude extracts for 24 hours and subsequently PGE_2 concentration in the medium will be determined. Extracts that contain anti-inflammatory compounds will decrease the amount of newly biosynthesized PGE_2 level when compared with the control without extracts. After testing a number of samples with this method, we recognized several problems resulting from exposing cells to foreign substances for 24 hours. First, crude extracts might have a global effects on cells resulting in an increase or decrease of cell metabolism which will affect the level of PGE_2 production. Thus, a decrease in prostaglandin biosynthesis might not represent a specific PGHS inhibition but rather a decrease in cell metabolism . Second, crude extracts might specifically induce PGHS-1 or PGHS-2 protein synthesis resulting in an increase in PGE_2 production. Third, crude extracts might affect the availability of PGHS substrate, arachidonic acid, by interfering with the production or the activity of phospholipase A_2 enzyme. To overcome these problems that might eventually lead to an inaccuracy of screening data, we have devised a new screening protocol using PGHS-1 null cells in the presence of excess arachidonic acid. Providing cells with excess arachidonic acid, either externally or internally via A23187, ensure that the amount of PGE_2 secreted into the medium exclusively represent the activity of PGHS-2. Therefore, any extract that reduces PGE_2 production from PGHS-1 null cells actually does so by inhibiting PGHS-2 activity.

Scaling down of the assay for measuring PGE_2 levels

As stated in the first progress reported submitted in July 1999, we have scaled down the RIA reaction to measure PGE_2 production to compose of 50 μl of sample, 50 μl of anti-PGE_2, and 50

μl of ^{3}H-PGE$_2$. However, we have found that further scaling down of the assay to 25 μl of each component yielded similar results and reduced the expense on each PGE$_2$ test by an additional 50%.

Test system using endogenous arachidonic acid versus exogenous arachidonic acid

In a previous study we characterized two cell lines lacking either PGHS-1 or PGHS-2 (21). In this study, we have shown that these cell lines provide a convenient and reliable whole cell assay system useful for testing compounds to determine their isozyme selectivity based on IC$_{50}$ and IC$_{80}$ values of PGHS-1 and PGHS-2. These cell lines have been thoroughly studied and were shown to express inherently higher levels of both PGHS-1 or PGHS-2 than levels expressed in control (wild-type) cells. Therefore, the levels of PGE$_2$ can be measured easily without the need of any treatment to induce PGHS-2 expression/activity. As reported previously, these cell lines are also very responsive to agonists including interleukin-1, tumor necrosis factor α, fibroblast growth factor, and phorbol esters (PMA). In an effort to distinguish between the preferred sources of AA utilized by PGHS-1 and PGHS-2, cells were provided with external arachidonic acid or treated with the calcium ionophore A23187 to mobilized AA from endogenous sources. The calcium ionophore A23187 stimulated the release of endogenous arachidonic acid from various internal lipid pools by increasing PLA$_2$ activity (22). Our results showed that PGHS-1 can use both external and internal AA, but exhibited significantly higher activity with external AA. PGHS-2 only exhibited a slightly higher activity with external AA at basal level. In addition, our data showed that IC$_{50}$ values for each NSAID measured when in the presence of exogenous AA were larger than those from cell utilizing endogenous substrate (A23187-derived) for both PGHS-1 and -2 (Table 2). It has been suggested earlier that PGHS-1 and PGHS-2 might preferrentially utilize different sources of AA (22). In our previous study, we suggested that AA was likely to be limiting in PGHS-2$^{-/-}$ cells but when cells were stimulated with phorbol ester, which increases the availability of endogenous AA, both PGHS-1$^{-/-}$ and PGHS-2$^{-/-}$ cells can use AAs from either exogenous or endogenous sources. Our present study supports the

previous data and confirmed that A23187 derived endogenous AA can be used as effectively as endogenous AA provided by a different stimulus.

In order to verify the potential value of the PGHS-null cell lines as a test system for screening PGHS-2 selective inhibitors, we used them to determine the IC_{50}s for three widely used NSAIDs and then compared both their IC_{50} and IC_{80} ratios of PGHS-2/PGHS-1 with previous reports. The PGHS-null cell system offers the advantage of being able to compare effects of drugs on PGHS isozymes in intact cells of the same lineage rather than comparing data gathered from among purified enzyme assays, cell-free extracts, cell fractions or different cell types. Of course, variables such as length of incubation with NSAIDs, sources and concentrations of substrate, type of drug vehicle used, and preincubation times with drugs also contributes to discrepancies in PGHS-2/PGHS-1 IC_{50} ratios among individual reports. As an example, aspirin PGHS-2/PGHS-1 IC_{50} ratios vary from 166 to 3.8 in different reports while those of indomethacin vary from 60 to 0.25 in others (18, 19, 23, 24). Therefore, it seems that the validity of the comparison may only be as good as the PGHS 'system' in which the drugs are compared. Clearly, the relative efficacy of a particular drug using the same system can be compared directly and ranked accordingly to help predict the effects of drugs in patients based on the assumption that the more selective the PGHS-2 inhibitor the less side effects it should generate.

In our PGHS-null, whole cell system, with exogenous AA added, indomethacin is the most potent PGHS-1 and PGHS-2 inhibitor among the three drugs, with IC_{50} values in the range of 10^{-9}-10^{-8} M whereas aspirin is the least potent inhibitor with the IC_{50} values in the range of 10^{-5} M. Ibuprofen is the most selective for PGHS-2, while indomethacin is less selective, and aspirin is the least selective in the presence of exogenous AA. This ranking of the IC_{50} ratio correlates well with the studies reported by Cryer and Feldman (25) using *ex vivo* whole blood assay and by Vane and Botting (23) also using a whole cell system. The study by Chulada and Langenbach (19) also indicated that with

exogenous AA, indomethacin is more selective to PGHS-2 than aspirin. In addition, Meade et al. (17) reported that ibuprofen is more selective to PGHS-2 than indomethacin. Thus, the findings obtained using our system yielded similar results to earlier studies on PGHS-2 selectivity in various test systems: ibuprofen > indomethacin >aspirin.

Our results from the experiments with A23187-derived endogenous AA showed that indomethacin is the most potent inhibitor for both PGHS-1 and PGHS-2 with the IC_{50} values in the range of 10^{-9} M while aspirin is the least potent inhibitor with the IC_{50} ratio of approximately 10^{-5} M. PGHS-2/PGHS-1 IC_{50} and IC_{80} ratios ranked ibuprofen as a better PGHS-2 selective inhibitor than aspirin, and indomethacin as the least selective. The results indicating aspirin is more selective than indomethacin did not agree with the data obtained from cells incubated with exogenous AA. However, we are not the first to report a discrepancy in selectivity ranking when cells are supplied with different sources of AA. It was shown previously that indomethacin is more selective for PGHS-2 than aspirin when cells were provided with external AA and the opposite results were observed when cells used internal AA pools (19). When compared with previous data in assay systems using A23187 derived AA (24), our ranking of PGHS-2 selectivity obtained from endogenous AA agreed with the ranking from that report. This ranking is also in agreement with the studies of NSAID-induced GI toxicity in humans showing that ibuprofen generated fewer side effects than either indomethacin or aspirin (26) and indicated that aspirin induced less toxicity than indomethacin (27). In order to efficiently predict the potential side effects of any NSAIDs in patients, it appears that A23187-derived AA should be the preferred source of substrate since in our test system, the data obtained with A23187 treated cells correlates best with clinical studies. Interestingly, none of the data from any of our experiments shows any correlation with the PGHS-2/PGHS-1 ratios obtained from assay systems employing broken cell extracts or purified enzymes (23).

It has been suggested that comparing IC_{80} and PGHS-2/PGHS-1 IC_{80} ratios of each NSAID is a better representation of the relevant physiological concentrations of NSAIDs in plasma (24). When the ratios of PGHS-2 IC_{80}/PGHS-1 IC_{80} of each NSAID were evaluated, there were no differences in the rankings based on the IC_{50} ratios. This similarity was also shown for the same three NSAIDs in an earlier report (24).

Other limitations in using different types of cells as sources of PGHS-1 and PGHS-2 enzymes could include differential abilities of potential inhibitors to enter the cells. Moreover, different types of cells might utilize different pathways in eliminating or sequestering foreign compounds, and when cells with transfected PGHS-1 or PGHS-2 are used, the possibility that alternate PGHS isozymes are present cannot be completely ignored. Discrepancies such as these could easily account for the variability in calculating IC_{50} values using different assay systems. Although results obtained from pure enzyme screening is fast, it may not represent the real physiological conditions under which drug molecules interact with the target enyzme in the cytosolic milieu.

In summary, we have presented a comparison of pharmacological profiles for three different widely used NSAIDs utilizing a PGHS-null, whole cell assay system. From these studies we conclude that with A23187-derived AA, the inhibition profiles we obtained are very similar to the previously reported data from other whole cell assay systems. However, our whole cell assay system offers the following advantages: 1) cells used as sources of PGHS-1 and PGHS-2 are the same type of cell (lung fibroblasts) and each completely lacks the alternate form of PGHS allowing for direct comparison of PGHS-1 and -2 activity in cells exhibiting similar physiological properties, 2) PGE2 is by far the predominant eicosanoid produced by these two cell lines so that only one type of eicosanoid measuring system is required, and 3) each respective PGHS isozyme is expressed at high levels eliminating the need to stimulate cells in order to induce the expression of PGHS-2.

Screening of plant extracts

We have tested total of 184 plant samples. Plant list was first generated from known medicinal properties of antipyretic, anti-inflammation, and analgesic according to Thai traditional usage. Some of these can be grouped into 18 families. Samples were first tested in *PGHS-1*$^{-/-}$ cells for an inhibition of PGHS-2 at the concentration of 10^{-5} and 10^{-7} g/ml. Positive samples, those that show inhibition of PGE2 synthesis at 10^{-5} g/ml, were selected for further testing in *PGHS-2*$^{-/-}$ cells. Samples that showed selectivity for PGHS-2 over PGHS-1 were additionally subjected to IC_{50} determination. Two plant samples, *Zingiber officinale* and *Artemisia scoparia*, exhibited lower IC_{50} values of PGHS-2 than PGHS-1 which specifies the preferential selectivity for PGHS-2.

There are no reports on the use of *Artemisia scoparia* in treating pain, fever and inflammation. However, there are many reports on the use of *Zingiber officinale* to relieve inflammation and its associated symptoms in vivo (28-31). Some of pure compounds extracted from *Zingiber officinale* have already been shown to inhibit prostaglandin inhibition by inhibiting PGHS enzyme without discriminating between PGHS-1 and PGHS-2 (32).

Screening of fungal extracts

Fungal extracts were selected to represent various groups of fungi. From 30 samples we tested, non showed the ability to inhibit either PGHS-1 or PGHS-2. Due to many contaminants in the extracts, it is generally recommended that both cells and medium should be extracted with several solvents and each fraction tested separately.

Screening of pure compounds

We tested 7 pure compounds prepared by the Bioresource Unit. Although SAEW05 showed the potential to be a selective PGHS-2 inhibitor, we need to repeat the experiment to confirm the results of SAEW05 in both cell lines.

Conclusion

In summary, we have established a reliable method in determining selective PGHS-2 inhibitors. We presented a comparison of pharmacological profiles of three different widely used NSAIDs generated from our whole cell assay to verify the system. From our comparisons we conclude that with A23187-derived AA, the profile is closely similar to the previously reported data from other whole cell assay systems. However, our system has the following obvious advantages:

1) cells used as sources of PGHS-1 and PGHS-2 are the same type of cells (lung fibroblasts) and that each cell line is lacking the alternate form of PGHS. Direct comparison of PGHS-1 and -2 activity is done between cells of similar physiological properties.
2) PGE_2 is the dominant eicosanoid produced from these two cell lines. Therefore, only one type of eicosanoid measuring system is required.
3) The existing PGHS is expressed at high levels. Thus, the stimulation of the enzyme by LPS or cytokines is not needed.

When we used this assay system to test plant extracts, fungal extracts, and pure compounds we found that 1)*Zingiber officinale* and *Artemisia scoparia* methanol extracts showed preferential inhibition of PGHS-2 over PGHS-1, and 2) SAEW05 has a potential to preferentially inhibit PGHS-2 more than PGHS-1.

The rate limitting step of this method in screening many samples is the PGE_2 determination step. Currently we are working on improving the measurement of PGE_2 to a rapid throughput level.

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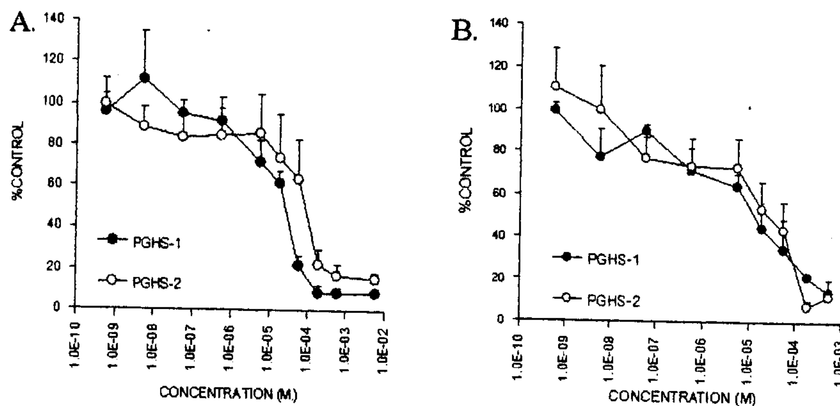

Fig. 1. Dose response curves for the inhibition of PGE_2 production by aspirin in mouse PGHS-1 or PGHS-2-null cell lines containing only PGHS-2 or PGHS-1, respectively. Cells were incubated with different concentrations of aspirin for 30 minutes before replacing with new medium containing aspirin and 20 μM AA (a) or 2 μM A23187 (b) and incubated for an additional 30 minutes. The PGE_2 concentration of the medium was then measured. Each point shows the mean percent control (± SD) of at least three different experiments with 2-3 replicates in each experiment.

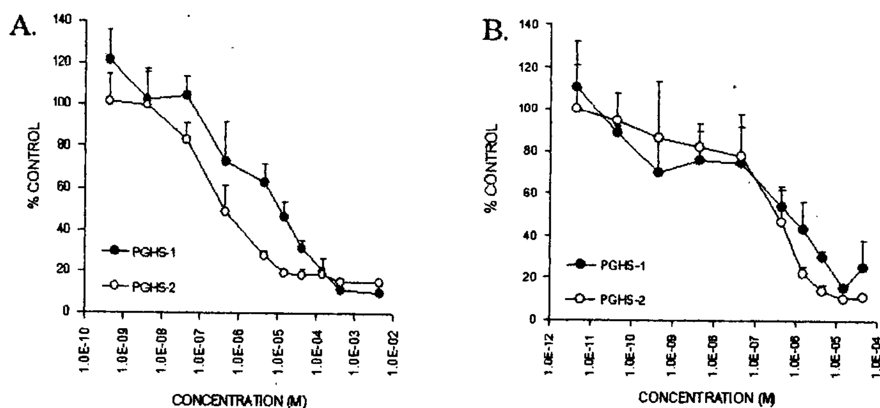

Fig. 2. Dose response curves for the inhibition of PGE_2 production by ibuprofen in mouse PGHS-1 or PGHS-2-null cell lines containing only PGHS-2 or PGHS-1, respectively. Cells were incubated with different concentrations of ibuprofen for 30 minutes before replacing with new medium containing ibuprofen and 20 μM AA (a) or 2 μM A23187 (b) and incubated for an additional 30 minutes. The PGE_2 concentration of the medium was then measured. Each point shows the mean percent control (± SD) of at least three different experiments with 2-3 replicates in each experiment.

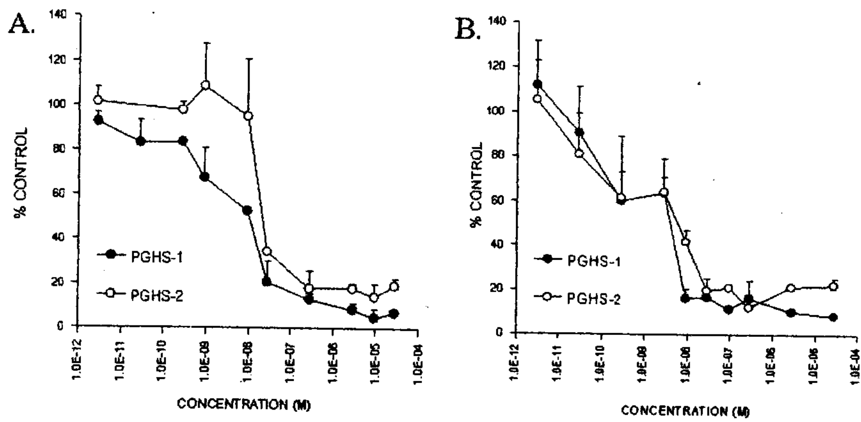

Fig. 3. Dose response curves for the inhibition of PGE_2 production by indomethacin in mouse PGHS-1 or PGHS-2-cell lines containing only PGHS-2 or PGHS-1, respectively. Cells were incubated with different concentrations of indomethacin for 30 minutes before replacing with new medium containing indomethacin and 20 μM AA (a) or 2 μM A23187 (b) and incubated for an additional 30 minutes. The PGE_2 concentration of the medium was then measured. Each point shows the mean percent control (± SD) of at least three different experiments with 2-3 replicates in each experiment.

Table 1

PGHS enzyme activity in PGHS-1 or PGHS-2-null cell lines using exogenous AA or A23187-derived endogenous AA.

Cell line	PGE_2 (pg/10^3 cells)	
	Exogenous AA	A23187-derived AA
PGHS-2$^{-/-}$	84.3±3.7	24.5±7.3
PGHS-1$^{-/-}$	32.8±8.6	29.3±7.8

Each value represents the mean ± SD of at least three different experiments with 2-3 replicates in each experiment.

Table 2

IC_{50} and IC_{80} values of NSAIDs for PGHS-1 or PGHS-2-null cell lines

	PGHS-1		PGHS-2		IC_{50}		IC_{80}	
	IC_{50} (M)	IC_{80} (M)	IC_{50} (M)	IC_{80} (M)	PGHS-2 /PGHS-1	rank	PGHS-2 /PGHS-1	rank
Exogenous AA								
Aspirin	2.02×10^{-5}	9.07×10^{-5}	7.48×10^{-5}	3.09×10^{-4}	3.7	3	3.4	3
Ibuprofen	7.68×10^{-6}	2.22×10^{-4}	4.31×10^{-7}	1.60×10^{-5}	0.06	1	0.07	1
Indomethacin	9.46×10^{-9}	3.31×10^{-8}	2.40×10^{-8}	4.20×10^{-8}	2.5	2	1.3	2
A23187								
Aspirin	1.33×10^{-5}	2.49×10^{-4}	1.84×10^{-5}	2.27×10^{-4}	1.4	2	0.9	2
Ibuprofen	3.40×10^{-7}	3.56×10^{-6}	2.90×10^{-7}	2.56×10^{-6}	0.9	1	0.7	1
Indomethacin	1.14×10^{-9}	4.16×10^{-8}	1.86×10^{-9}	7.98×10^{-7}	1.6	3	19.2	3

Enzyme activities were measured and IC_{50}s and IC_{80}s of each enzyme were determined. Rank of each NSAID was calculated from the ratio of IC_{50} of PGHS-2/IC_{50} of PGHS-1 and the ratio of IC_{80} of PGHS-2/IC_{80} of PGHS-1 to represent the selectivity for PGHS-2.

Table 3

Results of plant sample screening

	Sample code	Plant code	Name	Scientific name	Family name	Part	Solvent	Cytotoxicity (µg/ml)	PGHS-2 specific	PGHS-1 IC50 µg/ml	PGHS-2 IC50 µg/ml	PGHS2/PGHS-1 IC50 ratios
S001	S001	SW940001	กรรณิการ์	*Nyctanthes arbor-tristis* Linn.	*Verbenaceae*	stem	Hexane	> 50	No			
S002	S002	SW940001	กรรณิการ์	*Nyctanthes arbor-tristis* Linn.	*Verbenaceae*	stem	Methanol	> 20	No			
S003	S003	SW940001	กรรณิการ์	*Nyctanthes arbor-tristis* Linn.	*Verbenaceae*	leaf	Hexane	> 20	No			
S004	S004	SW940001	กรรณิการ์	*Nyctanthes arbor-tristis* Linn.	*Verbenaceae*	leaf	Methanol	> 20	No			
S013	S013	SW940005	แค	*Sesbania grandiflora* (L.) Pers.	*Leguminosae*	stem bark	Hexane	> 20	No			
S014	S014	SW940005	แค	*Sesbania grandiflora* (L.) Pers.	*Leguminosae*	stem bark	Methanol	> 20	No			
S043	S043	SW940019	มะตูม	*Aegle marmelos* (L.) Corr.	*Rutaceae*	leaf	Hexane	> 2	No			
S044	S044	SW940019	มะตูม	*Aegle marmelos* (L.) Corr.	*Rutaceae*	leaf	Methanol	> 50	No			
S045	S045	SW940019	มะตูม	*Aegle marmelos* (L.) Corr.	*Rutaceae*	stem	Hexane	> 20	No			
S046	S046	SW940019	มะตูม	*Aegle marmelos* (L.) Corr.	*Rutaceae*	stem	Methanol	> 50	No			
S047	S047	SW940020	หญ้าแฝกหอม	*Vetiveria zizanioides* (L.) Nash ex Small	*Graminae*	root	Hexane	> 2	No			
S048	S048	SW940020	หญ้าแฝกหอม	*Vetiveria zizanioides* (L.) Nash ex Small	*Graminae*	root	Methanol	> 20	No			
S049	S049	SW940021	เพกา	*Oroxylum indicum* (L.) Vent.	*Bignoniaceae*	stem bark	Hexane	> 20	No			
S050	S050	SW940021	เพกา	*Oroxylum indicum* (L.) Vent.	*Bignoniaceae*	stem bark	Methanol	> 50	No			
S054	S054	SW940023	กรุงเขมา	*Cissampelos pareira* Linn.	*Menispermaeae*	root	Methanol	> 20	No			
S055	S055	SW940024	มะเฟือง	*Averrhoa carambola* Linn.	*Oxalidaceae*	leaf	Hexane	> 20	No			
S056	S056	SW940024	มะเฟือง	*Averrhoa carambola* Linn.	*Oxalidaceae*	leaf	Methanol	> 50	No			
S057	S057	SW940024	มะเฟือง	*Averrhoa carambola* Linn.	*Oxalidaceae*	stem	Hexane	> 20	No			
S058	S058	SW940024	มะเฟือง	*Averrhoa carambola* Linn.	*Oxalidaceae*	stem	Methanol	> 20	No			
S061	S061	SW940026	ขมิ้นเครือ	*Arcangelisia flava* (L.) Merr.	*Menispermaeae*	stem	Hexane	> 20	No			
S062	S062	SW940026	ขมิ้นเครือ	*Arcangelisia flava* (L.) Merr.	*Menispermaeae*	stem	Methanol	> 50	No			
S065	S065	SW940028	ขิง	*Zingiber officinale* Roscoe	*Zingiberaceae*	tuber	Hexane	> 20	No			

S066	S066	SW940028	ขิง	*Zingiber officinale* Roscoe	*Zingiberaceae*	tuber	Methanol	> 20	YES	>20	6.009	<0.30
S067	S067	SW940029	ระงับพิษ	*Breynia gluca* Craib	*Euphorbiaceae*	leaf	Hexane	> 20	No			
S068	S068	SW940029	ระงับพิษ	*Breynia gluca* Craib	*Euphorbiaceae*	leaf	Methanol	> 20	No			
S069	S069	SW940029	ระงับพิษ	*Breynia gluca* Craib	*Euphorbiaceae*	stem	Hexane	> 20	No			
S070	S070	SW940029	ระงับพิษ	*Breynia gluca* Craib	*Euphorbiaceae*	stem	Methanol	> 50	No			
S079	S079	SW940032	พิมเสนต้น	*Pogostemon cablin* (Blanco) Benth.	*Labiatae*	leaf & stem	Hexane	> 20	No			
S080	S080	SW940032	พิมเสนต้น	*Pogostemon cablin* (Blanco) Benth.	*Labiatae*	leaf & stem	Methanol	> 20	No			
S081	S081	SW940033	น้ำนมราชสีห์	*Euphorbia hirta* L.	*Euphorbiaceae*	all	Hexane	> 20	No			
S082	S082	SW940033	น้ำนมราชสีห์	*Euphorbia hirta* L.	*Euphorbiaceae*	all	Methanol	> 20	No			
S088	S088	SW940036	กระวานเทศ	*Elettaria cardamomum* Mata	*Zingiberaceae*	seed	Methanol	> 20	No			
S089	S089	SW940037	เร่ว	*Amomum xanthioides* Wall.	*Zingiberaceae*	seed	Hexane	> 20	No			
S090	S090	SW940037	เร่ว	*Amomum xanthioides* Wall.	*Zingiberaceae*	seed	Methanol	> 50	No			
S103	S103	SW940034	คนสอ (คนทีสอ)	*Vitex trifolia* Linn.	*Verbenaceae*	leaf	Hexane	> 10	No			
S104	S104	SW940034	คนสอ (คนทีสอ)	*Vitex trifolia* Linn.	*Verbenaceae*	leaf	Methanol	> 20	No			
S107	S107	SW940046	โมกหลวง	*Holarrhena antidysenterica* Wall	*Apocynaceae*	stem bark	Hexane	> 10	No			
S108	S108	SW940046	โมกหลวง	*Holarrhena antidysenterica* Wall	*Apocynaceae*	stem bark	Methanol	> 20	No			
S109	S109	SW940047	ชุมเห็ดเทศ	*Cassia alata* Linn.	*Leguminosae*	leaf	Hexane	> 10	No			
S110	S110	SW940047	ชุมเห็ดเทศ	*Cassia alata* Linn.	*Leguminosae*	leaf	Methanol	> 20	No			
S111	S111	SW940047	ชุมเห็ดเทศ	*Cassia alata* Linn.	*Leguminosae*	stem	Hexane	> 20	No			
S112	S112	SW940047	ชุมเห็ดเทศ	*Cassia alata* Linn.	*Leguminosae*	stem	Methanol	> 20	No			
S115	S115	SW940049	เทียนเยาวพาณี	*Artemisia scoparia* Waldst&Kit	*Compositae*	seed	Hexane	> 50	No			
S116	S116	SW940049	เทียนเยาวพาณี	*Artemisia scoparia* Waldst&Kit	*Compositae*	seed	Methanol	> 2	YES	>20	7.53	<0.38
S117	S117	SW940050	พิลังกาสา	*Ardisia colorata*	*Myrsinaceae*	seed	Hexane	> 50	No			
S118	S118	SW940050	พิลังกาสา	*Ardisia colorata*	*Myrsinaceae*	seed	Methanol	> 50	No			
S119	S119	SW940051	พิษนาศน์	*Artemisia indica* Willd *var. heyneana* Pampan	*Compositae*		Hexane	> 50	No			
S120	S120	SW940051	พิษนาศน์	*Artemisia indica* Willd *var. heyneana* Pampan	*Compositae*		Methanol	> 20	No			
S121	S121	SW940052	โกฐจุฬาลำภา	*Artemisia pallens* Wall. ex Bess	*Compositae*	all	Hexane	> 20	No			
S122	S122	SW940052	โกฐจุฬาลำภา	*Artemisia pallens* Wall. ex Bess	*Compositae*	all	Methanol	> 20	No			

S125	S125	SW940054	ขมิ้นอ้อย	*Curcuma zedoaria* (Berg.) Rosc.	*Zingiberaceae*	tuber	Hexane	> 10	No			
S126	S126	SW940054	ขมิ้นอ้อย	*Curcuma zedoaria* (Berg.) Rosc.	*Zingiberaceae*	tuber	Methanol	> 10	No			
S127	S127	SW940055	ชุมเห็ดไทย	*Cassia tora* Linn.	*Leguminosae*	leaf	Hexane	> 20	No			
S128	S128	SW940055	ชุมเห็ดไทย	*Cassia tora* Linn.	*Leguminosae*	leaf	Methanol	> 50	No			
S129	S129	SW940055	ชุมเห็ดไทย	*Cassia tora* Linn.	*Leguminosae*	stem	Hexane	> 20	No			
S130	S130	SW940055	ชุมเห็ดไทย	*Cassia tora* Linn.	*Leguminosae*	stem	Methanol	> 50	No			
S131	S131	SW940056	มะเดื่ออุทุมพร	*Ficus racemosa* Linn.	*Moraceae*	stem	Hexane	> 50	No			
S132	S132	SW940056	มะเดื่ออุทุมพร	*Ficus racemosa* Linn.	*Moraceae*	stem	Methanol	> 50	No			
S133	S133	SW940057	พญาสัตบรรณ	*Alstonia scholaris* R.Br.	*Apocynaceae*	stem	Hexane	> 50	No			
S134	S134	SW940057	พญาสัตบรรณ	*Alstonia scholaris* R.Br.	*Apocynaceae*	stem	Methanol	> 50	No			
S135	S135	SW940057	พญาสัตบรรณ	*Alstonia scholaris* R.Br.	*Apocynaceae*	stem bark	Hexane	> 20	No			
S136	S136	SW940057	พญาสัตบรรณ	*Alstonia scholaris* R.Br.	*Apocynaceae*	stem bark	Methanol	> 50	No			
S139	S139	SW940059	ฟ้าด้น (ฟ้าทลายโจร)	*Andrographis paniculata* Nees	*Acanthaceae*	all	Hexane	> 20	No			
S140	S140	SW940059	ฟ้าด้น (ฟ้าทลายโจร)	*Andrographis paniculata* Nees	*Acanthaceae*	all	Methanol	> 20	No			
S143	S143	SW940061	ผักโขม	*Amaranthus gracilis* Desf.	*Amaranthaceae*	all	Hexane	> 50	No			
S144	S144	SW940061	ผักโขม	*Amaranthus gracilis* Desf.	*Amaranthaceae*	all	Methanol	> 50	No			
S151	S151	SW940064	พญายา	*Hesperethusa crenulata* Roem		stem	Hexane	> 20	No			
S152	S152	SW940064	พญายา	*Hesperethusa crenulata* Roem		stem	Methanol	> 20	No			
S153	S153	SW940065	ชิงช้าชาลี	*Tinospora cordifolia* Miers	*Menispermaeae*	เถา	Hexane	> 20	No			
S154	S154	SW940065	ชิงช้าชาลี	*Tinospora cordifolia* Miers	*Menispermaeae*	เถา	Methanol	> 50	No			
S161	S161	SW940069	ขันทองพยาบาท	*Suregada multiflorum* Baill.	*Euphorbiaceae*	stem only	Hexane	> 50	No			
S162	S162	SW940069	ขันทองพยาบาท	*Suregada multiflorum* Baill.	*Euphorbiaceae*	stem only	Methanol	> 50	No			
S165	S165	SW940071	คำฝอย	*Carthamus tinctorius* Linn.	*Compositae*	flower	Hexane	> 50	No			
S166	S166	SW940071	คำฝอย	*Carthamus tinctorius* Linn.	*Compositae*	flower	Methanol	> 50	No			
S173	S173	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	leaf	Hexane	> 10	No			
S174	S174	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	leaf	Methanol	> 50	No			
S175	S175	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	stem only	Hexane	> 20	No			
S176	S176	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	stem only	Methanol	> 50	No			

S177	S177	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	stem bark	Hexane	> 20	No			
S178	S178	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	stem bark	Methanol	> 50	No			
S179	S179	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	root	Hexane	> 50	No			
S180	S180	SW950074	พญาสัตบรรณ	*Alstonia scholaris* (L.) R.Br.	*Apocynaceae*	root	Methanol	> 20	No			
S189	S189	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	leaf	Hexane	> 20	No			
S190	S190	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	leaf	Methanol	> 2	No			
S191	S191	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	stem only	Hexane	> 20	No			
S192	S192	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	stem only	Methanol	> 50	No			
S193	S193	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	stem bark	Hexane	> 20	No			
S194	S194	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	stem bark	Methanol	> 50	No			
S195	S195	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	root	Hexane	> 20	No			
S196	S196	SW950076	กาสามปีก	*Vitex peduncularis* Wall. ex Schauer	*Verbenaceae*	root	Methanol	> 20	No			
S269	S269	SW950095	ตะไคร้น้ำ	*Homonoia riparia* Lour.	*Euphorbiaceae*	all	Hexane	ED50 = 14.4	No			
S270	S270	SW950095	ตะไคร้น้ำ	*Homonoia riparia* Lour.	*Euphorbiaceae*	all	Methanol	ED50 = 20	No			
S331	S331	SW950117	ผักปลาบ	*Commelina sp.*	*Commelinaceae*	all	Hexane	>50	No			
S332	S332	SW950117	ผักปลาบ	*Commelina sp.*	*Commelinaceae*	all	Methanol	>50	No			
S343	S343	SW950120	ก้างปลา	*Bridelia retusa*	*Euphorbiaceae*	leaf	Hexane		No			
S344	S344	SW950120	ก้างปลา	*Bridelia retusa*	*Euphorbiaceae*	leaf	Methanol		No			
S345	S345	SW950120	ก้างปลา	*Bridelia retusa*	*Euphorbiaceae*	stem	Hexane	>50	No			
S346	S346	SW950120	ก้างปลา	*Bridelia retusa*	*Euphorbiaceae*	stem	Methanol	>20	No			
S363	S363	SW950125	ผักกาดน้ำ ผักกาดนกกูด	*Crassocephalum crepidioides*	*Cruciferae*	all	Hexane	>20	No			
S369	S369	SW950127	ถอบแถบ	*Connarus cochinchinensis*	*Leguminosae*	leaf	Hexane	>50	No			
S370	S370	SW950127	ถอบแถบ	*Connarus cochinchinensis*	*Leguminosae*	leaf	Methanol	>50	No			
S371	S371	SW950127	ถอบแถบ	*Connarus cochinchinensis*	*Leguminosae*	ztem	Hexane	>20	No			
S372	S372	SW950127	ถอบแถบ	*Connarus cochinchinensis*	*Leguminosae*	stem	Methanol	>20	No			
S417	S417	SW950140	เปล้าใหญ่	*Croton cf. oblongifolius*	*Euphorbiaceae*	leaf	Hexane	>20	No			
S418	S418	SW950140	เปล้าใหญ่	*Croton cf. oblongifolius*	*Euphorbiaceae*	leaf	Methanol	>20	No			
S419	S419	SW950140	เปล้าใหญ่	*Croton cf. oblongifolius*	*Euphorbiaceae*	stem	Hexane	>10	No			

S420	S420	SW950140	เปล้าใหญ่	*Croton cf. oblongifolius*	*Euphorbiaceae*	stem	Methanol	>50	No			
S441	S441	SW950145	แกนมอญ	*Rhus chinensis*	*Anarcardiaceae*	leaf	Hexane	>50	No			
S442	S442	SW950145	แกนมอญ	*Rhus chinensis*	*Anarcardiaceae*	leaf	Methanol	>10	No			
S444	S444	SW950145	แกนมอญ	*Rhus chinensis*	*Anarcardiaceae*	stem	Methanol	>50	No			
S510	S510	SW950161	มะขามป้อม	*Phyllanthus emblica*	*Euphorbiaceae*	leaf	Methanol	>20	No			
S511	S511	SW950161	มะขามป้อม	*Phyllanthus emblica*	*Euphorbiaceae*	stem	Hexane	>50	No			
S512	S512	SW950161	มะขามป้อม	*Phyllanthus emblica*	*Euphorbiaceae*	stem	Methanol	>50	No			
S601	S601	SW950189	โด่ไม่รู้ล้ม	*Elephantopus scaper*	*Compositae*	all	Hexane	>50	No			
S602	S602	SW950189	โด่ไม่รู้ล้ม	*Elephantopus scaper*	*Compositae*	all	Methanol	>20	No			
S754	S754	SW950228	ผักกระสัง	*Peperomia pellucida Korth*	*Piperaceae*	all	Methanol	>50	No			
S801	S801	SW950245	ขมิ้น	*Curcuma longa Linn.*	*Zingiberaceae*	all	Hexane	>50	No			
S802	S802	SW950246	ขมิ้น	*Curcuma longa Linn.*	*Zingiberaceae*	all	Methanol	repeat	No			
S803	S803	SW950247	ข่า	*Alpinai nigra*	*Zingiberaceae*	leaf	Hexane	>50	No			
S804	S804	SW950248	ข่า	*Alpinai nigra*	*Zingiberaceae*	leaf	Methanol	>20	No			
S807	S807	SW950251	ทองพันชั่ง	*Rhinacanthus nasutus Kurz*	*Acanthaceae*	leaf	Hexane		No			
S808	S808	SW950252	ทองพันชั่ง	*Rhinacanthus nasutus Kurz*	*Acanthaceae*	leaf	Methanol		No			
S811	S811	SW950255	ฟ้าทะลายโจร	*Andrographis paniculata Wall.*	*Acanthaceae*	leaf	Hexane		No			
S812	S812	SW950256	ฟ้าทะลายโจร	*Andrographis paniculata Wall.*	*Acanthaceae*	leaf	Methanol		No			
S813	S813	SW950257	ฟ้าทะลายโจร	*Andrographis paniculata Wall.*	*Acanthaceae*	stem	Hexane		No			
S814	S814	SW950258	ฟ้าทะลายโจร	*Andrographis paniculata Wall.*	*Acanthaceae*	stem	Methanol		No			
S820	S820	SW950264	มะนาว	*Citrus aurantifloria Swing*	*Rutaceae*	leaf	Methanol		No			
S821	S821	SW950265	มะนาว	*Citrus aurantifloria Swing*	*Rutaceae*	stem	Hexane		No			
S822	S822	SW950266	มะนาว	*Citrus aurantifloria Swing*	*Rutaceae*	stem	Methanol		No			
S837	S837	SW950281	โมกหลวง	*Holarrhena antidisenterica Wall.*	*Apocynaceae*	leaf	Hexane		No			
S838	S838	SW950282	โมกหลวง	*Holarrhena antidisenterica Wall.*	*Apocynaceae*	leaf	Methanol		No			
S839	S839	SW950283	โมกหลวง	*Holarrhena antidisenterica Wall.*	*Apocynaceae*	stem	Hexane		No			
S840	S840	SW950284	โมกหลวง	*Holarrhena antidisenterica Wall.*	*Apocynaceae*	stem	Methanol		No			
S841	S841	SW950285	เพกา	*Oroxylum indicum Vent.*	*Bignoniaceae*	leaf	Hexane		No			

S842	S842	SW950286	เพกา	*Oroxylum indicum Vent.*	*Bignoniaceae*	leaf	Methanol		No			
S843	S843	SW950287	เพกา	*Oroxylum indicum Vent.*	*Bignoniaceae*	stem	Hexane		No			
S844	S844	SW950288	เพกา	*Oroxylum indicum Vent.*	*Bignoniaceae*	stem	Methanol		No			
S845	S845	SW950289	กระชาย	*Boesenbergia pandurata Holtt.*	*Zingiberaceae*	root	Hexane		No			
S846	S846	SW950290	กระชาย	*Boesenbergia pandurata Holtt.*	*Zingiberaceae*	root	Methanol		No			
S881	S881	SW950325	กระพี้เขาควาย	*Dalburgia Fusca Pierre*	*Leguminosae*	leaf	Hexane		No			
S882	S882	SW950326	กระพี้เขาควาย	*Dalburgia Fusca Pierre*	*Leguminosae*	leaf	Methanol		No			
S883	S883	SW950327	กระพี้เขาควาย	*Dalburgia Fusca Pierre*	*Leguminosae*	stem	Hexane		No			
S884	S884	SW950328	กระพี้เขาควาย	*Dalburgia Fusca Pierre*	*Leguminosae*	stem	Methanol		No			
S975	S975	SW950419	แจง	*Maerua siamensis* Kurz.	*Capparidaceae*	leaf	Methanol		No			
			กรุงเขมา	*Cissampelos pareira* Linn.	*Menispermaeae*	leaf	Hexane		No			
			กรุงเขมา	*Cissampelos pareira* Linn.	*Menispermaeae*	leaf	Methanol		No			
From Cell												
	P200	200	คูณ				H2O (10% DMSO)		No			
	P204	204	ราชพฤกษ์				H2O (10% DMSO)		No			
	P208	208	มะขามแขก				H2O (10% DMSO)		No			
	P212	212	ขี้เหล็ก				H2O (10% DMSO)		No			
	P216	216	แสมสาร				H2O (10% DMSO)		No			
	P219	219	ทรงบาดาล				H2O (10% DMSO)		No			
	P223	223	ชุมเห็ดเทศ	*Cassia alata* Linn.	*Leguminosae*		H2O (10% DMSO)		No			
	P363	363	ทรงกรด				H2O (10% DMSO)		No			
	P367	367	กานต์				H2O (10% DMSO)		No			
	P399	399	ชัยพฤกษ์				H2O (10% DMSO)		No			
	P403	403	กัลปพฤกษ์				H2O (10% DMSO)		No			
	P407	407	ขี้เหล็กอเมริกา				H2O (10% DMSO)		No			
	P414	414	ขี้เหล็กอเมริกา				Hexane		No			
	P426	426	ทรงบาดาล				Hexane		No			

	P434	434	กานต์				Hexane		No			
	P442	442	ทรงกรด				Hexane		No			
	P450	450	ชุมเห็ดเทศ	*Cassia alata* Linn.	*Leguminosae*		Hexane		No			
	P458	458	ชุมเห็ดไทย	*Cassia tora* Linn.	*Leguminosae*		Hexane		No			
	P466	466	ชัยพฤกษ์				Hexane		No			
	P530	530	ราชพฤกษ์				Methylene chlorite		No			
	P534	534	ขี้เหล็กมัน				Methylene chlorite		No			
	P538	538	ขี้เหล็กไทย				Methylene chlorite		No			
	P542	542	แสมสาร				Methylene chlorite		No			
	P546	546	คูณ				Methylene chlorite		No			
	P550	550	ขี้เหล็ก				Methylene chlorite		No			
	P558	558	มะขามแขก				Methylene chlorite		No			
Screened plants from Bala forest												
		B001	เอื้องหมายนา(หัว)				Hexane		No			
		B002	เอื้องหมายนา(หัว)				Methanol		No			
		B003	ตะขาบบิน				Hexane		No			
		B004	ตะขาบบิน				Methanol		No			
		B005	คล้าป่า(หัว)				Hexane		No			
		B006	คล้าป่า(หัว)				Methanol		No			
		B007	คล้าป่า(ใบ)				Hexane		No			
		B008	คล้าป่า(ใบ)				Methanol		No			
		B009	เอื้องหมายนา(ลำต้น)				Hexane		No			
		B010	เอื้องหมายนา(ลำต้น)				Methanol		No			
		B011	กระไดลิง				Hexane		No			
		B012	กระไดลิง				Methanol		No			

Table 4

Results of fungal sample screening

ACC code	Fermentation code	Scientific name	PGHS-2 specific
IF6	40609.4A	*Beauveria amorpha*	No
IF7	40609.9A	*Beauveria amorpha*	No
IF8	40609.3B	*Beauveria amorpha*	No
IF9	40609.4A	*Beauveria amorpha*	No
IF10	40609.8B	*Beauveria amorpha*	No
IF11	40609.10A	*Beauveria amorpha*	No
IF27	40651.4B	*Beauveria bassiana*	No
IF32	41247.3A	*Paecilomyces lilacinus*	No
IF33	41247.4A	*Paecilomyces lilacinus*	No
IF34	41247.9A	*Paecilomyces lilacinus*	No
IF42	401191.3B	*Hymenostilbe sphecocephala*	No
IF43	401220.4A	*Akanthomyces on Pentatomid*	No
IF46	40637.8B	*Verticillium heiptergenum*	No
IF53	40971.3A	*Hirsutella brunneapunctata*	No
IF58	401064.3A	*Hypocrela discoidea*	No
IF60	40641.3B	*Paecilomyces farinosus*	No
IF68	40981.3A	*Cordyceps konigsbergensis*	No
IF77	41825.4A	*Aschersonia tubulata*	No
IF83	401066.9A	*Aschersonia samoensis*	No
IF85	40880.9A	*Aschersonia samoensis*	No
IF86	41886.3A	*Aschersonia tubulata*	No
IF87	41886.3B	*Aschersonia tubulata*	No
IF89	41886.4B	*Aschersonia tubulata*	No
IF95	40974.3A	*Aschersonia placenta*	No
IF99	401075.9A	*Aschersonia cf samoensis*	No
IF101	41385.4A	*Aschersonia cf badia*	No
IF103	41386.3A	*Aschersonia hypocreoidea*	No
IF104	41386.4A	*Aschersonia hypocreoidea*	No
IF116	40619.4A	*Aschersonia oxystoma*	No
IF121	40998.9A	*Aschersonia oxystoma*	No