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Apoptosis induction in human breast cancer (MCF-7) cells by a novel venom L-amino acid oxidase (Rusvinoxidase) is independent of its enzymatic activity and is accompanied by caspase-7 activation and reactive oxygen species production

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Abstract We report the elucidation of a mechanism of apoptosis induction in breast cancer (MCF-7) cells by an L-amino acid oxidase (LAAO), Rusvinoxidase, purified from the venom of *Daboia russelii russelii*. Peptide mass fingerprinting analysis of Rusvinoxidase, an acidic monomeric glycoprotein with a mass of ∼57 kDa, confirmed its identity as snake venom LAAO. The enzymatic activity of Rusvinoxidase was completely abolished after two cycles of freezing and thawing; however, its cytotoxicity toward MCF-7 cells remained unaffected. Dose- and time-dependent induction of apoptosis by Rusvinoxidase on MCF-7 cells was evident from changes in cell morphology, cell membrane integrity, shrinkage of cells and apoptotic body formation accompanied by DNA fragmentation. Rusvinoxidase induced apoptosis in MCF-7 cells by both the extrinsic (death-receptor) and intrinsic (mitochondrial) signaling pathways. The former pathway of apoptosis operated through activation of caspase-8 that subsequently activated caspase-7 but not caspase-3. Rusvinoxidase-induced intrinsic pathway of apoptosis was accompanied by a time-dependent depolarization of the mitochondrial membrane through the generation of reactive oxygen species, followed by a decrease in cellular glutathione content and catalase activity, and down-regulation of expression of anti-apoptotic proteins Bcl-XL and heat-shock proteins (HSP-90 and HSP-70). Rusvinoxidase treatment resulted in increase of the pro-apoptotic protein Bax, subsequently leading to the release of cytochrome c from mitochondria to the cytosol and activating caspase-9, which in turn stimulated effector caspase-7. Rusvinoxidase at a dose of 4 mg/kg was non-toxic in mice, indicating that it may be useful as a model for the development of peptide-based anticancer drugs.

Keywords Catalase · Glutathione depletion · Heat-shock protein · Mitochondrial transmembrane potential · Russell's viper

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Introduction

A recent epidemiological survey conducted by the International Agency for Research on Cancer [1] highlighted an alarming increase in occurrence of breast cancer, which is the most frequently diagnosed cancer among women in 140 of 184 countries. Therefore, in order to decrease the global burden of cancer, research must continue to examine novel ways to inhibit cancer progression as well as to develop more effective chemotherapeutic agents against the malignancy. Further, exploring natural resources for potential therapeutic compounds [2] could lead to the development of novel, less toxic anticancer treatments with significantly less severe side effects than currently available treatments.

Cellular homeostasis is maintained via a tightly regulated apoptotic (programmed cell death) mechanism

[3].This is achieved via two major pathways—the extrinsic pathway that occurs through death receptors present in the outer membrane of the cell, and the intrinsic pathway, which is a mitochondria-dependent pathway [3]. Any critical defect in the apoptotic signaling pathways may result in uncontrolled proliferation and growth of cells which may ultimately lead to cancer, and the use of chemotherapeutic agents to induce apoptosis in cancer is one of the effective ways to overcome this deadly disease. However, despite the development of new therapies, acquired multidrug drug resistance in cancer cells has become one of the major impediments against successful treatment. Therefore, new anticancer drugs capable of targeting cancer through multiple mechanisms can provide a significant therapeutic advantage.

Snake venoms show promise in the treatment of several diseases, including cancer [4, 5]. Among the different components, L-amino acid oxidase (LAAO, E.C.1.4.3.2) is a well-studied, important component of snake venom which inhibits growth of mammalian cancer cells by induction of apoptosis and inhibition of angiogenesis, suggesting its potential as a lead compound for anticancer drug development [5, 6]. However, there is significant controversy regarding the anticancer mechanism(s) of snake venom LAAO; it has been shown that the cytotoxic activity of LAAOs is only partly dependent on H_2O_2 production, indicating the presence of LAAO-specific receptors or targets on the cell surface which are involved in the induction of apoptosis [5]. In contrast, it has been reported that the LAAO purified from Bothrops leucurus (Bl-LAAO) venom induces apoptosis in cancer cells through the generation of high amounts of H_2O_2 [7].

The venom of Russell's viper (Daboia russelii russelii) is rich in LAAO (which gives venom its characteristic yellow color [8]); however, at present no attempt has been made to characterize the anticancer potential and mechanism of apoptosis induction in breast cancer cells by LAAO purified from venom of D. r. russelii. In the present study, we explore the cytotoxic mechanism of a novel LAAO, named Rusvinoxidase, purified from venom of Russell's viper from Pakistan.

Materials and method

Venom of Daboia r. russelii was a gift from Kentucky Reptile Zoo, USA; venom was extracted from snakes originating in Pakistan. Protein concentration standard reagents were purchased from BioRad Inc., USA. Chromogenic and fluorogenic caspase substrates were purchased from Sigma-Aldrich, USA. Pre-cast NuPAGENovex® Bis–Tris gels, buffers and Mark 12 unstained molecular mass standards were obtained from Life Technologies (Invitrogen Inc.), USA. Primary antibodies against pro- and anti-apoptotic proteins were a gift from Bioss Antibodies, MA, USA. All other chemicals used were of analytical grade and procured from Sigma-Aldrich, USA.

Purification of a cytotoxic protein (Rusvinoxidase) from Russell's viper venom (RVV)

One hundred seventy-four mg (dry protein weight) of lyophilized D. r. russelii venom was dissolved in 1.5 ml of 25 mM HEPES buffer containing 100 mM NaCl and 5 mM $CaCl_2$, pH 6.8 and the solution was centrifuged at $10,000 \times g$ for 10 min (Microfuge 18 Centrifuge, Beckman Coulter, USA). The resulting clear yellow supernatant was fractionated through a Bio Gel P-100 gel filtration column (2.8 × 80 cm) previously equilibrated with the same buffer [9]. The gel filtration fractions showing cytotoxicity (peak 1) were pooled, desalted and lyophilized. The lyophilized proteins were dissolved in 1.0 ml of buffer A (20 mM Tris–HCl, pH 8.0) and loaded on a Mono Q 5/50 GL anion exchange column previously equilibrated with the buffer A. The column was washed with 3 column volumes of equilibration buffer to elute unbound proteins. The bound proteins were eluted with a linear gradient from 0 to 350 mM NaCl in 20 mM Tris–HCl, pH 8.0 (buffer B) at a flow rate of 0.75 ml/min for 80 min and the elution of proteins was monitored at 280 nm [10]. The protein peaks were desalted and each protein peak was then screened for cytotoxic activity against MCF-7 cells.

Determination of purity and molecular weight of Rusvinoxidase

The homogeneity and molecular mass of the protein showing cytotoxic activity was determined by SDS-PAGE analysis of 5 μg purified protein under both reduced and non-reduced conditions followed by staining with Coomassie Brilliant Blue R-250 [9]. Mobility of purified protein was compared with Mark 12 (Invitrogen) molecular weight markers (2.5–200 kDa) and a linear dependency of log MW versus migration distance of protein bands was observed.

Identification of Rusvinoxidase by peptide mass finger printing analysis

In-gel tryptic digestion of the protein was performed as described by Mukherjee and Mackessy [9]. For LC/MS/MS analysis, an aliquot of trypsin-digested peptide fragments was withdrawn from the digest and the peptides were purified and concentrated using an on-line enrichment column (Agilent Zorbax C18, 5 μm, 5 × 0.3 mm). LC/MS/MS was performed on a LTQ linear ion trap mass spectrometer (Thermo Scientific) using a reversed-phase

nanospray column (Agilent 1100 nanoHPLC, Zorbax C_{18}, 5 µm, 75 µm ID × 150 mm column).The peptides were eluted from the column with a 42 min linear gradient from 25 to 55 % buffer B (90 % acetonitrile, ACN, and 0.1 % formic acid) at a flow rate of 0.3 µl/min. Spectra were collected over a m/z range of 200–2000 Da using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Bioworks 3.0 software (Thermo Scientific) with an intensity threshold of 5000 and 1 scan/group. MS/MS spectra were searched against the NCBInr protein database using the Mascot database search engine (version 2.3). Scaffold 3 proteomic software (Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications.

The tryptic sequences of those peptides (obtained by LC/MS/MS) showing more than 95 % probability were subjected to a BLAST search in NCBInr data base, Swissprot protein sequences (swissprot), and Protein Databank (pdb) proteins against a snake venom protein database (snakes, taxid:8570) using the blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Assay of enzyme activity of Rusvinoxidase

The LAAO activity was assayed using L-kynurenine as a substrate [11]. The unit of LAAO activity is defined as one nano mole of kynurenic acid produced/min under the assay condition. Metalloprotease activity was assayed using azocasein as described by Aird and de Silva [12]. Esterolytic activity was assayed using N-α-p-Tosyl-L-arginine methyl ester hydrochloride (TAME) and N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates [13, 14].

Cell proliferation assay

In vitro cytotoxicity assays were conducted on the MCF-7 human breast adenocarcinoma cell line and human embryonic kidney cell (HEK 293) (ATCC; Manassas, VA, USA) cultured and maintained in Eagle's minimum essential medium (EMEM) and Dulbecco's modified eagle medium, respectively supplemented with 10 % heat-inactivated fetal bovine serum and 0.01 mg/ml bovine insulin at 37 °C in a humidified CO_2 incubator (5 % CO_2, 95 % air). For cytotoxicity assays, 100 µl aliquots of 1×10^5 cells/ml were plated into 96-well plates and treated with various concentrations (0–80.0 µg/ml) of Rusvinoxidase or cytosine-β-D-arabinofuranoside-HCl (AraC, an anticancer drug; 0–80 µg/ml, positive control), or treated with growth medium (negative control), and cells were incubated at 37 °C for 24 h. Complete medium without cells was used

for blank absorbance readings. After 24 h, cytotoxicity of Rusvinoxidase or AraC was measured using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay following manufacturer instructions (ATCC). A standard curve was generated for each assay performed, and the IC_{50} values were calculated from the regression analysis of growth curves of MCF-7 cells in the presence of Rusvinoxidase. All assays were performed in triplicate per treatment and repeated at least three times.

Cellular and nuclear morphological changes in MCF-7 cells induced by Rusvinoxidase

To study morphological changes induced by Rusvinoxidase, 1 ml of 5×10^5 MCF-7 cells were seeded in a 24 well plate and allowed to adhere overnight at 37 °C. The following day, the medium was replaced with complete fresh medium and after treatment with different doses of Rusvinoxidase ($\frac{1}{2} \times IC_{50}$, $1 \times IC_{50}$, and $2 \times IC_{50}$ doses; $IC_{50} = 83$ nM) for differing time periods (0–24 h), both the non-adherent and adherent cells were collected (by trypsinization). For every time point, a control was run in parallel where the cells were treated with only medium. The cells were washed with PBS, re-suspended in culture medium and then stained with 5 µl each of ethidium bromide and acridine orange (AO) (10 mg/ml in PBS). After 15 min of incubation at 37 °C in a humidified CO_2 incubator, the cells were washed twice in PBS and then observed under a fluorescence microscope at ×60 magnification.

Rusvinoxidase-induced nuclear damage in MCF-7 cells was observed by Hoechst 33258 staining [15, 16]. Briefly, after exposure to Rusvinoxidase ($\frac{1}{2} \times IC_{50}$–$2 \times IC_{50}$; $IC_{50} = 83$ nM), both non-adherent and adherent cells were collected, washed in PBS and then fixed in 1 % formaldehyde (in PBS) for 30 min at room temperature. A control was run in parallel in which cells were treated with only medium. After washing the cells with $1 \times$ PBS, they were re-suspended in 100 µl growth medium and incubated with 5 µl of Hoechst 33258 (10 mg/ml) for 30 min at 37 °C. The cells were then washed with PBS, placed on to a glass slide and observed under a fluorescence microscope at ×60 magnification. The percentage of apoptotic cells were counted at four randomly selected microscopic fields.

DNA fragmentation assay of Rusvinoxidase-treated cells

For qualitative assay of DNA fragmentation, 1 ml of MCF-7 cells were plated in 24 well plates at a density of 1×10^6 cells/well and allowed to adhere overnight at 37 °C. The next day, the medium was replaced with fresh medium containing an IC_{50} dose (83 nM) of Rusvinoxidase and

returned to 37 °C for an additional 24 h. The cells treated with only growth medium served as a control. The adherent cells were harvested by trypsinization and combined with non-adherent cells, washed in PBS and DNA was prepared from the pelleted cells following the procedure described by Herrmann et al. [17]. For the quantitative DNA fragmentation assay, the cells were cultured and treated with Rusvinoxidase as above. Following lysis of cells, the lysate was centrifuged at $11,400 \times g$ for 10 min to separate the fragmented DNA (supernatant) from the intact chromatin (pellet). Both fractions were treated with 1.0 ml of 0.5 M trichloroacetic acid (TCA) overnight at 4 °C. The next day, both mixtures were centrifuged and the pelleted DNA was treated with 160 μl of 5 % TCA at 90 °C for 15 min [18]. The DNA content of both fractions was estimated at 260 nm using a NanoDrop 2000 spectrophotometer. The percent fragmentation was determined by calculating the ratio of DNA in the supernatant to the total DNA recovered in the supernatant and pellet, multiplied by 100.

Flow cytometric analysis of apoptosis induction in MCF-7 cells by Rusvinoxidase

The induction of apoptosis in MCF-7 cells by Rusvinoxidase was detected using an APO-BrdU™ TUNEL Assay Kit (Invitrogen, USA). Briefly, 1 ml of 1×10^6 cells/well were plated in 24-well plate and incubated for 18 h at 37 °C, 5 % CO$_2$. Following incubation, the medium was replaced with complete fresh medium containing ½ ×, 1 ×, or 2 × IC$_{50}$ doses (IC$_{50}$ = 83 nM) of Rusvinoxidase, or growth medium (control) and incubated for additional 24 h at 37 °C. The adherent cells were harvested by trypsinization and combined with non-adherent (detached) cells. The cells were washed with PBS (pH 7.4) and then 5.0 ml of 1 % paraformaldehyde was added and cells were incubated on ice for 15 min. The cells were washed twice with PBS, pH 7.4 and then re-suspended in 0.5 ml PBS and stored in 70 % (v/v) ice-cold ethanol at −20 °C for 18 h before proceeding with the TUNEL assay, following the instructions of the manufacturer. The samples were analyzed using flow cytometry (FACscan, Becton–Dickinson, Bedford, MA, USA) within 3 h after staining with pro-pidium iodide.

Estimation of total glutathione and catalase activity in Rusvinoxidase-treated MCF-7 cells

One ml of MCF-7 cells were seeded in a 24-well plate at a density of 1×10^6 cells/well and then allowed to attach to the plate overnight at 37 °C. Culture medium was then replaced with new medium containing an IC$_{50}$ dose (83 nM) of Rusvinoxidase and incubated for additional 0–24 h. For every time point, a control was run in parallel where the cells were treated with only medium. After a specific time interval, both the free non-adherent and adherent cells were collected, and the cell lysate was used to determine total glutathione content and catalase activity [19]. Catalase activity or glutathione content per mg protein of control (untreated) MCF-7 cell lysate at each time point was considered as 100 % activity and treated values were compared to this control.

Assay for caspase activity in MCF-7 cells

MCF-7 cells (1×10^6 cells/ml) were treated with an IC$_{50}$ dose (83 nM) of Rusvinoxidase or growth medium (control) for various time periods (0–24 h); at the end of treatment, cells were lysed with RIPA lysis buffer (Sigma-Aldrich, USA). The cell lysate was centrifuged at $10,000 \times g$ for 10 min at 4 °C and 25 μl of the supernatant was assayed for caspase-3 or caspase-9 using Ac-Asp-Met-Gln-Asp-pNA or Ac-Leu-Glu-His-Asp-pNAas a substrate [9]. The unit of caspase activity (amidolytic activity) was defined as μmoles of 4-nitroaniline released per minute by the enzyme under the assay condition [9] and enzyme activity was expressed as units of enzyme activity per mg of protein. Caspase-8 and caspase-7 activities were assayed by using the fluorogenic substrate Ac-Val-Glu-Thr-Asp-AMCandAc-Asp-Glu-Val-Asp-AMC (20 μM, respectively). After 30 min incubation at 37 °C, release of methylcoumaryl-7-amine (AMC) was monitored in a spectrofluorimeter (Perkin Elmer) at an excitation wavelength of 380 nm and emission wavelength of 460 nm [20]. Caspase activity was expressed as units of enzyme activity per mg protein. For every time period, a control was run in which the cells were treated with growth medium only, and the experimental values were compared to that baseline value.

Determination of total ROS generation in MCF-7 cells

Intracellular reactive oxygen species (ROS) levels generated upon LAAO exposure to 1×10^6 cells were measured using the non-fluorescent compound 2′,7′-dichlorofluorescein-diacetate (DCFH-DA; Sigma-Aldrich, USA). MCF-7 cells were incubated with different doses of Rusvinoxidase or growth medium (control) for 4 h and thereafter both the adherent and free non-adherent cells were collected and washed twice with PBS, pH 7.4. The cells were incubated with 10 μM DCFH-DA at 37 °C for 30 min in the dark followed by washing twice with chilled PBS. The fluorescence intensity of 2′,7′-dichlorodihydrofluorescein (DCF) produced by intracellular reactive oxygen species was analyzed on a flow cytometer with excitation and emission at 480 and 530 nm, respectively.

Assay for mitochondrial transmembrane potential of MCF-7 cells

The toxin-induced change in mitochondrial transmembrane potential (MMP) in MCF-7 cells was determined using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (MitoProbe^{TM} JC-1 Assay kit, Sigma). After exposure of 1×10^6 cells to $1 \times IC_{50}$ (83 nM) or $\frac{1}{2} \times IC_{50}$ of Rusvinoxidase, or growth medium (control) for 0-24 h, the cells were treated with JC-1 or carbonyl cyanide m-chlorophenylhydrazone (CCCP; positive control) following the instructions of the manufacturer, and the fluorescence intensity was determined by flow cytometry (BD Accuri^{TM}C6 Cytometer) with an excitation at 488 nm and emission at 533 ± 30 nm (FL-1 green channel) and 585 ± 40 nm (FL-2 red channel). Cells were initially gated from debris using side scatter and forward scatter. Data were presented as percentage of cells with altered MMP. The Rusvinoxidase-induced change in MMP potential was also detected by observing the Rusvinoxidase-treated and control cells (JC-1 stained) with a confocal laser microscope (Olympus1X81).

Analysis of expression of pro- and anti-apoptotic proteins in MCF-7 cells after Rusvinoxidase treatment through Western blot analysis

For analyzing the expression of pro- and anti-apoptotic proteins, 1×10^6 MCF-7 cells were incubated with Rusvinoxidase (IC$_{50}$ dose, 83 nM) or growth medium (control) for 0–24 h, and both the detached and adhered cells were collected and washed with PBS, pH 7.4. Cells were lysed in 200 μl of RIPA lysis buffer (containing protease inhibitor cocktail) in ice and then centrifuged at $10,000 \times g$ for 20 min at 4 °C. The protein content of the supernatant was estimated by Bradford method (BioRad, Inc.) and 50 μg of protein from each sample was run in 12.5 % NuPAGENovex® Bis–Tris mini gels (Invitrogen). The proteins were electrophoretically transferred to a nitrocellulose membrane and non-specific sites were blocked by incubating the membrane for 1 h at room temperature with 5 % (W/V) skimmed milk in 20 mM Tris buffer saline (TBS), pH 7.4, containing 0.1 % Tween-20. After washing the membrane with TBS (pH 7.4), it was incubated with primary antibodies (1:750 dilution) against Bcl-XL, Bax, HSP-70, HSP-90, cytochrome c or β-actin (internal standard) for 1 h at room temperature and then overnight at 4 °C. The following day, the membrane was washed with TBS (pH 7.4) and then incubated with horseradish peroxidase-conjugated mouse anti-rabbit secondary antibodies (1:3000) for 1 h at room temperature, and the Western blot was developed with SuperSignal^{TM} Western Blot Enhancer (Fisher Scientific). The signal was

recorded with a CCD camera and analyzed through Quantity One 1-D analysis software (VersaDoc^{TM} Imaging System, BioRad, USA). The experiment was repeated three times to assure reproducibility.

Determination of in vivo toxicity of Rusvinoxidase on mouse model

All experimental protocols for animal use were approved by the Institutional Animal Care and Use Committee, UNC (protocol 9401). For determining lethal toxicity, Rusvinoxidase was dissolved in 0.2 ml of PBS, pH 7.4 and injected *i.p* (1.0–4.0 μg/g body weight) into a group of three non-Swiss albino (NSA) mice weighing between 18 and 20 g. Control animals received only 0.2 ml of PBS, pH 7.4. The animals were observed at regular intervals up to 48 h post-injection for death or any physical or behavioral changes [9, 10].

Statistical analysis

To determine any significant differences, data were analyzed by a Student's t test using the software SigmaPlot 11.0 for Windows (version 7.0), with $p \leq 0.05$ considered as statistically significant.

Results

Purification and identification of a cytotoxic protein (Rusvinoxidase) from Russell's viper venom

Fractionation of crude RVV through size exclusion resulted in separation of twelve protein/peptide peaks, named GF1-GF12 (supplementary Fig. S1). The pooled fractions of peak GF1 (showing highest cytotoxic activity) were fractionated into eight peaks following anion exchange FPLC (supplementary Fig. S2). The FPLC fraction (shown with an arrow in Fig. S2) demonstrating significant cytotoxic activity against MCF-7 cells (Table 1) was found to be homogenous by 12 % SDS-PAGE (Fig. 1), displaying a single band with an apparent molecular weight of 57.5 kDa (reduced) and 55.4 kDa (non-reduced). This protein, named Rusvinoxidase (Russell's Viper ʟ-amino acid oxidase), represents 0.3 % of the total protein of crude RVV. A summary of the purification of Rusvinoxidase from crude RVV is shown in Table 1.

The LC–MS/MS analysis of the Rusvinoxidase trypsin digest peptides unambiguously demonstrated its identity (100 % probability, rank 1, 11 % sequence coverage) as an LAAO with high homology to an LAAO (accession no. Q4F867) purified from *D. r. siamensis* venom. BLASTP analysis of 5 unique tryptic peptide sequences of Rusvinoxidase against the NCBI snake venom protein database

Table 1 Summary of purification of Rusvinoxidase, a cytotoxic L-amino acid oxidase from RVV

Purification steps	Total protein (mg)	Protein yield (%)	Cytotoxicity (% cell death)*	Specific activity (% cell death/mg protein)	Purification (fold)
Crude RVV	174	100	13.8	2760.0	111.0
GF-I	17.9	10.4	25.9	5180.0	1.9
FPLC-3 (Rusvinoxidase)	0.6	0.3	48.5	9700.0	3.5

The data represent a typical experiment

* Cytotoxicity (5 µg/ml) against MCF-7 cells after 24 h incubation at 37 °C, 5 % CO_2. Control cells (treated with growth medium) under identical experimental conditions showed 99 % cell viability
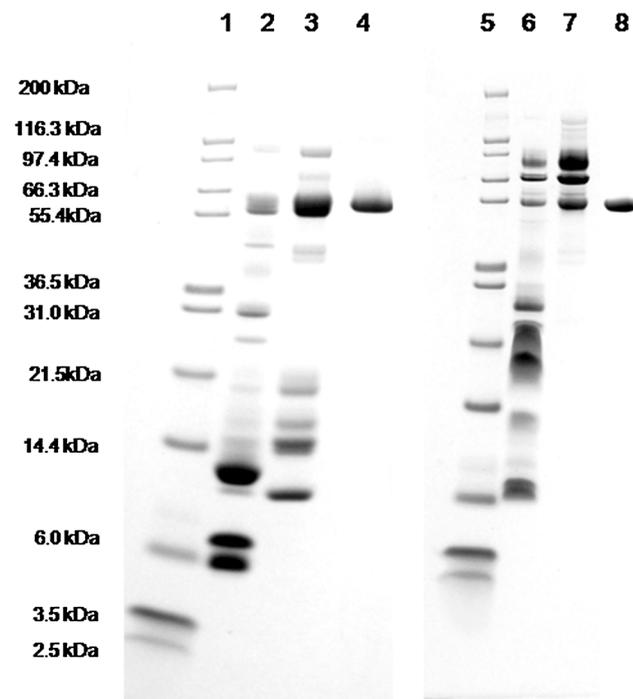

Fig. 1 Determination of the purity and molecular mass of Rusvinoxidase by 12 % SDS-PAGE. *Lanes 2–4* reducing conditions; *lanes 6–8* non-reduced conditions. *Lanes 1* and *5* protein mass standards (Dalton Mark 12); *lanes 2* and *6*, crude RVV (20 µg); *lanes 3* and *7*, gel filtration fraction (GF1) (15 µg); *lanes 4* and *8*, Rusvinoxidase (5.0 µg)

demonstrated that this protein has significant similarity with LAAOs isolated from other snake venoms, especially with those isolated from other viperid venoms (Table 2).

Biochemical characterization of Rusvinoxidase

Rusvinoxidase was yellow in color due to presence of flavin adenine dinucleotide, and it exhibited LAAO specific activity of 23.8 U/mg protein. The LAAO activity diminished progressively after storage at 4 °C yet cytotoxic activity of Rusvinoxidase was not affected (supplementary Fig S3). The LAAO enzyme activity of Rusvinoxidase

following two freeze and thaw cycles was completely abolished and after six cycles of freeze and thaw, the enzyme activity could not be regained after incubation for 24 h at 37 °C; again, its cytotoxic property remained unaffected. Rusvinoxidase did not show protease (azocaseinolytic, fibrinolytic, fibrinogenolytic), phospholipase A_2, TAME- or BAEE-esterase activities.

Rusvinoxidase significantly inhibits proliferation of MCF-7 breast cancer cells

Rusvinoxidase demonstrated significantly higher ($p < 0.05$) dose-dependent cytotoxic activity toward MCF-7 cells compared with the commercial anticancer drug cytosine-β-D-arabinofuranoside (AraC), an antitumor agent which selectively inhibits DNA synthesis (Fig. 2). From regression analysis, the IC_{50} value of Rusvinoxidase towards MCF-7 cells, after 24 h incubation, was 5.5 µg/ml (83 nM). After 24 h of treatment with Rusvinoxidase at a dose of 10 µg/ml ($\sim 2 \times IC_{50}$), MCF-7 cell viability was zero (Fig. 2). Following a 24 h treatment with Rusvinoxidase at its IC_{50} dose, most of the MCF-7 cells were detached from the culture flasks and were found non-adherent in the culture media.

In a sharp contrast, Rusvinoxidase at a concentration of 5 and 10 µg/ml inhibited 6 ± 1.2 and 12 ± 2.1 % (mean $\pm$ SD, n = 3), respectively of HEK cells after 24 h treatment suggesting it has marginal cytotoxicity against normal human cells.

Rusvinoxidase induces morphological changes, chromatin condensation and DNA fragmentation in MCF-7 cells

Microscopic analysis also revealed a dose-dependent and time-dependent decrease ($p < 0.01$) in the population of Rusvinoxidase-treated MCF-7 cells as compared to controls (Fig. 3a). Induction of apoptosis in MCF-7 cells by Rusvinoxidase was evident from the light microscopic observations of changes in cell morphology, loss of cell

Table 2 Homology of Rusvinoxidase tryptic peptide sequences generated by LC–MS/MS

| MS/MS derived peptide sequence	Accession	Homologous protein/reference sequence	Maximum identity (%)	Source organism
HDDIFAYEK	P81375	L-amino-acid oxidase	100	*Macrovipera lebetina*
	P0CC17	L-amino-acid oxidase (Batrox LAAO)	100	*Bothrops atrox*
	DQ104365.1	L-Amino acid oxidase, partial	100	*Daboia russelii siamensis*
	Q4F867	L-Amino-acid oxidase	100	*Daboia russelii siamensis*
	EU663622.1	L-Amino acid oxidase precursor	100	*Daboia russelii*
	Q6TGQ8	L-Amino-acid oxidase (BmooLAAO-I)	100	*Bothrops moojeni*
VTVTYQTTQK	AAZO8620.1	L-Amino acid oxidase	100	*Daboia russelii siamensis*
	Q4F867.2	L-Amino-acid oxidase (DRS-LAAO)	100	*Daboia russelii siamensis*
	ACF70483.1	Secreted L-amino acid oxidase precursor	100	*Daboia russelii*
FWEDDGIQGGK/ KFWEDDGIQGGK	AAZO8620.1	L-Amino acid oxidase	100	*Daboia russelii siamensis*
	Q4F867.2	L-Amino-acid oxidase (DRS-LAAO)	100	*Daboia russelii siamensis*
	ACF70483.1	Secreted L-amino acid oxidase precursor	100	*Daboia russelii*
	B5U6Y8	L-Amino-acid oxidase	100	*Echis ocellatus*
SAGQLYQESLGK	AAZO8620.1	L-Amino acid oxidase	100	*Daboia russelii siamensis*
	Q4F867.2	L-Amino-acid oxidase (DRS-LAAO)	100	*Daboia russelii siamensis*
	ACF70483.1	Secreted L-amino acid oxidase precursor	100	*Daboia russelii*
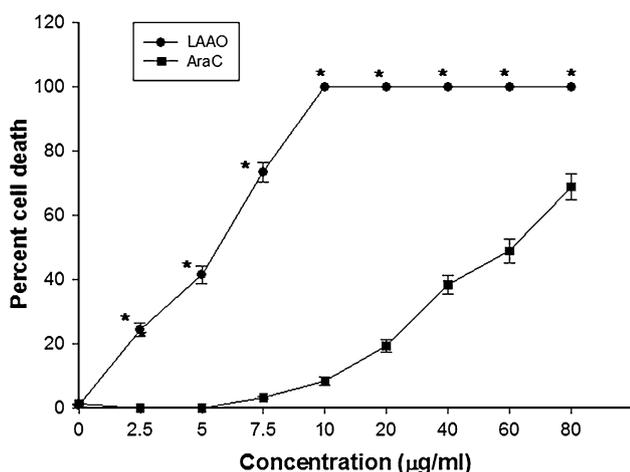
Fig. 2 A comparison of the dose-dependent cytotoxicity of Rusvinoxidase and cytosine-β-D-arabinofuranoside hydrochloride (AraC) towards MCF-7 cells (1×10^5 cells/ml) after 24 h incubation at 37 °C, 5 % CO_2. Data represent mean ± SD of three determinations. The cytotoxic effect of Rusvinoxidase was significantly higher (*p < 0.01) compared to AraC under identical experimental conditions

membrane integrity, and shrinkage of cells; apoptotic body formation was detected in a dose-and time-dependent manner (Fig. 3a). Twenty-four hour incubation of MCF-7 cells with $2 \times IC_{50}$ of Rusvinoxidase resulted in pronounced apoptosis, and apoptotic cells had undergone secondary necrosis (Fig. 3a).The changes in nuclear morphology of treated breast cancer cells were evident from chromatin condensation and formation of apoptotic cells (Fig. 3a). Further, using Hoechst 33258 staining, dose- and

time-dependent apoptosis induction in MCF-7 cells by Rusvinoxidase was observed (Fig. 3b). In addition to being dependent on Rusvinoxidase concentration, percent apoptosis of MCF-7 cells induced by Rusvinoxidase increased with exposure time (Fig. 3c).

Incubation of MCF-7 cells with Rusvinoxidase resulted in an increase in DNA fragmentation of cancer cells as compared to controls (Fig. 4a). This result confirmed that apoptosis was accompanied by DNA fragmentation in MCF-7 cells after exposure to Rusvinoxidase. Using the APO-BrdUTuNEL assay, a routine method to quantify the extent of apoptosis induction by anticancer agents, Rusvinoxidase was found to induce apoptosis (DNA fragmentation) dose-dependently in treated cancer cells as compared with control cells (Fig. 4b).

Rusvinoxidase induces apoptosis in MCF-7 cells via activation of caspases-8, 9 and 7

Increase or decrease in caspase-9, caspase-8, caspase-7 and caspase-3 expression as compared to control (untreated) cells was determined by release of chromophore/fluorophore from their respective chromogenic/fluorogenic substrates by cell lysates of Rusvinoxidase-treated (at $1 \times IC_{50}$ dose) MCF-7 cells. Caspase-9 activity of MCF-7 cells marginally increased after 60 min of treatment with Rusvinoxidase, and peak activity for caspase-8 and caspase-9 was attained 1 and 3 h after treatment, respectively (Fig. 5). Thereafter, a gradual decrease in the level of these enzymes was observed; however, the caspase-9 activity in Rusvinoxidase-treated MCF-7 cells was significantly
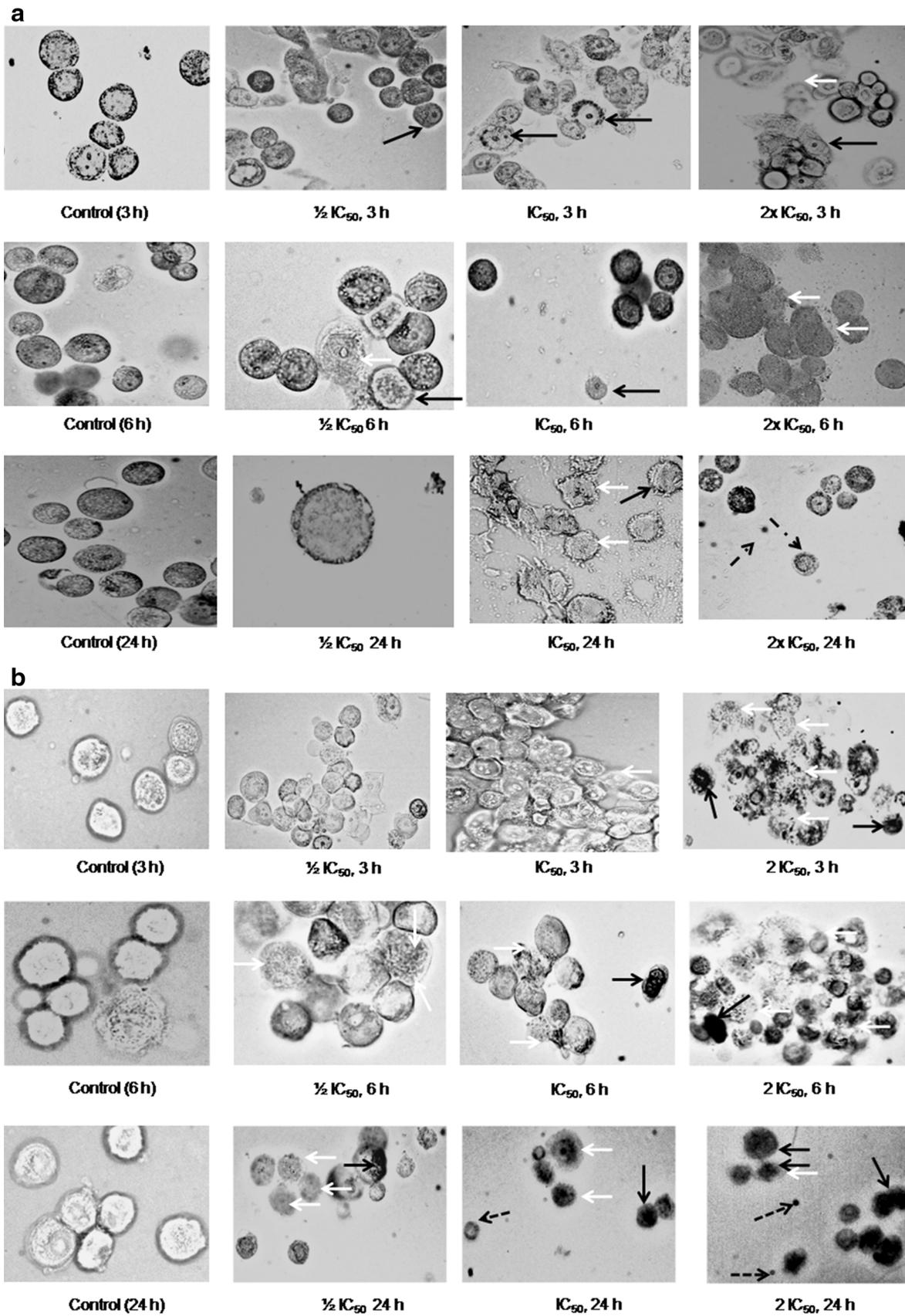
a

Control (3 h)   ½ IC$_{50}$, 3 h   IC$_{50}$, 3 h   2x IC$_{50}$, 3 h

Control (6 h)   ½ IC$_{50}$ 6 h   IC$_{50}$, 6 h   2x IC$_{50}$, 6 h

Control (24 h)   ½ IC$_{50}$ 24 h   IC$_{50}$, 24 h   2x IC$_{50}$, 24 h

b

Control (3 h)   ½ IC$_{50}$, 3 h   IC$_{50}$, 3 h   2 IC$_{50}$, 3 h

Control (6 h)   ½ IC$_{50}$, 6 h   IC$_{50}$, 6 h   2 IC$_{50}$, 6 h

Control (24 h)   ½ IC$_{50}$ 24 h   IC$_{50}$, 24 h   2 IC$_{50}$, 24 h

◄**Fig. 3 a** Dose- and time-dependent morphological changes induced by Rusvinoxidase in MCF-7 cells. Light micrographs were obtained after ethidium bromide-acridine orange staining (×60 magnification). **b** Dose- and time-dependent nuclear changes induced by Rusvinoxidase in MCF-7 cells. The cells were observed under a light microscope after Hoeschst 33258 staining (×60 magnification). The *white arrows* indicate cells with membrane blebbing and shrunken nuclei, the *black (solid)* arrows show chromatin condensation, and *black dashed arrows* indicate secondary cellular necrosis. **c** Quantitation of dose and time-dependent apoptosis induction in MCF-7 cells by Rusvinoxidase. The percentage of apoptotic cells (control as well as Rusvinoxidase-treated) after Hoeschst 33258 staining was counted from four random microscopic fields at ×60 magnification for each treatment. Data represent mean ± SD of three determinations. Significant differences with respect to controls are indicated by *different letters* *$p < 0.05$; **$p < 0.01$
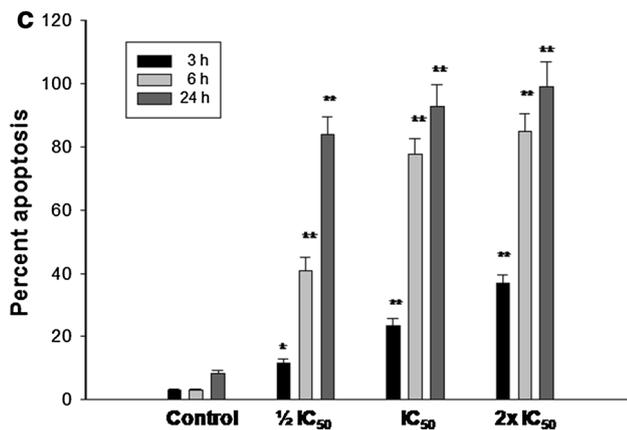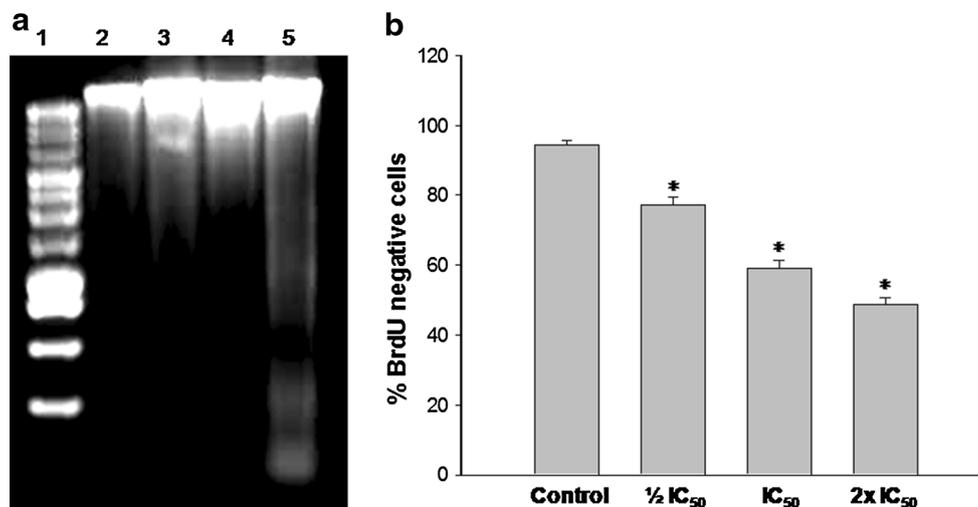
higher than the baseline value (untreated MCF-7 cells) after 24 h of treatment (Fig. 5). Treatment of MCF-7 cells with Rusvinoxidase resulted in a time-dependent decrease in the level of caspase-3 compared to the same enzyme activity displayed by control (untreated) MCF-7cells (Fig. 5). Conversely, extracts of MCF-7 cells treated with
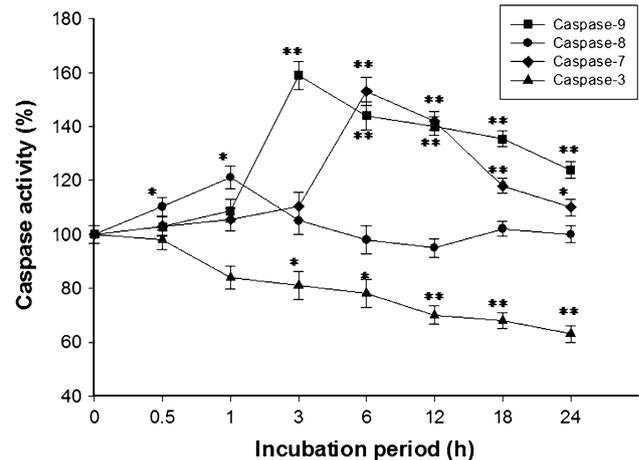

Fig. 5 Analysis of the time-dependent activation of caspases in MCF-7 cells after Rusvinoxidase treatment. MCF-7 (1×10^6) cells were treated with an IC_{50} dose (83 nM) of Rusvinoxidase for various time periods (0–24 h) at 37 °C, 5 % CO_2. The activity of control (cells treated with growth medium) at each time point was considered as baseline and other values were compared to that. Values are mean ± SD of three determinations. Significant differences with respect to controls are indicated by *different symbols* *$p < 0.05$; **$p < 0.01$

Fig. 3 continued

Fig. 4 a DNA fragmentation analysis in Rusvinoxidase-treated MCF-7 cells. The MCF-7 cells (1×10^6 cells/well) were incubated with an IC_{50} dose of Rusvinoxidase (83 nM) or growth medium (control) for 6 or 24 h at 37 °C in a humidified CO_2 incubator. After exposure, DNA fragmentation was analyzed by agarose gel electrophoresis. *Lane 1* 1 kb DNA ladder, *lane 2* DNA from control MCF-7 cells (6 h); *lane 3* DNA from Rusvinoxidase-treated MCF-7 cells (6 h); *lane 4* DNA from control MCF-7 cells (24 h); *lane 5* DNA

from Rusvinoxidase-treated MCF-7 cells (24 h). **b** Analysis of apoptosis (DNA fragmentation) by APO-BrdUTuNEL assay. MCF-7 cells (1×10^6 cells/well) were plated in a 24-well plate and incubated with different doses (½IC_{50}, IC_{50}, and $2 \times IC_{50}$) of Rusvinoxidase for 24 h at 37 °C. The samples were analyzed by flow cytometry within 3 h after staining with PI. Values are mean ± SD of three experiments.*$p < 0.01$ compared to controls

Rusvinoxidase exhibited a time-dependent increase in caspase-7 activity after 3 h of treatment and the peak value was observed 6 h post treatment (Fig. 5). Thereafter, a progressive decrease in caspase-7 activity was observed, although its activity remained significantly higher ($p < 0.05$) in Rusvinoxidase-treated MCF-7 cells compared to control (untreated MCF-7) cells 24 h after treatment (Fig. 5).

Effect of Rusvinoxidase on the level of cellular glutathione and catalase activity in MCF-7 cells

Rusvinoxidase at its IC_{50} value depleted the cellular glutathione (GSH) of treated-MCF-7 cells in a time-dependent manner as compared to baseline values (considered as 100 %) shown by control MCF-7 cells (Fig. 6). Total glutathione levels in Rusvinoxidase-treated MCF-7 cells were lowest at 3 h post-treatment; however, glutathione levels progressively increased with increasing treatment time beyond 3 h (Fig. 6). At 18 h glutathione levels were at ~ 85 % of baseline and they returned to baseline values 24 h after Rusvinoxidase-treatment (Fig. 6). Treatment of MCF-7 cells with Rusvinoxidase resulted in a similar time-dependent decrease in catalase activity of cell-free extract compared to the same activity of control cells (considered as 100 % activity) (Fig. 6). Catalase activity in Rusvinoxidase-treated MCF-7 cells was lowest at 3–6 h post-treatment and thereafter a gradual increase in activity was observed (Fig. 6). Nevertheless, catalase activity was still lower ($p < 0.05$) than that of control cells 24 h after treatment.
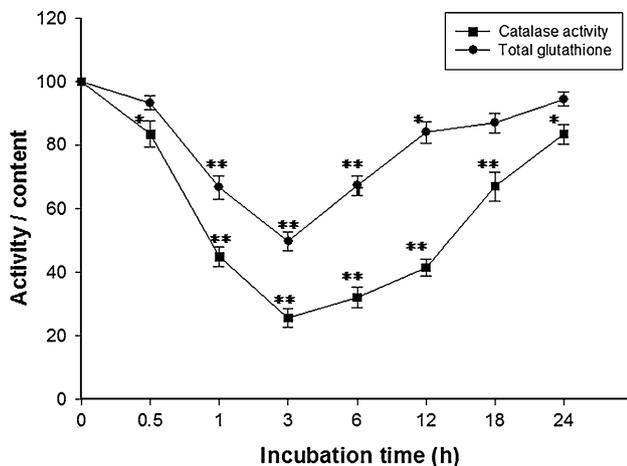

Fig. 6 Determination of time-dependent decrease in GSH and catalase activity of MCF-7 cells after exposure to Rusvinoxidase. MCF-7 (1×10^6) cells were treated with an IC_{50} dose (83 nM) of Rusvinoxidase for various time periods (0–24 h) at 37 °C, 5 % CO_2. The cellular GSH and catalase activity of the untreated MCF-7 cell extract at each time point was considered as 100 % activity and other values were compared to that. Values are mean ± SD of three determinations. Significant differences with respect to controls are indicated by *different symbols* *p < 0.05; **p < 0.01

Fig. 7 a Flow cytometric analysis of Rusvinoxidase-induced changes ▶ in mitochondrial transmembrane potential in MCF-7 cells. After exposure of 1×10^6 MCF-7cells to different doses of Rusvinoxidase for 4 h, cells were treated with JC-1 and CCCP (positive control) and the fluorescence intensity was determined with excitation at 488 nm and emission at 530 nm (monomeric form of JC-1, indicating unhealthy mitochondria or dissipation of mitochondrial membrane potential) and 585 nm (aggregated form of JC-1, indicating healthy mitochondria or intact mitochondrial membrane potential). In each graph, the *lower left* quadrant (Q3) represents endogenous fluorescence of MCF-7 mitochondria, used to set the gating to determine changes in mitochondrial potential in response to treatments. The *lower right* quadrant (Q4) shows the green-fluorescing population of MCF-7 cells that exhibit reduced mitochondrial potential, an indicator of possible apoptotic cells. The *upper right* quadrant (Q2) illustrates MCF-7 cells in which mitochondria are red-fluorescing as an indication of high mitochondrial membrane potential, suggestive of healthy, non-apoptotic cells. The *bar* diagram represents mean ± SD from three independent experiments. *p < 0.01 compared to controls. b Confocal laser microscopic appearance of JC-1 stained (*a*) control MCF-7 cells, and (*b*) Rusvinoxidase-treated (IC_{50} dose, 4 h) MCF-7 cells. c Flow cytometric analysis of Rusvinoxidase-induced ROS generation in MCF-7 cells after 4 h of Rusvinoxidase (½IC_{50}, IC_{50}, 2 × IC_{50} dose) treatment. The ROS level in control MCF-7 cells was considered as baseline (100 %) and other values were compared to that. Data represent mean ± SD of three determinations. *p < 0.01 compared to control

Rusvinoxidase increases the mitochondrial transmembrane potential and ROS generation in MCF-7 cell

Flow cytometric analysis of mitochondrial membrane depolarization using the MitoProbe™JC-1 assay demonstrated that Rusvinoxidase dose- and time-dependently enhanced the depolarization of mitochondrial membranes of treated MCF-7 cells as compared to control cells (Fig. 7a). Confocal laser microscope images of MCF-7 cells stained with JC-1 showed the loss of red J-aggregate fluorescence following exposure to Rusvinoxidase, indicating loss of mitochondrial transmembrane potential of MCF-7 cells after Rusvinoxidase treatment (Fig. 7b).

Rusvinoxidase dose-dependently increased the total ROS production in MCF-7 cells after a 4 h incubation with Rusvinoxidase; at a dose of 2 × IC_{50}, reactive oxygen species' concentrations had increased threefold as compared with controls (Fig. 7c).

Rusvinoxidase increases the expression of pro-apoptotic proteins and down-regulates the expression of anti-apoptotic proteins in MCF-7 cell

Investigation of the time-dependent expression of pro- and anti-apoptotic proteins in MCF-7 cells following exposure to Rusvinoxidase (at $1 \times IC_{50}$ dose)
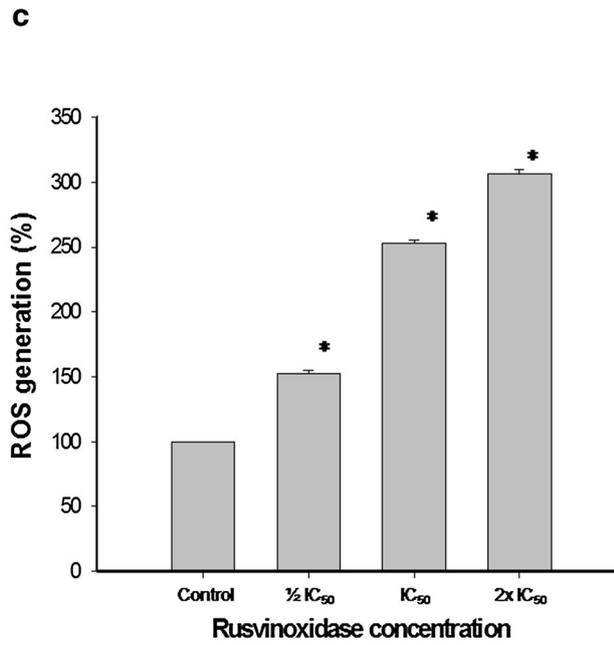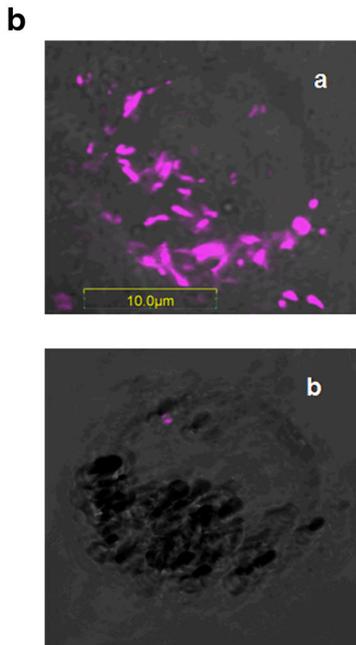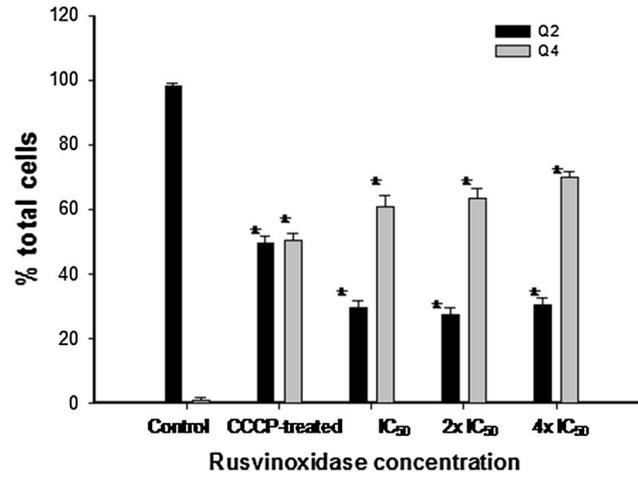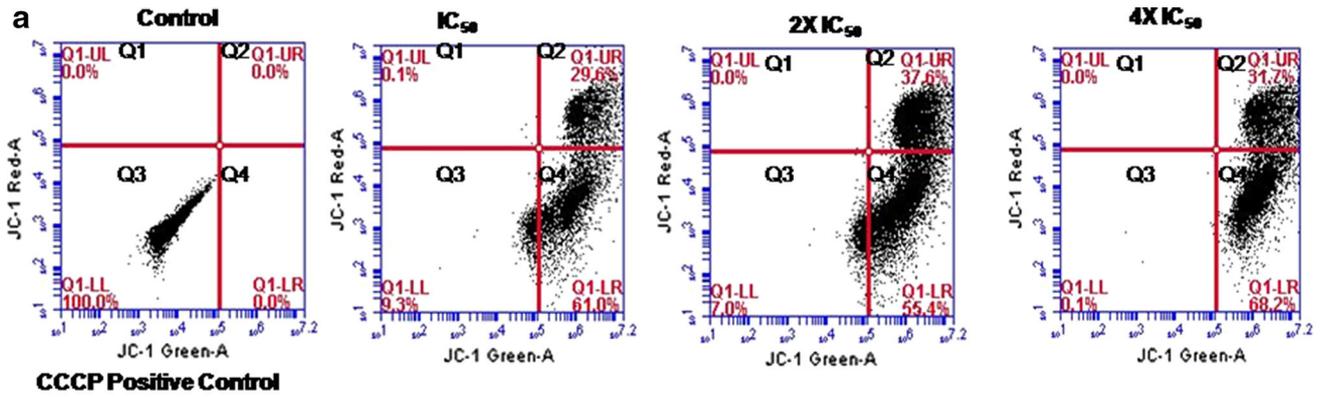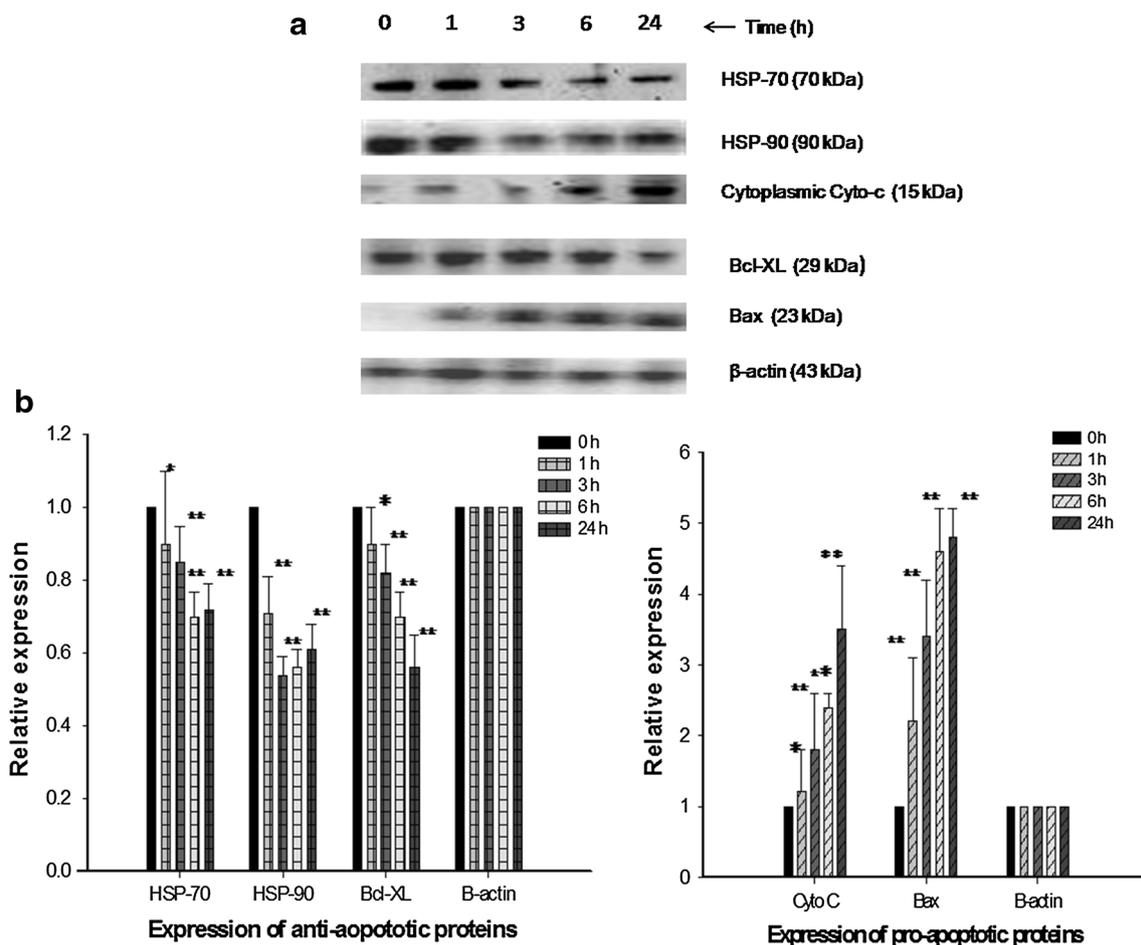
Fig. 8 a Time-dependent expression of pro-(Bax, Cyt C), and anti-apoptotic (Bcl-XL, Hsp90, Hsp70) proteins, and cytochrome c in Rusvinoxidase-treated ($1 \times IC_{50}$) MCF-7 cells. After treatment of the cells for the indicated time period, the 50 μg of cell extracts were separated by 12 % SDS-PAGE and the expressions of proteins were detected by Western blot analysis using antibodies against the targeted proteins. **b** The expression of pro- and anti-apoptotic proteins was normalized to β-actin using ImageJ software and the figures show the mean ± SD of normalized values for three different experiments. Significance of difference compared to control *p < 0.05; **p < 0.001

demonstrated that expression of the anti-apoptotic proteins HSP-70, HSP-90 and Bcl-XL decreased with time; conversely, an increase in the expression of the apoptotic protein Bax was observed (Fig. 8a, b). The release of cytochrome c from the mitochondria to the cytosol of MCF-7 cells was detected as early as 60 min after exposure to Rusvinoxidase but was most apparent by 6 and 24 h after treatment (Fig. 8a, b).

Rusvinoxidase does not show in vivo toxicity in a mouse model

Rusvinoxidase at 4.0 μg/g body weight (i.p.) was not lethal to NSA mice nor did it induce any behavioral changes/adverse effects in treated animals.

Discussion

Snake venom LAAOs are thermolabile proteins; an exception to this rule was shown by an LAAO isolated from *Naja naja oxiana* venom [21] which retains enzymatic activity after repeated freezing and thawing. Repeated freeze–thaw cycles led to a loss in enzymatic activity of Rusvinoxidase, but this did not compromise its cytotoxic properties, indicating that significant differences in substrate-specific properties exist among various members of this family of venom enzymes. The molecular mass of Rusvinoxidase is lower than the molecular masses of LAAO isoenzymes (60–63 kDa) isolated from venom of *D. r. russelii* of eastern India origin [8]. This suggests geographic variation in LAAO enzymes occurs in RVVs.

The cytotoxic activity of snake venom LAAOs has been shown to be linked to production of H_2O_2, a reactive oxygen species, which accumulates on the cell surface and triggers oxidative stress in cancer cells, leading to apoptosis [22, 23]. Nevertheless, recent studies suggest that apart from production of H_2O_2-stimulated cell apoptosis, SV-LAAOs may induce apoptosis via intrinsic (mitochondrial) or extrinsic (death-receptor) pathways [24, 25]. Because Rusvinoxidase lost its enzymatic activity but retained cytotoxicity after freezing and thawing, apoptosis induction by this protein may not be correlated directly with H_2O_2 production on the MCF-7 cell surface. Similar to H_2O_2 production by doxorubicin, an anticancer drug used to treat solid tumors, Rusvinoxidase may also induce perturbation of the cellular redox system that leads to an increase in intracellular H_2O_2 production [26].

Because programmed cell death may follow either the apoptotic or necrotic pathway [3], the mechanism of cell death induced by an anticancer drug prior to its pre-clinical trial should be critically assessed. Rusvinoxidase-induced death of MCF-7 cells demonstrated some hallmarks of apoptosis; however, after prolonged (>24 h) incubation with Rusvinoxidase, MCF-7 cells that have shown apoptotic signs may also show some of the morphological phenotypes associated with necrosis [27]. Moreover, as an indicator of its high potency against MCF-7 cells, the IC_{50} value of Rusvinoxidase was significantly lower than the IC_{50} values of anticancer drugs commonly used to treat breast cancer, such as AraC (present study), cisplatin [28], doxorubicin [29] and tamoxifen [30].

The induction of apoptosis through both the death receptor (extrinsic) and mitochondrial (intrinsic) pathways provides opportunity to develop potent therapeutic molecules against cancer, particularly against multidrug resistant cancers. Both pathways activate a cascade of proteolytic enzymes, caspases that participates in the cleavage of aspartic acid-containing motifs and ultimately results in induction of apoptosis [31]. The extrinsic pathway of apoptosis is triggered by the binding of apoptosis-inducing ligands with their cognate death receptors [32] that results in formation of the death-inducing signaling complex (DISC). Consequently, through a series of events, procaspase-8 is activated to caspase-8 which is then released from the DISC to further activate the effecter caspase-3 or caspase-7 [33]. Notably, activation of inactive procaspase-8 to active caspase-8 occurs through the death receptor, while caspase-9 activation is associated with mitochondrial pathway [31].

The results of present study provide evidence that Rusvinoxidase can induce apoptosis in MCF-7 cells by both the extrinsic and intrinsic pathways; nevertheless, a significantly higher level ($p < 0.01$) of expression of caspase-9 compared to caspase-8 suggests that the intrinsic pathway is more significant in Rusvinoxidase-induced apoptosis in MCF-7 cells. Rusvinoxidase may promote apoptosis through interaction with putative "death receptors" in the plasma membrane of MCF-7 cells that results in initial cleavage of procaspase-8 and activation of subsequent downstream events, leading to apoptosis by activating procaspase 3/7 to caspase-3 or caspase-7 [31, 33]. However, our results agree with the findings of McGee et al. [20] who demonstrated that PBOX-6-induced apoptosis in MCF-7 cells was accompanied by DNA fragmentation due to activation of caspase-7 rather than activation of caspase-3; Rusvinoxidase-treated MCF-7 cells showed a similar increase in caspase-7 and a decrease in caspase-3 activities. It has been suggested that the key role of caspase-3 is regulation of apoptotic DNA fragmentation via proteolysis of caspase-activated DNase [31], but the decrease of caspase-3 activity in MCF-7 cells in response to Rusvinoxidase suggests that caspase-3 may not be essential for execution of apoptosis in MCF-7 cells [20, 34].

The intrinsic pathway of apoptosis has been demonstrated to be associated with key mitochondrial events such as depolarization of the mitochondrial membrane through generation of reactive oxygen species (ROS). This is accompanied by depletion of cellular glutathione that subsequently leads to release of cytochrome c from the mitochondria to the cytosol, activating caspase-9, which is an integral mechanism for initiating and regulating the caspase cascade [31]. The initiator caspase-9 in turn activates effecter caspase-3 or caspase-7 [35]. Under normal cellular conditions, production of excess ROS is downregulated by an antioxidant enzyme such as catalase, as well as by non-protein antioxidants such as GSH, which provide electrons for glutathione peroxidase to reduce H_2O_2 to H_2O [35, 36]. Therefore, reduction in catalase activity as well as depletion of GSH (a hallmark of apoptosis) in MCF-7 cells following exposure to Rusvinoxidase results in the accumulation of excess ROS within the cytoplasm, which in turn induces apoptosis [37, 38]. Glutathione depletion was initially correlated with its oxidation by ROS generated during oxidative stress; however, more recently it has been demonstrated that activation of death receptors also leads to glutathione depletion, involving its extrusion across the plasma membrane [37]. Therefore, Rusvinoxidase-mediated dose- and time-dependent initial depletion of total cellular glutathione may result via both intrinsic and extrinsic pathways of apoptosis.

The time-dependent increase in the level of catalase enzyme activity and cellular glutathione contents in MCF-7 cell at approximately 4-6 h after Rusvinoxidase-treatment may be correlated with the increased level of ROS production. Anticancer drug resistance in human cancer cell lines is found to be associated with marked increase of glutathione synthesis as well as enhanced stability of

mRNA of catalase, the major antioxidant defense systems in cells, to down-regulate the excess ROS production [39, 40]. Therefore, it is suggested that MCF-7 cells may have attempted to counteract Rusvinoxidase-induced apoptosis by increasing (insufficiently) cellular glutathione content and catalase activity; however, the precise mechanism of action still needs to be elucidated. Treatment with curcumin, a pro-apoptotic antitumor agent, has also resulted in elevation of glutathione levels and/or prevention of glutathione depletion, as well as inhibition of caspase-3 activation in human lymphoid Jurkat cells [41].

Apoptosis is regulated by the Bcl-2 protein family, including the anti-apoptotic proteins such as Bcl-XL and pro-apoptotic proteins such as Bax. A balance between the expression levels of both Bcl-XL and Bax is a significant determinant for cell survival or death [42]. It has been well documented that Bcl-XL inhibits apoptotic processes by acting upstream of caspase-3 activation and preventing the release of cytochrome c from the mitochondria, while Bax acts in the mitochondria to cause the release of cytochrome c, leading to the activation of caspase-9 and the subsequent activation of caspase-3 (or caspase-7 in the case of MCF-7 cells) [43, 44]. Therefore, it is expected that diminished expression of Bcl-XL and increased regulation of expression of Bax, leading to alteration of mitochondrial transmembrane potential along with the discharge of cytochrome c to the cytosol, are key events associated with the intrinsic mechanism of apoptosis induction in MCF-7 cells by Rusvinoxidase.

Heat shock proteins (Hsp) are molecular chaperones and have been demonstrated to be inhibitors of apoptosis [45]. Hsp90 is regarded as the inhibitor of oligomerization of the Apaf-1 complex to halt apoptosis, while the function of Hsp70 is to inhibit the signaling pathway from surface receptors to retard apoptosis [45], implying that Hsp could serve as pharmaceutical targets to modulate apoptosis in cancer cells. In the present study, the observed time-dependent down-regulation of expression of Hsp70 and Hsp90 by Rusvinoxidase are likely involved in induction of apoptosis in MCF-7 cells. Furthermore, Rusvinoxidase (4 mg/kg) does not show toxicity or adverse pharmacological effects in mice as well as against normal (non-cancerous) HEK cells, indicating the suitability of Rusvinoxidase as a model compound for the development of peptide-based drugs for the treatment of breast cancer.

Conclusion

Our study suggests that Rusvinoxidase induces apoptosis in MCF-7 cells via both intrinsic and extrinsic pathways; however, the mitochondrial intrinsic pathway predominates. Apoptosis in MCF-7 cells is accompanied by DNA fragmentation due to activation of caspase-7 by Rusvinoxidase rather than activation of caspase-3. Moreover, apoptosis induction by Rusvinoxidase may not be correlated directly with H_2O_2 production on the MCF-7 cell surface. Assessment of anticancer potential, including pharmacokinetics study of Rusvinoxidase in experimental animals, is the goal of our next study.

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