

Sanguinarine suppresses IgE induced inflammatory responses through inhibition of type II PtdIns 4-kinase(s)



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ABSTRACT

The effects of sanguinarine on IgE mediated early signaling mechanisms leading to inflammatory mediators release were investigated. Pretreatment of RBL 2H3 cells with sanguinarine inhibited IgE induced activation of type II PtdIns 4-kinase activity. Concomitant with type II PtdIns 4-kinase inhibition, sanguinarine also inhibited IgE induced degranulation and β hexosaminidase release in RBL 2H3 cells. *In vitro* assays showed sanguinarine inhibited type II PtdIns 4-kinase activity in a dose dependent fashion with no effect on PtdIns 3-kinase activity. Fluorescence spectroscopic studies suggested that sanguinarine binds to type II PtdIns 4-kinases α and β isoforms with a K_d of 2.4 and 1.8 μ M, respectively. Kinetic studies showed that sanguinarine competes with PtdIns binding site of type II PtdIns 4-kinase β . These results suggest that the anti-inflammatory effects of sanguinarine on PtdIns 3-kinase signaling pathway are more likely an indirect effect and emphasize the importance of the cross talk between type II PtdIns 4-kinases and PtdIns 3-kinases.

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Introduction

Sanguinarine, a plant derived quaternary benzo[c]phenanthridine alkaloid (Fig. 1) has several biological properties such as anti-inflammatory, anti-cancer, anti-oxidant, anti-bacterial, anti-fungal, anti-viral and anti-protozoan activities [1–5]. The physiological target molecules for sanguinarine have not been clearly identified. Several key molecules and pathways involved in inflammatory responses, cell cycle and apoptosis are affected by sanguinarine [5–9]. Pretreatment of neutrophils with sanguinarine has shown to inhibit degranulation, oxidative burst and phagocytosis [10–12]. In human prostate cancer cell lines, sanguinarine induces cell cycle arrest at G0/G1 and trigger apoptosis [13]. Sanguinarine also inhibits VEGF induced AKT phosphorylation and angiogenesis suggesting that PtdIns 3-kinase signaling pathway may be preferentially affected [14].

However, the effects of sanguinarine on classical phosphatidylinositol² 4,5P₂ (PtdIns 4,5P₂) cycle are largely unknown. This classical pathway contributes to two important signaling pathways (i) PLC mediated hydrolysis of PtdIns 4,5P₂ and generation of second

messengers, inositol 1,4,5 trisphosphate and 1,2 diacyl glycerol (ii) PtdIns 3-kinase mediated signaling pathway in which PtdIns 3,4,5P₃ are generated and activation of AKT [15]. Following hydrolysis, hormone/receptor sensitive PtdIns 4,5P₂ pool is rapidly resynthesized by phosphatidylinositol 4-kinases (PtdIns 4-kinases) and phosphatidylinositol phosphate kinases. Inhibition of these enzymes may lead to defective signaling in PLC mediated signaling and/or PtdIns 3-kinase signaling cascades [16].

Type II PtdIns 4-kinase(s) are shown to be part of many immune cell receptors including Fc ϵ RI, TCR-CD3, CD4 and CD7 receptor signaling [17–21]. Inhibition of type II PtdIns 4-kinase activity has been shown to abrogate degranulation and oxidative burst in neutrophils and IgE induced mediator release in RBL 2H3 cells [19, 22]. These results mimic inhibition of PtdIns 3-kinase signaling pathways and suggest that some of the sanguinarine effects may be due to inhibition of type II PtdIns 4-kinases and their cross signaling pathways with PtdIns 3-kinases. This idea has been investigated in the present manuscript. The results show that sanguinarine indeed inhibits type II PtdIns 4-kinase activities with no effect on PtdIns 3-kinase activity and underlines the importance of cross signaling pathways mediated by type II PtdIns 4-kinases and PtdIns 3-kinases.

Materials and methods

Chemical reagents and antibodies

All the chemicals unless otherwise mentioned were obtained from Sigma–Aldrich chemicals, Bangalore India. DMEM

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² Abbreviations used: PtdIns, phosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium.

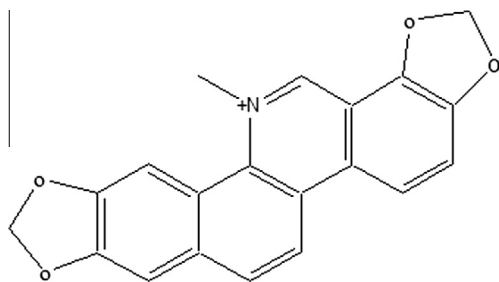


Fig. 1. Structure of Sanguinarine.

(Dulbecco's Modified Eagle's Medium) was from Himedia laboratories, India. Phenylmethylsulfonylfluoride was from SRL India, [γ - ^{32} P] ATP was from Board of Radiation and Isotope Technology, Hyderabad, India. Ni-NTA beads were from Qiagen. Glutathione beads were from GE health care life sciences. P6 resin was from Bio-Rad.

Cell culture

RBL 2H3 cells were maintained as monolayer cultures in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 U/mL), streptomycin (25 μ g/mL), amphotericin B (0.25 μ g/mL), in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were harvested with trypsin–EDTA solution (0.05% trypsin–0.2% EDTA) at 37 °C for 3–5 min. Cell suspension was washed twice with complete DMEM prior to the experiments.

Cell stimulation and lysis

RBL 2H3 cells were stimulated with IgE DNP-HSA as described earlier [19]. Briefly, RBL 2H3 cells (1×10^6 cells in 0.2 mL of complete DMEM) were incubated with DNP-specific-IgE (1 μ g) at 37 °C for 3 h. IgE sensitized RBL 2H3 cells were washed twice with DMEM and the bound IgE were cross-linked with DNP-HSA (1 μ g/mL) in a final volume of 200 μ L for 3 min. The reaction was terminated by adding 1 mL of ice cold DMEM and immediately transferred into an ice bucket. Cells were washed once with ice cold phosphate buffered saline prior to lysis in 0.2 mL of lysis buffer [Triton X-100 (1%), Tris–HCl (25 mM pH 7.4), NaCl (150 mM), EGTA (2 mM), sodium orthovanadate (0.25 mM) and PMSF (1 mM)]. The lysates were centrifuged at 30,000g at 4 °C for 30 min. The supernatants were assayed for PtdIns 4-kinase activity or used for immunoprecipitation.

PtdIns 4-kinase assay

PtdIns 4-kinase activity was assayed as described earlier [23]. Briefly the enzymatic reaction was carried out in final volume of 50 μ L at room temperature ($\sim$ 25 °C) in 50 mM Tris (pH 7.6), 10 mM MgCl₂, 0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μ g/mL phosphatidylinositol, 100 μ M [γ - ^{32} P] ATP and 0.4% Triton X-100 with 10 μ L of cell lysate. The reaction was initiated by adding [γ - ^{32} P] ATP and incubated for 6 min. The reaction was terminated by adding 50 μ L of 12 N HCl. Phospholipids were extracted with 500 μ L of chloroform: methanol: water (15:15:5, v/v/v), washed once with methanol: 1 N HCl (1:1 v/v) and analyzed on thin layer chromatography. Thin layer chromatography plates were pre-treated with 2% sodium potassium tartrate and 60 mM EDTA in 50% ethanol and dried in oven before loading samples. The chromatograms were developed with chloroform: methanol: water: ammonium hydroxide (90:90:20:7, v/v/v). Labeled phospholipids

were visualized by autoradiography and quantified using scintillation counting.

Immunoprecipitation and PtdIns 3-kinase activity

RBL 2H3 cell lysates were prepared as described above [19]. Cell lysates were incubated with p85 or p110 antibodies at 4 °C for 90 min. At the end of incubation, protein A agarose beads were added and further incubated for 60 min. The protein A agarose beads were washed thrice with ice cold phosphate buffer saline (pH 7.4) and assayed for PtdIns 3-kinase activity in 100 μ L reaction volume containing 50 mM Tris (pH 7.6), 10 mM MgCl₂, 0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μ g/mL PtdIns, 100 μ M [γ - ^{32} P] ATP. The reaction was initiated with the addition of [γ - ^{32} P] ATP and incubated at room temperature ($\sim$ 25 °C) for 6 min. The reaction was terminated with 50 μ L of 12 N HCl. The labeled phospholipids were extracted and separated as described in PtdIns 4-kinase assay. The phospholipids were visualized by autoradiography and quantified by scintillation counting.

Purification of His-tagged proteins

His-tagged human type II PtdIns 4-kinase α and β were cloned in pET 28 vectors and were expressed in *Escherichia coli* BL 21 cells [21]. Mid log culture of bacterial cells were induced with 0.5 mM IPTG at 37 °C for 3 h. At the end of incubation, cells were centrifuged at 7000 rpm for 15 min and lysed on ice with lysis buffer [50 mM Na₂HPO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, and lysozyme 100 μ g/mL]. After 15 min incubation, cells were sonicated for 1 min in presence of 1 mM PMSF. The lysate was centrifuged at 23,469g for 20 min. The supernatant was loaded on Ni-NTA column. The Ni-NTA column was washed with 6 column volumes of wash buffer [50 mM Na₂HPO₄ (pH 8.0), 300 mM NaCl, 70 mM imidazole 1%]. Bound PtdIns 4-kinases were eluted with elution buffer [50 mM Na₂HPO₄ (pH 8.0), 300 mM NaCl, 150 mM imidazole, 1%]. Imidazole is removed from purified protein solution using P6 column.

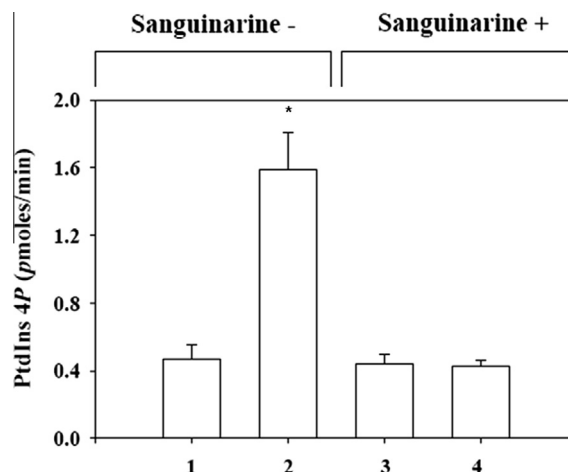


Fig. 2. Sanguinarine inhibits PtdIns 4-kinase activation in FcεRI cross linked RBL 2H3 cells. RBL 2H3 cells were pre-incubated with sanguinarine and FcεRI receptors were cross linked with IgE and DNP-HSA. Control and stimulated cells were lysed and cell lysates were assayed for PtdIns 4-kinase activity. Lane 1 and 2, PtdIns 4-kinase activity in unstimulated and antigen stimulated cell lysates in the absence of sanguinarine. Lane 3 and 4, PtdIns 4-kinase activity in unstimulated and antigen stimulated cell lysates, which were preincubated with sanguinarine. Data points represent the mean $\pm$ SEM ($N \geq 3$). Statistical analysis was done to compare PtdIns 4-kinase activity upon stimulation and decrease in PtdIns 4-kinase activity upon sanguinarine treatment with graph pad prism and P value is lesser than 0.05 (one way ANOVA).

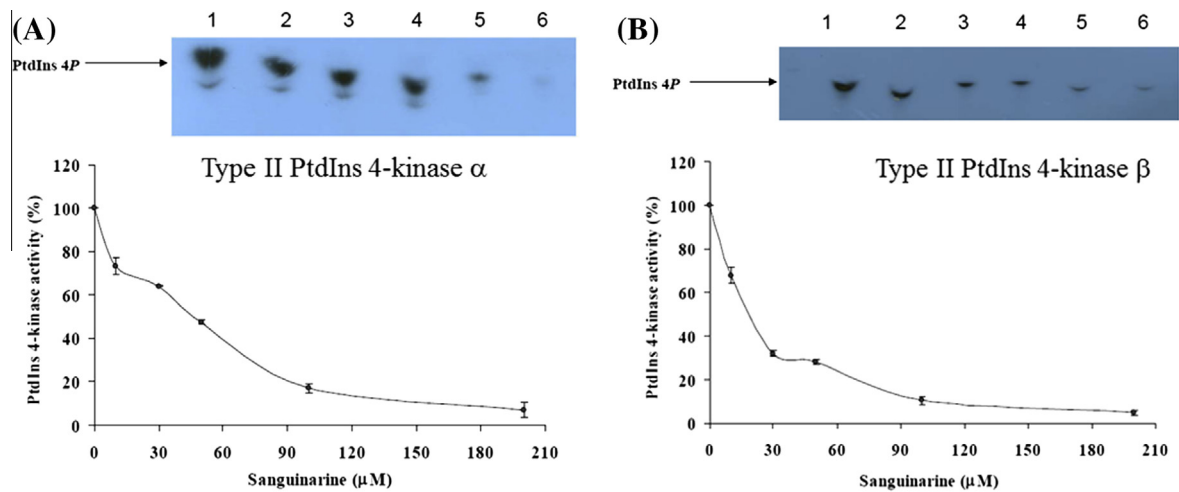


Fig. 3. Sanguinarine inhibits recombinant type II PtdIns 4-kinase activity. (A) Purified recombinant type II PtdIns 4-kinase α was incubated with increasing concentration of sanguinarine and assayed for PtdIns 4-kinase activity. The upper panel shows the autoradiograph and the numbers 1–6 correspond to sanguinarine concentrations of 0, 10, 30, 50, 100 and 200 μ M. The lower graph shows the quantification of the data. (B) Purified type II PtdIns 4-kinase β was incubated with increasing concentration of sanguinarine and assayed for PtdIns 4-kinase activity. The upper panel shows the autoradiograph and the numbers 1–6 correspond to sanguinarine concentrations of 0, 10, 30, 50, 100 and 200 μ M, respectively. The lower graph shows the quantification of the data. Data points represent the mean $\pm$ SEM ($N \geq 3$). Statistical analysis was done with graph pad prism and P value is lesser than 0.05 (one way ANOVA).

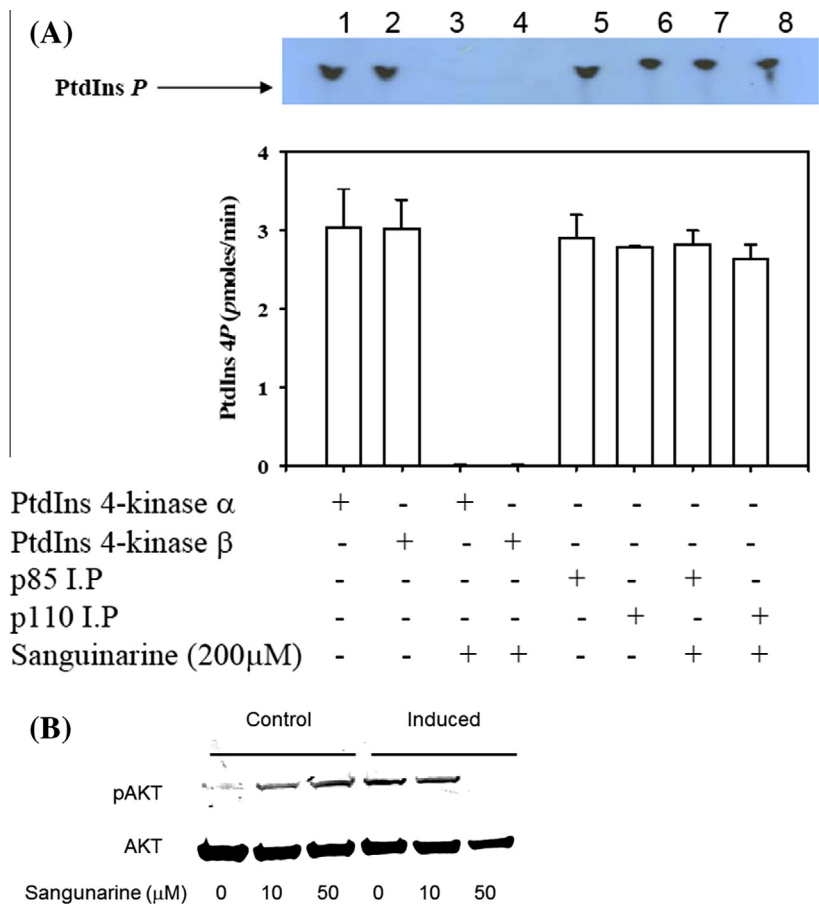


Fig. 4. Sanguinarine does not inhibit PtdIns 3-kinase but inhibits Akt phosphorylation. (A) Purified type II PtdIns 4-kinase α , β and immunoprecipitates of p85 and p110 from IgE stimulated RBL 2H3 cell lysates were incubated with sanguinarine (200 μ M) and assayed for PtdIns kinase activity. Lane 1 and 2, Type II PtdIns 4-kinase α and β activities in the absence of sanguinarine respectively. Lane 3 and 4, Type II PtdIns 4-kinase α and β activities in presence of sanguinarine respectively. Lane 5 and 6, PtdIns 3-kinase activity of p85 and p110 immunoprecipitates in the absence of sanguinarine. Lane 7 and 8, PtdIns 3-kinase activity of p85 and p110 immunoprecipitates in presence of sanguinarine respectively. Upper panel shows autoradiograph and the lower graph shows the quantification of the data. Data points represent the mean $\pm$ SEM ($N \geq 3$). Statistical analysis was done with graph pad prism and P value is lesser than 0.05 (one way ANOVA). (B) Cells were pretreated with different concentrations of sanguinarine for 30 min and Fc ϵ R1 receptors were cross linked for 30 min. Cell lysates were immune blotted for pSer473-AKT and total AKT as shown a representative of three repeats.

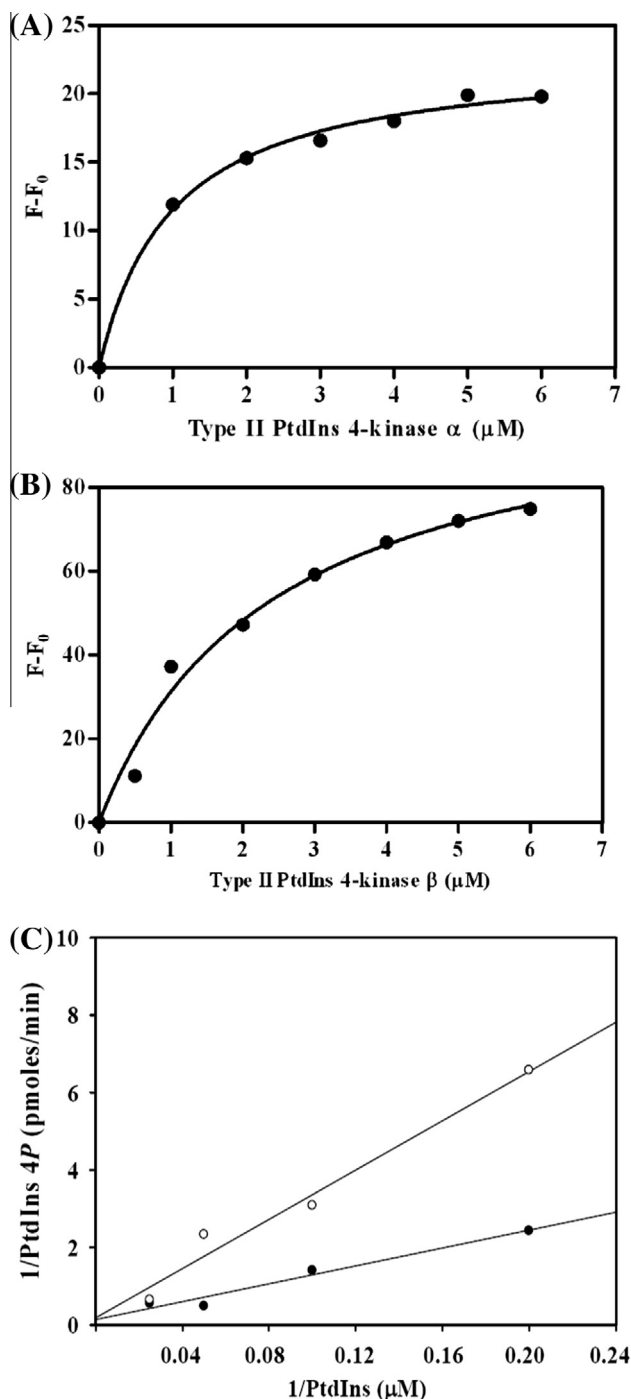


Fig. 5. Sanguinarine binding to type II PtdIns 4-kinases. (A) Increasing concentrations of purified human type II PtdIns 4-kinase α was incubated with sanguinarine (1 μM). The fluorescence intensity of sanguinarine at 400 nm was measured. (B) Increasing concentrations of purified human type II PtdIns 4-kinase β was incubated with sanguinarine (1 μM). The fluorescence intensity of sanguinarine at 400 nm was measured. (C) Type II PtdIns 4-kinase activity was assayed with increasing concentrations of PtdIns in the absence (solid circle) and in presence of 50 μM sanguinarine (open circle). Data points represent the mean $\pm$ SEM ($N \geq 3$). Statistical analysis was done with graph pad prism and P value is lesser than 0.05 (one way ANOVA).

Sanguinarine treatment of RBL 2H3 cells

RBL 2H3 (5×10^6) cells were suspended in 200 μL of DMEM containing sanguinarine and incubated for 20 min at 37 $^\circ\text{C}$. Cells were washed twice with PBS. The cells were either used for β -hex-

osaminidase release (degranulation) assay or lysed in 200 μL of lysis buffer [25 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 0.25 mM sodium orthovanadate, 1 mM PMSF]. The lysate was assayed for PtdIns 4-kinase activity.

β -Hexosaminidase assay

β -Hexosaminidase release from Fc ϵ RI cross-linked cells was used as a measure of degranulation [19]. Briefly, 2.5×10^5 cells in 200 μL were primed with 3 $\mu\text{g}/\text{mL}$ of anti-DNP-mouse monoclonal IgE for 2 h. Unbound IgE was removed from RBL 2H3 cells by washing twice with extracellular buffer (5 mM KCl, 125 mM NaCl, 20 mM HEPES, 1.5 mM MgCl_2 , 1.5 mM CaCl_2 , 1 mM Dextrose) and stimulated with DNP-HSA (100 ng/mL) for 3 min. Cell stimulation was terminated by placing the tubes in an ice bucket. Cells were centrifuged at 200g for 4 min and the supernatant was collected. The amount of β -hexosaminidase in the supernatant was assayed. 20 μL of cell supernatant was incubated with 200 μL of 1 mM p -nitrophenyl- N -acetyl- β -D-glucosaminide in 0.05 M sodium citrate buffer (pH 4.5) for 45 min at 37 $^\circ\text{C}$. The reaction is terminated by adding 0.5 mL of 0.05 M sodium carbonate buffer (pH 10.0) and absorbance was read at 405 nm.

TNF alpha and IL6 assay

IgE induced release of TNF alpha and IL6 were measured in culture supernatants of RBL 2H3 cells with the help of ready set go ELISA kits from Ebioscience according to the manufacturer's protocol. Purified anti-TNF alpha or IL6 were coated on maxisorp plates for overnight. Plates were washed and incubated with cell supernatant overnight. Bound antigen (TNF alpha or IL6) was assayed by detection antibodies and HRP streptavidin substrates and plates were measure by plate reader at 450 nm.

Sanguinarine binding studies

Fluorescence studies were performed using a JASCO fluorescence spectrophotometer as described in [24]. Spectra were taken by multiple scans and buffer blanks were subtracted from all measurements. A quartz cuvette of 3 mm path length was used. The band pass for excitation and emission monochromators was 5 and 10 nm respectively. Emission spectra were recorded over a range of 375–475 nm using 325 nm as excitation wavelength and fluorescence intensity at 400 nm was used to calculate the dissociation constant. The dissociation constant of sanguinarine for type II PtdIns 4-kinase α and β were determined by using protein (type II PtdIns 4-kinase α or β) titration procedures. Free sanguinarine has a fluorescence maximum around 400 nm, upon binding to type II PtdIns 4-kinase α or β the fluorescence intensity increases several fold. Increased fluorescence of 1 μM sanguinarine at 400 nm, upon binding with varying concentrations of type II PtdIns 4-kinase α or β (1–6 μM) was used to determine the dissociation constants. Fluorescence of various concentrations of protein solution in the absence of sanguinarine was subtracted from experimental data. The dissociation constant of sanguinarine and type II PtdIns 4-kinase was calculated using the formula: $\Delta F = \Delta F_{\text{max}} L / (K_d + L)$, where ΔF is change in fluorescence intensity of Sanguinarine upon binding to type II PtdIns 4-kinase, ΔF_{max} is the maximum change in the fluorescence intensity of sanguinarine when it is fully liganded with type II PtdIns 4-kinase α or β and L is the concentration of type II PtdIns 4-kinase. The ΔF_{max} value was calculated by using GraphPad Prism 5 software. ΔF was calculated by subtracting the fluorescence intensity of sanguinarine and free protein (type II PtdIns 4-kinase α or β) from the fluorescence intensity of liganded form (sanguinarine bound to type II PtdIns 4-kinase α and β). The

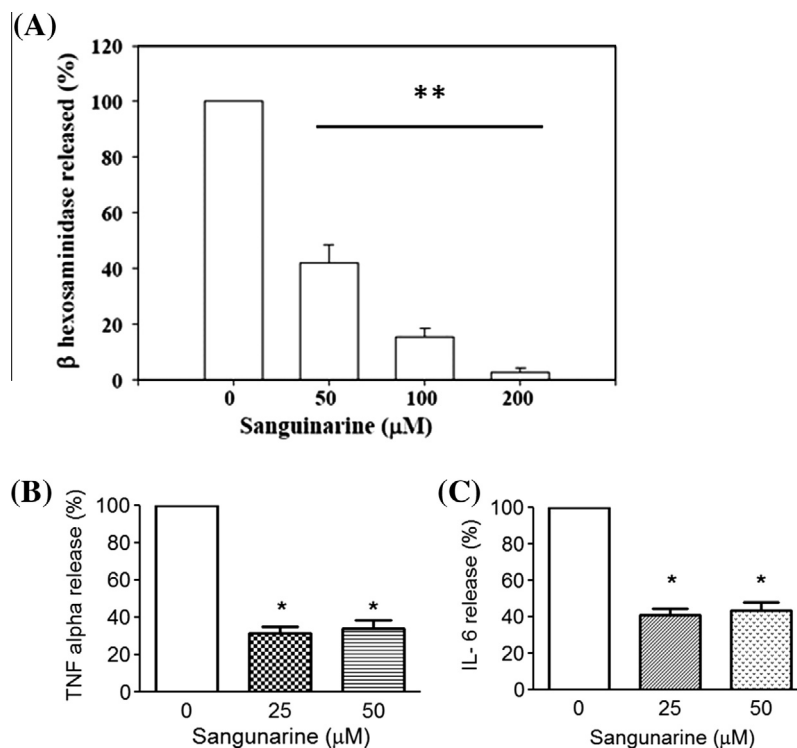


Fig. 6. Sanguinarine inhibits pro inflammatory molecules release in RBL 2H3 cells (A) RBL 2H3 cells were incubated with different concentrations of sanguinarine. Fc ϵ RI were cross linked with IgE and DNP-HSA for 3 min. Cell supernatants were assayed for β -hexosaminidase release. The amount of β hexosaminidase released in 3 min stimulated RBL 2H3 cells in the absence of sanguinarine was taken as 100% and all other values were in comparison to this value. (B) RBL 2H3 cells were pre incubated with sanguinarine for 30 min and receptors were cross linked with IgE and DNP-HSA for 12 h. Supernatants were assayed for TNF alpha release and (C) IL6 release. The amount of TNF alpha/IL6 released in absence of sanguinarine was taken as 100%. Data points represent the mean $\pm$ SEM ($N \geq 3$). Statistical analysis was done with graph pad prism and P value is lesser than 0.05(*) and lesser than 0.01 (**) (one way ANOVA).

data were analyzed and curve fitted using GraphPad Prism 5 software.

Results

In situ inhibition of type II PtdIns 4-kinases by sanguinarine

Effects of sanguinarine on type II PtdIns 4-kinase activity were studied in RBL 2H3 cells. As reported earlier, stimulation of RBL2H3 cells with IgE and its antigen showed an increase in type II PtdIns 4-kinase activity [19]. Pretreatment of RBL 2H3 cells with sanguinarine inhibited Fc ϵ RI mediated type II PtdIns 4-kinase activation (Fig. 2). The effect of sanguinarine on type II PtdIns 4-kinases may be direct or through indirect mechanisms. To distinguish these possibilities, purified human type II PtdIns 4-kinase α and β isoforms were assayed in presence of sanguinarine. Both isoforms were inhibited by sanguinarine in concentration dependent fashion suggesting direct inhibition of type II PtdIns 4-kinases by sanguinarine (Fig. 3 A and B). Type II PtdIns 4-kinase β was more strongly inhibited than the α isoform. The IC₅₀ for type II PtdIns 4-kinase α and β isoforms were 40 μ M and 20 μ M, respectively.

PtdIns 3-kinase activity is not affected by Sanguinarine

Sanguinarine has been suggested to affect PtdIns 3-kinase signaling events [25]. The effect of sanguinarine on PtdIns 3-kinase activity *per se* was assayed *in vitro*. Even very high concentrations of sanguinarine failed to inhibit PtdIns 3-kinase activity in both anti p85 and anti p110 immunoprecipitates from RBL 2H3 cells, suggesting that PtdIns 3-kinases are resistant to sanguinarine

inhibition. In contrast, at this concentration of sanguinarine, type II PtdIns 4-kinases were completely inhibited (Fig. 4A).

Next we addressed the effect of type II PtdIns 4-kinase(s) inhibition on AKT phosphorylation in IgE sensitized RBL 2H3 cells. Even though PtdIns 3-kinase was not inhibited by sanguinarine, pretreatment of RBL 2H3 cells with sanguinarine inhibited AKT phosphorylation in IgE induced RBL 2H3 cells (Fig. 4B).

Sanguinarine binds directly to type II PtdIns 4-kinase α and β

The affinity of sanguinarine for type II PtdIns 4-kinase α and β were studied using fluorescence spectroscopy. In presence of type II PtdIns 4-kinases (α and β), the sanguinarine fluorescence was increased, suggesting a direct interaction of sanguinarine with type II PtdIns 4-kinases. The dissociation constants of sanguinarine for type II PtdIns 4-kinase α and β were determined to be 2.4 and 1.8 μ M respectively (Fig. 5A and B). Binding of sanguinarine to type II PtdIns 4-kinases was affected by increasing concentrations of exogenously added phosphatidylinositol. Lineweaver–Burke plot suggests that sanguinarine compete with PtdIns binding to type II PtdIns 4-kinases (Fig. 5C). However, increase in ATP concentration has no effect on sanguinarine binding to PtdIns 4-kinases (results not shown).

Sanguinarine inhibits β -hexosaminidase, TNF alpha and IL6 release in RBL 2H3 cells

Previous studies had implicated a role for type II PtdIns 4-kinase(s) in Fc ϵ RI mediated signaling including β hexosaminidase release in RBL 2H3 cells [19, 26]. Pre-incubation of RBL 2H3 cells with sanguinarine inhibited IgE induced β -hexosaminidase release.

Sanguinarine also significantly reduced IgE induced release of other pro-inflammatory molecules TNF α and IL6 (Fig. 6A–C). These results suggest that part of the anti-inflammatory effects of sanguinarine may be due to inhibition of type II PtdIns 4-kinases and implicate them as potential targets for developing anti-inflammatory agents in type I hypersensitive reactions.

Discussion

PtdIns phosphates play a critical role in various cellular processes. While PtdIns *P* kinases, PtdIns 3-kinases and type III PtdIns 4-kinases have taken the central stage in these signaling cascades, very little is known about the role of type II PtdIns 4-kinases [27–29]. PtdIns 4-kinases catalyze the formation of PtdIns 4*P*, which is a precursor molecule for biosynthesis of PtdIns 4,5*P*₂, PtdIns 3,4*P*₂, and PtdIns 3,4,5*P*₃ [30–32]. PtdIns 3,4,5*P*₃ and the hydrolytic products of PtdIns 4,5 *P*₂ (Inositol 1,4,5 *P*₃ and 1,2 diacyl glycerol) have been shown to regulate receptor mediated signaling pathways that lead to T cell and mast cell activation, secretion of inflammatory mediators and apoptosis [26, 33].

In addition to the above signaling cascades, PtdIns 4*P* has its own role in diverse cell signaling pathways and is shown to be key component in replication of RNA viruses and cell motility [27, 28, 34]. Type II PtdIns 4-kinases act upstream of PtdIns *P* kinases and PtdIns 3-kinases and have the potential to cross talk with PLC and PtdIns 3-kinase mediated signaling pathways.

Type II PtdIns 4-kinases have been shown to play a role in early signaling events in T cell and mast cell activation and are suggested to be potential targets for developing knowledge based immunomodulators [17,19,23,35]. To explore this line of thought, effects of sanguinarine on type II PtdIns 4-kinases were investigated. Sanguinarine inhibited Fc ϵ RI mediated activation of type II PtdIns 4-kinase activity in RBL 2H3 cells.

In vitro experiments have shown that, sanguinarine competes with phosphatidylinositol binding to type II PtdIns 4-kinases and inhibits their activities. The dissociation constants of sanguinarine binding were determined to be in the range of 2–3 μ M. The strong correlation between inhibition of type II PtdIns 4-kinases and mediator release support a role for type II PtdIns 4-kinases in Fc ϵ RI signaling cascades. Vesicular trafficking plays an important role in mediator release [36]. Calcium and phosphoinositides have been suggested to play a role in vesicular fusion with plasma membrane and exocytosis. Inhibition of type II PtdIns 4-kinases may affect this process either directly or indirectly and needs further studies [37].

Inhibition of PtdIns 3-kinase signaling pathways including AKT signaling and MAP kinases has been suggested to be a mechanism for sanguinarine mediated anti-inflammatory, anti-angiogenic and pro-apoptotic effects. However, the results presented in the manuscript suggest that sanguinarine does not inhibit PtdIns 3-kinase catalytic activity *per se*, but is a strong inhibitor of type II PtdIns 4-kinases and Akt phosphorylation. These results are similar to our earlier studies with resveratrol, where resveratrol has been shown to inhibit type II PtdIns 4-kinase activity and not PtdIns 3-kinases and inhibit β hexosaminidase release [19]. Earlier Chu et al. have observed that knock down of type II PtdIns 4-kinase α inhibited EGF induced phosphorylation of AKT [38] in COS 7 cells with increased apoptosis of the cells. In contrast to COS7 cells, in MDA-MB231 cells they have seen an increase in AKT phosphorylation in type II PtdIns 4-kinase α knock down cells, suggesting cell type specific effects. However, the mechanisms that regulate AKT phosphorylation were not clearly understood and may not dependent upon the production of PtdIns 4*P* alone. These studies clearly demonstrate additional regulatory mechanisms between type II PtdIns 4-kinases and PtdIns 3-kinase signaling pathways exist and warrants further investigations.

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