Sanguinarine is a potent inhibitor of oxidative burst in DMSO-differentiated HL-60 cells by a non-redox mechanism

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Abstract

Sanguinarine (SA), a member of the benzo[c]phenanthridine isoquinoline alkaloids, has been shown to possess antimicrobial, anti-inflammatory, and antioxidant properties. We examined the effects of SA on oxidative burst in DMSO-differentiated HL-60 cells, an excellent model for studying oxidative burst. SA inhibited both *N*-formyl-Met–Leu–Phe (fMLP) and phorbol 12-myristate 13-acetate (PMA)-induced oxidative burst with half-maximal concentration for inhibition (IC_{50}) of 1.5 and 1.8 μM, respectively. Despite suggestions of SA antioxidant activity this inhibition cannot be ascribed to radical scavenging property of SA because the IC_{50} for superoxide dismutase-like activity in a non-cellular system was 60 μM. TROLOX, a water-soluble vitamin E analog, had IC_{50} of 3 μM in the same system. Moreover, cyclic voltammetry measurements show that SA is not an easily oxidisable species, with a peak anodic potential at 700 mV, as compared to TROLOX with peak anodic potential at 200 mV. On the other hand, TROLOX, when used in cell suspension, was much poorer inhibitor of oxidative burst than SA. When testing direct effect of SA on NADPH oxidase in the post-granular fraction of disrupted cells, the IC_{50} was found to be 8.3 μM. It is higher than that observed in whole cells, however, the shift may be ascribed to SDS effect on SA activity. We conclude the SA inhibition of oxidative burst is not caused by SA redox activity but most likely is a result of SA affecting the activity of NADPH oxidase directly and in part by preventing the formation of NADPH oxidase protein complex.

1. Introduction

Toxicity of alkaloids has always hampered their more frequent use in current medical preparations intended for internal use. However, their antimicrobial, anti-inflammatory, and antioxidant properties make them often desirable in external use or topical medications. Also important is the potential use of alkaloids, or their derivatives, as anticancer drugs.

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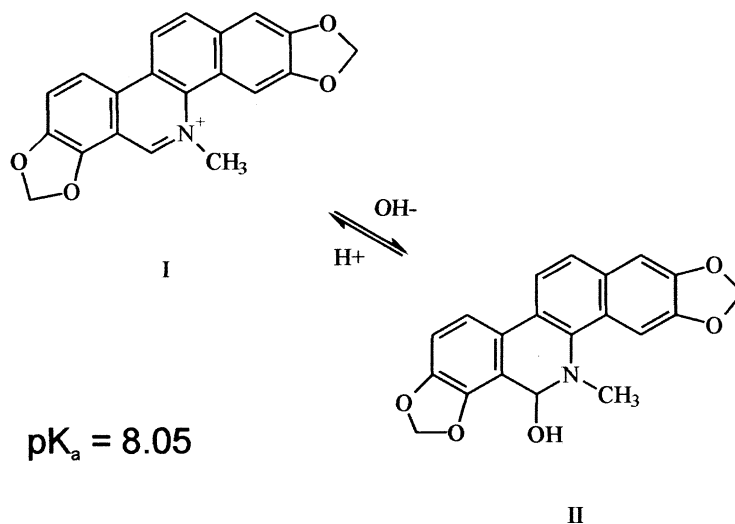
Fig. 1. Structure of sanguinarine. The iminium (structure I) and "pseudobase" (structure II) forms of sanguinarine are shown.

Majority of these features apply to sanguinarine (SA), a benzo[c]phenanthridine alkaloid isolated from the root of *Sanguinaria canadensis* (Fig. 1). Extract of the plant has been used in toothpastes and oral rinse products [1–3] but its safety is occasionally questioned because of past toxic outbreaks ascribed to SA [4]. It is also considered for cancer therapy thanks to its effect on cancer cell lines [5,6]. SA inhibits broad spectrum of enzymes with variable potency, especially enzymes containing active sulfhydryl groups [7,8]. Chemical reactivity of SA involves nucleophilic attack on the iminium form (Fig. 1) [9]. The iminium form may participate in either oxidant scavenging or enzyme inhibition or both. It can reversibly react with hydroxyl anion thus forming a "pseudobase", an electroneutral substance which is the form expected to penetrate cells where it can act as is or dissociate back into the positively charged iminium form of SA.

Anti-inflammatory and antioxidant properties of substances are often linked to the oxidative burst and inflammatory agents production by polymorphonuclear neutrophils (PMN). These particular cells, as well as other peripheral cell types, are among the subjects of SA biological activity. The phenomenon of oxidative burst inhibition in PMN may be a result of direct scavenging of reactive oxygen species (ROS), e.g. by flavonolignans [10], or inhibition of signaling pathways leading to ROS formation involving effects on cell receptors, G-protein, phospholipase C, cytosolic calcium ion level, and protein kinase C (PKC). SA was reported to scavenge superoxide radical with IC_{50} of $10\,\mu M$. The same report also claims that SA inhibits fMLP-stimulated oxidative burst with IC_{50} of $16\,nM$ [11]. The 1000-fold difference suggests involvement of SA in an enzymatic reaction or signaling pathway. The last but one protein in a signaling pathway leading to oxidative burst, initiated by various stimuli, is protein kinase C [12]. SA has been shown to inhibit PKC but the reported IC_{50} varied greatly: $217\,\mu M$ for PKC in rat brain [13] and $16\,\mu M$ for PKC in rat liver [14]. The most important enzyme in oxidative burst is the cytoplasmic membrane NADPH oxidase complex. Composed of five subunits, it is the superoxide production unit catalyzing transfer of electron from NADPH onto molecular oxygen [15]. The membrane spanning flavocytochrome b_{558}, the main subunit, is likely responsible for the catalysis and it is a likely target for inhibitors due to binding/active sites content: NADPH binding site, FAD site, and two hemes b. For example anti-inflammatory activity of ebselen was linked to NADPH oxidase as well as PKC inhibition [16]. SA was shown to inhibit phorbol myristate (PMA)-induced oxidative burst, involving NADPH oxidase activation through PKC, but the affinity did not correspond to the IC_{50} for PKC inhibition [17].

The discrepancies in SA specificity led us to investigate whether the inhibition of oxidative burst is the result of ROS scavenging activity or an enzyme inhibition by SA. We used DMSO-differentiated HL-60 cells, largely considered a useful model for studying oxidative burst [18], and sensitive chemiluminescent detection of superoxide generation. The data obtained support the hypothesis that ROS scavenging by SA is a negligible factor; it is most likely the effect on NADPH oxidase activity or assembly which leads to the oxidative burst inhibition by SA.

2. Materials and methods

2.1. Chemicals

RPMI-1640 medium, L-glutamine, penicillin–streptomycin solution, fetal bovine serum heat inactivated, flavin adenine dinucleotide (FAD), dithiotreitol (DTT), 2-mercaptoethanol, reduced glutathione (GSH), diisopropyl fluorophosphate, guanosine $5'$-[γ-thio] triphosphate (GTP-γ-S), chemotactic peptide *N*-formyl-Met–Leu–Phe (fMLP), phorbol 12-myristate 13-acetate (PMA), phenylmethylsulfonyl fluoride, diphenylene iodonium (DPI), superoxide dismutase from bovine erythrocytes (SOD), cytochrome *c* from bovine heart, horse radish peroxidase (HRP), and anti-rabbit IgG alkaline phosphatase conjugated antibody were obtained from Sigma Chemical (St. Louis, MO). Sanguinarine (SA) was isolated in 98.1% purity (mp 279–282 °C) as described previously [19]. Complete™ protease inhibitor cocktail was purchased from Roche Molecular Biochemicals (Mannheim, Germany). L-Cysteine and sodium dithionate were purchased from Lachema (Brno, Czech Republic). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) was purchased from Aldrich (Milwaukee, WI). 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA) was purchased from Molecular Probes (Eugene, OR). Polyclonal phospho-(Ser) PKC substrate antibody was purchased from Cell Signaling Technology (Beverly, MA). Sodium dodecyl sulfate molecular biology grade and ChemStar alkaline phosphatase chemiluminescent substrate were obtained from Bio-Rad (Hercules, CA). All other chemicals were of the highest grade commercially available.

2.2. HL-60 cell line maintenance and differentiation into neutrophils

Human promyelocytic cell line HL-60 (ECACC No. 98070106) was maintained in RPMI-1640 medium containing 5 mM L-glutamine, penicillin–streptomycin, and 10% fetal bovine serum in a humidified incubator at 37 °C and 5% CO_2. Cell density was maintained between 2 and 9×10^5 cells/ml as per supplier instructions. Prior to differentiation cells were harvested by centrifugation ($400 \times g$, 3 min), resuspended in fresh medium and diluted to density 5×10^5 cells/ml. DMSO was added to reach 1.25% and the cells were cultured for 7 days in a humidified incubator at 37 °C and 5% CO_2. On day four fresh medium, again containing DMSO, was added to prevent cell starvation.

Differentiated cells were harvested by centrifugation as described above, washed once in phosphate buffered saline (PBS) pH 7.4, and finally resuspended in PBS+ at a cell density 10^7 cells/ml. PBS+ contains as additives 30 mM glucose, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Cells were then kept on ice and used in further experiments only when capable of superoxide generation, which was sensitive to inhibition by DPI and SOD.

2.3. Detection of superoxide formation

2.3.1. Chemiluminescent detection

The harvested DMSO-differentiated HL-60 cells (final density in assay 10^6 cells/ml) were transferred into a fluorescence quartz cuvette containing PBS+ buffer pre-warmed to 37 °C. An aqueous solution of SA (final concentrations 0.01–10 μM) or an ethanol solution of TROLOX (final concentrations 1–80 μM) or 10 μM DPI or 100 U/ml SOD were added 5 min prior to cell stimulation. Four micromolar MCLA was added 30 s prior to start of measurement. The background chemiluminescence was obtained and thereafter automatically subtracted from the readings. Superoxide generation was initiated by fMLP (5 μM) or PMA (62.5 ng/ml) addition 60 s after the start of measurement. Final volume of sample was 2 ml, organic solvent concentration, i.e. DMSO and/or ethanol, did not exceed 0.5% in any assay.

The time course of MCLA-$O_2{}^-$ chemiluminescence was monitored on Perkin–Elmer LS50B luminometer

equipped with total emission mirror and revolving cuvette holder allowing thermostating at 37 °C and continuous stirring of sample (Perkin–Elmer Corp., Norwalk, CT). The instrument was set as follows: luminescence mode (lamp off), delay time 0.03 ms, cycle time 100 ms, gate time 90 ms, and flash count 1. Emission slit was set to 20, emission filter to 350 nm, and photomultiplier voltage was 775 V.

The area under chemiluminescence peak was integrated and then used in calculation of half-maximal concentration of SA (IC_{50}) necessary for inhibition of oxidative burst. Activity detected in samples containing 100 U/ml SOD was used as 100% inhibition.

When testing the influence of reducing agents on SA effect on oxidative burst, SA was first incubated with a 50-fold higher concentration of a reducing agent for 5 min. Cell suspension was then added to reach the same final volume of sample and cell density as described above. Control samples contained appropriate concentration of each reducing agent only.

2.3.2. Spectrophotometric detection

The harvested DMSO-differentiated HL-60 cells (final density in assay 10^6 cells/ml) were transferred into a microcuvette containing 32 μM cytochrome *c* in PBS+ buffer pre-warmed to 37 °C. Aliquots of aqueous SA solution or 100 U/ml SOD were added 5 min prior to cell stimulation. The samples were mixed by a teflon stirrer immediately after PMA (62.5 ng/ml) addition. Absorbance at 550 nm, the absorption peak for reduced cytochrome *c*, was monitored for 4 min at 30 s intervals on Shimadzu UV1606 spectrophotometer equipped with a thermostated cuvette holder (Shimadzu GmbH, Vienna). The absorbance change per minute was used in calculating half-maximal concentration of SA (IC_{50}) necessary for inhibition of oxidative burst. Activity detected in samples containing SOD ± SA was used as 100% inhibition.

2.4. Superoxide oxidase assay in a non-cellular system

The method using $NADPH:MnCl_2:2$-mercaptoethanol system published by Paoletti and Mocali [20] was utilized. An aqueous solution of SA (final concentrations 1–100 μM) or an ethanol solution of TROLOX (final concentrations 1–800 μM) or SOD (final concentration 0.1–5 U/ml) was added to the 37 °C warm triethanolamine–diethanolamine buffer, pH 7.4, 5 min prior to 2-mercaptoethanol addition which is the start of reaction. Decrease in absorbance at 340 nm, i.e. oxidation of NADPH, was monitored continuously for 20 min on the same instrumentation as described in Section 2.3.2. Linear portion of the curves, from 11 to 20 min, were used for calculation of absorbance change. Control sample containing no additives was taken as 0% inhibition, sample containing 10 U/ml of SOD was taken as 100% inhibition.

2.5. NADPH oxidase activity in the post-granular fraction

Suspension of DMSO-differentiated HL-60 cells (10^7 cells/ml) in PBS+ buffer (137 mM NaCl, 2.7 mM KCl, 9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 30 mM glucose, pH 7.4) was incubated for 20 min with 1 mM diisopropyl fluorophosphate at room temperature, 500 rpm in thermomixer Comfort (Eppendorf, Germany). Cells were subsequently centrifuged in glass test tube for 5 min at $200 \times g$ and resuspended in RB^{EGTA} buffer (10 mM HEPES, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and 1.25 mM EGTA, pH 7.3) and Complete^{TM} protease inhibitor cocktail solution at 2×10^7 cells/ml. Immediately after addition of 1 mM phenylmethylsulfonyl fluoride cells were sonicated by sonic dismembrator Dynatech (Artek, USA) for 5 s, with power setting of 35% while holding the tube in ice water. Unbroken cells and nuclei were separated by centrifugation for 5 min at $200 \times g$, no brake. Post-granular supernatant containing 2×10^7 cell equivalents/ml was obtained by centrifugation of post-nuclear supernatant for 30 min at $12\,000 \times g$, 4 °C.

For superoxide generation, 2×10^6 cell equivalents were combined in the presence or absence of inhibitor with 10 μM FAD, 10 μM GTP-γ-S, and 100 μM SDS in broken cell buffer (10 mM Na_2HPO_4, 130 mM NaCl, 2 mM EGTA, 2 mM $MgCl_2$, and 2.7 mM KCl, pH 7.2). After a 2.5-min incubation with SDS, 4 μM MCLA was added and reaction was started after another 30 s by addition of 200 μM NADPH into a final volume of 1 ml. Formation of superoxide was measured at room temperature for 200 s on luminometer Luminova 1254 (Bio Orbit, Finland). Background measured in the absence of NADPH was subtracted from all other sample readings.

2.6. Cyclic voltammetry

Cyclic voltammetry measurements were performed using the Potentiostat/Galvanostat Model 273 cyclic voltammetry apparatus (EG&G Princeton Applied Research, USA). A three electrode system, consisting of a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, USA), a platinum-wire auxiliary electrode, and a Hg/Hg_2Cl_2/saturated KCl reference electrode, was used. The potentials mentioned throughout this work are referred to against this electrode. All measurements were performed at laboratory temperature and 200 mV/s scan rate. The tested substances: TROLOX, final concentration 100 μM, added as an ethanol solution, and SA, final concentration 100 μM, added as a DMSO solution, were diluted in 0.1 M PBS, pH 7.4, or 0.1 M triethanolamine–diethanolamine–HCl buffer, pH 7.4. The working electrode was polished with aluminum oxide prior to each measurement.

2.7. Immunodetection of phospho-(Ser) PKC substrates

Differentiated HL-60 cells were treated with SA (0.12–5 μM concentrations) for 5 min and then stimulated by PMA as described above. Following 10 min incubation cells were collected by centrifugation for 4 min at $2380 \times g$, room temperature. Resulting pellet was resuspended in 200 μl of 0.2% SDS (w/v) supplemented with Complete^{TM} protease inhibitor cocktail to lyse the cells. Cell lysate was frozen and stored at $-20\,^{\circ}C$ until further use. Protein content of the samples was estimated by the method of Lowry et al. [21]. Five micrograms of total protein sample per well were loaded into 10% polyacrylamide gel, subjected to SDS-PAGE electrophoresis, and subsequently blotted onto nitrocellulose membrane. The presence of phosphorylated PKC substrates was revealed using primary polyclonal phospho-(Ser) PKC substrate antibody and chemiluminescent detection. Photographs of the membranes were scanned and stored as graphic Tagged Image Files.

2.8. Statistical analysis

Results were expressed as mean ± standard deviation. One-way ANOVA was used for statistical analysis with the aid of NCSS statistical software (Kaysville, UT, USA).

3. Results

3.1. Effect of SA on fMLP and PMA-induced oxidative burst

Oxidative burst in neutrophils can be elicited by a number of stimulants. The final step for any of the stimulants is the assembly of NADPH oxidase complex and superoxide generation [12]. Human promyelocytic cell line HL-60 is capable of differentiating into functional neutrophils in the presence of DMSO as well as other effectors. The DMSO-differentiated cells respond to chemotactic peptide (fMLP) stimulation by a rapid rise in superoxide generation followed by a quick return to the original background level (Fig. 2A, trace fMLP). It is indeed a brief burst lasting no more than 100 s and can be completely inhibited by superoxide dismutase (Fig. 2A, trace SOD) or diphenylene iodonium (DPI) (not shown). Therefore the stimulus results in formation of extracellular superoxide, inhibited by SOD, and involves NADPH oxidase, inhibited by DPI.

PMA stimulation, on the other hand, results in superoxide production lasting more than 30 min. The apparent fading of chemiluminescence signal (Fig. 2B, trace PMA) is likely due to the depletion of the MCLA probe rather than the decrease in superoxide generation. Addition of fresh MCLA to the activated cells brings the signal to the level gained upon the original stimulus (data not shown). As was the case for fMLP stimulation, SOD and DPI inhibited the PMA-mediated oxidative burst.

Both fMLP (trace SA, Fig. 2A) and PMA-stimulated bursts are inhibited by micromolar concentration of SA (trace SA, Fig. 2B). This inhibition is dose-dependent with IC_{50} of 1.5 ± 0.2 and IC_{50} of 1.8 ± 0.3 μM SA, respectively (Fig. 3, Table 1). Hill slope of the dose–response curve is 2.7 and 2.1 for fMLP and PMA stimulation, respectively, suggesting an interaction of SA with two or more sites. Very similar IC_{50} of 0.9 ± 0.1 μM was calculated from spectrophotometric data using cytochrome *c* reduction (data not shown). Because the fMLP-stimulated burst was inhibited by SA with essentially the same
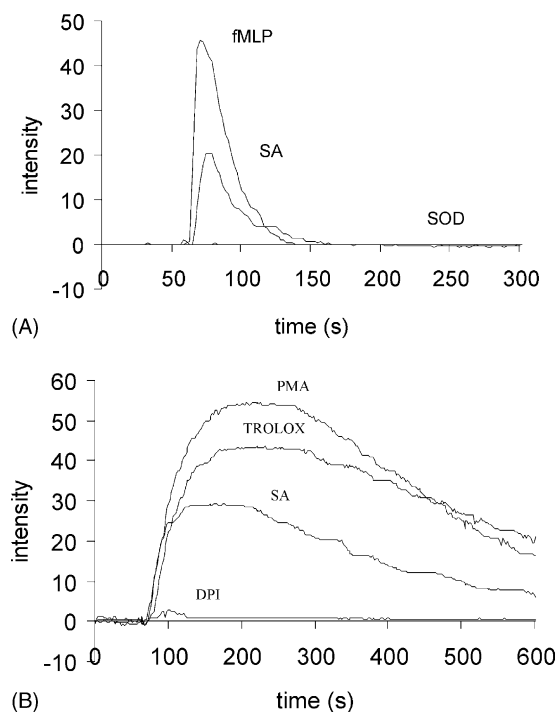
(A)

(B)

Fig. 2. Time course of oxidative burst in DMSO-differentiated HL-60 cells. Actual chemiluminescent traces of MCLA-detected superoxide production in differentiated HL-60 cells are shown. Panel A 10^6 cells/ml were stimulated by fMLP alone (trace fMLP), or pre-incubated with $1 \mu M$ SA prior to fMLP stimulation (trace SA), or pre-incubated with $100 U$/ml superoxide dismutase prior to fMLP stimulation (trace SOD). Panel B 10^6 cells/ml were stimulated by PMA alone (trace PMA), or pre-incubated with $1 \mu M$ SA prior to PMA stimulation (trace SA), or pre-incubated with $80 \mu M$ TROLOX prior to PMA stimulation (trace TROLOX), or pre-incubated with $10 \mu M$ diphenylene iodonium (trace DPI).

(A)

(B)

Fig. 3. Dose–response curves for sanguinarine inhibition of fMLP or PMA-stimulated oxidative burst. 10^6 cells/ml were pre-incubated with variable concentrations of SA for 5 min prior to stimulation. Inhibition was calculated from chemiluminescence trace areas where 0% inhibition represents superoxide generation in the presence of a stimulus alone (fMLP or PMA) and 100% inhibition in the presence of $100 U$/ml SOD and stimulus. The derived IC_{50} was $1.5 \pm 0.2 \mu M$ SA for fMLP stimulation (panel A) and $1.8 \pm 0.3 \mu M$ SA for PMA stimulation (panel B).

IC_{50} as for PMA (Table 1), we hypothesized that SA affects the final step in oxidative burst rather than the signaling pathway leading to it.

TROLOX, a water-soluble analog of vitamin E and a potent antioxidant, was much less effective in inhibiting PMA-stimulated oxidative burst than SA (trace TROLOX, Fig. 2B). TROLOX was included in these and subsequent experiments as a comparison substance to estimate potential ROS scavenging activity because it is expected to function primarily as an ROS scavenger. The low potency of TROLOX, as compared to SA in the whole cell system, may imply SA as a very potent antioxidant.
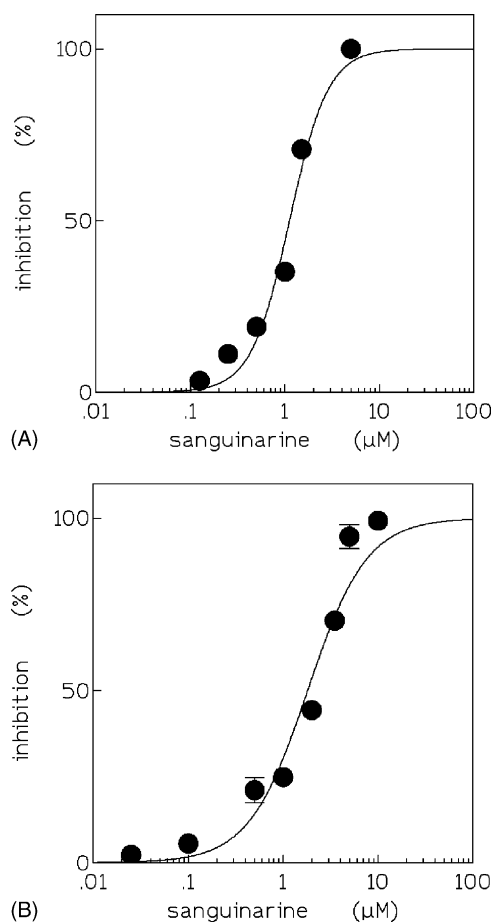
3.2. ROS scavenging activity of SA

An inhibitory effect of a substance on oxidative burst can be ascribed to ROS scavenging ability or enzyme inhibition. The two activities are impossible to distinguish in the overall effect because inhibition of enzymatic activity preceding superoxide formation by NADPH oxidase, i.e. any one of G-protein, phospholipase C, or protein kinase C depending on the initial

Table 1
Summary of IC_{50}s obtained for SA and TROLOX inhibition of various activities

Activity	Detection	Substance	IC_{50} (μM)
fMLP-stimulated oxidative burst	Chemiluminescent	SA	1.5
PMA-stimulated oxidative burst	Chemiluminescent	SA	1.8
	Photometric	SA	0.9
	Chemiluminescent	TROLOX	60.0
NADPH oxidation (non-cellular)	Photometric	SA	60.0
		TROLOX	3.0
Singlet oxygen generation	Chemiluminescent	SA	>100
		TROLOX	3.0
Cell-free NADPH oxidase	Chemiluminescent	SA	8.3

The different activities summarized were measured as described in Section 2. Dose–response curves for SA or TROLOX were constructed and their respective IC_{50}s were calculated.

stimulus, has the same result as scavenging of superoxide formed—it prevents the reaction of the radical with its target, e.g. MCLA probe. Therefore a powerful antioxidant may show the same IC_{50} for inhibition of oxidative burst as a potent inhibitor of, for example, PKC. Previous report claims that SA is capable of superoxide scavenging with IC_{50} of 10 μM correlating this concentration to the scavenging ability [11]. There are several methods published and frequently used for assessing the ability of a substance to scavenge ROS. Each one of the methods has its pros and cons with none giving a definite answer. If SA is indeed a more powerful antioxidant than TROLOX, as may be deduced from the whole cell experiments described above, it should display this property in other systems as well. Therefore we used a combination of two independent methods to investigate the ability of SA to scavenge ROS.

3.2.1. SOD-like activity in a purely chemical system using NADPH oxidation

Originally developed to evaluate SOD activity in biological samples, this system uses purely chemical chain of reactions resulting in NADPH oxidation [20]. Advantage of the system is that it contains no enzyme and can be used to assess SOD-like activity of a substance.

Addition of 2-mercaptoethanol into the triethanolamine–diethanolamine–HCl buffer:$MnCl_2$ system triggers chain of reactions at the end of which the present NADPH is oxidized showing a marked decrease in absorbance at 340 nm (Fig. 4, trace A). This activity is inhibited by SA (Fig. 4, trace B) with

the dose–response yielding IC_{50} of 60 μM (Table 1). TROLOX, a water-soluble homologue of vitamin E, caused inhibition of the same system (Fig. 4, trace C) and the dose–response yielded IC_{50} of 3 μM (Table 1). SOD at 5 U/ml caused complete inhibition of the system as well (Fig. 4, trace D).

3.2.2. Redox activity of SA by cyclic voltammetry

The second set of experiments involved cyclic voltammetry measurements. While cyclic voltammetry cannot completely rule out a substance as an antioxidant, it demonstrates the ability of a substance to undergo oxidation/reduction. Most antioxidants fall within the range of 0–350 mV versus SCE for their peak anodic potential, some have a negative
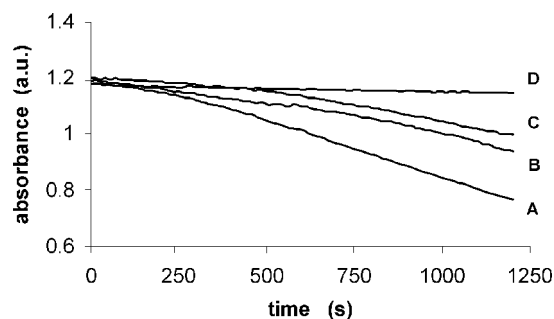

Fig. 4. Time course of SOD-like activity in a purely chemical system. Actual absorbance change at 340 nm due to NADPH oxidation monitored over time is shown. Triethanolamine:diethanolamine buffer pH 7.4 contained $MnCl_2$ and NADPH oxidation of which was initiated by 2-mercaptoethanol addition. Additions prior to reaction start were: none (trace A), 50 μM SA (trace B), 1 μM TROLOX (trace C), and 5 /ml SOD (trace D).
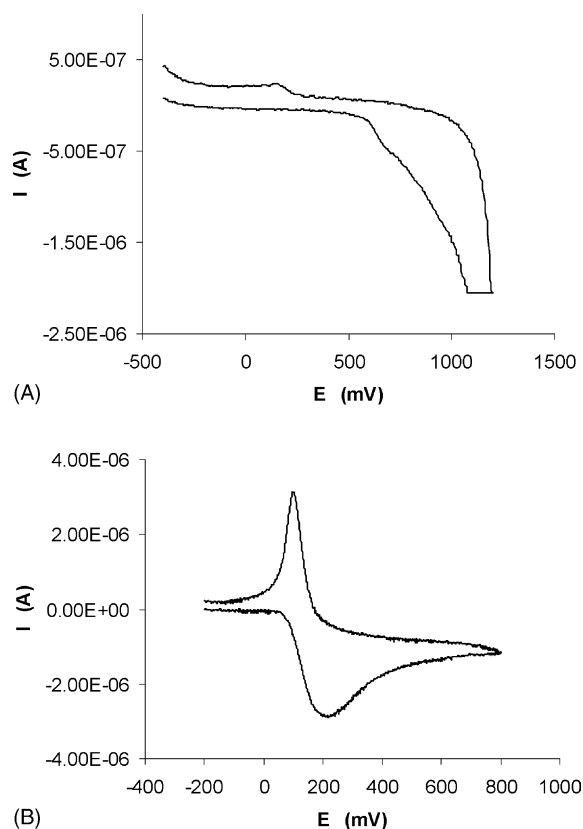
Fig. 5. Cyclic voltammograms. Actual traces of cyclic voltammetry performed at 200 mV/s are shown. All substances tested were dissolved in PBS buffer pH 7.4. (A) 100 μM sanguinarine, (B) 100 μM TROLOX.

peak anodic potential, for example glutathione or *N*-acetylcysteine around −200 mV.

Cyclic voltammetry conducted in PBS buffer pH 7.4 at a scan rate of 200 mV/s yielded a peak anodic potential 700 mV for SA (Fig. 5A), corresponding to its one electron oxidation. The oxidation of SA is followed by a chemical reaction (the so called CE mechanism), therefore the value of SA E'_0 potential will lie around 600–700 mV. Further oxidation of SA primary oxidation products follows at higher potentials. It can be concluded that observation of such a high value of SA peak anodic potential allows us to rule out SA as a superoxide scavenger, since it is thermodynamically impossible to convert SA into its radical cation by superoxide. On the contrary to SA, the peak anodic potential for TROLOX was 200 mV (Fig. 5B) ($E'_0 =$ 150 mV at pH 7.4). Similar measurements conducted

in the triethanolamine–diethanolamine–HCl buffer pH 7.4, the same buffer used in the SOD assay experiments described above, showed the same peak anodic potential for TROLOX at 200 mV and no detectable peak for SA in the range up to 800 mV (data not shown). The narrower scan range is due to oxidation of the triethanolamine–diethanolamine–HCl buffer alone at higher potentials which may mask an SA peak arising at potentials higher than 800 mV.

In addition to the SOD assay and cyclic voltammetry experiments we also utilized a singlet oxygen generating system. In this case we chose to use a system involving a single enzyme, horseradish peroxidase (HRP), rather than the non-enzymatic system using HClO [22]. Disadvantage of the system is the acidic pH 4.5 of the assay buffer. Nevertheless, TROLOX displayed singlet oxygen scavenging ability with IC_{50} of 3 μM and SA demonstrated very poor scavenging with IC_{50} exceeding 100 μM (Table 1).

3.3. Effect of reducing agents on SA inhibition of oxidative burst

It was proposed that SA easily reacts with cysteine residues within a protein [7] as well as directly interacting with GSH [23]. Therefore, reducing agents are likely to affect the SA inhibition of PMA-stimulated oxidative burst. We tested five different reducing agents (Table 2) by pre-incubating 50 μM of a particular agent with 1 μM SA for 5 min and then testing the mixture on differentiated HL-60 cells oxidative burst activity. Besides dithionate, which is considered the most powerful reducing agent, DTT significantly diminished the SA inhibition of oxidative burst. Surprisingly, effect of the remaining reducing agents was not significant somewhat undermining the proposed direct interaction between SA and GSH. Influence of all agents tested alone in control experiments differed widely: while mercaptoethanol and dithionate elevated the intensity of oxidative burst, DTT had negligible effect, and cysteine and GSH diminished oxidative burst confirming their antioxidant abilities.

3.4. Effect of SA on NADPH oxidase in the post-granular fraction

PMA stimulation of oxidative burst proceeds by activation of PKC which in turn causes phosphorylation

Table 2
Effect of reducing agents on sanguinarine inhibition of PMA-stimulated oxidative burst

Agent	SA inhibition (%)
None	28.9 ± 6.8
L-Cysteine	25.2 ± 5.6 n.s.
GSH	22.9 ± 9.7 n.s.
2-Mercaptoethanol	20.9 ± 4.8 n.s.
SDS	17.3 ± 3.5*
DTT	7.1 ± 1.6***
Sodium dithionate	3.4 ± 0.6***

$1 \mu M$ sanguinarine was incubated with $50 \mu M$ concentration of a particular agent for 5 min and the mixture was then added to 10^6 cells/ml which were then stimulated with 62.5 ng/ml PMA. Inhibition is the difference between the superoxide generating activity detected in the presence or absence of a reducing agent and the activity in the presence of SA plus a reducing agent. Data are from at least three independent experiments. n.s., not significant; significantly different from SA alone.

* $P < 0.05$.
*** $P < 0.001$.

of primarily one of NADPH subunits, p47[PHOX], resulting in NADPH oxidase assembly and production of superoxide by this protein. Because SA is much poorer scavenger of ROS than TROLOX in non-cellular systems but more effective in an oxidative burst inhibition in whole cell system, it is likely affecting one or more proteins involved in PMA-stimulated oxidative burst. All the data obtained thus far suggest the NADPH oxidase as an obvious target for SA. The simplest test of this hypothesis is to use SA in a cell-free NADPH oxidase activity assay. However, this assay requires SDS, or another amphiphile, to be present for NADPH oxidase functioning. During our experiments with reducing agents affecting SA inhibition of oxidative burst we found that SDS affects it as well (Table 2). Nevertheless, we proceeded with the experiments expecting a shift in the IC_{50} for SA inhibition. Indeed, the dose–response yielded IC_{50} of $8.3 \mu M$ (Fig. 6, Table 1), approximately five-fold higher than that observed for either stimulus in whole cells. Hill slope value of 2.1 corresponds to that for SA inhibition of oxidative burst supporting again an interaction of SA with more than one site.

The SA inhibition of oxidative burst may be a result of the alkaloid affecting the assembly of NADPH oxidase under physiological conditions. Once associated, the complex can undoubtedly dissociate again as
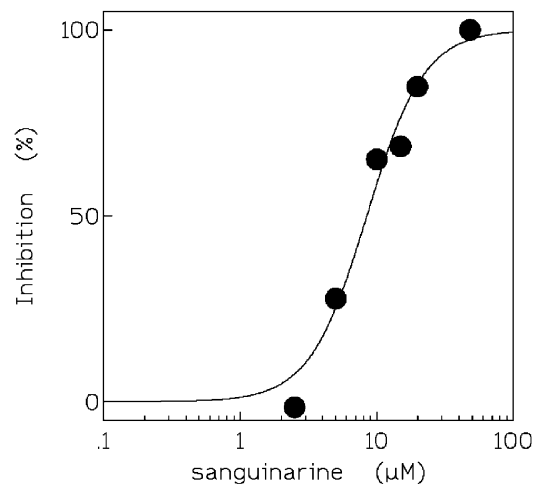

Fig. 6. Dose–response curve for sanguinarine inhibition of NADPH oxidase in post-granular fraction. 10^6 cell equivalents prepared by differential centrifugation from disrupted DMSO-differentiated HL-60 cells were incubated in the absence or presence of variable SA concentrations. NADPH oxidase activity was monitored as superoxide generation in the presence of NADPH by chemiluminescent detection. Photon counts taken for 200 s at 20 s intervals were used for calculation of SA inhibition. 0% inhibition represents activity in the presence of NADPH alone and 100% inhibition represents activity in the presence of 100 U/ml SOD. The derived IC_{50} was $8.3 \mu M$ SA.

seen, for example, in the fMLP-stimulated cells where the superoxide generation is not continuous. To estimate the ability of SA to inhibit a flavoprotein which does not undergo association of multiple subunits in order to become catalytically active, we used human liver microsomes containing the NADPH:CYP reductase which may serve as primary source of superoxide radicals [24]. Activity of NADPH:CYP reductase is easily detected by monitoring absorbance change in cytochrome *c* reduction in the presence of NADPH. This activity is inhibited by SA in dose-dependent manner with IC_{50} of $21.8 \mu M$ (Table 1).

3.5. Effect of SA on PKC substrate phosphorylation in PMA-stimulated cells

Addition of PMA results in activation of PKC which then phosphorylates a number of substrates including at least two of the NADPH oxidase subunits followed by assembly of the protein complex and superoxide generation [15]. Inhibition of PKC activity
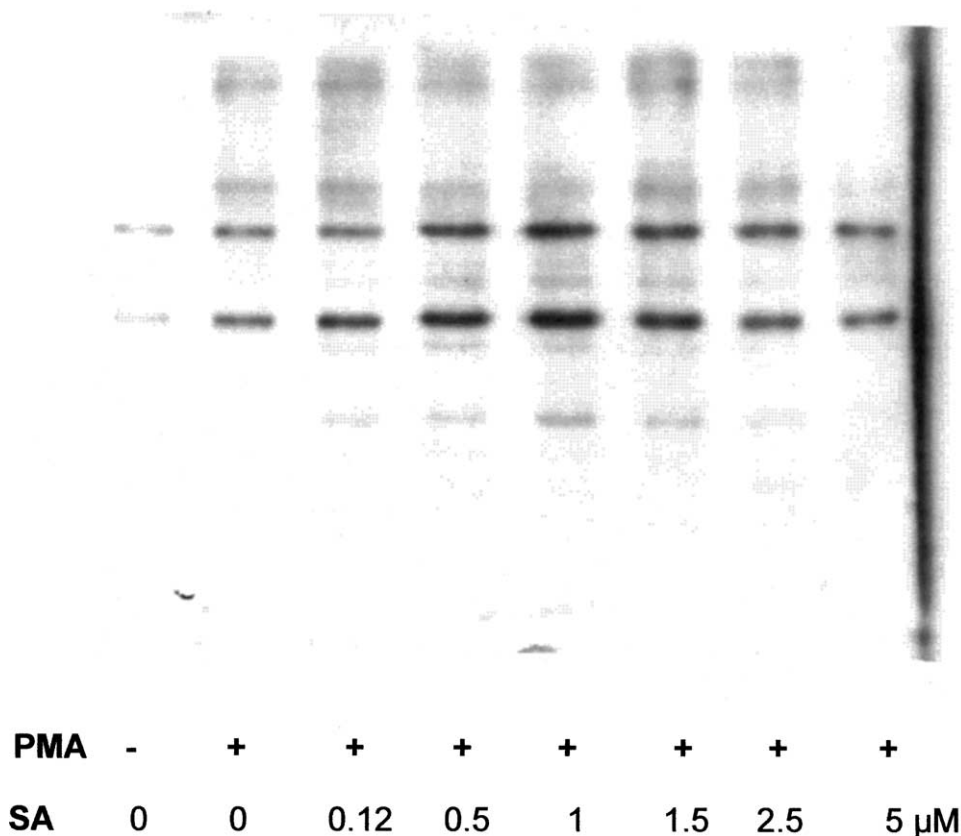
Fig. 7. Effect of SA on phosphorylation of PKC substrates. DMSO-differentiated HL-60 cells were pretreated without (lane 0 SA) or with 0.12–5 μM SA followed by PMA stimulation (lanes + PMA) or untreated (lane 0 SA, − PMA). After 10 min were the cells lysed using 0.2% SDS (w/v); proteins were first separated by SDS-PAGE electrophoresis and then blotted onto nitrocellulose membrane. Phosphorylated PKC substrates were detected by primary phospho-(Ser) PKC antibody visualized by chemiluminescent detection.

will obviously result in decreased phosphorylation of its substrates and most likely failure of the active NADPH oxidase complex to assemble.

To examine whether SA inhibits phosphorylation of PKC substrates under our experimental conditions, we used immunodetection of phosphorylated PKC substrates. In this case inhibitory effect is expected to appear as a decrease in number and intensity of bands detected in the Western blot. We chose to use a low amount of protein loaded per well to make the changes more discernible as a large number of proteins is expected to be phosphorylated upon PMA stimulation. Indeed, addition of PMA increases the number and intensity of protein bands detected in whole cell lysate (Fig. 7, lanes − PMA and + PMA). Even the high-

est concentration of SA used in these experiments, 5 μM, shows very little effect on PMA-stimulated cells (Fig. 7, lane 5 μM) as compared to PMA alone (Fig. 7, + PMA). The lack of SA effect corresponds to our choice of concentration range which is below the IC_{50}s described in the literature for PKC inhibition by SA [13,14].

4. Discussion

Anti-inflammatory properties of SA have been demonstrated in rat paw tests [25] and are readily interpreted as modulation of neutrophil activities [17], including inhibition of oxidative burst [11].

We demonstrate that SA is indeed capable of potent inhibition of fMLP and PMA-stimulated oxidative burst and this inhibition is most likely a result of SA affecting NADPH oxidase activity and/or assembly.

TROLOX, an effective antioxidant, displays much better SOD-like activity than SA in a purely chemical system. The redox capability of TROLOX is also much better than that of SA at physiological pH as revealed by cyclic voltammetry. However, SA is about 40-fold more effective in inhibiting oxidative burst than TROLOX. Perhaps the antioxidant ability of SA may be different in a cellular system. In such a system the more lipophilic SA would associate with cellular membrane and thus would be in proximity to the site of superoxide generation. We do not think this is the case because the electrochemical properties of SA would have to be changed dramatically as well. In a phosphate buffered solution of SA such a change can be achieved in the presence of oxidation potential approaching 900 mV (Jan Hrbáč, submitted to J. Electroanal. Chem.). When SA is subjected to this potential it is transformed into a species which possesses a reversible pair of peaks with $E'_0 = 180$ mV at pH 7.4. The corresponding cathodic peak can be observed at 200 mV on SA voltammogram (Fig. 5A). Detailed analysis of SA electrochemistry reveals that formation of a redox active film from SA oxidation product occurs. Conditions of this kind, however, are unlikely to arise in a cellular system.

The case of silybin supports our hypothesis that peak anodic potentials are reliable indicators of the antioxidant property of a substance. Silybin, the major constituent in *Silybum marianum* extract and a good antioxidant, inhibits PMA-stimulated oxidative burst but is less effective than vitamin E [10]. There are two peak anodic potentials for silybin, 230 and 490 mV, both lower than those obtained for its glycoside analogs [26]. The same report demonstrates that silybin, while weaker radical scavenger than its analogs as shown by DPPH and TEAC assays, is much better in inhibiting lipoperoxidation of subcellular fractions [26].

Judging from a long list of enzymes inhibited by SA [23] it is more likely that inhibition of oxidative burst is a result of enzyme inhibition. NADPH oxidase, of course, is a primary suspect thanks to its role in superoxide generation. Our data of SA inhibiting NADPH oxidase activity in the post-granular fraction support this hypothesis.

Comparison of inhibition of NADPH oxidase and NADPH:CYP reductase, another flavoprotein present in microsomes, offers a more detailed explanation because of the features of the two enzymes. Active NADPH:CYP reductase is a single enzyme anchored by its amino terminus in the endoplasmic reticulum membrane [27] whereas active NADPH oxidase consists of five subunits one of which spans the cytoplasmic membrane [15]. Electron flow in the NADPH:CYP reductase goes from NADPH to FAD to FMN to a final electron acceptor [27]. The membrane spanning cytochrome b_{558} unit of NADPH oxidase has, besides a consensus NADPH binding site and FAD, also two hemes b with electron flow going in that order [15]. The NADPH binding site of NADPH:CYP reductase contains two important cysteine residues [28] which are an ideal attack site for SA as an implication of reducing agent effects on SA presented here and elsewhere [7,8]. Because it is a consensus sequence, cysteine residues are present within the NADPH binding site of flavocytochrome b_{558} as well [15]. Important cysteine residues were also found on the regulatory p47[PHOX] subunit of the NADPH oxidase [29]. While the data with sulfur-containing compounds affecting SA activity support the hypothesis of direct interaction between the alkaloid and cysteine residues within a protein, they are not conclusive. Reducing agents may act as promoters of SA reaction with other sites or substances capable of nucleophilic attack, thus indirectly causing depletion of SA. However, the evidence allows us to speculate that the effect of SA on NADPH oxidase may be due in part to inhibition of flavocytochrome b_{558} and in part to interaction with p47[PHOX].

Lower on the list of target enzymes for SA inhibition are PKC and the two remaining NADPH oxidase subunits found in the cytosol p67[PHOX] and Rac. PKC is inhibited by SA [13,14] but the IC_{50} concentrations are much higher than that we show to be effective in oxidative burst inhibition. Our data on immunodetection of phosphorylated PKC substrates (Fig. 7) show that SA concentrations effective on oxidative burst have little effect on PKC activity. Therefore phosphorylation of NADPH oxidase subunits is not impaired under our experimental conditions. Moreover, the fMLP-stimulated extracellular production

of superoxide was shown to be PKC-independent [30] and because both fMLP- and PMA-stimulated bursts were inhibited by the same concentration of SA (Table 1), we propose that SA affects NADPH oxidase activity or assembly. If PKC is unaffected by low micromolar concentrations of SA, assembly of NADPH oxidase complex may fail due to interaction of SA with membrane associated components of the complex. Even though it carries a positive charge, SA is quite lipophilic with $\log P$ of 2.67 which suggests interactions with more hydrophobic proteins located within a lipid environment. The shift in IC_{50} for SA inhibition of superoxide generation in the post-granular fraction versus whole cells may be caused by SA interacting with the amphiphile present in the former system. Such an interaction, e.g. formation of micellar structures containing SA, changes and apparently weakens the affinity of SA towards its target, most likely flavocytochrome b_{558}. Although not ruled out, PKC probably ranks among minor targets for SA inhibition. The same applies to $p67^{PHOX}$ and Rac proteins.

In summary, we present data supporting the role of SA as an enzyme inhibitor rather than ROS scavenger. We also present evidence suggesting the NADPH oxidase is the primary target for SA in oxidative burst modulation and the mechanism of SA effect is most likely a combination of inhibition of NADPH oxidase assembly and activity of flavocytochrome b_{558}.

Acknowledgements

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