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Evaluation of [3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2, 3-dihydro-1H-indole-5-sulfonamide] (OXSI-2), as a Syk selective inhibitor in platelets

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Abstract

In the present study, we characterized OXSI-2 ([3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2, 3-dihydro-1H-indole-5-sulfonamide], a putative inhibitor of Syk, and determined its specificity and selectivity in platelets. We found that OXSI-2 completely abolished convulxin-induced platelet functional responses. In order to determine whether OXSI-2 inhibited Src family kinase-mediated platelet responses, we evaluated its effect on Src family kinase (SFK)-mediated signaling events in platelets, viz. Lyn-mediated phosphorylation of Y352 on Syk, LAT- Y191 phosphorylation by Syk, and protease-activated receptor (PAR)-mediated phosphorylation of ERK. In the present work, we report that convulxin mediated Syk tyrosine 352 phosphorylation is not inhibited by OXSI-2, whereas piceatannol and PP2 abolished it. Syk-mediated Y191 LAT phosphorylation is abolished by all the three inhibitors. AYPGKF-induced phosphorylation of ERK was marginally inhibited by OXSI-2, whereas treatment with PP2 and piceatannol completely abolished it. However, PAR mediated thromboxane generation (an event mediated by ERK) was potentiated by OXSI-2 whereas PP2 and piceatannol brought thromboxane to basal levels. Protein kinase C (PKC) inhibitors are known to potentiate PAR-mediated thromboxane generation in platelets. In contrast, OXSI-2, unlike PKC inhibitors, did not inhibit secretion. Therefore, we conclude that OXSI-2 is not a Syk-selective inhibitor in platelets because of its unexplained non-specific effects.

Index words

OXSI-2; Syk; Src; GPVI signaling; PAR and Piceatannol

1. Introduction

Platelets play a crucial role in the processes of hemostasis and thrombus formation. Platelets are activated after agonists bind to their membrane receptors, and initiate intracellular signaling events causing reorganization of platelet cytoskeleton, aggregation, granule secretion and thromboxane generation.

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Upon vessel wall injury, platelet activation is triggered by collagen in subendothelial matrix (Alberio and Dale, 1999; Barnes et al., 1998; Nieswandt and Watson, 2003). When platelets adhere to collagen, they release granular components and activate other platelets in the vicinity, which in turn amplify platelet responses and mediate thrombus formation. Collagen signals via Glycoprotein (GP) VI and $\alpha 2\beta 1$ receptor (Alberio and Dale, 1999; Nieswandt and Watson, 2003). Collagen-related peptide (CRP) and convulxin, a snake venom lectin, are other GPVI agonists (Nieswandt and Watson, 2003). Collagen mediated activation of platelets results in the generation of thromboxane A_2 (TXA₂) in the cell and generation of thrombin on the activated platelet surface. TXA₂ activates platelets through prostanoid TP receptors (Hourani and Cusack, 1991; Murugappan et al., 2004a; Paul et al., 1999; Shen and Tai, 1998) whereas thrombin activation of platelets is mediated by protease-activated receptors (PARs) (Coughlin, 2000). Human platelets express PAR1 and PAR4 which are activated by their activating peptides SFLLRN and AYPGKF, respectively (Coughlin, 2000; Sambrano et al., 2001). Collagen, TXA₂, and thrombin cause the release of dense granule contents from platelets and this event has been shown to be mediated through protein kinase C (PKC) activation (Murugappan et al., 2004b; Werner et al., 1991).

Syk (Spleen Tyrosine Kinase) is a 72 kDa non-receptor tyrosine kinase that contains tandem SH2 domains. Syk exhibits a widespread expression pattern and is implicated in various signaling cascades both in hematopoietic and non-hematopoietic cells (Yanagi et al., 1994). Upon phosphorylation, Syk binds to immunoreceptor tyrosine based activated motif (ITAM) of FcR γ chain and mediates downstream signaling (Sada et al., 2001; Siraganian et al., 2002; Yanagi et al., 1994). For example, activation of Syk in platelets by thrombin and TXA₂ results in its translocation to cytoskeleton (Clark et al., 2003; Sada et al., 2001). Syk then undergoes additional phosphorylation by Src family kinases (SFKs) for its activation (Kurosaki et al., 1994). Lyn, a member of Src family kinases, is one of the first kinases to be activated upon GPVI activation, leading to phosphorylation of ITAM motifs on FcR γ chain, recruitment of Syk to the ITAM and subsequent phosphorylation of Syk on tyrosine 352 (Bruyninckx et al., 2001). Functionally, Syk plays an important role in inflammatory cell signaling in mast cells and basophils, phagocytosis in macrophages and a variety of other functions in immune cells (Law et al., 1999). Syk^{-/-} mice have a lethal phenotype associated with petechiae, diffuse hemorrhage and chylous ascites (Cheng et al., 1995; Law et al., 1999; Siraganian et al., 2002). In platelets, Syk is known to be activated downstream of PAR and GPVI receptors (Yanaga et al., 1995; Yanagi et al., 1994). However gene deletion of Syk had no effect on fibrinogen induced signaling (Law et al., 1999).

Currently, piceatannol (3,4,3', 5'-tetrahydroxy-trans stilbene) (Zheng and Ramirez, 1999) is the only available Syk inhibitor. However, its use as a pharmacological inhibitor of Syk is limited by its non-selective effects on Src family kinases (Law et al., 1999; Miura et al., 2001; Mocsai et al., 2000). Recently, an oxindole compound [3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2, 3-dihydro-1H-indole-5-sulfonamide] (OXSI-2) has been reported as potent inhibitor of Syk (Law et al., 1999). In this present study, we evaluated the effect of OXSI-2 on platelet functional responses and intracellular signaling events. Our work shows that OXSI-2 inhibits Syk-mediated events in platelets; however, OXSI-2 also causes some non-selective effects. Therefore, we conclude that OXSI-2 is not suitable as a Syk selective inhibitor for analysis of its role in platelets.

2. Materials and Methods

2.1 Materials

2MeSADP, Apyrase grade VII, human fibrinogen, acetylsalicylic acid, GR144053 were obtained from Sigma (St.Louis, MO). Luciferin-luciferase reagent was purchased from Chrono-Log (Havertown, PA). Hexapeptide AYPGKF was custom synthesized at Invitrogen

(Carlsbad, CA). Convulxin was purchased from Centerchem Inc. (Norwalk, CT). Phospho-specific antibodies against Y352 Syk, LAT Y191 and ERK, and total ERK antibodies were obtained from Cell signaling technologies (Beverly, MA). Antibodies against total PKC δ , LAT and Syk were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). PP2 and PP3 were purchased from Biomol (Plymouth Meeting, PA). Phospho-PLC γ antibody that recognizes p-Y753 and p-Y759 was prepared in our laboratory (Ozdener et al., 2002). All other reagents were of reagent grade.

2.2. Preparation of washed human platelets

Whole blood was drawn from healthy, human volunteers selected from students, staff or workers at the Temple University with informed consent. Donated blood was collected in tubes containing one-sixth volume of acid citrate dextrose (ACD) (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of de-ionized water). Citrated blood was centrifuged (Eppendorf 5810R centrifuge, Hamburg, Germany) at 230 RCF for 20 min at room temperature (RT) to obtain platelet-rich plasma. If required, the platelet rich plasma was incubated with 1 mM acetylsalicylic acid (aspirin) for 30 min at 37°C, and then allowed to remain at RT for 15 min. The platelet rich plasma was then centrifuged for 10 min at 980 RCF at RT. Platelet pellet was re-suspended in calcium-free Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 0.42 mM NaH₂PO₄, 5 mM glucose, 10 mM HEPES, and 0.2% bovine serum albumin adjusted to pH 7.4) containing 0.01 units/ml apyrase. Cells were counted using the Z1 Coulter Particle Counter and adjusted to the desired concentration.

2.3. Aggregometry

Aggregation of 0.5 ml of aspirin treated, washed platelets were analyzed using a PICA lumiaggregometer (Chrono-log, Havertown, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37°C (Garcia et al., 2005). Agonists were added simultaneously for platelet stimulation. However, platelets were preincubated with each inhibitor (where noted) as follows, DMSO (vehicle control), OXSI-2 (2 μ M), Piceatannol (20 μ g/ml), PP3 (10 μ M), PP2 (10 μ M) for 5 min at 37°C. Each sample was allowed to aggregate for at least 3 min. Aggregation tracings are representative of results obtained from 3 separate experiments using platelets from 3 different donors.

2.4. Measurement of platelet dense granule secretion in human platelets

ATP secretion from platelet dense granules was determined in aspirin-treated, washed human platelets by using the Luciferin-Luciferase assay (Murugappan et al., 2004b). The platelets were stimulated in a lumi-aggregometer at 37°C with stirring at 900 rpm, and the corresponding luminescence was measured. The data is represented in the form of actual secretion tracings.

2.5. Western blotting

Aliquots of aspirin-treated, washed human platelets were lysed using Laemmli buffer in presence of dithiothreitol (DTT) (100 mM) and boiled for 10 min. The platelet lysates were loaded on to a 12% -Tris-glycine gel, subjected to SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), and transferred to PVDF membrane. Nonspecific binding sites were blocked by incubating the membrane in Tris-buffered saline-Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% (vol/vol) Tween 20) containing 3% (wt/vol) bovine serum albumin (BSA) and 5% (vol/vol) Irish cream for 30 min at RT, followed by incubating it overnight at 4°C with gentle agitation in the primary antibody (1:1000 dilution for Anti-Syk Y352, Anti-LAT Y191, Anti-phospho-ERK, Anti-Syk, Anti-LAT Anti-PKC δ , and Anti-ERK in TBST with 3% BSA). After washing with TBST, the membranes were probed with an alkaline phosphatase-labeled secondary antibody (1:5000 dilutions in TBST with 3% BSA) for 1 hour at RT. After additional washing steps, membranes were incubated with

chlordiazepoxide (CDP)-Star® chemiluminescent substrate (Tropix, Bedford, MA) for 10 min at RT, and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-3000 CH; Tokyo, Japan).

2.6. Measurement of TXA₂ generation in human platelets

Washed human platelets (500 µl brought to a concentration of 2×10^8 platelets/ml) were stimulated with AYPGKF (500 µM) as mentioned in an lumi-aggregometer at 37°C with stirring at 900 rpm, in the presence or absence of Piceatannol (20 µg/ml), OXSI-2 (2 µM), PP3 (10 µM), and PP2 (10 µM). After 3.5 min of stimulation, the reaction was stopped by quickly freezing the sample in a dry ice-methanol bath. Samples were stored at -80°C and were brought to RT prior to analysis (Shankar et al., 2006). The samples were centrifuged for 10 min at 4°C at 15,000g to remove lysed platelets. The supernatants of human platelets were diluted with standard diluent (assay buffer). The level of TXB₂, the stable metabolite of TXA₂ was measured in duplicates using the Thromboxane B₂ Enzyme Immunoassay Kit according to the manufacturer's instructions (Assay Designs Inc, Ann Arbor, MI). Data represents mean of at least three experiments ± standard deviation.

3. Results

3.1 Effect of OXSI-2 on Convulxin-induced platelet functional responses

OXSI-2 is a recently identified Syk inhibitor (Law et al., 1999). We evaluated its effects in platelets using convulxin, a GPVI agonist, as Syk plays an important role downstream of GPVI signaling (Zheng and Ramirez, 1999). We investigated the minimal concentration of the inhibitor required for complete blockade of Syk-mediated platelet functional responses. As shown in Fig. 1A, OXSI-2 completely inhibited convulxin-induced platelet aggregation and shape change at 2 µM. At this concentration OXSI-2 also completely blocked GPVI-mediated dense granule release Fig.1B. At 100 nM, OXSI-2 did not affect the platelet functional responses induced by convulxin (Fig.1), and modest shape change was still evident at 1 µM.

3.2. Comparison of OXSI-2 with other Syk and Src family kinase inhibitors in platelet functional responses

Syk and SFKs are involved in the phosphorylation and activation of PLCγ2, downstream of GPVI signaling (Mangin et al., 2003; Ozdener et al., 2002). We evaluated the effect of OXSI-2 on PLCγ2 phosphorylation in comparison with other Syk and SFK inhibitors. Phospho-PLCγ2 antibody was prepared in our laboratory (Ozdener et al., 2002), which is an activation state-specific antibody that recognizes p-Y753 and p-Y759. As shown in Fig. 2A, OXSI-2 (2 µM), piceatannol (20 µg/ml) and PP2 (10 µM) completely inhibited convulxin-induced platelet aggregation. At these concentrations, both OXSI-2 and PP2 completely blocked PLCγ2 tyrosine phosphorylation, whereas the control compound PP3 had no effect. The non-selective Syk inhibitor, piceatannol significantly, but not completely, blocked PLCγ2 tyrosine phosphorylation. These data indicate that OXSI-2 inhibits PLCγ2 tyrosine phosphorylation downstream of GPVI signaling. However, its effects on SFKs cannot be entirely ruled out, as SFKs regulate Syk activity since OXSI-2 inhibits PLCγ2 phosphorylation to the same extent, as does PP2.

3.3. Effect of OXSI-2 on Convulxin -induced tyrosine phosphorylation of Y 352 of Syk

Lyn is known to phosphorylate Syk on Y352 (Bruyninckx et al., 2001). To determine if OXSI-2 exerts inhibitory effects on SFKs, we evaluated whether OXSI-2 treatment blocks Lyn-mediated phosphorylation of Syk at Y352. As shown in Fig. 3, convulxin-induced Y352 phosphorylation is not inhibited by OXSI-2 (2 µM), and the lack inhibition was evident from the densitometric analyses which showed a P value of 0.3. In contrast, piceatannol (20 µg/ml),

and PP2 (10 μ M) completely abolished Lyn mediated Syk Y352 phosphorylation. These data indicate that OXSI-2 does not inhibit Lyn; it is, however, unclear whether OXSI-2 inhibits other SFKs expressed in platelets.

3.4. Effect of OXSI-2 on Syk-mediated LAT Y191 phosphorylation

Adaptor protein LAT is a known substrate of Syk Kinase (Galli et al., 2005). In order to determine the specificity of OXSI-2, we tested the effect of OXSI-2 on Y191 phosphorylation of LAT. Of the inhibitors tested, PP2, piceatannol and OXSI-2 completely inhibited LAT Y191 phosphorylation (Figure 4). These results suggest that OXSI-2 inhibits Syk mediated events in platelets.

3.5. Effect of AYPGKF induced ERK phosphorylation and thromboxane generation

ERK 1/2 has been shown to be phosphorylated by SFKs, downstream of G protein-coupled receptor agonists in platelets and plays a key role in thromboxane generation (Garcia et al., 2005; Shankar et al., 2006). Hence we evaluated the effect of OXSI-2 on ERK phosphorylation and compared it to the treatments with piceatannol and PP2. OXSI-2 marginally inhibited AYPGKF-induced ERK 2 phosphorylation ($P=0.12$), whereas both piceatannol and PP2 dramatically blocked ERK 2 phosphorylation (Fig 5A). Moreover, piceatannol and OXSI-2 had exact opposite effects on AYPGKF-induced thromboxane generation. Whereas OXSI-2 potentiated thromboxane generation (Fig. 5B), piceatannol completely blocked thromboxane generation. PP2 (10 μ M) also inhibited AYPGKF-induced thromboxane generation. These data indicate that OXSI-2 elicits this effect through non-specific inhibition of some hitherto uncharacterized signaling molecules.

3.6. Effect of OXSI-2 on PAR-mediated dense granule release in platelets

Our previous studies demonstrated that inhibition of protein kinase C potentiates thromboxane generation (Shankar et al., 2006). As OXSI-2 seems to potentiate thromboxane generation, we evaluated its effect on PKC using dense granule release as a read out. AYPGKF-induced dense granule secretion was completely blocked by pan-PKC inhibitor GF109203X (10 μ M), whereas OXSI-2 did not affect ATP secretion (dense granule release) as shown in Fig. 6. These results indicate that OXSI-2 does not inhibit PKCs.

4. Discussion

Syk signaling is markedly complex and is not completely understood in platelets. Syk is an important signaling molecule downstream of GPVI/FcR γ chain, FcRIIA, and GPIb in platelets (Yanaga et al., 1995). In addition, Syk is activated downstream of PARs and integrins in platelets (Law et al., 1999; Yanagi et al., 1994). The main impeding factors in thorough understanding of Syk signaling are the lack of specific pharmacological inhibitors (Law et al., 1999) and the embryonic lethality of the Syk null mice. The most commonly used Syk inhibitor, piceatannol (Zheng and Ramirez, 1999), has been reported to inhibit FAK, Src, and other tyrosine kinases in platelets (Law et al., 1999). Further, Piceatannol also failed to inhibit Syk in other cell systems such as basophils and neutrophils, and also exhibits other non-specific effects in these cells (Miura et al., 2001; Mocsai et al., 2000). Recently a new class of Syk inhibitor OXSI-2 (Law et al., 1999) was reported, and here we characterized its specificity towards Syk in platelets. We could not compare OXSI-2 with another Syk inhibitor R406 as we were unable to procure the inhibitor from the manufacturer.

OXSI-2 at the minimal concentration (2 μ M) abrogated several known Syk-dependent platelet functions and signaling events downstream of GPVI. In order to confirm that the inhibitory effects of OXSI-2 are at Syk but not at upstream SFKs, we evaluated its effect on known SFK dependent tyrosine phosphorylations in platelets. OXSI-2 did not inhibit Lyn mediated Y352

phosphorylation of Syk. OXSI-2, PP2, and piceatannol inhibited convulxin-induced phosphorylation on Y191 of LAT. Furthermore, ERK phosphorylation was marginally inhibited by OXSI-2, whereas PP2 and piceatannol dramatically inhibited ERK phosphorylation. In addition, PAR-mediated TXA₂ generation was potentiated by OXSI-2 whereas piceatannol and PP2 inhibited thromboxane generation. However, OXSI-2 did not have any effect on dense granule secretion in which PKCs are known to play an important role.

The inhibitory effects of OXSI-2 on GPVI-mediated events could occur due to its specific inhibition of Syk as well as possible non-specific effects on SFKs or other downstream kinases. For example OXSI-2 does not inhibit Lyn mediated Syk Y352 phosphorylation but on other hand marginally inhibits SFK-dependent ERK phosphorylation downstream of PARs. In contrast, OXSI-2 potentiates thromboxane generation downstream of PARs, whereas PP2 and piceatannol exert the exact opposite effects. These results are a clear indication that OXSI-2 acts on poorly understood cellular targets, other than the inhibition of Syk. We have shown that pan PKC inhibitors could potentiate thromboxane generation in platelets (Shankar et al., 2006). However, the potentiating effects of OXSI-2 on thromboxane generation are not due to its effects on PKCs, as this inhibitor did not affect dense granule secretion, a PKC mediated event. We conclude that OXSI-2 has non-specific effects in platelets, other than its effects on Syk, and hence may not be used as a selective Syk inhibitor in platelets.

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Fig. 1. Effect of OXSI-2 on Convulxin-induced platelet functional responses

Aspirin-treated, washed platelets were pre-treated with various concentrations of OXSI-2 for 5 min at 37°C and stimulated with Convulxin (100 ng/ml). A) Aggregation and B) dense granule secretion were measured and representative tracings are shown.

Fig. 2. Comparison of OXSI-2 with other Syk and Src family kinase inhibitors in platelet functional responses

Aspirin-treated, washed platelets were pre-treated with OXSI-2 (2 μ M), Piceatannol (20 μ g/ml), PP3 (10 μ M), and PP2 (10 μ M) for 5 min at 37°C then stimulated with convulxin (100 ng/ml) and A) aggregation and B) PLC γ 2 phosphorylation were measured as outlined in the methods section. For western blotting, platelets were stimulated for 1 minute under stirring conditions in presence of a fibrinogen receptor antagonist GR144053 (200 nM) and the reaction was stopped by using 3X Lamelli buffer and the samples were western blotted for phospho-PLC γ 2. Total PKC δ was used as lane loading control from the same blots.

Fig. 3. Effect of OXSI-2 on Convulxin-induced tyrosine phosphorylation of Y 352 of Syk
 Aspirin-treated, washed human platelets were stimulated with convulxin (100 ng/ml) in the presence of GR144053 (200 nM) for 1.0 minute at 37°C under stirring conditions in presence and absence of DMSO (vehicle control), OXSI-2 (2 µM), Piceatannol (20 µg/ml), PP3 (10 µM) and PP2 (10 µM). The reaction was stopped by using 3X sample Lamelli buffer. The lysates were then subjected to western blotting analysis and probed with anti- phospho- Syk (Y352), and total Syk antibodies as lane loading control from same blots.

Fig. 4. Effect of OXSI-2 on Syk (Y 352) mediated LAT (Y 191) phosphorylation

Aspirin-treated, washed human platelets were stimulated with Convulxin (100 ng/ml) in the presence of GR144053 (200 nM) for 1.0 minute at 37°C under stirring conditions in presence and absence of DMSO (vehicle control), OXSI-2 (2 μM), Piceatannol (20 μg/ml), PP3 (10 μM) and PP2 (10 μM). The reaction was stopped by using 3X Lamelli buffer. The lysates were then subjected to western blotting analysis and probed with anti- phospho- LAT (Y 191), and total LAT antibodies as lane loading control from same blots.

Fig. 5. Effect of OXSI-2 on agonist-induced ERK phosphorylation and thromboxane generation
Aspirin-treated, washed human platelets were stimulated with AYPGKF (500 μ M) in the presence of GR144053 (200 nM) for 1.0 minute at 37°C under stirring conditions in the presence and absence of DMSO (vehicle control), OXSI-2 (2 μ M), Piceatannol (20 μ g/ml), PP3 (10 μ M) and PP2 (10 μ M). The reaction was stopped by using 3X Lamelli buffer. A) The lysates were subjected to western blotting analysis and probed with antibodies against phospho-ERK and total ERK.

Washed, non- aspirin treated human platelets were stimulated with AYPGKF (500 μ M) for 3.5 min at 37°C under stirring conditions in the presence and absence of DMSO (vehicle control) OXSI-2 (2 μ M), piceatannol (20 μ g/ml), PP3 (10 μ M), and PP2 (10 μ M), reactions were stopped

by snap freezing after 3.5 min and B) TXB₂ levels were analyzed as described in “Materials and Methods”. The data are represented as the TXB₂ (pg/ ml) generated.

Fig. 6. Effect of OXSI-2 on PAR-mediated dense granule release in platelets

Aspirin treated, washed platelets were stimulated with AYPGKF (500 μ M) in the presence of DMSO (vehicle control), GF109203X (10 μ M), or OXSI-2 (2 μ M). The corresponding ATP release in each sample was recorded and represented as tracings.