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The Akt Pathway Inhibitor Degeulin Prevents Staphylococcal Enterotoxin B Induced Splenocyte Proliferation and Inflammation

Sarah Joanne Christine Whitfield[1*], Jane Elizabeth Risdall[1,2], Gareth Griffiths[1], Ethel Diane Williamson[1], Alun James Carter[1,3]

[1]Department of Biomedical Sciences, Defence Science and Technology Laboratory, Porton Down, Wiltshire, UK
[2]Department of Anaesthesia, University of Cambridge, Cambridge, UK
[3]Department of Medicine, Washington University, St. Louis, Missouri, USA
Email: *sjwhitfield@dstl.gov.uk

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Abstract

Staphylococcal Enterotoxin B (SEB) is considered a potential biological weapon. It is toxic by both inhalation and ingestion. Effects of ingestion include fever, vomiting and diarrhoea, while inhalation may additionally result in chest pain, dyspnoea, pulmonary oedema and respiratory failure. Severe exposure may be fatal and treatment relies on symptomatic support. At a cellular level, SEB up-regulates T-cell proliferation leading to a pathological inflammatory response. Deguelin, a rotenoid isolated from the African plant *Mundulea sericea* (Leguminosae), has been shown to reduce cellular proliferation by inhibiting the phosphoinositide 3-kinase/Akt (PI3K/Akt) signalling pathway. Using isolated murine splenocytes, we have demonstrated that treatment with deguelin reduces SEB inducing T cell proliferation by 60%. Deguelin treatment also decreased IL-2 and CCL2 secretion by splenocytes exposed to SEB. We demonstrate that targeting cellular proliferation can significantly reduce inflammation after SEB exposure and suggest that anti-proliferatives may have a role as potential generic medical counter measures if superantigens are used as biological weapons.

Keywords

Staphylococcal Enterotoxin B, Deguelin, Therapy, Inflammation, Biological Weapon

1. Introduction

Staphylococcal enterotoxin B (SEB), an enterotoxin produced by the bacterium *Staphylococcu saureus*, is classed as a bacterial superantigen (BSAg) [1] [2]. It is

most commonly associated with food poisoning, but can also cause widespread systemic damage and toxic shock syndrome [3] [4] [5] [6] [7]. SEB is stable to aerosolisation and is toxic via the inhalation of even small amounts. In this form, it causes severe lung pathology, shock and death in man [8] [9]. For this reason, it is considered a potential biological weapon [10] [11]. Clinical signs of intoxication are to some extent dependent on the route of exposure with onset starting 1 to 6 hours after exposure [12]. Signs of inhalational intoxication include fever, respiratory problems, gastrointestinal (GI) symptoms, toxic or septic shock and death [13].

Staphylococcal enterotoxins are extremely potent activators of T-cells and they bind directly to the major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APCs) and to variable β chains on T-cell antigen receptors [14] [15] [16]. Under normal conditions, specific proliferation, co-stimulatory recognition and verification signals are required for T-cell activation. Under normal conditions, the percentage of T-cells recruited to affect a response to an antigen is small, <0.01% of the T-cell population. In the presence of SEB, there is a significant increase in T-cell activation (approximately 20% of the T-cell population) [17] [18]. The resultant activation of T-cells leads to the induction of a systemic inflammatory response characterised by the production of significant levels of cytokines, often referred to as the "cytokine storm" [19]. The systemic inflammatory response induced by BSAgs results in numerous immune-pathologies characteristics of superantigen intoxication.

The use of superantigens as potential biological weapons presents military forces with significant challenges in terms of medical countermeasures (MedCMs). The two potential approaches for protection against the effects of weaponised BSAgs are the prophylactic administration of a MedCM prior to exposure or post-exposure treatment. Recent research has looked at the development of effective post-exposure treatments, including agent specific medical countermeasures (e.g. anti-toxins) and wider-spectrum therapies (e.g. anti-inflammatory agents) to target the resulting immunopathology [20]-[26]. One of the more successful broad-spectrum superantigen MedCMs is rapamycin, which targets the PI3K/mTOR pathway, specifically inhibiting mTOR complex 1 [27] [28]. Inhibition of the PI3K/mTOR pathway and subsequent reduction in BSAg toxicity indicates that specific intracellular pathways offer potential therapeutic targets. One potential intracellular target, up-stream of the mTOR complex 1, is Protein Kinase B (Akt). Akt, like mTOR, is involved in the regulation of cellular growth, differentiation, apoptosis and pro-inflammatory signalling [29] [30]. However, modulation of Akt may have greater utility in reducing BSAg pathology, since it has wider involvement in the intracellular signalling processes [31].

Deguelin, a rotenoid from the African plant *Mundulea sericea* (Leguminosae), has been shown to have anti-carcinogenic properties, including the inhibition of cellular proliferation and induction of apoptosis [32]. These potent effects have been attributed to its inhibitory effect on PI3K/Akt signalling [33]. This study investigates the *in vitro* efficacy of deguelin, as an inhibitor of the Akt pathway,

in reducing splenocyte proliferation and the subsequent pro-inflammatory cytokine production.

2. Materials and Methods

2.1. Toxin & Therapeutics

SEB toxin was obtained from the Health Protection Agency, Porton Down, Wiltshire, UK and used at a concentration of 0.5 μg/ml. Deguelin was purchased from Tocris, UK, and used at concentrations up to 160 nmol/l. Con A and LPS were purchased from Sigma Aldrich and used at concentrations of 3.13 μg/ml and 5 μg/ml respectively. Deguelin was initially dissolved in 10% DMSO. All remaining reagents and DMOS diluted deguelin were prepared in phosphate buffered saline (Gibco, UK). Working solutions were made on the day of the study carried out using sterile RPMI-1640 media containing 15% (v/v) fetal calf serum (Sigma-Aldrich, Poole, Dorset, UK), 1% (v/v) Penicillin/Streptomycin solution and 1% (v/v) L-Glutamine (Sigma-Aldrich, Poole, Dorset, UK).

2.2. Splenocyte Preparation

Fifteen 42 - 49 days old male Balb/C mice (Charles River Laboratories Ltd., Margate, Kent, UK) were killed by cervical dislocation and their spleens aseptically removed. The splenic tissue was then passed through a 10 μm cell strainer and the recovered splenocytes re-suspended in supplemented RPMI-1640 medium. Cell suspension was centrifuged for 10 minutes at 350 g and the supernatant discarded. Red blood cells in the tissue pellet were lysed in 3 ml of red blood cell lysing buffer (Sigma, Dorset UK) for 1 minute. Sterile supplemented RPMI-1640 medium was added to give a final volume of 30 ml and the resulting cell suspension centrifuged as above. The supernatant was again discarded. Four milliliters of sterile supplemented RPMI-1640 medium was added, the tissue pellet resuspended and the cell count quantified using a Neubauerhaemocytometer. The volume of the cell suspension was adjusted with supplemented RPMI-1640 medium to give a final cell count of 1.0×10^6 cells/ml.

2.3. Cell Cytotoxicity Assay

A baseline cytotoxicity assay was conducted to establish the relative toxicities of SEB and deguelin, individually and in combination, on splenocytes cultured for 48 hours. Splenocytes were seeded into 96-well flat-bottomed cell culture plates (B. E. Thompson Supplies, Andover, UK) at a cell density of 5×10^4 cells per well and incubated under the following conditions (Table 1).

Table 1. Slenocyte treatment summary for cytoxicty assay and cytokine analysis.

Splenocyte treatment	Treatment group			
	Cells	Deguelin + SEB	Degueli[a]	SEB
Treatment	PBS	Deg 160 nm/l + SEB 0.5 μg/ml	Deg 160 nm/l	SEB 0.5 μg/ml

a. Deguelin treatment for cytotoxicity assay only.

After 48 hours, cell death was determined using a Promega Multi-Fluor LDH cytotoxicity assay (Promega, USA), in accordance with the manufacturer's instructions. Dead cell numbers were determined by fluorescence at 485 nm excitation and 535 nm emission 3 hours after the addition of the Multi-Fluor reagent. Results were expressed as mean fluorescent intensity (MFI) for each group.

2.4. SEB-Induced Cell Proliferation (MTT Assay)

SEB characteristically causes a dose dependent proliferation of T-lymphocytes. The ability of deguelin to inhibit this proliferative effect was measured using a modified cell proliferation technique. Splenocytes were seeded into 96-well flat-bottomed cell culture plates at a cell density of 5×10^4 cells per well. They were then treated with PBS (negative control), SEB (positive control) or SEB and deguelin together. A twofold dilution series of deguelin was used to determine a dose response curve (160 nmol/l to 5 nmol/l). After 48 hours incubation, cell proliferation was measured using an MTT assay (Promega, USA). In brief 10 µl of the MTT reagent was added to each plate and these were incubated at 37˚C for a further 4 hours. One hundred microliters of detergent reagent was then added and the plates incubated for a further 3 hours. Cell proliferation was then determined by measuring the absorbance at 570 nm. For each experimental run, the proliferation response of deguelin treated cells was normalised to the negative control (PBS) and the positive control (SEB) at 0% and 100% respectively. Analysis was performed on the optical density values and the positive control (SEB) compared with splenocytes treated with SEB and deguelin together. Splenocytes were also separately incubated with Con A as a positive proliferation control and a pro-inflammatory endotoxin control (LPS).

2.5. ELISA for Cytokines IL-1β, IL-2 and MIP-1 Determination

Quantitative ELISAs (Quantikine) were performed using the cell culture supernatants to determine the effect of deguelin treatment on the production of interleukin-1β (IL-1β), interleukin-2 (IL-2) and CCL2 by the isolated splenocytes following SEB exposure. Cell culture supernantant were prepared by centrifugation of cultures at 1000 g for 10 mins at room temperature. Measurement of cytokines by ELISA was performed according to manufacturer's instructions. Splenocytes were also stimulated with the positive proliferation control (Con A) for IL-2 and a pro-inflammatory endotoxin control (LPS) for IL-1β and CCL2. IL-1β, IL-2 and CCL2 levels expressed by splenocytes were analysed by comparing SEB-exposed, deguelin treated responses to negative control (PBS) and positive control (SEB) concentrations of cytokines

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Normality of values was determined using a Kolmogorov-Smirnov normality test and appropriate analysis was performed. For the proliferation and cytotoxicity assays, A570 and MFI values of the deguelin treated cells were compared to the SEB (positive con-

trol) or PBS (negative control) respectively. Matched analysis was performed using a Repeated Measure ANOVA with a Dunnett's post-test. For cytokine analysis, treated cells were compared to PBS (negative control) and SEB, Con A and LPS (positive controls) respectively. For normally distributed data (IL-1β and CCL2) a student T-test was used to determine significance. For non-parametric data (IL-2) a Mann-Whitney test was used to determine statistical significance.

3. Results

3.1. Cell Proliferation Assay

A reduction of SEB-induced proliferation was observed in cells treated with deguelin, which reached statistical significance at a deguelin concentration of 160 nmol/l ($p < 0.01$) (Figure 1).

3.2. Cell Cytotoxic Assay

Treatment of splenocytes with SEB, deguelin or a combination with SEB did not appear to cause any overt cytotoxicity. The MFI measured by the Promega Multi-Fluor cytotoxicity assays after 48 hours is shown in Figure 2. Data are presented as the mean and 95% CI. No significant differences were observed between untreated splenocytes and the treatment groups ($p = 0.0970$ and $p = 0.3739$, respectively).

3.3. Cytokine Analysis

SEB-exposed positive controls secreted 1216 ± 628 pg/ml (mean ± 95% CI) IL-2, significantly higher than untreated splenocytes which produced 146 ± 101 pg/ml (mean ± 95% CI, $p = 0.0017$) (Figure 3(a)). The treatment of splenocytes with

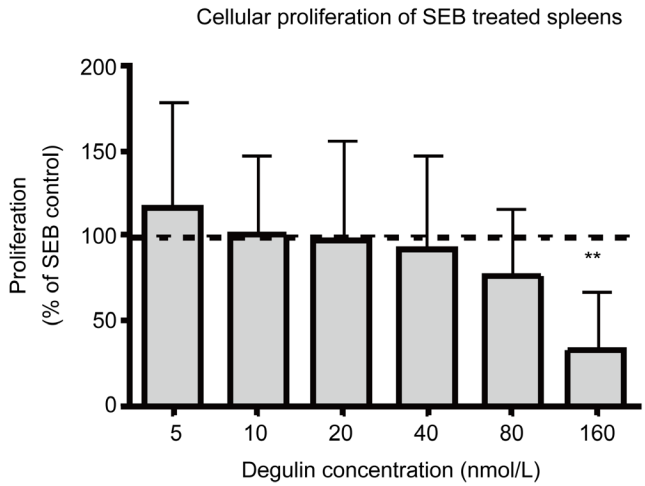

Figure 1. Concentration dependent inhibition of normalised murine splenocyte proliferation by deguelin. Repeated measures ANOVA showed that the effect of treatment was significant differences were observed between treatments ($p = 0.0011$). Pos hoc analysis using Dunnett's multiple comparison test are indicated as ** = $p < 0.01$. Mean and 95% CI (n = 5).

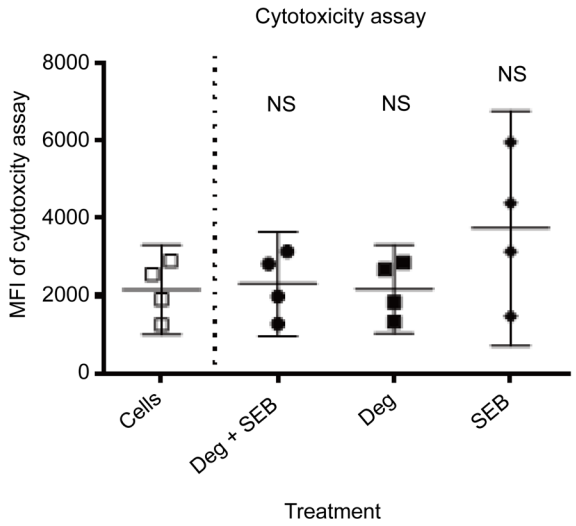

Figure 2. Cytotoxicity measured by MFI. No significant difference was observed in toxicity between control cells and treatments (ns = $p < 0.05$). Bars represent mean MFI and 95% CI (n = 4).

deguelin in the presence of SEB resulted in a significant inhibition of IL-2 production compared to SEB treated control splenocytes (239 ± 159 pg/ml p = 0.0079). Stimulation of splenocytes with Con A as a control resulted in production of IL-2 (4282 pg/ml) (data not shown).

A significant reduction in CCL2 secretion was observed between SEB treated cells and deguelin treated cells ornegative control cells (p = 0.0159 and p = 0.0317 respectively) (**Figure 3(b)**). The levels of CCL2 secreted by splenocytes in the deguelin treated group, SEB positive control group and unstimulated negative control group was 66 ± 89 pg/ml (mean ± 95% CI), 679 ± 810 pg/ml (mean ± 95% CI) and 73 ± 150 pg/ml (mean ± 95% CI) (**Figure 3(b)**).

The levels of IL-1β production in deguelin treated cells and negative control cells were not significantly different from the levels of cytokines produced by SEB control cells (p = 0.1392 and p = 0.3898 respectively) (**Figures 3(c)**). Whilst SEB did not significantly increase IL-1β in splenocytes, there was a trend in reduction of IL-1β between the deguelin treated splenocytes and unstimulated cells as compared to the SEB treated cells (p = 0.1392 and p = 0.3898) **Figures 3(c)**. Levels of IL-1β and CCL2 production were greatest when splenocytes were stimulated with LPS as a control (109 pg/ml and 2392 pg/ml respectively) (data not shown).

4. Discussion

The aim of this study was to determine the efficacy of deguelin in preventing cellular proliferation and pro-inflammatory cytokine release following SEB exposure *in vitro*.

There was no apparent cytotoxicity of deguelin in our *in vitro* system, as evidenced by the result of the cytotoxicity assay. Deguelin treatment of SEB-exposed splenocytes significantly reduced the production of IL-2, a cytokine

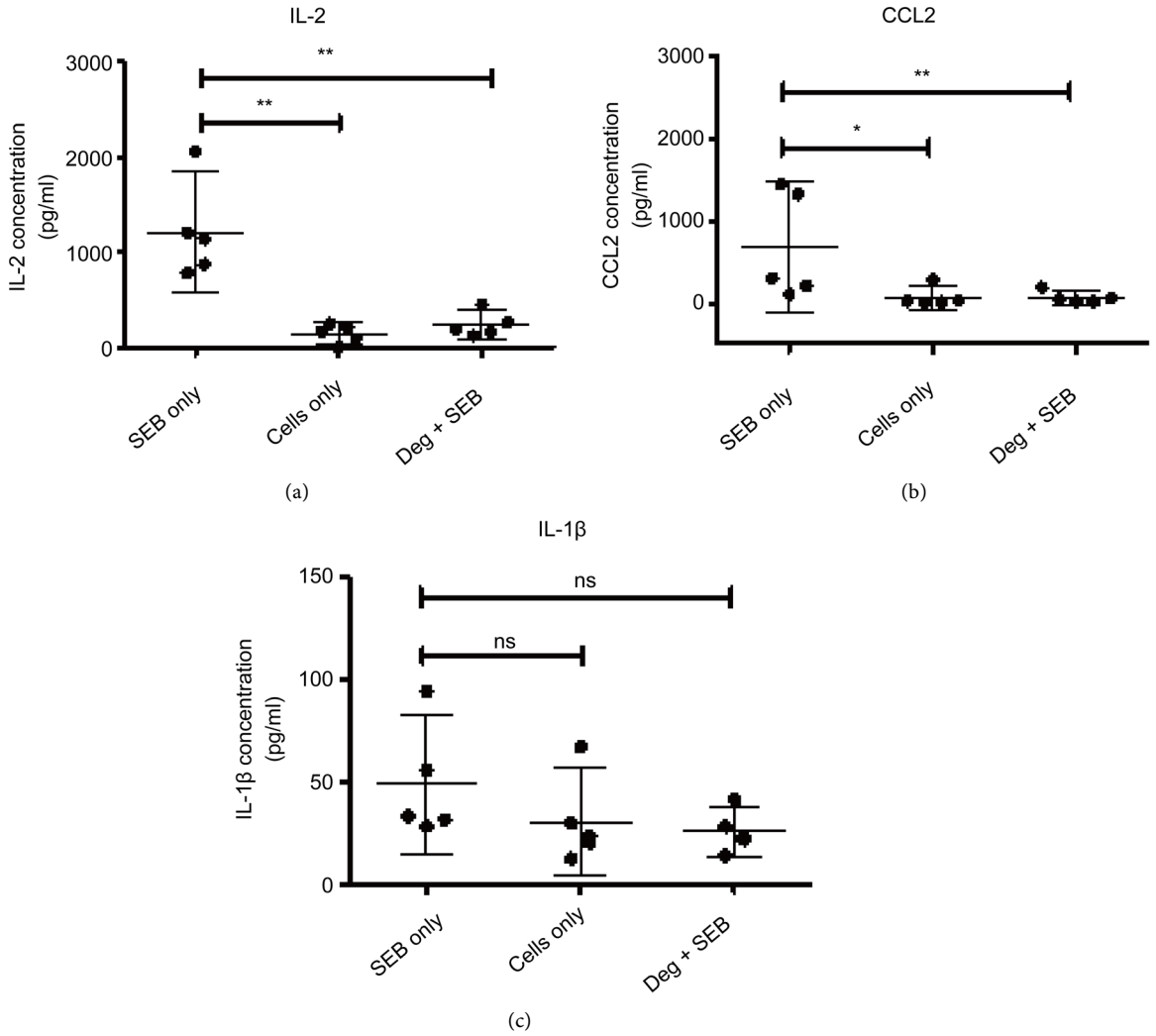

Figure 3. Murine splenocyte production of IL-2, CCL2 and IL1β following SEB exposure. Isolated splenocytes were treated with PBS (negative control), SEB (positive control) or with SEB and deguelin together. For each experiment, the production of (a) IL-2, (b) CCL2 and (c) IL-1β was measured for the negative control (PBS only), the positive control (SEB only), combined SEB and deguelin treatment (Deg + SEB), a positive proliferation control (Con A) or an endotoxin inflammatory positive control (LPS) (data not shown). Significant differences are indicated ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$. Bars represent mean and 95% CI (n = 5).

essential for T-cell proliferation and activation, and the chemokine CCL2, which initiates the migration of monocytes into systemic tissues [34] [35] [36]. Whilst deguelin did not significantly reduce IL-1β, a pro-inflammatory cytokine involved in the acute phase response, a trend in cytokine section was observed. The biological mechanism of SEB toxicity is well defined. SEB interacts with the T-cell/APC immune synapse (MCH class II molecules on APCs, the β-chain on T-cell receptors) by passing the need for T-cells to recognize antigen [37] [38]. Secondary signals are required for T-cell survival and proliferation, and production of pro-inflammatory cytokines resulting in superantigen toxicity [39] [40] [41] [42].

Targeting T-cell proliferation would seem a reasonable approach to counteract the effects of SEB exposure. However, the prevention of cellular prolifera-

tion and differentiation of T-cells has largely been ignored. PI3K/mTOR intracellular signaling regulates proliferation. This pathway is dependent upon chemokine, IFN-γ and IL-2 receptor cell signaling for proliferation and T-cell activation [43] [44] [45]. Rapamycin is one of the few therapeutics that have been shown to be effective at preventing superantigen toxicity *in vivo* [23].

The mechanism by which deguelin reduces proliferation has been extensively studied and is identified as a potential therapeutic due to its effect on Akt. Deguelin binds directly to Hsp90 inhibiting its biological activity [46]. Hsp90 stabilises signaling molecules include Akt and PI3K, inhibiting the PI3K/Akt pathway [47]. This pathway is involved in the regulation of many cellular processes including cellular growth, differentiation, cell survival and apoptosis [47]. We hypothesised that by blocking the intracellular signaling associated with this pathway, it should be possible to prevent T-cell proliferation and this would reduce the inflammatory response. This study demonstrates both a reduction in cellular proliferation and in inflammatory cytokine production following deguelin treatment of splenocytes exposed to SEB *in vitro*.

PI3K/Akt signaling is necessary for numerous pro-inflammatory processes including cytokine receptor signaling, the induction of pro-inflammatory cytokine production, increased expression of adhesion molecules (intergrins and selectins), superoxide production, neutrophil degranulation and the expression of IFN-inducible proteins [29]. The observed reduction in pro-inflammatory cytokines following deguelin treatment may be due to inhibition of this signaling pathway. PI3K/Akt/mTOR intracellular signaling also modulates inflammatory receptor signals, including the immune synapse complex, interleukin 1 receptor, Toll-like receptors, TNF receptor, chemokine receptors and interferon receptor, all of which have been implicated in superantigen toxicity [48] [49]. The resultant pro-inflammatory cellular responses lead to increased production of cytokines and chemokines including IL-1β, TNF-α, IL-6, IL-12, CCL2, CCL5 and IFN-γ [50] [51]. Furthermore, deguelin has been previously shown to inhibit IκBα kinase activation and reduce nucleoporin Nup88 expression, both of which suppress of NF-κB dependent gene expression [52]. NF-κB is considered a key transcription factor for the production and regulation of the pro-inflammatory response. Deguelin suppression of NF-κB transcription of pro-inflammatory genes may also explain reduced pro-inflammatory cytokine production following SEB exposure.

From our studies, it would appear that deguelin reduced SEB-induced cellular proliferation and pro-inflammatory cytokine production. Although showing promising anti-cancer activity in the laboratory, deguelin has not been developed for clinical use. Several other Akt inhibitors, such as VQD-002, perifosine and miltefosine, have been developed for use in the treatment of cancer and protozoal infections [53] [54]. These drugs may represent potential treatments for SEB intoxication.

Additional cellular targets influencing the PI3K/Akt pathway include inhibitors of IL-2 receptor signaling, such as basiliximab, daclizumab and inhibitors of

CD28 signaling, such as abatacept [55]. Further research will be required to demonstrate the efficacy of these agents in preventing superantigen toxicity *in vivo*. However, targeting PI3K/Aktsignaling and/or T-cell proliferation in BSAg intoxication may prevent the inflammatory response progressing with positive effects on morbidity and mortality.

Here we have demonstrated for the first time, that a PI3K/Akt pathway inhibitor is effective in preventing superantigen-induced cellular proliferation. This *in vitro* system also has the potential to offer rapid screening of other putative therapeutic agents for use in SEB intoxication.

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