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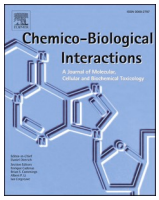

Research paper

Inactivation of ricin by constituents present in a skin decontamination lotion

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A B S T R A C T

Ricin is a proteinaceous toxin, listed on the schedules of both the chemical and biological weapons conventions. The ease of accessibility to the *Ricinus communis* plant and toxin extraction makes ricin a viable concern for use of intentional release and causal effects. The adverse effects following exposure to the toxin are caused by the bipartite molecular structure of ricin which allows binding to the mammalian cell surface, enter via endocytic uptake, and deliver the catalytically active polypeptide into the cell cytosol where it irreversibly inhibits protein synthesis, causing cell death. In the present study, the inactivation effectiveness of RSDL® (Reactive Skin Decontamination Lotion) and its individual inactivating constituents (Potassium 2,3-butanedione monoximate (KBDO) and 2,3-butanedione (DAM)) was evaluated for ricin using a number of read out systems including a cytotoxicity assay, quantitative sandwich ELISA test, and a mass spectrometry-based assay. The results demonstrate that RSDL is able to abolish ricin activity after an incubation time of 30 min as determined in the cytotoxicity assay, and after 2 min as determined in the ELISA assay. Mass spectrometric analysis provided evidence that RSDL is able to induce cleavage of the disulfide linkage between the A- and B-polypeptide chain of ricin which is crucial to the inactivation of the toxin, but this seems not the only mechanism of inactivation. Follow on studies would assist to elucidate the details of the toxin inactivation because it is possible that additional generic mechanisms are in place for denaturation with the RSDL lotion components. This may also provide a promise for testing and inactivation with RSDL of other protein toxins.

1. Introduction

The protein toxin ricin (Ricinus communis agglutinin 60; RCA60) is a toxic protein found in the castor bean plant, *Ricinus communis*. This toxin belongs to the Class II Ribosome Inhibiting Proteins (RIP-II) like other toxins such as abrin and modeccin [1–3]. Active ricin consists of an A- and B-polypeptide chain coupled via a single interchain disulfide (S–S) bond. The B-chain of the protein is associated with binding and internalization into the cell structure, and its cellular trafficking via the Golgi complex and endoplasmatic reticulum (ER), while the A-chain inhibits protein synthesis by modifying the 22S subunits of ribosomes [4]. Ricin, classified as a Category B threat agent and Schedule 1 chemical warfare agent, has gained much attention after a thwarted terrorist plot in Cologne, Germany (2018) with >3000 castor beans and >80 mg isolated ricin being confiscated by the German police [5].

The widespread accessibility of the castor beans, and the absence of an effective antidote for ricin intoxications are important reasons for a quick and accurate detection and decontamination capability. Despite the occurrence of several ricin incidents in the recent past, which have been well documented (see, e.g. Response to ricin contamination | US EPA, and [6], information about decontamination of ricin is scarce. For instance, the Centers of Disease Control & Prevention (CDC) only provides a list of available chemical decontaminants that might be applied to ricin but does not give specific recommendations other than soap and water and provides guidance that the most important factor is getting ricin off or out of the body as quickly as possible. (https://emergency.cdc.gov/agent/ricin/pdf/ricin_protocol.pdf, and https://emergency.cdc.gov/agent/ricin/facts.asp). The Johns Hopkins Center for Health Security (Ricin Toxin (centerforhealthsecurity.org)), proclaims the use of 0.1% sodium hypochlorite for 30 min, but without providing scientific literature sources supporting that statement.

Although ricin presumably does not penetrate the intact skin [7], it is important to enable adequate removal from the skin to prevent cross contamination and secondary exposure via ingestion or inhalation. In general, decontamination efficacy relies on two different mechanisms: physical removal and inactivation of the agent. Dissociation of the A- and B-chain renders the protein unable to enter the cell, making it inactive.

The objectives of the present study were to evaluate the inactivation potential of RSDL (Reactive skin Decontamination Lotion) against the toxin and attempt to elucidate the potential mechanism(s) of inactivation, employing a number of complementary read out systems, i.e. a

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cytotoxicity assay [8,9], a quantitative sandwich ELISA test [10,11], and a mass spectrometry-based assay (see, e.g. Ref. [12] for a recent example).

2. Materials and methods

2.1. Chemicals and consumables

Ricin was isolated from Castor beans according to Lin and Li [13], and subsequently purified in the TNO High Tox facility (Rijswijk, The Netherlands). The purity was determined by SDS-PAGE and found to be >90% (one band).

Antibodies R18 (1.39 mg/mL) and R109 (2.05 mg/mL) for the ELISA assay were kindly provided by Dr. B. Dorner, RKI, Berlin, Germany. The secondary antibody (R18) was biotinylated according to standard procedures.

The ricin-derived bridged peptide A-S-S-B (C*APPPSSQF – ADVC*MDPEPIVR, in which the C* residues are linked through a disulfide bridge was a generous gift from OPCW laboratory (Rijswijk, The Netherlands).

The RSDL kit contains a viscous amber-coloured, low-volatility lotion and a specially designed nonreactive sponge. The lotion component of the RSDL kit is composed of a nucleophilic salt, potassium 2,3-butanedione monoximate (KBDO, 1.25 molal) and its corresponding oxime, 2,3-butanedione monoxime (0.58% w/w) as a buffering agent, dissolved in a semi-aqueous solvent of polyethylene glycol monomethyl ether [14].

The training version of RSDL (t-RSDL) is an inactive neutral physical simulant of RSDL, which does not contain the active ingredients KBDO and 2,3-butanedione monoxime; we used t-RSDL as a surrogate negative control, assuming USP/EP/BP Glydant Plus does not have any activity. The training version of RSDL lotion consists of a semi-aqueous solvent of Polyethylene Glycol 600 NF and 0.2% USP/EP/BP Glydant Plus) and was provided as kits by Emergent BioSolutions (Gaithersburg, MD, USA).

The lotion was extracted from sponges from fresh packets, and stored in the dark at room temperature (RT). To evaluate the decontamination efficacy of RSDL, ricin was incubated in different compositions of RSDL ingredients or RSDL prior to measuring its presence and biological activity, as described in detail in the subsections below.

Noncritical reagents were obtained from renowned commercial suppliers.

2.2. Cytotoxicity assay

2.2.1. Propagation and maintenance of Vero cell culture

The cytotoxicity assay was carried out according to Lemmer et al. [8] and Pauly et al. [9]. African green monkey kidney epithelial (Vero) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were quickly thawed by gently swirling in a 37 °C water bath. Then the cells were transferred to a 15-mL centrifuge tube and 10 mL culture medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 1% penicillin and streptavidin) was added slowly with gentle swirling. The cells were centrifuged at 200×*g* for 6 min. The pellet was resuspended in 4 mL culture medium and the suspension was transferred to two 25-cm^2 tissue culture flasks with vented caps and cultured at 37 °C in a humidified CO_2 incubator. The next day cells were monitored and culture medium was replaced by fresh medium. When cells were grown to 60–90% confluent monolayers, the cells were trypsinized with 0.5 mL Trypsin/EDTA solution (0.25%/0.02% (w/v)) in phosphate buffered saline (PBS) for 3 min at 37 °C in a CO_2 incubator and complemented with 3.5 mL culture medium. Then, the cells were transferred to 15-mL centrifuge tubes and centrifuged at 200×*g* for 6 min and resuspended in 4 mL culture medium. Cells were cultured in a 1:20 and 1:40 dilution.

2.2.2. CellTiter 96® non-radioactive cell proliferation assay

To increase cell yield, cultures were continued in 75-cm^2 tissue culture flasks. When cells were grown to 60–90% confluent monolayers, the cells were trypsinized with 1.5 mL Trypsin/EDTA solution (0.25%/0.02% (w/v)) in PBS for 3 min at 37 °C in a humidified CO_2 incubator and complemented with 10.5 mL culture medium. The cells were transferred to 15 mL centrifuge tubes, centrifuged for 6 min at 200×*g* and resuspended in 5 mL culture medium. Cells were counted with a Bürker counting chamber and a 50,000 cells/mL solution was made. Then, 5,000 cells/well were cultured in a 96-well plate for 18 h at 37 °C in a CO_2 incubator. Cells were treated with ricin standard dilutions or samples and incubated for 2 h. Cells were washed 3 times with PBS, 100 μl culture medium was added, and cells were further incubated for 44 h. To determine the level of proliferation, 15 μl dye solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT, Promega, Madison, WI, USA) was added to each well and incubated for 4 h at 37 °C in a CO_2 incubator followed by addition of 100 μl solubilization/stop solution (Promega, Madison, WI, USA) to each well. The cells were left standing for 1 h and the absorbance was recorded at 570 nm (referenced to 690 nm) using the 96-well plate reader.

2.2.3. Incubation of ricin samples with RSDL and t-RSDL, followed by cytotoxicity assay

A stock solution of purified ricin (2.12 mg/mL in PBS) was diluted to 11.2 μg/mL in PBS and further diluted to 112 ng/mL in culture medium. Standards (112 ng/mL – 0.35 ng/mL) were made by 1:3.16 serial dilutions in culture medium. Cells were exposed to ricin (2 h) by addition of 30 μl standards to 75 μl fresh culture medium yielding final concentrations of 32–100 ng/mL.

RSDL or t-RSDL was spiked with ricin at a 35 μg/mL level (incubation time approximately 30 min) and subsequently the samples were further diluted to 350 ng/mL in culture medium. Samples (350 ng/mL – 1.11 ng/mL) were made by 1:3.16 serial dilutions in culture medium. Subsequently, cells were exposed to these pre-treated ricin samples by addition of 30 μl sample to 75 μl fresh culture medium yielding final theoretical ricin concentrations in the range of 0.3 ng/mL – 100 ng/mL. Incubation and evaluation of results was performed as described in the previous paragraph.

2.3. Quantification of ricin using ELISA

The ELISA assay was carried out according to Simon et al. [10] and Worbs et al. [11]. Unless mentioned differently, 96-well plates were washed 4 times with 300 μl PBS-Tween 20 (PBS-T, 0.1%, vol/vol) between incubation steps, chemicals were diluted in casein blocking buffer (Senova, Weimar, Germany) and incubation steps were performed using a volume of 50 μl at room temperature unless mentioned otherwise. MaxiSorp microtiter plates were coated with ricin-specific monoclonal antibody R109 (2.5 μg/mL in PBS) overnight at 4 °C. Following washing, the plates were blocked with 200 μl casein buffer for 1 h. Ricin standards, positive controls and samples in PBS, 0.1% bovine serum albumin (BSA) were added and incubated for 2 h. Next, the sandwich ELISA was continued by incubation with the biotin-labelled secondary monoclonal antibody R18 (1 μg/mL in casein buffer) for 1 h, followed by labeling with streptavidin-PolyHRP40 (Senova, 0.2 μg/mL) for 30 min. After washing the plates 8 times, color was developed by adding 100 μl substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Seramun diagnostica, Heidesee, Germany) for 15 min and stopped with 100 μl 0.25 M sulphuric acid. The absorption was measured photospectrometrically at 450 nm (referenced to 620 nm) using a microtiter plate reader (Synergy™, BioTek instruments, Winsooki, VA, USA).

2.3.1. Incubation of ricin samples with RSDL and individual constituents, followed by quantification by ELISA

A stock solution of purified ricin (2.12 mg/mL in PBS) was diluted to 100 ng/mL in PBS, 0.1% BSA. Standards (100 ng/mL – 0.32 pg/mL) and

positive controls (400, 125 and 50 pg/mL) were prepared in 0.1% BSA. To determine the effects of RSDL and its components, ricin was incubated in different compositions of RSDL components (1.25 M DAM or KBDO) in Milli-Q water (MQ) or 90% MPEG; alkaline solutions (2 M or 20 mM potassium hydroxide (KOH)) in MQ or 90% MPEG or RSDL and incubated for 2 or 30 min (final ricin concentration was 100 ng/mL). To evaluate matrix effects on assay performance, all samples were independently spiked with 125 pg/mL ricin after dilution in PBS, 0.1% BSA and measured in parallel. For quantification of ricin, only samples were included in which the resulting ricin content was between 100 and 150 pg/mL, indicating no negative or positive effects of the matrix in the assay.

For accurate pH determinations within this series of experiments, samples were diluted with water (100x), and subsequently 100 μl of the diluted sample was applied on a CupFET 3200-010 probe, after which the pH was read from the probe by the Sentron SI 400 pH meter (Leek, The Netherlands).

2.4. Incubation of synthetic A-S-S-B peptide with RSDL and constituents

Reference peptide A-S-S-B (C*APPPSSQF – ADVC*MDPEPIVR, in which the C* residues are linked through a disulfide bridge; MH^+, 2275) was spiked in RSDL and in different compositions of RSDL lotion constituents (1.25 M KBDO in 90% MPEG, 2 M KOH in 90% MPEG) or PBS at a level of 50 μg/mL, and left for 30 min at room temperature. Samples were then diluted 10x with 5% formic acid. Separation of peptide material from the RSDL components was performed by extraction through a ZipTip C18 pipette tip (Millipore, ZTC18S096, Sigma -Aldrich). A volume of 10 μL was applied to the tip according to the manufacturer's instructions. The tip was washed with 0.1% trifluoroacetic acid (TFA) in H_2O and subsequently the tip was eluted with 10 μL acetonitrile/water (1/1), containing 0.2% TFA. Samples were diluted 10x with 0.1% TFA before analysis with LC-MS/MS. Samples were analyzed directly after work-up, as well as after prolonged standing.

2.5. Mass spectrometric analyses

2.5.1. Incubation of ricin with RSDL or RSDL constituents followed by solvent-assisted tryptic digestion

A stock solution of ricin (2.12 mg/mL in PBS) was diluted to 275 μg/mL in PBS. 5 μl of the dilution was added to 50 μl different compositions of RSDL lotion components (1.25 M DAM or KBDO) in MQ or 90% MPEG; alkaline solutions (2 M or 20 mM KOH) in MQ or 90% MPEG or RSDL lotion and incubated for 30 min (final ricin concentration was 25 μg/mL). Then 50 μl of these mixtures were added to 450 μl 50 mM ammonium bicarbonate (ABC) and 400 μl of the samples was filtered on centrifugal filter units (MWCO 10000, Millipore) and centrifuged at 14,000×*g* for 20 min. The sample in each centrifugal filter unit was washed 2 times with 400 μl 50 mM ABC. The samples were concentrated to a minimal volume and were brought to 100 μl with 50 mM ABC and transferred to clean Eppendorf tubes. 100 μl methanol was added to the samples followed by 10 μl of a freshly prepared trypsin (TPCK treated from bovine pancreas, Sigma Aldrich) solution (1 mg/mL in 50 mM ABC) was added. The cleavage reaction was allowed to proceed at 25 °C overnight. The peptides were recovered by spinning down the digest solution on centrifugal filter units (MWCO 30000, Sartorius, Göttingen, Germany) to a minimal volume and twice washing of the filter with 50 μl 50 mM ABC. Then the samples were dried in a vacuum centrifuge and the digests were dissolved in 50 μl 10% acetonitrile/1% formic acid, before injection into the LC-MS/MS system.

2.6. Protocol for higher ricin concentrations and on-filter trypsin digestion

A stock solution of ricin (2.12 mg/mL; 5 μl) was spiked in different compositions of RSDL lotion ingredients: KBDO in 90% MPEG, 2 M KOH in 90% MPEG, RSDL, or PBS (control sample) and incubated for 30 min at room temperature (final concentration of ricin was 190 μg/mL); a blank (PBS) sample without ricin was also included. 50 μl of each mixture was added to 450 μl 50 mM ABC and 450 μl of each sample was filtered on centrifugal filter units (MWCO 10000) and centrifuged at 14,000×*g* for 20 min. The samples were washed twice with 400 μl 50 mM ABC. The samples were concentrated to a minimal volume and brought up to a volume of 100 μl with 50 mM ABC. Then 100 μl methanol was added to the samples. The samples were incubated overnight with 10 μl of a freshly prepared trypsin solution (1 mg/mL in 50 mM ABC) at 25 °C. The tryptic digest was collected by spinning down the digest solution on the same centrifugal filter units (14,000×*g* for 20 min), followed by washing (2x) of the filter with 100 μl 50 mM ABC. The filtrates were concentrated in a vacuum centrifuge and subsequently dissolved in 50 μl 10% aqueous acetonitrile/1% formic acid, before analysis with LC-MS/MS.

2.6.1. Targeted LC-MS/MS analysis of A-S-S-B peptide

The peptide digests obtained after treatment were injected (1 μL inj. volume) in a LC-system containing a Waters HSS-T3 Column (100 mm × 2.1 mm x 1.8 μm) at 21 °C. A gradient was used according to the following programme: 100% A in 20 min–60% A, in 5 min–20% A, in 0.1 min–100% A 4.9 min, at a steady flow rate of 100 μL/min, with A = 0.2% formic acid in water, and B = 0.2% formic acid in acetonitrile.

The LC-system was coupled to a Xevo-TQS triple quad mass spectrometer (Waters, Milford, MA). Peptides were ionized via electrospray, and scanned in selected reaction monitoring (SRM) mode, using 1 mass unit resolution. MS/MS measurements were performed in the positive ion multiple reaction monitoring (MRM) mode, making use of the parameters mentioned in Table 1, using argon as the collision gas (at an indicated flow rate of 0.15 mL/min). The samples were screened for presence of the bridged peptide C*APPPSSQF – ADVC*MDPEPIVR ("RIP-peptide"), in which the C* residues are linked through a disulfide bridge. This peptide, abbreviated as A-S-S-B represents the A-chain T24 tryptic fragment, linked to the B-chain T1 tryptic fragment through a disulfide bridge and is a diagnostic peptide for the presence of intact ricin.

2.7. Targeted analysis of additional ricin derived peptides

In addition to the bridged peptide, two other tryptic peptides (LEQLAGNLR and YTFAFGGNYDR, both from the A-subunit) were analyzed after solvent assisted trypsin digestion, followed by mass spectrometric analysis. Mass spectrometric conditions are shown in Table 2.

3. Results

3.1. Effect of RSDL on cytotoxicity of ricin as determined through Vero cell viability assay

First, a viability assay was implemented to enable evaluation of the ricin toxicity. To that end, African green monkey kidney epithelial (Vero) cells were incubated with different concentrations of ricin for 2 h. Viability of the cells was assessed at 20 or 44 h after the end of the incubation period by quantifying reduction of MTT in metabolically active or living cells.

It was observed that the initial number of cells did not affect the viability assay result, whereas an increase of the delay time after ricin incubation from 20 to 44 h substantially increased the sensitivity of the assay. From these experiments, an initial cell number of 5,000 cells/well and a post incubation delay of 44 h until determination of metabolic activity was determined to be a valid method to measure toxicity of ricin (see Fig. 1).

After incubation of 100 ng/mL purified ricin with RSDL lotion or t-RSDL lotion for approximately 30 min, the remaining toxicity of these samples after both treatments was compared in the Vero cell viability

Table 1
Mass spectrometric settings for the analysis of A-S-S-B peptide.

Peptide	Ricin chain	MH^+	Prec ion MH_3^{3+}	Transition			CE (eV)	Cone voltage (V)	Dwell time (ms)
				1	2	3			
C*APPPSSQF -ADVC*MDPEPIVR[#]	A(T24) -ss-B(T1)#	2275	759.2	710	484	166	25	20	150

[#]C* residues are connected through a disulfide bridge.

Table 2
Mass spectrometric settings for the analysis of Ricin Proteospecific Tryptic peptides.

Peptide	MH^+	MH_2^{2+}	transition			Collision energy (eV)	Cone voltage (V)	Dwell time (ms)
			1	2	3			
LEQLAGNLR	1013	507.0	771	643	530	13	20	87
YTFAFGGNYDR	1311	655.7	1046	828	192	20	5	87

assay. The results are shown in Fig. 2. The results indicate that active RSDL, but not the negative control (t-RSDL), inactivates ricin.

3.2. Effect of RSDL lotion and its individual components on ricin, as determined through ELISA activity

Initial experiments revealed that after treatment of 100 ng/mL ricin with RSDL lotion for approximately 30 min, ricin could no longer be detected by means of the ELISA assay, while after incubation with t-RSDL ricin could still be detected (see Fig. 3). Note that samples had to be diluted before performing the ELISA test; in case samples were measured undiluted, the response in the immunoassay was significantly lower. This preliminary experiment indicates that the absence of ELISA-detectable ricin, as indicated by decreased or absent ELISA activity, coincides with a decreased cytotoxicity, as shown above. To determine the contribution of different components and properties of RSDL to the decrease in ELISA activity, purified ricin was mixed at 100 ng/mL in different compositions of RSDL components (1.25 M DAM or KBDO) in MQ or 90% MPEG; alkaline solutions (2 M or 20 mM KOH) in MQ or 90% MPEG or RSDL and incubated for 2 or 30 min. The pH of each solution was determined after dilution (100x) with water (see Table 3).

The samples were then quantified for the presence of ricin using the ELISA assay. To correct for possible matrix effects in the ELISA, only results in which a standard addition of ricin fell within acceptable quantification limits (100–150 pg/mL for the 125 pg/mL standard) were included. The results are shown in Fig. 4. It is shown that the standard curves were highly reproducible. In addition, it is clear that treatment with RSDL in all cases completely abolished ELISA activity after 2 min. The same result, complete absence of ricin activity, was observed at a high alkaline solution of 2 M KOH, both in MQ as well as in 90% MPEG. Whereas in the RSDL samples the standard addition still yielded detectable amounts, indicating that the absence of ricin activity resulted from RSDL interaction, for the 2 M KOH solutions the conditions immediately appear to interfere with ricin/antibody activity. After incubation with a slightly less alkaline solution, 20 mM KOH in either MQ or 90% MPEG, ricin could be detected in a number of cases. Whereas KBDO in MQ, or DAM in 90% MPEG did not affect activity of ricin in all cases, KBDO in 90% MPEG produced a similar effect as RSDL. In none of these cases, ricin could be detected using ELISA in the samples that were incubated with 1.25 M KBDO in 90% MPEG. The results indicate that effects, if present, occur within a short time frame and that KBDO and MPEG should both be present to achieve a comparable result to RSDL, at a similar pH.

3.3. Mass spectrometric analyses

A similar set of ricin samples (i.e., treated with RSDL or its individual constituents), using higher ricin concentrations (25–190 μg/mL), was prepared for mass spectrometric analysis. The aim was to investigate whether the inability to detect ricin by ELISA after treatment of ricin by RSDL is caused by RSDL-induced changes within the primary structure

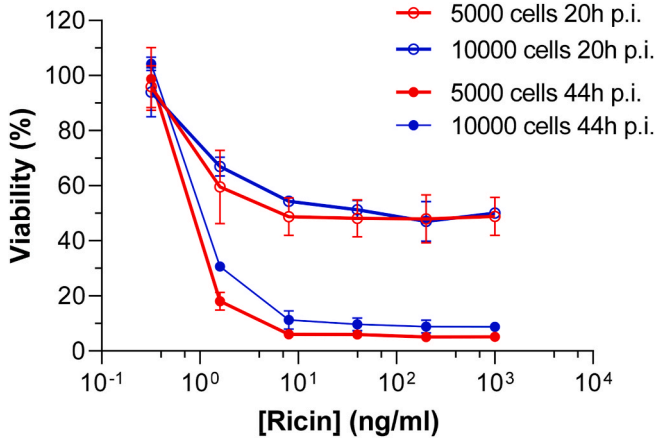

Fig. 1. Optimization of the MTT assay for cellular viability. Viability of Vero cells was assessed at 20 or 44 h after a 2 h incubation time with various final concentrations of ricin; p.i. = post incubation.

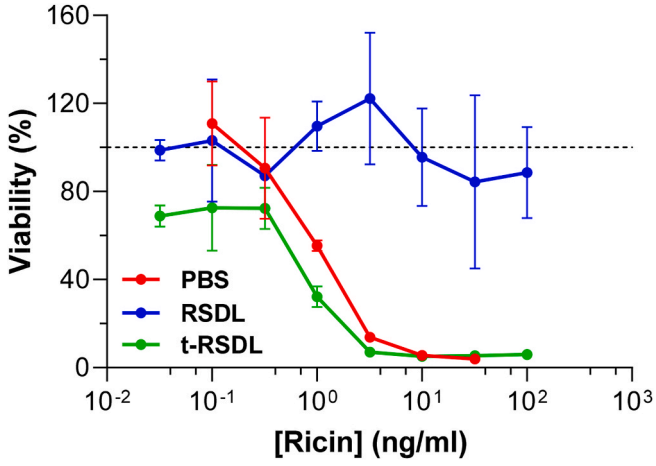

Fig. 2. Viability of Vero cells measured after a 2-h incubation with various concentrations of ricin, which had been pre-treated with RSDL, t-RSDL or PBS for approximately 30 min. After the 2-h incubation period, cells were washed, and after addition of fresh medium the cells were incubated for 44 h and viability was determined via the MTT assay. Results are shown as mean for n = 2 and error bars are indicative for the SD.

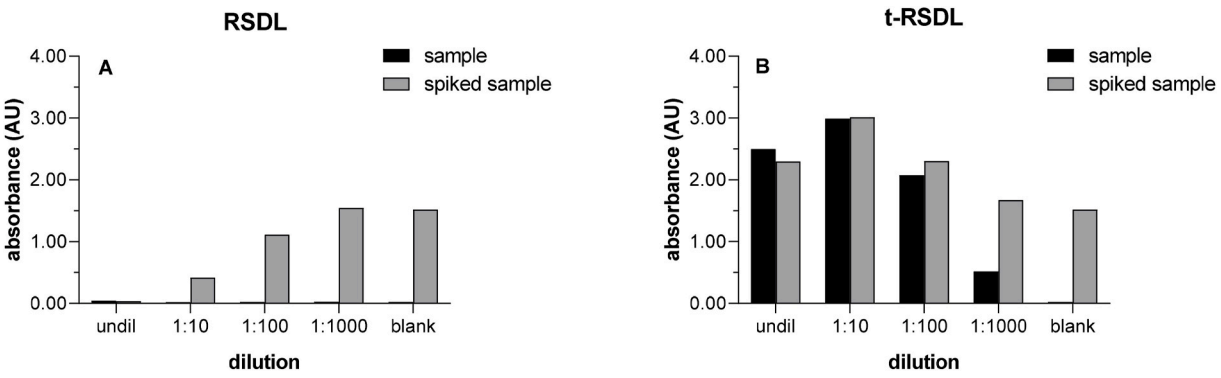

Fig. 3. Quantitative determination of ricin after incubation (100 ng/mL) in RSDL (panel A) or t-RSDL (panel B) for approximately 30 min, using the ELISA assay. Samples were diluted in a serial dilution and analyzed by ELISA (black bars). In addition, samples were spiked with ricin (end concentration 125 pg/mL; grey bars) after the actual dilution, in order to serve as controls for ruling out the effect of RSDL or t-RSDL on the ELISA assay. The blank samples represent PBS (black bar, not visible) or PBS spiked with ricin at 125 pg/mL (grey bar). After sandwich ELISA the absorbance was measured at 450 nm.

Table 3
Matrices tested to assess effect on ricin.

Matrix	Compound	Concentration (M); w/v%	MQ/ MPEG % v/v	pH, after 100x dilution with water
1	DAM	1.25/~12%	10/90	6.8
2	KBDO	1.25/~12.5%	10/90	10.6
3	KBDO	1.25/~12.5%	100/0	10.6
4	KOH	2 M	10/90	12.6
4a	KOH	20 mM	10/90	9.3
5	KOH	2 M	100/0	12.8
5a	KOH	20 mM	100/0	11.0
6	RSDL	N/A		10.2

of the protein. In the first experiments, the absence of the bridged disulfide peptide A-S-S-B (C*APPPSSQF – ADVC*MDPEPIVR, in which the C* residues are linked through a disulfide bridge), and which is a diagnostic peptide for intact ricin [16], was monitored carefully. The results of the mass spectrometric analyses are shown in Fig. 5. The level of the bridged disulfide peptide seemed to decrease upon treatment of ricin with RSDL and KBDO/MPEG, and in case of KOH/MPEG it was completely absent. In case of DAM/90% MPEG, the disulfide peptide could still be detected. Reanalysis of the samples on an Orbitrap high resolution LC/MS with improved chromatography still showed significant levels of the bridged peptide in the RSDL and KBDO/MPEG samples, but not in the KOH/MPEG treated samples. On average, the absence of activity in the ELISA assay, is accompanied by (partial) absence of the bridged disulfide peptide. This might imply that either the disulfide bridge is cleaved under agency of RSDL lotion or of its components, or that RSDL leads to precipitation of ricin. In order to check for possible precipitation, new sets of samples were prepared and were also analyzed for two additional, ricin-derived peptides, i.e. LEQLAGNLR and YTFAFGGNYDR which are both resulting from the A-chain [15]. In samples where the bridged disulfide peptide could no longer be found, these two peptides could still be analyzed, indicating that ricin was still in solution, but in a form that was no longer active in the ELISA assay.

Based on these initial results we hypothesized that the disulfide bridge in ricin, linking the ricin A- and B-chain, appears sensitive towards nucleophiles like DAM/KBDO, the active components of RSDL, leading to inactivation of ricin. However, the disulfide bridge is not that sensitive that it is converted instantaneously. In order to further investigate this finding on a more molecular level, we performed experiments with synthetic A-S-S-B peptide. Synthetic A-S-S-B was spiked in RSDL lotion and in its individual constituents, followed by incubation at room temperature for 30 min. After work-up on ZipTip pipette tips in order to remove RSDL components, samples were diluted with 0.1% TFA before analysis with LC-MS/MS. The mass spectrometric results show a clear decrease in the amount of the disulfide peptide after incubation with RSDL, KBDO/MPEG, and KOH/MPEG, with a complete disappearance of this peptide in the latter case (results not shown).

4. Discussion

In case of an incident with toxic chemicals, rapid decontamination of surfaces and the skin of possibly exposed victims is of utmost importance as a mitigation measure. In general decontamination is based on two mechanisms: removal of the agent and chemical conversion of the agent into a less harmful compound. Apart from the earlier mentioned chlorine bleach based decontamination of ricin referenced in first response protocols, very few scientifically substantiated methods for inactivation of ricin have been published. The available literature does not provide information on decontamination procedures for skin and the sourced literature focusses on mechanism of action for ricin with chemical compounds for decontamination on material substrates. To elaborate, the effect of calcium oxide (CaO) has been described by Meneguelli de Souza et al. [17], demonstrating that an alkaline CaO solution (pH > 12) cleaved the protein at e.g., asparagine and glutamine residues and affected the tertiary structure of ricin. In a report of the US Environmental Protection Agency [18], the inactivation of ricin on various surfaces using vapor phase hydrogen peroxide was evaluated, showing that 99% inactivation was achievable, depending on the duration of the treatment. While the authors did not elaborate on the chemistry behind the inactivation, the mechanism is probably based on oxidation of crucial cysteine residues. Cole et al. [19] described the effect of sodium hypochlorite and monochloramine on ricin, with sodium hypochlorite being much more effective. Whereas chemical means of inactivation seem to be quite effective, heat inactivation was not very effective, with ricin activity still present after heating at 60 °C. Wei et al. [20] described the inactivation of ricin by Nanosecond Pulsed Electric Fields. From the above it is evident that most of these methods are not suitable for (mild) skin decontamination purposes. Milder forms of inactivation of ricin are based on e.g. glycotechnology. For instance, synthetic glycolipids, which cause precipitation of ricin, have been described by Dohi et al. [21]. Similarly, Nagatsuka et al. [22] reported on the deteriorating effect of lactose-containing polyacrylamide-based glycopolymers on ricin activity. In the current paper, we have focused on the effect of RSDL on ricin, predominantly because RSDL is widely used with emergency responders for the decontamination of intact skin following exposure to chemical warfare agents and T-2 toxin.

As a first step to study the effect of RSDL on ricin activity, cytotoxicity and ELISA activity after treatment of ricin with RSDL, or its inactive physical surrogate, i.e. t-RSDL were investigated. Pre-treatment of ricin with RSDL led to reduction of cytotoxicity, whereas t-RSDL pre-

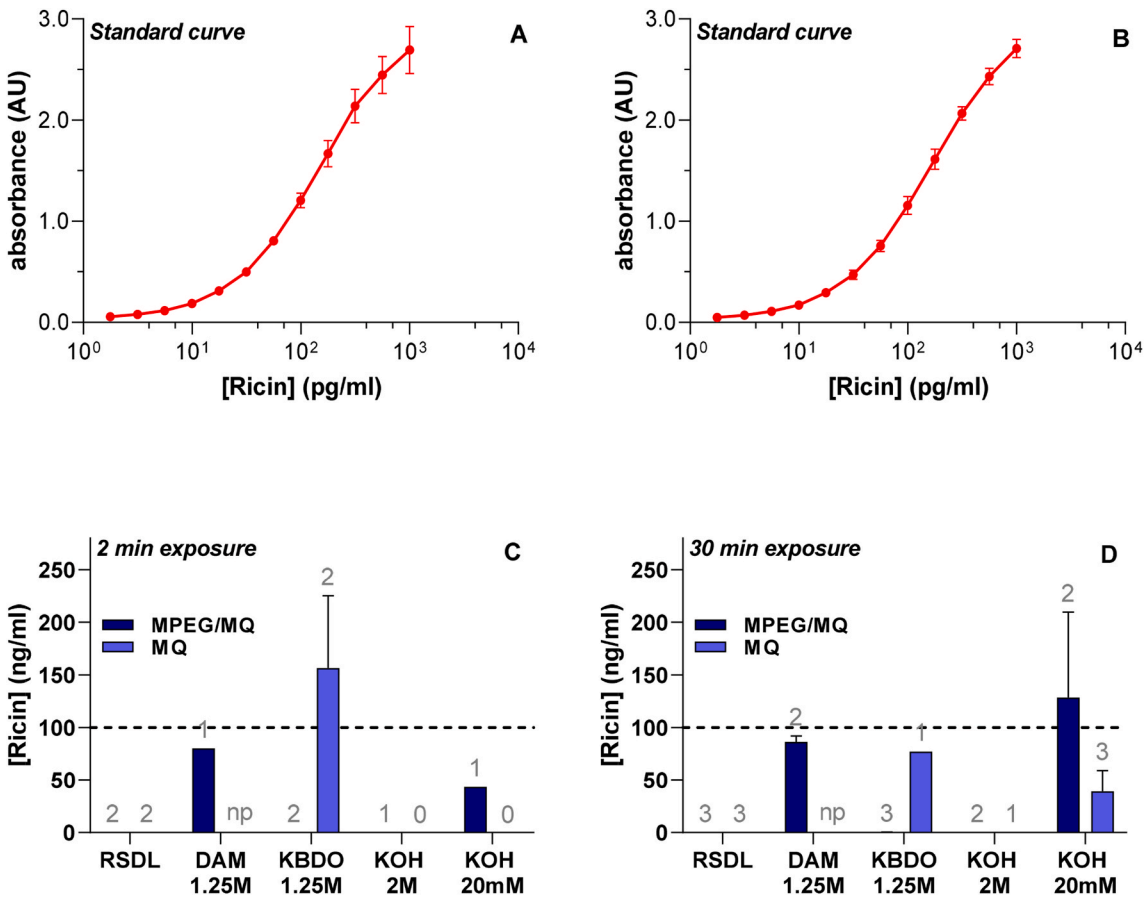

Fig. 4. Effect of RSDL or its constituents on ricin activity as determined with an ELISA assay. Panels A and B represent calibration curves for ricin over a range of 1.8–1000 pg/mL. Panels C and D show the amount of ricin recovered after a 2 (panel C) or 30 (Panel D) minute incubation with RSDL or the individual RSDL constituent in either 90% MPEG or MQ (dark blue and light blue bars, respectively). The grey numbers represent the number of incubations (n = 2 or n = 3) based on measurements passing the quantification criteria for the standard addition. Results are shown as mean for n = 2 and error bars are indicative for the SD.

incubation did not. In addition, it was determined that treatment of a solution of ricin with RSDL completely abolished detectability of ricin by the ELISA assay, while incubation with t-RSDL did not. These results suggest that ricin was inactivated by the active ingredients in RSDL, i.e. KBDO/DAM.

In the subsequent phase of the study, the effects of separate properties and separate ingredients of RSDL on ricin were determined, using the ELISA assay for read out. RSDL completely mitigated ELISA activity. It was shown that DAM (1.25 M in 90% MPEG), i.e. the protonated form of KBDO, which is one of the reactive components, did not break down ricin. KBDO (1.25 M), led to a complete reduction of ricin activity when dissolved in 90% MPEG, whereas the inactivation by KBDO (1.25 M) in water was less effective. To compare this effect to a solution with a similar pH, several KOH solutions were prepared in MQ or 90% MPEG. In a 2 M KOH solution in MQ or 90% MPEG, ricin lost its activity in the ELISA assay. However, in case of incubation with 20 mM KOH (in 90% MPEG or in MQ), at a pH in the range of RSDL (9–11, after 100x dilution with water), ricin remained detectable in the ELISA assay in a number of samples, indicating a less apparent effect than after RSDL or 2 M KOH treatment. It must be noted that properties of the solutions, such as high viscosity and the alkaline pH did affect the performance of the assay to some extent. These effects were accounted for by correcting via standard addition; this indicated that the conditions in a well were not suitable for ELISA ricin positive results, based on measurements passing the quantification criteria for the standard addition. In spite of this, it can be concluded that a high pH contributes to a decrease of ricin activity in the ELISA assay, and that the presence of KBDO is crucial (except for the 2 M KOH samples). Also, for KBDO to be active, it needs to be dissolved in 90% MPEG, and not MQ. The fact that DAM in 90% MPEG was not active can be explained by the fact that the DAM, i.e. the acidic form of KBDO is fully protonated and therefore not nucleophilic enough to exert its action under the applied conditions.

Based on these results, the second part of the study was initiated to better understand the mechanism of action as well as the possible structural modification of ricin underlying this inactivation. To that end, a mass spectrometric approach to assess the molecular effects of RSDL on ricin, in particular the effect on the disulfide linkage between the ricin A and B chain, was employed. Dissociation of the A- and B-chain should render the protein unable to gain entry into the cell, making it inactive. It was hypothesized that this disulfide bridge might be sensitive towards nucleophiles like DAM/KBDO, the typical components of RSDL, leading to inactivation of ricin. Therefore, secondary experiments were carried out at higher concentrations of ricin, because of the lower sensitivity of the method compared to the ELISA. Mass spectrometry results pointed towards a decrease in the level of the bridged disulfide peptide in case of incubation with RSDL and KBDO/MPEG, and a complete disappearance of this peptide in KOH/MPEG. Two other diagnostic peptides, i.e.,T11a (LEQLAGNLR) and T10a (YTFAFGGNYDR), which have been reported by Worbs et al. [15], could also still be detected, supporting the observation that RSDL treatment did not lead to precipitation of ricin (which might have been an alternative explanation for the fact that no cytoxicity or ELISA activity was observed). When a solution of the bridged disulfide peptide was incubated with RSDL or its individual components, conversion of this peptide could be monitored, by the concomitant formation of the B–S peptide, which indeed points to a certain lability of the disulfide bridge (data not shown). When a

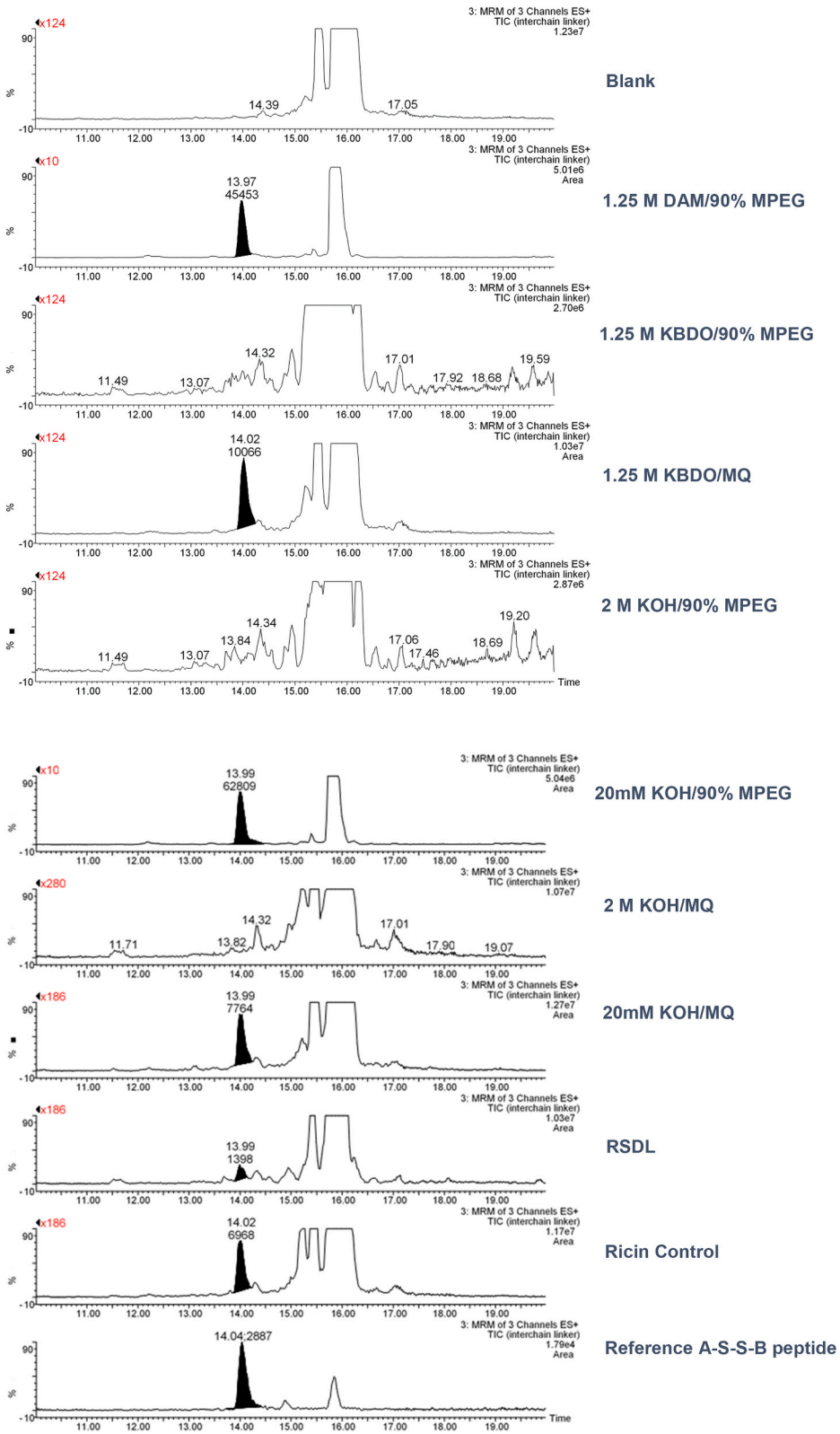

Fig. 5. LC/MS analysis of the A-S-S-B bridged disulfide peptide in a solvent assisted trypsin digest of ricin, after treatment of ricin with RSDL, its individual constituents, or relevant basic conditions. The reference standard of the A-S-S-B peptide elutes around 14.0 min.

processed sample was analyzed directly after incubation (30 min) and work-up, only in case of the KOH/MPEG matrix the complete disappearance of the peptide was observed, with decreased signals for the KBDO and RSDL samples. Upon prolonged standing (>24 h) of the worked-up samples, the bridged peptide could only be observed in the control samples. In general, reaction at the disulfide bond occurs less readily at the protein level, than at the peptide level. This is logical from a chemical point of view, because within the protein the disulfide bond is less readily accessible. Disulfide cleavage by OH- has been described in the literature [23], which in case of a cysteine residue results in an

alkene and a thiolate ion. However, it should be emphasized that this phenomenon cannot be generalized to all O-nucleophiles; within this context, cleavage of disulfides by oximates has not been reported. Irreversible reduction of protein disulfide bonds has also been reported in the literature [24], making use of a mixture of a reducing agent, a denaturant and hydroxide ion. In this respect, the efficacy of RSDL with regards to protein toxins might be increased by the addition of a reducing agent.

5. Conclusions

In summary, the results described herein demonstrate that RSDL is able to effectively inactivate ricin after an incubation time of 2 min, as evidenced by the results of the ELISA activity assays. These results are also supported by the findings of the viability assay, after pre-incubation of ricin in RSDL for 30 min. The precise mechanism by which inactivation occurs could not yet be completely resolved. Although mass spectrometry experiments provided evidence that RSDL is able to induce cleavage of the disulfide linkage between the A- and B-chain of ricin, catalyzed by a combination of high pH and a nucleophile oximate (KBDO) in a matrix of 90% MPEG, it seems unlikely that this is the only inactivation mechanism in place. In the event that there are also additional generic inactivation mechanisms taking place, e.g., based on denaturation of the protein, this holds promise for RSDL inactivation of other protein toxins as well.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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