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Silibinin as a potential therapeutic for sulfur mustard injuries

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ABSTRACT

Sulfur mustard (SM) is a vesicating chemical warfare agent causing skin blistering, ulceration, impaired wound healing, prolonged hospitalization and permanent lesions. Silibinin, the lead compound from *Silybum marianum*, has also been discussed as a potential antidote to SM poisoning. However, its efficacy has been demonstrated only with regard to nitrogen mustards. Moreover, there are no data on the efficacy of the water-soluble prodrug silibinin-bis-succinat (silibinin-BS).

We investigated the effect of SIL-BS treatment against SM toxicity in HaCaT cells with regard to potential reduction of necrosis, apoptosis and inflammation including dose-dependency of any protective effects. We also demonstrated the biotransformation of the prodrug into free silibinin.

HaCaT cells were exposed to SM (30, 100, and 300 μM) for 30 min and treated thereafter with SIL-BS (10, 50, and 100 μM) for 24 h. Necrosis and apoptosis were quantified using the ToxiLight BioAssay and the nucleosome ELISA (CDDE). Pro-inflammatory interleukins-6 and -8 were determined by ELISA. HaCaT cells, incubated with silibinin-BS were lysed and investigated by LC–ESI MS/MS.

LC–ESI MS/MS results suggest that SIL-BS is absorbed by HaCaT cells and biotransformed into free silibinin. SIL-BS dose-dependently reduced SM cytotoxicity, even after 300 μM exposure. Doses of 50–100 μM silibinin-BS were required for significant protection. Apoptosis and interleukin production remained largely unchanged by 10–50 μM silibinin-BS but increased after 100 μM treatment.

Observed reductions of SM cytotoxicity by post-exposure treatment with SIL-BS suggest this as a promising approach for treatment of SM injuries. While 100 μM SIL-BS is most effective to reduce necrosis, 50 μM may be safer to avoid pro-inflammatory effects. Pro-apoptotic effects after high doses of SIL-BS are in agreement with findings in literature and might even be useful to eliminate cells irreversibly damaged by SM. Further investigations will focus on the protective mechanism of silibinin and its prodrug and should establish an optimum concentration for treatment.

1. Introduction

Despite intense efforts on chemical disarmament, the chemical warfare agent sulfur mustard (SM) remains reason for strong concern. It was repeatedly used in armed conflicts during the 20th century from World War I to the Iran–Iraq war in the 1980s. Stockpiles still exist and even if all known stockpiles were successfully disarmed, the agent is comparatively easy to synthesize. Moreover, no causative treatment for SM injuries is known despite a century of medical research. Numerous pharmaceutical compounds had been investigated for their potential to reduce SM-induced cytotoxicity, including *N*-acetylcysteine [1–3], glutathione [4,5], glutathione derivatives [6] and doxycycline [7]. Some protective effects were identified; in particular when drugs were administered prior to SM exposure. However, efficacy in cells already exposed to SM was limited. Moreover, Rappeneau et al. also noted that "the level of protection against SM was always weak compared to HN2" (a nitrogen mustard) [7].

Recently, however, silibinin has been proposed as a potential therapeutic compound against SM injures. Tewari-Singh et al. reported protective effects against toxicity induced by 2-chloroethyl ethyl sulfide (CEES, or hemi mustard) [8] and nitrogen mustard [9]. However, data on the efficacy against the actual agent SM were missing. Moreover, those studies had been conducted using silibinin which is poorly soluble in water. In a clinical case of SM poisoning, rapid, systemic intervention using a water-soluble pro-drug of silibinin would be desirable.

Silibinin (Fig. 1A), a flavonolignan, is the lead compound of the extract from *Silybum marianum* seeds. The dried *S. marianum* extract, standardized for its silibinin content is approved for the

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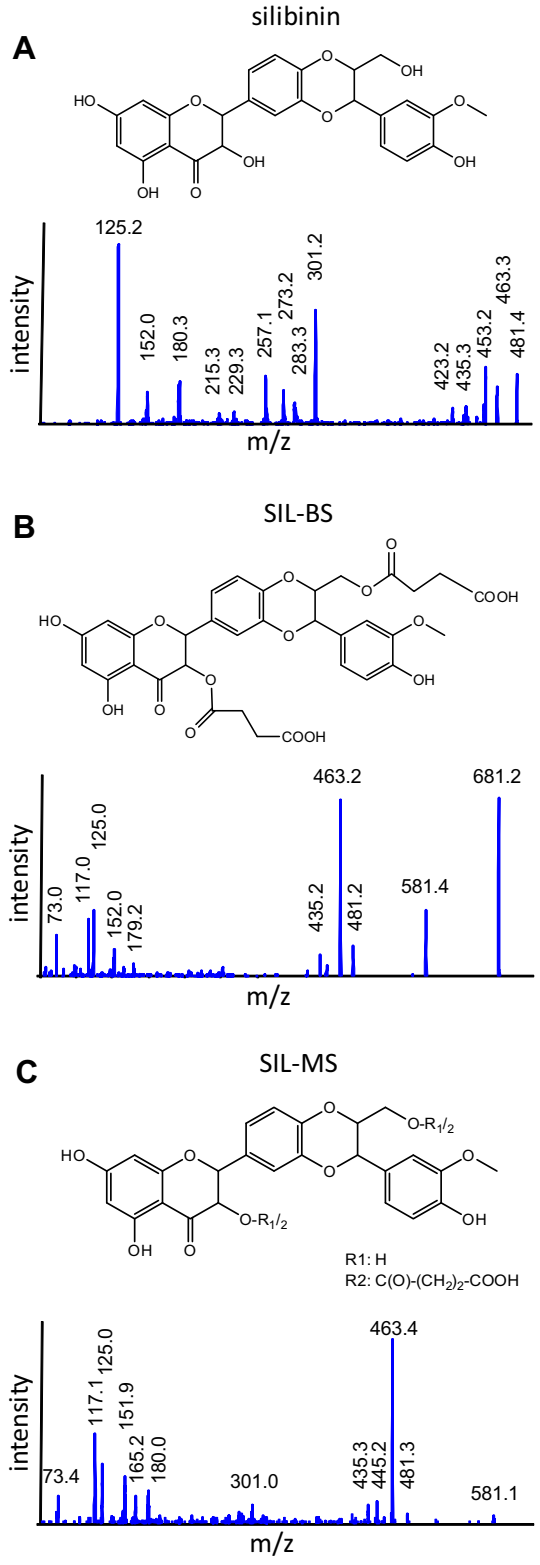

Fig. 1. Chemical structures and MS/MS spectra of silibinin-derived analytes. All MS/MS spectra were obtained after negative electrospray ionization. Deprotonated analyte molecules, $[M-H]^-$, were chosen as precursor ions and fragmented by collision-induced dissociation (CID) in a triple quadrupole mass spectrometer. Transition to the most intense product ions were used for quantification as listed in Table 1. (A) Silibinin, $[M-H]^-$ m/z 481.4; (B) silibinin bis-succinate (SIL-BS), $[M-H]^-$ m/z 681.1; (C) silibinin mono-succinate (SIL-MS), $[M-H]^-$ m/z 581.1.

supportive therapy of chronic liver diseases [10,11]. This extract, prepared for peroral application, is not sufficiently effective in case of acute poisonings. For treatment of acute liver poisoning, in particular, ingestion of *Amanita phalloides* (Green deathcap mushroom), the derivative silibinin-bis-succinate (SIL-BS) (Fig. 1B) has been developed and approved [12]. Derivatization with two residues of succinic acid has introduced two carboxy groups. These carboxy groups can be deprotonated, the resulting sodium salt constitutes a highly water-soluble compound, suitable for i.v. administration in case of acute poisoning.

While the protective activity of silibinin against *A. phalloides* poisoning is primarily attributed to an inhibition of OATP transporters (preventing the uptake of the toxin, alpha-amanitin in hepatocytes [13], its ability to protect against a less specific noxae, including nonalcoholic steatohepatitis [11], liver transplantation injury [14], chronic ethanol abusus [15] has been attributed to antioxidative properties, increased activity of superoxide dismutase and peroxidase as well as membrane inhibition of prostaglandin synthesis [16] and suppression of NFkB activation.

Silibinin is known to be a very safe and non-toxic substance in mammalian organisms. In the most susceptible species, i.e. rabbits and dogs, an LD_{50} of 140 mg/kg had been found [17]. It has also been reported to inhibit apoptosis in cells damaged by a number of noxae, including UV-B radiation [18,19] or arsenic compounds [20].

In this study, we investigated the protective effect of SIL-BS in a cell culture model after SM challenge with special focus on dose–effect relationships, both for the toxic compound (SM), the candidate substance (SIL-BS) and the combination thereof. Our aim was to assess overall cytotoxicity, apoptosis and inflammation. In accordance with Arroyo et al. [21] who had reported interleukin-6 (IL-6) production as an important inflammatory mechanism in SM-exposed cells, we determined IL-6 levels in supernatants. Another pro-inflammatory cytokine in SM-exposed keratinocytes is interleukin-8 (IL-8) [22] which was also determined in our study.

HaCaT cells are a well-established model to test cutaneous toxicity [23,24] as well as pharmacologic effects in skin cells [25]. Our model enables us to rapidly assess a variety of parameters, i.e. necrosis, apoptosis and inflammation and was thus considered suitable for this investigation.

Moreover, we aimed to demonstrate the biotransformation of the prodrug SIL-BS into free intracellular silibinin by LC–ESI MS/MS analysis. To our knowledge this issue was never addressed before in a cell culture model, however, in order to understand a potential protective mechanism of silibinin, a prerequisite is to verify the presence of the compound.

2. Material and methods

2.1. Cell culture

HaCaT cells [26,27] purchased from cell lines service (cls, Eppelheim, Germany) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Karlsruhe, Germany), supplemented with 5% Fetal Calf Serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2. No antibiotics were used. 50,000 HaCaT cells per well were seeded on a 96-well-plate which was then incubated for approx. 24 h, allowing cell adherence and proliferation.

2.2. SM exposure

SM was made available by the German Ministry of Defence. A purity of at least 99% purity was confirmed by NMR analysis.SM doses were chosen to include doses above and below the LD_{50} for SM, observed in HaCaT cells over 24 h which is approx. 140 μM. A concentration of 100 μM SM is also a dose known to inflict acute toxic effects, including skin blistering [28]. Exposure time was in