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# Development and validation of a phosphorylated SMAD *ex vivo* stimulation assay

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**Abstract**

Assessing the pharmacodynamics (PD) of a potential therapeutic through the use of a downstream biomarker is essential. This is traditionally performed in the target tissue but limited volume and invasiveness of sampling pose challenges with solid tumours. Currently, there are several small molecule receptor kinase inhibitors and large molecule therapeutic antibodies in clinical trials that interfere with TGFβ signalling to treat various forms of cancer. With the advent of these new therapies, there is a need for a surrogate tissue that is easily accessible and indicative of tumour response. We propose the use of an *ex vivo* $TGF\beta_1$ stimulation of peripheral blood mononuclear cells (PBMCs) coupled with the measurement of phosphorylated SMAD2 (Sma/Mothers Against dpp, a downstream transcriptional activator) using a sandwich ELISA. TGFβ is involved in many different cellular responses, such as proliferation, angiogenesis, migration, invasion and immunomodulation. SMAD2 and SMAD3 are phosphorylated as a result of the canonical cascade through ligand binding and receptor kinase activation. These phosphorylated SMADs (pSMAD) associate with SMAD4, a co-SMAD, and transcriptionally activate TGFβ-mediated genes. This paper describes the novel method for measuring the downstream effects of inhibiting canonical TGFβ signalling using *ex vivo* stimulation of surrogate tissue to predict tumour response. In addition, we present the assay validation rationale and data. This novel, validated assay can be used to gain insight into clinical trials regarding TGFβ signal modulation by multiple inhibitor platforms for both large and small molecules.

**Keywords:** *pSMAD, tSMAD, TGFβ receptor I kinase inhibitor, cancer, ex vivo stimulation, LY2157299*

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## Introduction

In early clinical investigation, there is a growing need to determine pharmacodynamic (PD) effects of a novel drug in the first clinical trial. One approach invokes the use of surrogate tissue samples to test for PD. The sample should be readily accessible, amenable to a moderate throughput format, and ideally show a correlation with

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activity in the tumour. Peripheral blood mononuclear cells (PBMCs) meet the first two criteria. PBMCs can be easily obtained by blood collection into a CPT tube, thus are readily accessible. These mononuclear cells reside in the peripheral blood for 1–3 days (Goldsby et al. 2000, Lawrence 1992) after which the cells migrate into the tissues and differentiate. The cells are then replenished in the periphery by new PBMCs. PBMCs can be processed into a lysate in a moderate throughput manner. Others have used PBMCs of cancer patients as surrogate tissues in assessing various oncolytic therapies. Bible et al. (2005) used PBMCs to determine if flavopiridol, a cyclin-dependent kinase inhibitor, transcription inhibitor and DNA interacting agent, was affecting its molecular targets *in vivo* as seen *in vitro*. pERK was assessed using leukocytes that were *ex vivo* stimulated with PMA from cancer patients treated with a small molecule inhibitor of MEK-1 and MEK-2, CI-1040 (Lorusso et al. 2005). $p70^{s6}$ kinase inhibition was monitored using PBMCs from cancer patients treated with CCI-779, an inhibitor of mTOR (Peralba et al. 2003). In this paper, we present a method using *ex vivo* stimulated PBMCs to assess signalling through the canonical transforming growth factor-β 1 ($TGF\beta_1$) pathway (see reviews Massague 1996, Massague & Chen 2000, Shi & Massague 2003) that correlates with tumour response in animal models.

One molecular target that has gained attention as an oncolytic and an antifibrotic is the $TGF\beta_1$ signalling network (Yingling et al. 2004, Wick et al. 2006). This cytokine elicits a diverse range of responses. It is a potent growth inhibitor in epithelial cells (Feng et al. 2000). Yet, TGFβ can also stimulate growth in cells of mesenchymal origin including fibroblasts and osteoblasts. In addition, this cytokine appears to have a role in controlling angiogenesis (Wick et al. 2006).

The canonical $TGF\beta_1$ pathway begins with $TGF\beta_1$ binding a TGFβ receptor type II (RII) located in the plasma membrane. The binding of the ligand to RII allows the TGFβ receptor type I (RI) also located in the plasma membrane to recognise and associate with the complex (Wrana et al. 1992). The RII, a constitutively active kinase, phosphorylates RI (Wrana et al. 1994). The RI kinase is activated and phosphorylates SMAD2 (Sma/Mothers Against dpp, a downstream transcriptional activator) and SMAD3. SMAD2 and SMAD3 are receptor-activated SMADs (R-SMADs) (Zhang et al. 1996). These R-SMADs are released from the receptor complex and associate with SMAD4, common SMAD or co-SMAD. This phosphorylated SMAD heterotrimer (pSMAD) then translocates from the cytoplasm to the nucleus (Lagna et al. 1996, Nakao et al. 1997). Once in the nucleus, the SMAD trimer is able to interact with SMAD response elements and activate or repress certain TGFβ target genes (Feng et al. 2000, Qing et al. 2000, Stopa et al. 2000).

The phosphorylation of SMAD2/3 was chosen as the target for measurement allowing for one assay to be used when analysing any compound that will interfere upstream of the phosphorylation. Therefore, this assay can be used with large molecule antibodies that act as $TGF\beta_1$ antagonists and small molecules that act as TGFβ RI kinase inhibitors. In this paper, we describe using PBMCs *ex vivo* stimulated with $TGF\beta_1$ coupled with a pSMAD sandwich ELISA as a biomarker to assess the PD of $TGF\beta_1$ signalling modulating therapeutics. The performance characteristics and the rational approach for validating assays of this type are discussed.

## Materials and methods

### *Study design*

*Time course of LY2157299 treatment in tumours and PBMCs in rats.* Twenty-eight female, Fisher344 rats were obtained from Taconic (Hudson, NY, USA). The weight range of the rats was 150–160 g. Rat mammary adenocarcinoma cells (13762RMC) were injected into the flanks subcutaneously. Tumours were allowed to achieve a volume of 500–600 $mm^3$ prior to treatment with compound. The rats were administered either saline or 300 mg $kg^{-1}$ of LY2157299, a TGFβ RI kinase inhibitor, as a single dose p.o. The rats were euthanised at 0.5, 1, 1.5, 2, 4, 8 or 16 h after dosing using $CO_2$ asphyxiation and cervical dislocation. Each cohort consisted of three rats. Blood and tumours were collected.

*Dose response of LY2157299 treatment in mouse xenografts.* Sixteen female, nu/nu mice were obtained from Charles River (Wilmington, MA, USA). The weight range of the mice was 26–28 g. Calu6 cells (HTB-56, human lung carcinoma cells) were injected into the flanks subcutaneously. After the xenografts achieved a volume of 200–300 $mm^3$, the mice were administered PBS, 10, 30, 100 or 200 mg $kg^{-1}$ of LY2157299 as a single dose p.o. The mice were euthanised 2 h after dosing using $CO_2$ asphyxiation and cervical dislocation. The control group consisted of four mice while the other cohorts contained three mice. Xenografts were collected.

*Human studies: dose response inhibition of TGFβ$_1$-activated pSMAD to LY2157299 in human PBMCs measured by Western blot or ELISA.* Human PBMCs or THP-1 cells, a human acute monocytic leukemia cell line, were incubated with 0–20 μM of LY2157299 for 30 min and then activated with 100 pM TGFβ$_1$ for 1 h at 37°C. Samples were washed with PBS three times and lysed.

### *Sample requirements and processing*

*Tumours/xenografts.* Using liquid nitrogen, the tumours were homogenised and transferred to a microcentrifuge tube. To this tube, a volume of 500–600 μl of lysis buffer was added. After addition of lysis buffer, the tube was placed in ice water and sonicated. The samples were then centrifugated for 15–20 min at 15 000*g*. The supernatants were transferred to new, labelled tubes.

*PBMCs.* Cell preparation tubes (CPT) from Becton Dickinson (Franklin Lakes, NJ, USA) were stored upright at room temperature prior to sample collection. CPT tubes with either sodium heparin or sodium citrate anticoagulant were used for blood collection. Within 2 h of blood collection, the tube was centrifuged at ~800*g* for 25 min at room temperature. The tube was then refrigerated at 4°C.

*Reagents.* Recombinant human TGFβ$_1$ was purchased from R&D Systems, (catalog # 240-B, Minneapolis, MN, USA). BupH Carbonate-BiCarbonate Buffer (Pierce 28382, Rockford, IL, USA) was prepared according to the manufacturer's instructions. Lysis stock buffer contained 50 mM HEPES, 149.2 mM NaCl, 20 mM NaF, 20 mM NaPP, 5 mM EDTA, 5 mM EGTA and 1% Triton X-100. All reagents for the lysis buffer were purchased from Sigma (St. Louis, MO, USA). A complete protease inhibitor mini tablet from Roche (Indianapolis, IN, USA; catalog # 11836153) along

with 100 μl each of phosphatase inhibitor cocktail I (Sigma P-2850) and phosphatase inhibitor cocktail II (Sigma P-5726) were added to each 10 ml of lysis buffer just before use. One component TMB substrate (BioFx, Owings Mills, MD, USA) (TMBW-0100) was used in these colorimetric sandwich ELISAs. Ten-fold concentrated Tris Buffered Saline (Fisher BP2701 Fair Lawn, NJ, USA) was diluted to 1 × with MilliQ water and Tween-20 (Sigma P-7949) was added to a final concentration of 0.05%. The Tris Buffered Saline with 0.05% Tween-20 wash buffer (TBST) for the ELISA assays was made fresh daily. Buffer A used in preparation of the standard consisted of 50 mM phosphate, pH 7.5, 300 mM NaCl, 2 mM beta-mercaptoethanol, 5 mM imidazole, 10% glycerol, 0.1 μM okadiac acid. Buffer B used in the preparation of the standard consisted of 20 mM Tris, pH 7.5, 25 mM NaCl, 10 mM NaF, 1 mM DTT and 10% glycerol. Total-SMAD 2/3 (tSMAD) monoclonal antibody (for use as a capture antibody on tSMAD and pSMAD ELISA assays) was purchased in a special bulk lot without BSA or azide (BD Transduction Laboratories, 621948, San Jose, CA, USA). pSMAD 2/3 rabbit polyclonal antibody (for use as a conjugate antibody in the pSMAD ELISA) was produced by Zymed using CKKS-pS-M-pS-COOH, a multi-serine phosphorylated fragment of SMAD2 (Zymed (Invitrogen), Carlsbad, CA, USA; ELJD7+/8- Rabbit #2 and bulk lot 40302). tSMAD 2/3 polyclonal antibody (for use as a conjugate antibody in the tSMAD ELISA) was also manufactured at Zymed in a bulk lot without BSA or azide (Zymed ELX51-1300 lot # 40788860). The detection antibody donkey anti-rabbit HRP was purchased from Amersham Biosciences, (Piscataway, NJ, USA; NA934). For Western blotting, a polyclonal tSMAD 2/3 antibody was obtained from Upstate (Billeriea, MA, USA). Both polyclonal tSMAD 2/3 antibodies were comparable when used for Western blotting. After evaluating multiple antibodies in the ELISA during assay optimisation, the Zymed polyclonal tSMAD 2/3 antibody was chosen based on dilutional linearity and availability of bulk material (data not shown).

*R-SMAD standard.* To prepare a standard for the R-SMAD immunoassays, the insect cell line Sf-9 was used to produce recombinant protein. The full-length SMAD2 gene was inserted into the pFastBacHTa vector (Invitrogen) (His-SMAD2, construct B310). In addition, TGFβ RI was also inserted into a baculovirus vector (Construct T204D). Sf-9 cells were co-infected with His-SMAD2 (B310) and TGFβ RI (T204D) recombinant baculoviruses. At 45 h post-infection (hpi), okadaic acid was added to a final concentration of 0.1 μM. Cells were harvested at 48 hpi, washed with PBS, and frozen at −80°C. Cell pellets were re-suspended in cold buffer A containing 0.1% Triton X-100 and EDTA-free protease inhibitor cocktail mini tablets (Roche Catalog #11836170001) and rotated at 4°C for 30 min. The ratio of cell pellet to cold buffer A was 25:1. The cells were then homogenised and centrifuged at 15 000 rpm for 30 min at 4°C. The supernatant was collected and mixed for 2 h at 4°C with Talon beads (BD Biosciences) that were pre-washed with water and buffer A containing 0.1% Triton X-100. The beads were centrifuged, packed into a column, and washed with cold buffer A. Proteins were eluted at 1 ml $min^{-1}$ with gradient of buffer A containing 100 mM imidazole. Fractions were pooled, and diluted tenfold with buffer B. The diluted pool was loaded onto a Mono Q (1 ml) column (GE Healthcare, Piscataway, NJ, USA) and eluted with a gradient (0–100%) of buffer B containing 1 M NaCl at 0.5 ml $min^{-1}$. Suitably sized aliquots of appropriately diluted Sf9 R-SMAD were frozen at −80°C. These aliquots were thawed fresh to prepare the

standard curves by diluting into lysis buffer containing 1% BSA (Sigma A-7888). For validation experiments, standard curves were prepared on ice, just prior to setting up the ELISA. The unit for the concentration of the standard is ng $ml^{-1}$. For the purposes of this assay, the concentration is defined from the total protein concentration of the standard. The standard will be subsequently referred to as HIS-SMAD2.

*Ex vivo TGF$\beta_1$ stimulation of PBMCs and cell lysis procedure*. PBMCs were re-suspended into the separated plasma by gently inverting the previously spun CPT tube several times. Two 1.5 ml fractions of the supernatant located above the polymer barrier were transferred into two 12 × 75 mm polypropylene tubes. TGF$\beta_1$ stock was added into the TGF$\beta_1$ (+) tube to achieve a final concentration of 100 pM. An equivalent volume of PBS w/1% BSA was added to the TGF$\beta_1$ (−) tube. Tubes were incubated at 37°C in a Dubnoff metabolic water bath (Precision Scientific, Chicago, IL, USA) for 60 min with shaking at 60 rpm. Samples were centrifuged for 5 min at 800*g* at 4°C. Supernatant was immediately removed by aspiration. The cell pellet was washed with 1 ml of ice cold PBS, centrifuged, and the wash aspirated. This wash step was repeated two additional times. Following the three PBS washes, 750 µl of ice-cold lysis solution containing protease and phosphatase inhibitors was added to the PBMCs. During the lysis step, the cells were incubated on ice for 30 min and vortexed periodically. The lysate was then transferred to a microfuge tube, and centrifuged at 16 000 rpm for 5 min at 4°C. The supernatant was transferred to a newly labeled tube, and stored at −70°C for analysis.

*Western blot procedure*. Protein concentrations were measured by Bio-Rad DC assay. Control and treated lysates (50–60 µg total protein) were boiled for 5 min in 1 x SDS sample buffer and clarified by centrifugation approximately 3 min at room temperature. Equal amount of proteins were resolved by 10% Tris-Glycine gel (Novex (Invitrogen) Carlsbad, CA, USA). Two identical gels were run for analysis with pSMAD and tSMAD primary antibodies. After running for 2 h at 135 (V), the gels were transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dried milk in TBS plus 0.5% Tween 20 (TBST) for approximately 30 min at room temperature. The membranes were incubated with the primary antibodies (pSMAD or tSMAD) overnight at 4°C diluted 1:750–1000 in 5% milk TBST. On the following day, the membranes were washed three times for 15 min with TBST. The membrane was then incubated with anti-rabbit IgG-HRP in 5% milk in TBST antibody for 1–2 h. Immunoreactive reagents were detected by chemiluminescence using Supersignal West Pico (Pierce 34080). Images were captured and analysed on a Lumi-Imager F1 (Roche, Indianapolis, IN, USA).

*ELISA procedure*. Two ELISA procedures were optimised to measure both phosphorylated and SMAD 2/3. Plates were coated by adding monoclonal tSMAD antibody at a concentration of 2.5 µg $ml^{-1}$ to each well. The plates were covered and incubated rocking overnight at ~4°C. At the completion of each incubation step, five washes of 250 µl/well with TBST using a plate washer (Bio-tek Instruments, Inc. Winooski, VT, USA) occurred. The plate was blocked by adding TBS w/1% BSA to each well and incubating at room temperature rocking for at least 2 h. Sample, standard or control was added in duplicate to appropriate wells. The plate was sealed, and incubated overnight at ~4°C. To each well of the respective plates, pSMAD 2/3

or tSMAD 2/3 polyclonal antibodies diluted to 2.5 μg $ml^{-1}$ in lysis solution w/1% BSA were added. The plates were incubated at room temperature on a rocker for 2 h. Donkey anti-rabbit HRP antibody (Amersham # NA934V) was diluted 1:10 000 in TBS w/1% BSA, added, and incubated at room temperature rocking for 1 h. TMB substrate (BioFx) was added to each well, and incubated at room temperature on a rocker for 30 min. Phosphoric acid (2M diluted in water) was added to each well to stop the colorimetric reaction. The plate was read at 450 nm on Spectramax 384 plate reader (Molecular Devices). Total protein was assessed prior to the development of the tSMAD ELISA using the Pierce BCA Protein Assay kit per the manufacturer's instructions.

*Assay validation*

*Calibration curve.* Calibration curves were prepared in lysis buffer with 1% BSA. For the pSMAD analysis, an initial point of 5900 ng $ml^{-1}$ HIS-SMAD2 was serially diluted threefold to 2.7 ng $ml^{-1}$ resulting in an eight-point calibration curve. For tSMAD analysis, an initial point of 218 ng $ml^{-1}$ HIS-SMAD2 was serially diluted threefold to 0.1 ng $ml^{-1}$ resulting in an eight-point calibration curve. Validation experiments consisted of six runs: two analysts completing three runs each.

*Accuracy and precision (LLQ/ULQ).* Validation samples were prepared by spiking reference standard (HIS-SMAD2) into lysis buffer containing 1% BSA and inhibitors at the following concentrations: 25, 100, 600, 1500, 3300 and 4500 ng $ml^{-1}$ for pSMAD; and 1, 5, 15, 30, 50 and 75 ng $ml^{-1}$ for tSMAD. These levels were chosen by determining the $EC_{50}$ of the ELISA assay. From this figure, the $EC_{20}$ and $EC_{80}$ were calculated. These values served as the low and high samples of the validation concentration ranges. Validation samples were dispensed into aliquots and stored frozen at about $-70^{\circ}C$. For each validation run, validation sample aliquots were removed from the freezer and thawed on ice. The validation samples were analysed for immunoreactive pSMAD and tSMAD concentrations to generate three reportable results per concentration by two analysts each completing three assays in greater than 3 days. A total of 18 results were generated for each validation sample concentration.

*Dilutional linearity.* A concentrated stock solution (10 000 ng $ml^{-1}$) of reference standard (HIS-SMAD2) was prepared in lysis buffer containing 1% BSA plus inhibitors to evaluate dilutional linearity. The dilutional linearity samples were dispensed into aliquots and then stored frozen at about $-70^{\circ}C$. For each validation run, a dilutional linearity aliquot was removed from the freezer, thawed on ice, and diluted 1:5, 1:10 and 1:25 in lysis buffer containing BSA plus inhibitors to yield concentrations, which were expected to fall within the anticipated validated range of the assay standard curve (2000, 1000 and 400 ng $ml^{-1}$, respectively). Two analysts analysed the dilutional linearity validation samples to generate three reportable results per dilution across two assays.

*Stability.* Stability validation samples were prepared by spiking reference standard (HIS-SMAD2) at 600 and 1500 ng $ml^{-1}$ into assay buffer with inhibitors. Additionally, an extracted pool of normal human PBMCs stability validation sample was prepared. An *ex vivo* stimulation was performed on the extracted normal human PBMCs. The stability samples were pooled and dispensed into aliquots and stored

frozen at −70°C. For each validation run, stability validation sample aliquots were removed from storage and thawed on ice or at 2–8°C for analysis. Freeze–thaw stability of pSMAD and tSMAD was evaluated following three freeze–thaw cycles. Stability samples were analysed, for each concentration and condition, to generate three reportable results per concentration and condition across one assay by one analyst. In addition, long-term stability was assessed. Periodically, an aliquot of the stability sample was analysed out to 143 days.

*Biological precision.* Biological precision was assessed by analysing pSMAD and tSMAD in normal human PBMCs from 16 donors collected once a day over a 3-day period. An *ex vivo* stimulation with TGFβ$_1$ was performed on the samples from the 16 donors. The assays were carried out at two sites and ten donors were from one site and six donors from the other site. The samples were analysed to generate ≥1 reportable result per donor, per day for 3 days.

*Statistics.* Inter-donor and intra-donor components of variance of pSMAD concentrations were estimated for each site and pooled across both sites. Total variability in the samples is equivalent to the inter-donor coefficient of variation. Previous experience indicated that pSMAD concentrations should be normalised by their corresponding tSMAD concentrations prior to analysis to estimate components of variance. The normalisation applied was to divide pSMAD by tSMAD$^{0.6}$ instead of the usual tSMAD$^{1}$ as there was evidence that pSMAD did not increase proportionally with tSMAD. The normalised pSMAD values were then log (base e) transformed prior to analysis to try and ensure approximate symmetry of their distribution and to minimise any variance–mean relationship. A random effects model using SAS v8.2 was fitted to the normalised pSMAD data to estimate the components of variance, with donor as a random effect and day as a repeated measure. Coefficients of variance were estimated using the relationship; $CV\% = (\sqrt{(e^{\sigma^2} - 1)})*100$ where $\sigma^2$ is the estimated component of variance on the log scale.

## Results

### *Time course of inhibition of TGFβ$_1$-activated pSMAD measured in tumours and PBMCs in rats*

To test the feasibility of using pSMAD measurement in PBMCs as a biomarker in a surrogate tissue for tumour response, a rat model was utilised. Rats were injected with rat mammary carcinoma cells (13762RMC) subcutaneously and allowed to form tumours before administering either PBS or 300 mg kg$^{-1}$ of LY2157299. Tumours and blood were simultaneously collected at the various time points. The PBMCs were isolated and stimulated with TGFβ$_1$ before lysis. After treatment, pSMAD levels in tumours decreased gradually to almost zero at 2 h with no further changes at longer time points (Figure 1A, top gel). The PBMCs responded almost immediately after dosing (Figure 1B, top gel). The tSMAD value was used for normalisation and was a measurement verifying the consistency in sample processing. This study shows a correlation between the *in vivo* tumour response and the *ex vivo* PBMC response. Thus, this inhibitor is disrupting the canonical TGFβ$_1$ pathway in a tumour model and the stimulated PBMCs.

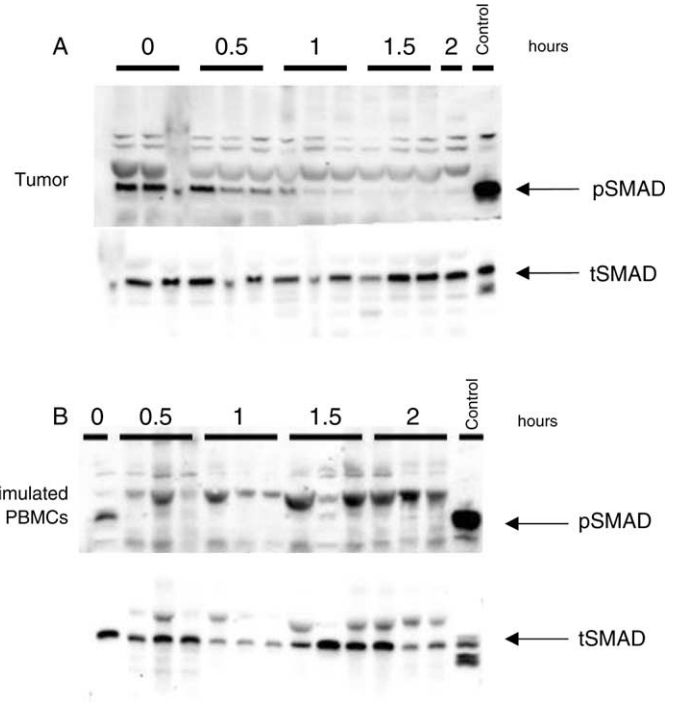


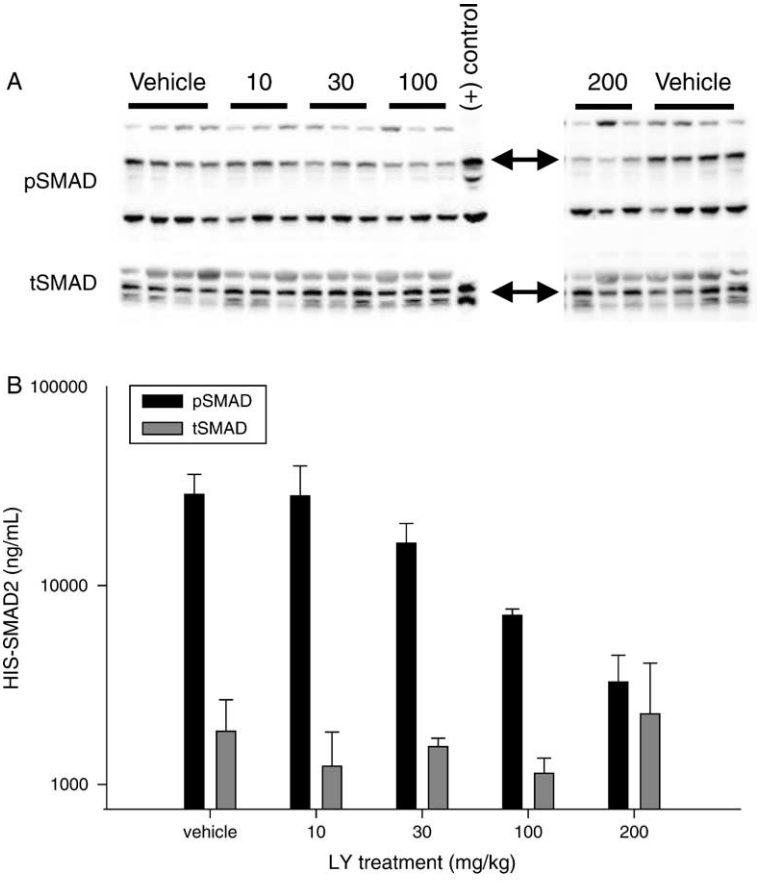

Figure 1. Time course inhibition of $TGF\beta_1$-activated pSMAD by 300 mg $kg^{-1}$ TGFβ receptor type I (RI) kinase inhibitor in rats. Rat mammary carcinoma cells were injected subcutaneously and allowed to form tumours. Rats were dosed with either vehicle (PBS) or 300 mg $kg^{-1}$ of TGFβ RI kinase inhibitor. Samples were collected at various time points. (A) Tumour lysates analysed by Western blot for pSMAD and tSMAD. (B) *Ex vivo* stimulated rat PBMC lysates analysed by Western blot.

*Dose response of inhibition of $TGF\beta_1$-activated pSMAD measured in mouse xenografts by Western blot and ELISA*

To assess the correlation of the Western blot measurement to the ELISA assay measurement, human xenografts were grown in mice using human lung carcinoma cells to a predetermined volume. The mice were treated with various doses of LY2157299. The xenografts were collected 2 h post-treatment and processed. These lysates were analysed using both techniques (Figure 2A,B). The percentage inhibition of each dose was similarly measured in both assays (Figure 2C). The percentage difference between techniques in the pSMAD measurement was $<25\%$. When the p/tSMAD ratio between techniques was compared, the% inhibition values differed by $<15\%$. Thus, the ELISA method showed relative agreement to the Western blot. Using the normalised pSMAD value (p/t ratio) best controlled for the sample processing and sample loading which yielded improved agreement between the methods.

*Dose response inhibition of $TGF\beta_1$-activated pSMAD in human PBMCs and THP-1 cells*

The optimised stimulation procedure was tested by incubating human PBMCs with increasing doses (0–20 μM) of LY2157299 for 30 min prior to addition of 100 pM $TGF\beta_1$. These samples were analysed by Western blot. The pSMAD signal was

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| | % inhibition | | | |
|---|---|---|---|---|
| | WB | ELISA | WB | ELISA |
| LY mg/kg | pSMAD | | p/tSMAD | |
| Vehicle | 0 | 0 | 0 | 0 |
| 10 | 6 | 2 | 22 | 36 |
| 30 | 32 | 43 | 46 | 53 |
| 100 | 51 | 75 | 61 | 71 |
| 200 | 71 | 89 | 72 | 85 |

Figure 2. Dose response inhibition of TGFβ$_1$-activated pSMAD by TGFβ receptor type I (RI) kinase inhibitor in mouse xenografts measured by Western blot (A) and ELISA (B). Human lung epithelial carcinoma cells (Calu6) were injected subcutaneously and allowed to form tumours. Mice were dosed with 0, 10, 30, 100 or 200 mg kg$^{-1}$ of TGFβ RI kinase inhibitor. Samples were collected 2 h post-treatment. (A) Xenograft lysates were analysed by Western blot. The positive control was TGFβ-stimulated Calu6 cell lysate. (B) Xenograft lysates were analysed by ELISA (p- and tSMAD). (C) Table comparing percentage inhibition by Western blot (A) and ELISA (B).

inhibited to nearly 0 with 20 μM of LY2157299 (Figure 3A). The dose response experiment was repeated using human PBMCs along with THP-1 cells, a human cell line used as a mononuclear cell model. These data were generated using the pSMAD ELISA using normalisation to lysate sample total protein as determined by the BCA protein determination kit (Pierce). Lysate total protein allows for the variance in cell

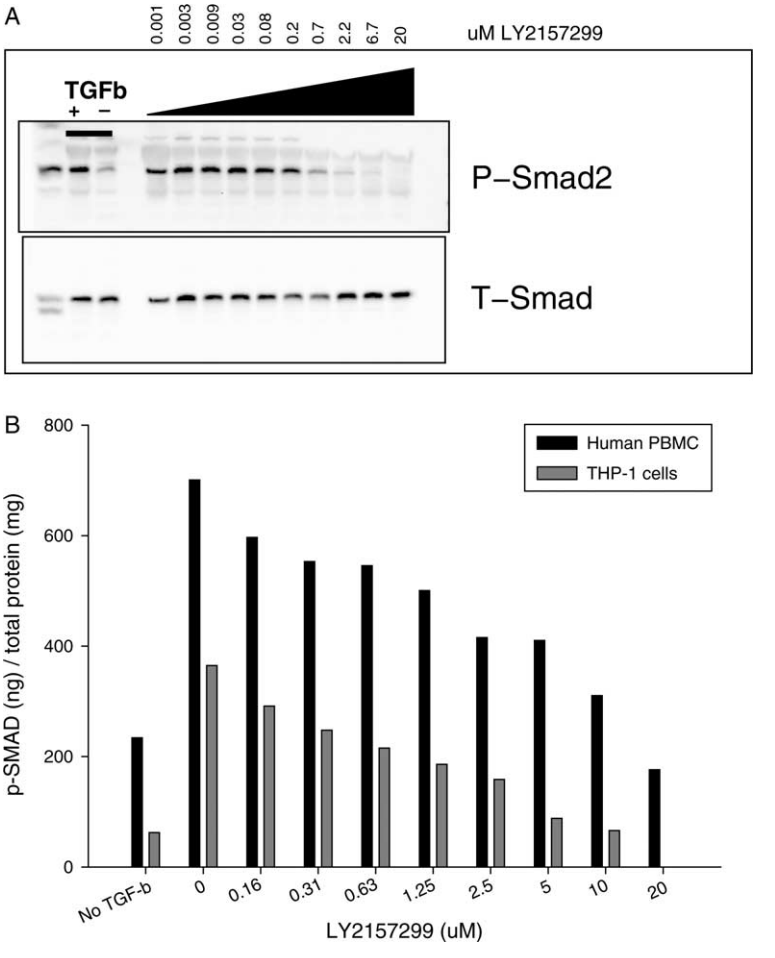


Figure 3. Dose response inhibition of TGFβ$_1$-activated pSMAD by TGFβ receptor type I (RI) kinase inhibitor in humans measured by western blot (A) and ELISA (B) in human PBMCs. (A) Human PBMCs were incubated with increasing doses (0, 0.001, 0.003, 0.009, 0.03, 0.08, 0.2, 0.7, 2.2, 6.7 or 20 μM) of LY for 30 min prior to addition of 100 pM TGFβ$_1$. Following the 1 h incubation at 37°C, cells were washed three times with PBS, lysed with 2 × sample buffer, western blotted. (B) Human PBMCs and THP-1 cells were incubated with increasing doses (0–20 μM) of LY for 30 min prior to addition of 100 pM TGFβ$_1$. Following the 1 h incubation at 37°C, cells were washed three times with PBS, lysed, and measured in the pSMAD ELISA. The black bars represent the human PBMC lysate and the grey bars represent the THP-1 cells.

numbers in each sample. These data were generated prior to the development of the tSMAD ELISA. The data show a reduction of relative pSMAD signal in both the human PBMCs and the THP-1 cell line down to background levels (Figure 3B). Both techniques showed comparable results.

*Assay validation*

*Calibration curve.* Calibration curves were prepared in lysis buffer with 1% BSA ranging from 2.7 to 5900 ng ml$^{-1}$ for the pSMAD assay and from 0.1 to 218 ng ml$^{-1}$ for the tSMAD assay. A representative pSMAD standard curve was

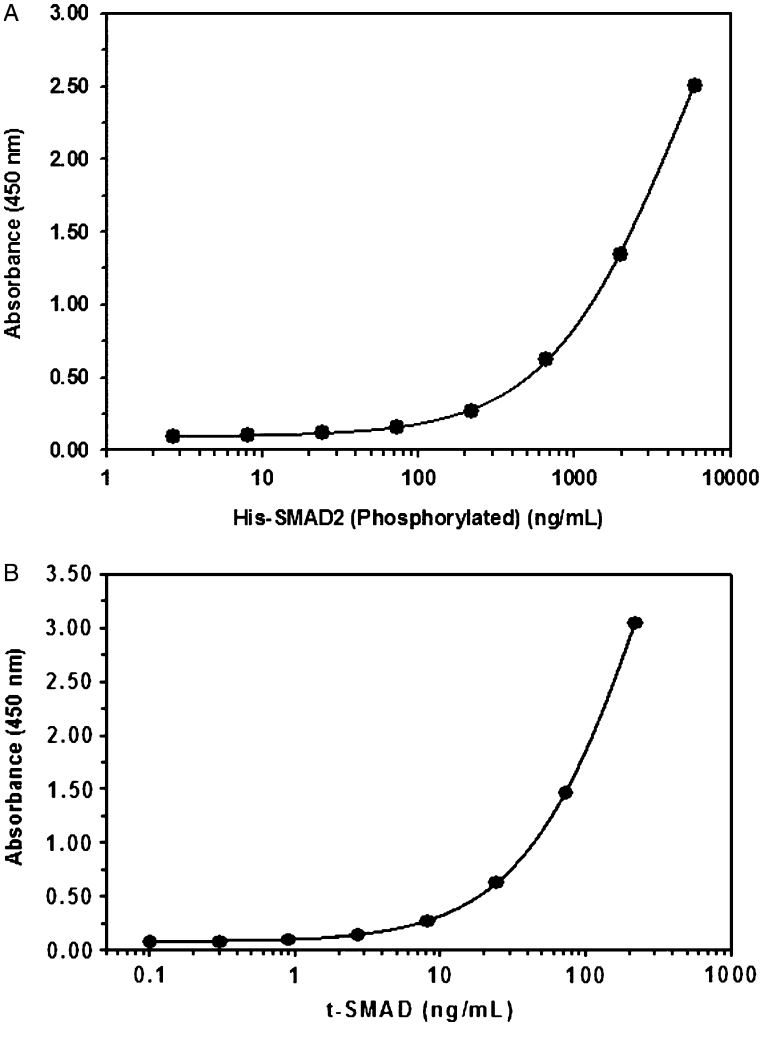


Figure 4. Representative standard curves from p- and tSMAD. (A) The graph represents a typical standard curve in the pSMAD ELISA using the detection antibody specific for pSMAD. (B) The graph represents a typical standard curve for the tSMAD ELISA using the detection antibody specific for tSMAD. The *x*-axis represents increasing concentration of the recombinant standard and the *y*-axis is optical density at 450 nm.

plotted in Figure 4A, and the representative tSMAD values were plotted in Figure 4B. Calibration curve levels were chosen by first determining the approximate $EC_{50}$ of the ELISA. After this was done, the $EC_{20}$ and $EC_{80}$ values were calculated. These values served as the low and high samples of the validation concentration ranges. The precision profile data were generated based on three runs each by two separate analysts spanning at least six levels for both p- and tSMAD. Analysis of these data via an Excel macro template indicated that the assay working range for the pSMAD assay was 46.68–1040.14 ng $ml^{-1}$ and the tSMAD working range was 0.35–46.14 ng $ml^{-1}$. Using the guidance set forth by Dudley et al. (1985), this working range provided a 'best guess' of standard curve performance on which to base the validation sample range.

*Accuracy and precision.* In order to assess the accuracy and precision of these ELISAs, six concentration levels of validation samples were prepared and stored frozen at −70°C. Accuracy (mean bias) was determined by comparing the measured analyte concentration to the nominal level. Precision (intra- and inter-assay) was determined by calculating the coefficient of variation (%CV) between the reportable results generated for each validation sample concentration level within and between the six assays. The *a priori* criteria for the inter- and intra-assay precision were set at ±35%. The limits of quantification were determined to be at the lowest and highest validation sample value that met the *a priori* criteria. At all concentrations for the pSMAD assay, the mean bias (%RE) ranged from −19.4 to −3.0. The intra-assay precision (%CV) ranged from 3.1 to 17.2, and the inter-assay precision (%CV) ranged from 5.9 to 31.5 (Table I). For the pSMAD assay, the lower limit of quantification (LLQ) was determined to be 25 ng $ml^{-1}$ and the upper limit of quantification (ULQ) was determined to be 4500 ng $ml^{-1}$. For all tSMAD concentrations, the mean bias (%RE) ranged from −23.3 to −13.0. The intra-assay precision (%CV) ranged from 2.1 to 7.1, and the inter-assay precision (%CV) ranged from 14.0 to 23.9 (Table I). These data demonstrate that the validated range of the tSMAD method is from 1 ng $ml^{-1}$ (LLQ) to 75 ng $ml^{-1}$ (ULQ). Both assays met the predetermined criteria set forth in the validation. It should be noted that pooled PBMC samples were not run in these experiments, and variability of these types of the samples might be higher. This was subsequently addressed in biological precision experiments described below.

*Dilutional linearity.* In the event that a sample would exceed the working range of the assay, sample dilution would be required to provide a valid result. The linearity assessment demonstrated that the%RE for pSMAD ranged from −11.5 to −6.5 with the cumulative%CV being 7.2. The tSMAD%RE ranged from −5.7 to 3.5 with the cumulative%CV being 9.4. These results were within the acceptance criteria of ±30% of the nominal value. In addition, these data demonstrated that the dilution of these

Table I. Accuracy and precision of pSMAD and tSMAD assays.

| Validation sample (ng $ml^{-1}$) | Intra-assay precision (%CV) | Inter-assay precision (%CV) | Relative error (%) | Total error (%) |
|---|---|---|---|---|
| pSMAD | | | | |
| 25 | 17.2 | 31.5 | −5.3 | 36.8 |
| 100 | 12.3 | 20.9 | −3.0 | 23.9 |
| 600 | 4.8 | 7.0 | −19.4 | 26.4 |
| 1500 | 3.1 | 5.9 | −15.3 | 21.2 |
| 3300 | 3.6 | 7.7 | −16.3 | 24.0 |
| tSMAD | | | | |
| 1 | 7.1 | 23.9 | −13.0 | 36.9 |
| 5 | 3.9 | 20.5 | −15.7 | 36.2 |
| 15 | 6.6 | 18.3 | −13.5 | 31.8 |
| 30 | 2.1 | 14.0 | −23.3 | 37.5 |
| 50 | 6.3 | 17.2 | −20.3 | 37.5 |

Values represent $n = 18$ results over 3 days with two operators.

samples is valid up to a 1:25 dilution. This extends the maximum quantifiable limits for the pSMAD assay to 112 500 ng $ml^{-1}$ and the tSMAD assay to 1875 ng $ml^{-1}$.

*Stability.* In order to prove that samples could be repeated in the event of a failed run, freeze–thaw stability data were needed. A stimulated PBMC lysate was prepared at a concentration of 724.5 ng $ml^{-1}$ pSMAD and 57.1 ng $ml^{-1}$ of tSMAD. The percentage difference from the reference value was calculated and reported for each level and condition. Samples demonstrated freeze–thaw stability up to three cycles for both the p- and tSMAD analytes in the recombinant samples. The pSMAD human PBMC lysate freeze–thaw data demonstrated a one time loss of immunoreactivity but no progressive loss compared to the reference samples (−23.2%, −28.0% and a −21.6%). The total SMAD assay demonstrated a similar loss of immunoreactivity (−16.7%, −22.7% and −18.7%). It was noted that the pSMAD/tSMAD ratio remained fairly constant across sequential freeze–thaw cycles. Samples can be repeated if needed with little loss of analyte. Stability samples were thawed and tested at periodic intervals after being stored for up to 143 days after stimulation. The samples can be stored for at least 4 months with little loss of either analyte (data not shown).

*Biological precision.* In an effort to better characterise the imprecision, we defined the different components that lead to the overall variation in the $TGF\beta_1$ *ex vivo* stimulation procedure. Sixteen donors had three samples collected, activated and measured over a 3-day span. The average of the reportable results per donor per day formed the raw data. Figure 5 shows the normalised data from each site. Each line

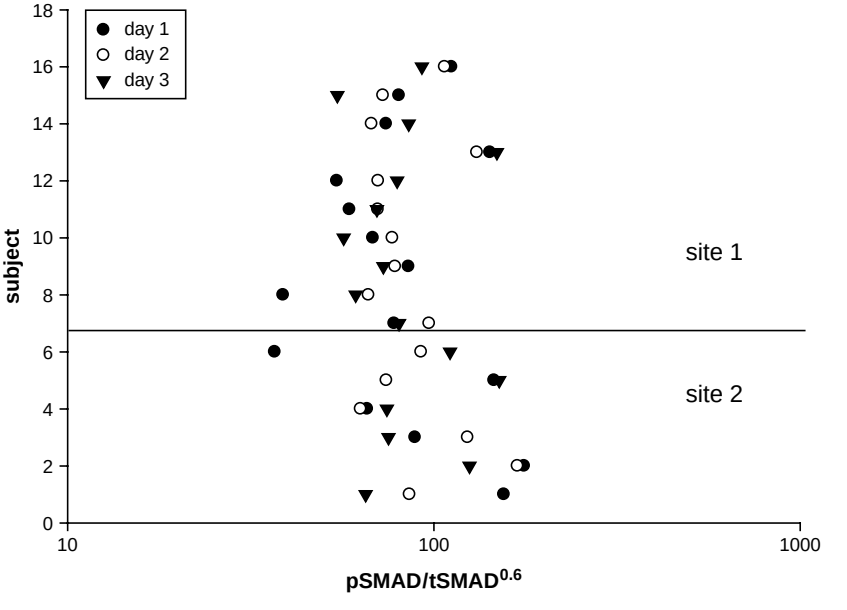


Figure 5. Normal subject pSMAD/tSMAD ratio distribution. Each human subject is one line with PBMC stimulated samples isolated over three different days. Day 1 is represented by a closed circle (●). Day 2 is represented by an open circle (○) while day 3 is represented by a closed inverted triangle (▼). Samples were taken, stimulated and processed at two different sites.

represents three samples from the same individual. Two components of variation (intra- and inter-donor) were estimated and expressed as coefficients of variation along with the overall inter-donor coefficient of variation, the total of the two components. The intra-donor component of variance is a combination of the intra-assay and intra-donor components, since the duplicate data from each sample were not available for analysis. The intra-donor component coefficient of variance for site 1 was 15.7% while site 2 was 38.1%. The pooled intra-donor component across sites was 26.1%. Inter-donor component coefficient of variance for site 1 was 26.4% while site 2 was 25.4%. The pooled inter-donor component across both sites was 26.0%. The overall variation for site 1 was 31.0% and site 2 was 46.8%. The pooled overall variation across sites was 37.5% in normal subjects.

## Discussion

Activity biomarkers are essential for understanding a drug's mechanism of action, establishing exposure response models, and setting an efficacious dose. With an increasing number of kinase inhibitors moving forward as cancer therapies (Mead et al. 2003, Uhl et al. 2004, Yingling et al. 2004, Schlingensiepen et al. 2006), an acute need exists for valid assays to measure phosphoprotein substrates that are downstream of the potential drug targets. SMAD phosphorylation as a result of TGF-$\beta_1$ receptor binding has been described (Wrana et al. 1992, Wrana et al. 1994, Lagna et al. 1996, Massague 1996, Nakao et al. 1997, Shi & Massague 2003) and was a logical biomarker for demonstrating target inhibition for this kinase inhibitor, LY2157299.

In developing a biomarker assay, it was important to establish the biological relevance of the marker. The marker not only needs to be predictive of the drug efficacy in modulating its target, but also indicative of the target tissue response, the tumour. The correlation of the alternate tissue response and the target tissue response is essential. We were able to show that the *ex vivo* stimulation of PBMCs was indicative of the tumour response *in vivo* in rats to the TGF$\beta_1$ kinase inhibitor. pSMAD levels decreased in a time-dependent manner in both the tumour and PBMCs. In addition, the tSMAD levels were unaffected by the treatment (Figure 1).

In early development efforts, Western blotting was the method of choice for multiple reasons including confirmation of specificity, use of only one antibody, and lack of throughput limitations. As LY2157299 continued its clinical development, an effort was made to develop a more clinically feasible approach to measuring the target of interest. One significant advantage that the ELISA approach offered was the utilisation of a reference standard. The addition of a reference standard allowed for plate to plate comparisons and more importantly quantification. In Figure 2, we were able to demonstrate the correlation of the Western blot data to the ELISA data through analysis of one set of samples in both assays. In the Western blot (Figure 2A), there is a reduction in the pSMAD band with increasing dose of LY2157299, while the tSMAD levels remain relatively constant. The ELISA data show a similar dose response with the pSMAD levels decreasing with increasing LY2157299. The tSMAD levels in the ELISA are relatively constant, note the overlapping error bars (Figure 2B). The percentage inhibition calculated relative to the vehicle control was very similar across assays. The pSMAD inhibition varied less than 25% between assays, while the p/tSMAD varied less than 15% (Figure 2C). The techniques are correlative

and comparable. The most important consideration for these assays is to demonstrate compound inhibition of the molecular target. Trends due to compound can be seen with both assays.

The animal models were successful at showing activity of the compound, correlation between PBMC and tumour response, and correlation between the two techniques. In order to transition into clinical trial use, human samples needed to be evaluated. The normal human PBMCs responded in a dose-dependent manner with the pSMAD values decreasing with increasing amounts of compound. This was shown by both Western blot and ELISA (Figure 3). The ELISA data was normalised to total protein to account for cell number differences. This analysis was done prior to the development of the tSMAD ELISA. After development of the tSMAD ELISA, comparison of normalisation using either total protein or tSMAD levels was performed via bridging experiments showing correlated results with either method of normalisation (data not shown). The trend of the pSMAD measurement is comparable to the response shown in rats and mice. THP-1 cells were used to show that the same effect could be achieved in a human mononuclear cell line. Human PBMCs responded in a dose-dependent manner as predicted by the animal models.

The assay was biologically relevant but still needed to be validated. We applied the recommendations made by Lee and colleagues (Lee et al. 2006) for quasi-quantitative assays for verifying the assay. This route was chosen because of the multiple components of the assay: *ex vivo* stimulation of the PBMCs and the ELISAs. The stimulation component lacks a reference standard and therefore would be considered quasi-quantitative. Both ELISAs utilised a relative reference standard, not completely representative of the endogenous pSMAD, and therefore was considered to be a relative quantitative assay. In this case, the validation plan would default to the limiting assay thus quasi-quantitative validation standards. Assays of this type require an assessment of selectivity, accuracy and precision, and stability. Selectivity was previously discussed. The inhibitor shows a decrease in the downstream marker as seen in rats, mice and humans (Figures 1–3). This validation determined that both the pSMAD and tSMAD ELISA assays were able to measure with both accuracy and precision (Table I). As with most immunoassays, the greatest component of variation is between assays (inter-assay precision). Therefore, a decision was made to measure all samples from a subject on one plate. The variation within the assay was less than 18% for both assays. The pSMAD assay exhibited a range of 3.1–17% CV while the tSMAD assay exhibited a range 2.1–7.1%CV with the greatest imprecision at the lower limit of quantification. Both assays clearly met the established criteria of $\pm$ 35%CV for inter- and intra-assay precision. In addition, the assays demonstrated both accurate and reproducible results following sample dilution. This is an important assay characteristic because the samples could exceed the dynamic range of the assay and need to be diluted. Stability data indicated that the HIS-SMAD2 standard was stable up to three freeze–thaw cycles for both analytes. In addition, the human PBMC lysate samples were tested for both freeze–thaw and long-term stability. The data showed that the lysates can tolerate up to three freeze-thaw cycles without successive loss of immunoreactivity and that pSMAD and tSMAD are stable for up to 143 days when stored at $-70^{\circ}$C. Biological precision was tested in normal donors. The linear regression of pSMAD to tSMAD on a log/log scale yielded a line with a slope of 0.6. This was consistent with the preclinical data collected (data not shown). This was

used as the factor of tSMAD to calculate the pSMAD:tSMAD ratio. The variation between donors was great, but the application of the assay is to detect changes exerted by the compound within the same individual. In order for the compound to be deemed effective, it would have to reduce the pSMAD level more than 26.1%, the pooled intra-donor variance. This analysis provided insight for the planning of the clinical trials. As a result of the fairly high total (analytical + biological with biological being the greater of the two) variability of the assay, it was realised that larger sample sizes would be needed to generate meaningful data. We are currently exploring other ways to decrease the variability of the assay, in addition to running all samples as batched patient sets on a single plate to minimise run-to-run variability for each patient.

This paper demonstrates that the novel assay we have designed is a valid biologically relevant assay that allows for the measurement of TGFβ-mediated pSMAD and tSMAD in a surrogate tissue, human PBMCs. The reference standard utilised here is unique in that Sf9 cells were co-transfected with SMAD construct along with an RI construct. The cells were grown in the presence of TGFβ which led to a constitutively phosphorylated SMAD. In addition, this is the first *ex vivo* stimulation of PBMCs to use $TGF\beta_1$ activation to determine downstream affects of modulating therapeutics. Our assay has been further evaluated in a method study involving cancer patients without drug onboard. This method study also yielded a pSMAD:tSMAD linear regression slope of 0.6 consistent with the preclinical and validation data. The intra-patient variability was 47%CV., much higher than 26.1% seen in the validation using normals. Processing the samples in a clinical environment versus the more tightly controlled laboratory environment might have contributed to the increased variation. The design of the method study had three sites with multiple people performing the sample processing. This could have also introduced additional variation. The validation was a nice estimate, but this shows the importance of testing in a cancer population. In addition, this method is currently being used to evaluate the TGFβ RI kinase inhibitor tested here in a Phase 1 clinical trial. There is now a less-invasive, predictive biomarker assay that can evaluate multiple TGFβ signalling modulators.

In the future, it would be advantageous to be able to measure pSMAD in unstimulated PBMCs. The less the sample is manipulated, the more robust the measurement is. This would require a more sensitive pSMAD ELISA in order to be able to detect the analyte at such low levels. We are currently working on a more sensitive pSMAD ELISA assay.

In the meantime, the current assay has undergone clinical validation and is supporting ongoing clinical trials. Currently, three levels of QC (pooled PBMC lysate) are run in the assay in duplicate with the acceptance criteria being that four out of six must be within 25% of their respective QC means and at least one QC from each level must be within range. Compared to the alternative of quantitative Western blotting, the ELISA offers much improved throughput, a broader dynamic range, and better precision, thus making it a suitable choice to support clinical trials enrolling large numbers of patients.



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