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Short communication

A direct ELISA assay for quantitative determination of the inhibitory potency of small molecules inhibitors for JNK3

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ABSTRACT

The c-jun N-terminal kinase 3 (JNK3) is a promising drug target for the treatment of neurological disorders. Here we report a direct ELISA including the optimization of a nonradioactive immunosorbent JNK3 activity assay to determine inhibitory potency of small-molecule inhibitors. Based on our previous JNK3 assay and our recently optimized p38α mitogen activated protein kinase (MAPK) protocol for monitoring the phosphorylation of activating-transcription factor 2 (ATF-2), we present a rapid and straightforward alternative to conventional radioactive and indirect ELISA kinase assays. To validate the assay with the optimized assay conditions we used reference compounds and achieved well comparable IC_{50} results to published data. The use of a linked monoclonal antibody increased the specificity and the sensitivity of the assay, reducing the required antibody concentration by approximately 100-fold. The novel protocol is an accurate, easy-to-handle and robust screening assay for JNK3 and the assay performance was reduced from 7.5 to 3 h.

1. Introduction

The c-jun-N-terminal kinases, members of the stress activated protein kinase family, consist of three isoforms, JNK1, JNK2 and JNK3. The JNKs were first identified as the enzymes responsible for the phosphorylation of the N-terminus of c-Jun, which is a component of the AP-1 transcription factor complex. Other downstream targets of JNK include ATF-2, AP-1, Elk1, p53, and Bcl-2 [1]. Activated JNKs play an important role in a wide range of diseases e.g. neurodegenerative diseases, brain, heart and renal ischemia and inflammatory disorder. JNK3 is primarily expressed in the brain and has been associated with neurological damage such as Parkinson's disease, Alzheimer's disease and stroke, therefore JNK3 is an attractive target for the treatment of neurological disorders [2,3]. Due to the research effort toward the development of small molecule inhibitors in treatment of such neurological disorders, we needed to establish a rapid and reproducible assay protocol for the quantitative determination of the inhibitory potency of small molecule inhibitors for JNK3. Although similar to existing ELISA and radioisotope methods and therefore not novel, this assay has advantages that include good precision, is easier to perform, more accurate (no cross reactivity of the secondary antibody), faster and no need for radioisotopes. For quantitative non-radioactive kinase assays, ELISA is a convenient and frequently used method. The direct detection of the ATF-2 phosphorylation with a peroxidase-labeled antibody was successfully established in a similar p38 MAPK assay [4]. However, assays dealing with bisubstrate enzyme reactions result in (apparent) K_m values for each substrate, which are not constant and mechanistic considerations as part of the assay evaluation have to be made. Important parameters that have to be considered in this assay are the enzyme and the ATP concentration. The IC_{50} values obtained by using the optimized parameters were however not comparable with reference data from literature. Therefore, a fine-tuning of all crucial parameters (concentrations of ATF-2 substrate, ATP, enzyme, antibody and incubation time) was necessary.

2. Experimental

2.1. Materials

Activated JNK3 kinase and the activating-transcription factor 2 (ATF-2) were purchased from ProQinase Tools & Tests GmbH (Freiburg, Germany). Monoclonal anti-phospho-ATF-2 (Thr$^{69/71}$) peroxidase antibody, bovine serum albumin (BSA), dithiothreitol (DTT) and Tween-20 from Sigma–Aldrich GmbH (Kappelweg, Germany). Reference Standard SP600125 and SB203580 were

Abbreviations: TBS, Tris-buffered saline; BB, blocking buffer; BSA, bovine serum albumin; TMB, Substrate A (solution containing hydrogen peroxide) and Substrate B (solution containing 3,3′,4,4′-tetramethylbenzidine); KB, kinase reaction buffer.

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synthesized in our laboratory (purity by HPLC > 98%, structural analysis by NMR, IR, and MS) [5,6]. 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagent set was obtained from BD Biosciences (San Diego, CA). Nunc Maxisorp flat-bottomed plates (96 wells) were purchased from VWR-International GmbH (Bruchsal, Germany). Titrisol® – 2 N sulfuric acid (H_2SO_4) and $MgCl_2 \cdot 6H_2O$ pure were purchased from Merck KGA (Darmstadt, Germany). Dimethylsulfoxide (DMSO), β-glycerolphosphate (ß-Gly), Adenosine-5′-triphosphate disodium salt hydrate (ATP), Tris–HCl, NaCl and Tris-base were purchased from Fluka (Buchs, Switzerland).

2.2. Assay optimization

The aim of this work was to develop and optimize a direct ELISA assay and to define balanced screening conditions for the routine screening of MAPK inhibitors. This method adapted the protocol developed earlier by Peifer et al. [7]. This assay used so far is an indirect ELISA assay using a primary polyclonal anti-phospho ATF-2 antibody to detect the substrate phosphorylation and a secondary polyclonal antibody conjugated with an alkaline phosphatase. To increase the specificity of the assay and eliminate cross reactivities of secondary antibodies, a labeled monoclonal antibody was used to detect the substrate phosphorylation directly, which recognizes a single epitope also contribute to the assay stability. The assay parameters optimized to achieve stable screening conditions included the coating of the ATF-2 substrate to the plate as well as the buffer used for the antibody dilution that guarantees the highest activity of the conjugated peroxidase and thus results in an increase of the signal–noise ratio of the antibody. Also, the amount of JNK3, ATF-2 and the monoclonal anti-phospho ATF-2 ($Thr^{69/71}$) peroxidase antibody in the assay, as well as the incubation time and the ATP concentrations were optimized. Each assay was carried out as described in the protocol by varying the parameter to be determined. The reaction volume added in each well and each step of the assay was 50 μl. The amount of ATF-2 was determined using a fixed, non-limiting ATP concentration (150 μM) keeping the kinase at 10 ng/50 μl reaction volume. The substrate concentrations were tested from 1.25 to 40 μg/ml. The monoclonal anti-phospho ATF-2 ($Thr^{69/71}$) peroxidase antibody was titrated (1:500 to 1:64,000), keeping the other reaction parameters constant. The K_m ATP was measured at a saturating concentration of substrate ATF-2 (20 μg/ml) keeping the kinase concentration constant at 200 ng/ml and varying the ATP concentration from 0.01 μM to 1000 μM. The enzyme concentration was determined by varying the concentrations from 6.25 to 1600 ng/ml, otherwise keeping the optimized assay conditions. Last but not least, the assay was run varying the incubation time and keeping the optimized assay parameters (10 μg/ml ATF-2, 200 ng/ml JNK3, 10 μM ATP and anti-phospho ATF-2 $Thr^{69/71}$ – 1:5000). To achieve the desired intensity of color from the peroxidase reaction, the pH of blocking buffer (BB) was adjusted with 1 M HCl for the antibody dilution. Three buffer solutions were prepared with pH values of 7.5, 7.0 and 6.5. To determine the best incubation time for immobilize the substrate ATF-2 onto the wells, plates were incubated overnight at 4 °C or for 1.5 h at 37 °C.

2.3. JNK3 assay protocol

A 96 well plate was coated with 0.5 μg/50 μl in each well of the JNK3 substrate ATF-2 (1:192 in TBS buffer pH 7.8) and incubated overnight at 4 °C. The plate was blocked with blocking buffer (BB) (TBS buffer (pH 7.8), 0.025% BSA and 0.05% Tween-20) for 30 min at room temperature. A kinase reaction buffer (KB) (500 μl of 1 M Tris (pH 7.5), 100 μl of 1 M $MgCl_2$, 100 μl of 1 M ß-Glycerolphosphate, 100 μl of 10 mg/ml BSA solution, 10 μl of 1 M DTT, 10 μl of 10 mM ATP and 10 μl of 100 mM Na_3VO_4 and 9170 μl double-distilled water) was prepared and used to diluted the kinase 1:1055 to generate a final concentration of 10 ng/50 μl. 50 μl/well were transferred to the plate with ATF-2 and incubated for 50 min at 37 °C. After this incubation time the plate was blocked for 15 min again. 60 min of incubation time were used to determine the individual parameters before determining the optimum incubation time. The ATF-2 phosphorylation was detected with a monoclonal anti-phospho ATF-2 ($Thr^{69/71}$) peroxidase antibody. The antibody was diluted 1:5000 in BB (TBS buffer – pH 6.5, 0.025% BSA and 0.05% Tween-20) and 50 μl of antibody solution added into each well and incubated for 1 h at 37 °C. After each incubation step the wells were washed three times again with double-distilled water and dried for 5 min at 37 °C. The TMB substrate (50 μl/well) used to detect the anti-phospho-ATF-2 complex was incubated 5 to 10 min at room temperature. The reaction was stopped with 25 μl of 2 N H_2SO_4 per well. The absorbance was measured at 450 nm using an ELISA reader equipped with SOFTmax PRO software.

2.4. Assay validation

After the determination of the optimal concentration of each assay parameters, the assay was validated using the conventional JNK3 inhibitors SP600125 and SB203580. A schematic representation of the assay performance is shown in Fig. 1. For the IC_{50} values determination, a stock solution of the reference standards (10 mM in DMSO) was diluted in a kinase reaction buffer (KB) with enzyme and ATP on ice to get the appropriate concentration (10 μM, 1 μM, 0.1 μM and 0.001 μM) and 50 μl of each concentration was dispensed in triplicate into the microplate wells. As positive control (STIM) KB with enzyme was used in triplicate, in the absence of inhibitor. To determine the non-specific binding (NSB) of the antibody to non-phosphorylated ATF-2 or other possible interferences, a KB without enzyme was used as negative control (NSB). The NSB value is the measured absorbance of unspecific bindings and was subtracted from the measured absorbance of STIM and samples. The percent of inhibition is calculated with the following equation:

$$\text{Inhibition}\,(\%) = 100 - \left(\frac{OD_{SAMPLE} - NSB}{OD_{STIM} - NSB}\right) \times 100$$

The IC_{50} values were determined and compared with the reported literature data. The intra-assay precision was assessed by analyzing 3 replicates of each sample in a single run. The inter-assay precision was analyzed in triplicate in 10 or 3 separate run for SP600125 and SB203580, respectively.

3. Results and discussion

3.1. Assay optimization

The first step of this assay is the adsorption of the antigen on the plate. With an overnight incubation a better immobilization could be determined. The signal after 1.5 h was good (OD 0.428 ± 0.042), however the signal increased by ~30% with an overnight incubation (OD 0.637 ± 0.022) probably due to better adsorption of the antigen to the plate surface and was therefore chosen for the assay. To achieve the desired intensity of color from the peroxidase reaction, the optimum pH of the blocking buffer used for the dilution of the antibody was determined. This guarantees the highest activity of the conjugated peroxidase and thus results in an increase of the signal–noise ratio of the antibody. At pH 6.5 the peroxidase reaction developed a good signal in fast time and was therefore selected as optimum for the substrate phosphorylation detection (Fig. 2A).

Assays dealing with bisubstrate enzyme reactions result in (apparent) K_m values for each substrate that are not constant and mechanistic considerations as part of the assay evaluation have

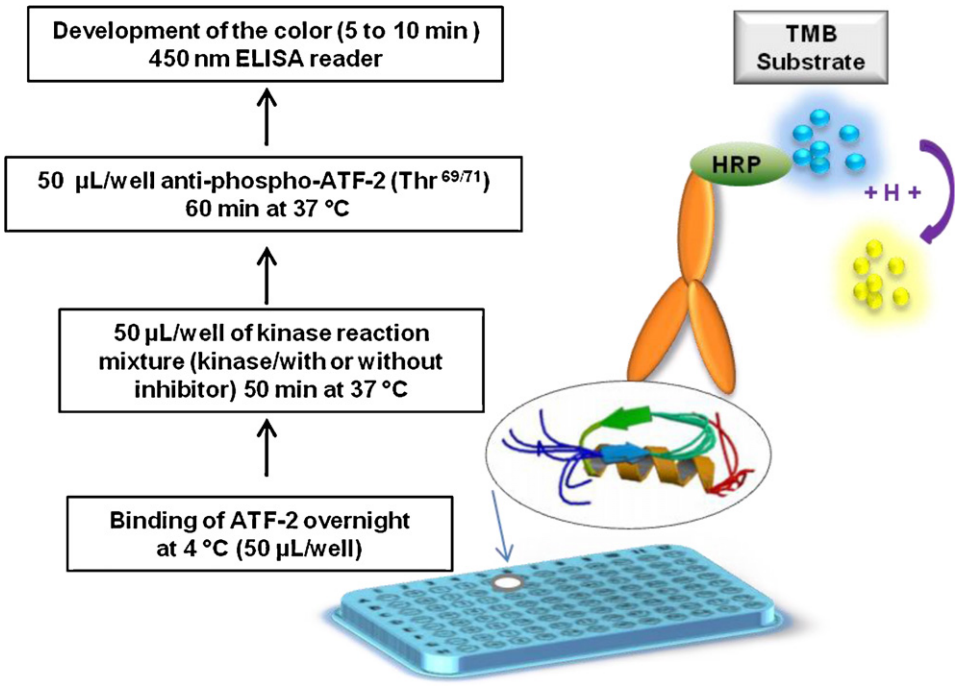

Fig. 1. Performance of the direct JNK3 ELISA assay: immobilization of ATF-2, addition of the kinase and samples, detection of the dually phosphorylated ATF-2 with a specific antibody and measurement of the developed signal by ELISA equipment.

to be made [8]. Ultimately, the degree of phosphorylated ATF-2 inversely correlates with the inhibitory potency of the inhibitor [9]. The concentration of ATF-2 must be high enough to act as phosphate acceptor for the kinase reaction and sufficient to be not a limiting factor. In this assay the concentration used so far can be maintained. 10 μg/ml of ATF-2 became limiting at the enzyme and incubation time chosen (Fig. 2B).

The use of a monoclonal antibody which recognizes a single epitope may also contribute to the assay stability when compared to polyclonal antibody preparations with varying affinities for an epitope [10]. Direct labelling of the primary antibody avoids the use of a secondary antibody, thereby eliminating potential cross-reactivity, issues and speeding up the protocol. So, a new monoclonal anti-phospho-ATF-2 ($Thr^{69/71}$) peroxidase antibody was selected, which reacts specifically with dually phosphorylated ATF-2 (at both Thr^{69} and Thr^{71}) detecting neither the mono nor the unphosphorylated substrate. A good linear increase of the signal was found over the dilution range tested (not saturated). A dilution of 1:5000 which is well in the linear range and yields a good signal–noise ratio was considered appropriate (Fig. 2C). The monoclonal antibody concentration (~40–80 ng/ml) required to detect the phosphorylated ATF-2 was reduced approximately 100-fold in comparison to the polyclonal antibody (40 μg/ml) used before.

Our assay system was set up to measure inhibitors that compete with ATP for the same binding pocket in the catalytic site of JNK3. For such competitive inhibitors the ATP concentration is critical. As different kinases have different ATP binding affinities, the particular concentration has to be optimized individually. A high ATP concentration results in a rapid phosphorylation of the substrate ATF-2 and high ATP concentrations also displace the inhibitor from the catalytic site. Thus, for each and every reaction the optimal ATP concentration used in an assay should be close to the K_m value or maximum the double of it. If a too low or too high ATP concentration is chosen false positive or false negative inhibitory activities of a given test compound may be detected [8]. The half saturation (50%) concentration lies at 5 μM; 10 μM were chosen for the final test setup, the double K_m value (Fig. 2D).

The optimum JNK3 concentration for this assay should be within the linear range to give the maximum sensitivity. The assay was linear from 12.5 up to 800 ng/ml; for screening purposes the enzyme concentration was set 200 ng/ml (Fig. 2E).

The incubation time should lie within the linear range of the reaction, in which the measurements are most sensitive in the presence of inhibitor [8,11]. If the incubation time is too long, no difference between the stimulation values (maximum enzyme activity) and the samples containing inhibitor is measurable. This leads to an increase in the IC_{50} values for the corresponding inhibitor. As shown in Fig. 2F, an absolute saturation is not achieved between 60 and 90 min. The incubation time of 50 min was selected as a good compromise between linearity and signal strength for this assay.

3.2. JNK3 inhibition assay validation

In a previously JNK3 assay [7] a K_m ATP of 5.54 μM was determined which agreed well with the determined in our experiments now (5 μM). However, by Peifer et al. for the initial characterization of the ATP-competitive inhibitors the assay was run at an ATP-substrate concentration of 10 μM (According to 1.8-fold K_m ATP) and the determined IC_{50} values did not match the published IC_{50} values. Therefore Peifer et al. used an ATP concentration of 1 μM to achieved IC_{50} values comparable to published reference compounds (Table 1).

After optimizing the assay parameters (10 μg/ml ATF-2, 200 ng/ml JNK3, 50 min of incubation time, 10 μM ATP (double of the K_m ATP) and monoclonal anti-phospho ATF-2 antibody diluted 1:5000 in BB – pH 6.5) the IC_{50} values for the reference compounds SB203580 [12,13] and SP600125 [3,7,14] were determined and compared with the literature data. The intra-assay precision for the reference compounds was determined by analyzing 3 replicates of a single run; the inter-assay precision using the results from 10 or 3 separate run for SP600125 and SB203580, respectively (each in triplicates). The obtained values for standard deviation (SD) are lower than 5% and a coefficient of variation (CV) ≤ 20% met the

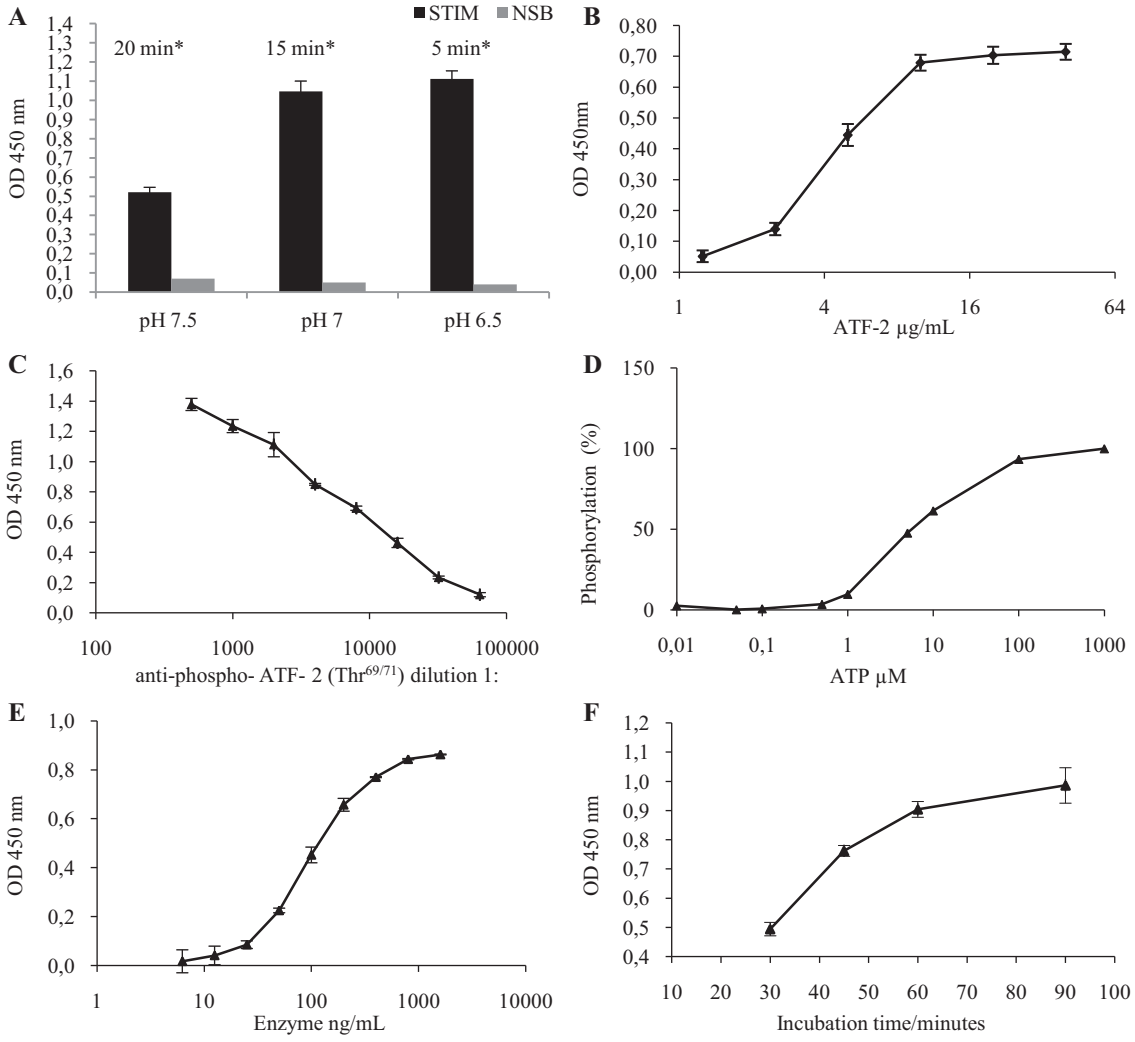

Fig. 2. Determination of the assay conditions: (A) Optimum pH for the antibody solution. A lower pH (pH 6.5) increased the peroxidase activity resulting in a higher signal and NSB values decreased with the pH. STIM values are represented as maximum activity achieved in the presence of JNK3 and ATP. NSB values are representing a non-specific binding in the absence of JNK3. *Time required to achieve the measured signal; (B) ATF-2 titration, 10 µg/ml; (C) titration of the monoclonal anti-phospho ATF-2 ($Thr^{69/71}$) peroxidase antibody, 1:5000; (D) K_m value for ATP, 5 µM; (E) titration of the kinase JNK3, 10 ng/50 µl; (F) determination of the incubation time for the JNK3 assay, 50 min.

acceptance criteria, thus establishing the quality of the assay. A good agreement with reported literature data could be achieved (Table 1). The determined IC_{50} values for the reference compound SB203580 are in better agreement with the literature as compared with the assay used previously.

Table 1
Determined IC50 values (µM), statistic and comparison of the data with the literature.

Reference compound	SP600125	SB203580
Literature data	0.150–0.190 [3,11]	0.790 ± 0.150 [9,10]
Peifer et al.	0.165 µM ± 0.03 (1 µM ATP) 0.210 µM (10 µM ATP)	1.11 µM ± 0.08 (1 µM ATP) 2.20 µM (10 µM ATP)
Optimized assay (mean µM)		
Inter-assay	0.186 (n = 10)	0.720 (n = 3)
Intra-assay	0.180 (n = 3)	0.727 (n = 3)
Standard deviation (SD)		
Inter-assay	0.02	0.11
Intra-assay	0.02	0.05
Coefficient of variation (CV)		
Inter-assay	9.83	15.31
Intra-assay	8.73	7.10

4. Conclusions

The present work describes the development and optimization of a direct ELISA assay for the determination of the inhibitory potency of small molecule inhibitors against JNK3. The use of a monoclonal anti-phospho ATF-2 ($Thr^{69/71}$) peroxidase antibody reduced the required antibody concentration to detect the substrate phosphorylation by ~100-fold, increasing the specificity and the sensitivity of the assay, making this method a direct ELISA assay. The ATP concentration, as a key parameter to screen ATP competitive inhibitors, was used in the recommended range and achieves results well comparable to literature data. Besides, the assay showed a good precision, is easier to perform, more accurate (no cross reactivity of the secondary antibody) and faster (3 h of total assay time) as compared to the reference assay. The method is expected to contribute to the investigation of neurodegenerative diseases through the identification of potent JNK3 inhibitors.

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