

A semiquinone glucoside derivative (SQGD) isolated from *Bacillus sp. INM-1* as a provider of antioxidant protection to male mice against CCNU-induced oxidative toxicity

Y. D. Karamalakova^{1*}, A. M. Zheleva¹, R. Kumar², G. D. Nikolova¹, V. G. Gadjeva¹

¹Department of Chemistry and Biochemistry, Medical Faculty, Trakia University, 11 Armeiska Str., 6000 Stara Zagora, Bulgaria

²Institute of Nuclear Medicine and Allied Sciences, Defense Research and Development Organization (DRDO), Brig S. K. Mazumdar Marg, Delhi-110054, India

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Present investigation was focused on evaluation of a semiquinone glucoside derivative (SQGD) isolated from radioresistant bacterium *Bacillus sp. INM-1* for its activity against CCNU-induced oxidative stress in healthy mice. Mice were divided into four groups, i.e., (I) untreated controls; (II) SQGD treated (40 mg/kg b. wt. i.p.) mice; (III) CCNU (40 mg/kg b.wt., i.p.); and (IV) SQGD (40 mg/kg b.wt., i.p.) administered 1 h prior to CCNU-administration (40 mg/kg b.wt., i.p.). Following treatment, liver homogenates and blood serum of the treated animals were subjected to ascorbate radical levels estimation and ROS production. Results indicated that SQGD+CCNU administration significantly ($p < 0.05$) reduced ascorbate radicals and ROS products in the liver and blood serum of mice as compared with CCNU-treated group. Reduction in oxidative disorders was observed in healthy mice which were treated with SQGD only, compared with controls. Further, the maximal concentration of free SQGD (a.u.) in the blood-flow was established at 30 min after i.p., and completely reduced after 240 min. The pharmacokinetic profile of free SQGD showed significant selective accumulation, mostly in liver and lungs (60 min), brain (90 min), followed by kidney, pancreas, spleen, blood and testicles. Thus, it can be concluded that SQGD treatment alone and in combination SQGD+CCNU neutralized oxidative toxicity caused by medicines not only by reducing lipid peroxidation but also by improving antioxidant status of organs and blood, and this effect may emphasize SQGD as a strong radical-scavenger and excellent natural protector.

Keywords: SQGD, CCNU, Pharmacokinetic profile, Lipid peroxidation, Protection

INTRODUCTION

Lomustine (CCNU) is a cytotoxic chemotherapeutic antitumor drug with an alkylating effect. Its mechanism of action involves the alkylation of DNA and RNA matrices, as well as the inhibition of key enzyme processes by altering the structure and function of many proteins and enzymes [1]. The main drawback limiting the clinical use of CCNU is the damaging effect of the gastrointestinal tract and the delayed cumulative dose-dependent hepatotoxicity [2]. Metabolism of CCNU results in the production of nitric oxide (NO•), hydrogen peroxide (H₂O₂), hydroxyl radicals (OH•) or peroxynitrite (ONOO⁻), thus increasing oxidative toxicity in the body by generating reactive oxygen species (ROS) [3-5]. Excessive use of medicines increases the generation of OH• and leads to a rapid initiation of lipid peroxidation, overproduction of lipids (L•), lipid peroxy (LOO•) and hydroperoxides (LOOH•) [6].

A series of radical reactions changes the functionality of biomembranes and generates potential toxic products of lipid peroxidation [7]. Therefore, it is important to look for protective agents [8] that can overcome the toxicity caused by

drugs and prevent lipid peroxidation in models *in vivo* and *in vitro*. From this point of view, we hypothesized that radioresistant microbial species that survive in an extreme radiation environment can synthesize new secondary metabolites with better adaptability in the presence of recorded oxidative disorders and be used as suitable protectors [8,9], neutralizing CCNU-toxicity. For this purpose, the new semiquinone glucoside derivative SQGD (deposited in NCBI gene bank with accession number EU 240544.1), isolated from the fermented broth of *Bacillus Sp. INM-1* [10], was evaluated for its protective activity against the modulation of oxidative lesions in liver and blood serum in male RCS/b mice. A number of authors commented on the antioxidant [10,11] and immuno-suppressive properties [12], as well as the radio protective activity of SQGD to the reproductive, gastrointestinal [8,13], and renal systems [14].

The present study focuses on blood clearance/serum resorption and organ bio-distribution of the natural protector SQGD investigated by electron paramagnetic resonance (EPR) assays. Oxidative toxicity in the body and ROS generation induced by CCNU treatment and their redox modulation after combination with SQGD were also analyzed by

* To whom all correspondence should be sent.

E-mail: ykaramalakova@gmail.com

examining levels of ascorbate radicals and residual lipid peroxide products in *ex vivo* models.

EXPERIMENTAL

Materials and instrumentation

N-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea (CCNU); 0.1M phosphate buffer saline; spin-trap *n*-tert-butyl-alpha-phenylnitron (PBN), dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co, St. Louis, USA. Ethanol was purchased from Haymann Chemicals, Essex, England.

EPR measurements were performed on an X-band EMX^{micro} spectrometer (Bruker, Germany), equipped with a standard resonator. Experiments were carried out in triplicate. The EPR spectrum was immediately registered at room temperature (305 K) in the organs and blood plasma/ serum (expressed in arbitrary units) with following settings: 3505 g centerfield, 6.42 mw microwave power, 5-10 G modulated amplitude, 1-5 scans. All experiments were made in triplicate and results were calculated by double integration.

Characterization of SQGD

Characterization (strong, single symmetrical signal, $g = 2.0056 \pm 0.0002$ registered in powder) of SQGD was carried out using EPR spectroscopy [15] and other extraction procedures described previously [10, 12]. The type strain (SQGD) (*Bacillus* sp. INM-1, MTCC No. 1026, IBG-21) was deposited at the Institute of Microbial Technology, Chandigarh and INMAS, Delhi, India as a reference.

Animals

Non-inbred 6 weeks old (26–30 g of body weight, specific pathogen-free, second line) male RCS/b mice maintained at the Vivarium, Medical Faculty, Stara Zagora, Bulgaria were used in the study. Mice were maintained under standard conditions (20–22°C and 12 h light/12 h dark) on animal feed, water *ad libitum* and housed in groups of 6 in polypropylene cages at standard humidity of 40–60%. The regulations and rules of Research Ethics Commission of the Medical Faculty, Trakia University and the European directive 210/63/EU from 22.09.2010, were strictly followed during the experimental process.

Treatment groups

Mice were divided into the following four groups: *Group I* (n = 6): Control mice treated with 300 µL of cold saline intraperitoneal (i.p.); *Group II* (n = 6): SQGD (40 mg/kg b.wt., i.p.); *Group III* (n = 6): CCNU (40 mg/kg b.wt., i.p.); *Group IV* (n = 6): SQGD (40 mg/kg b.wt., i.p.) administered 1 h prior

to CCNU-administration (40 mg/kg b.wt., i.p.). Upon completion of i.p. injection with SQGD, the different groups of animals were dissected after 10, 30, 60, 90, 240, 1440 min and subjected to EPR blood clearance and organ/ blood biodistribution assays. The complete set of experiments was repeated three times. The presented data are averaged by three independent experiments.

EPR blood clearance experiments

Blood resorption and SQGD toxicity were assessed after 10, 30, 60, 90, 240, 1440 min in blood samples taken from free-flowing blood and collected in heparinized tubes containing cold PBS (pH= 7-7.4). The presence of SQGD (determined in arbitrary units) was calculated by double integration of the plot of the registered spectrum and the g-factors were measured.

EPR ex vivo organ/tissue and blood distribution

Different groups of animals were decapitated at appropriate time points following injection (10, 30, 60, 90, 240, 1440 min) and dissected. 80-100 mg samples of lungs, liver, spleen, brain, kidneys, pancreas, testicles and blood were collected and kept in 0.1M phosphate buffer saline (10% w/v) and homogenized immediately using a hand-held homogenizer. The tissue homogenate was centrifuged at 2000×g for 15 min. Supernatant separated from tissue and blood was used for analysis. Biodistribution of SQGD (40 mg/kg; i.p.) was evaluated by EPR spectroscopy as described previously [16].

Ex vivo evaluation of ROS production

Liver tissue 100 mg (100 µl plasma) were homogenized with 900 µl of 50 mM spin-trap *n*-tert-butyl-alpha-phenylnitron (PBN) dissolved in DMSO using a sonicator at one cycle for 1 min. After 5 min of incubation in ice, the suspension was centrifuged at 4000 rpm for 10 min at 4°C. Supernatants were transferred into Eppendorf tubes and immediately analyzed. The real time formation of ROS products in the supernatant was estimated as described earlier [17] with some modifications [15].

Ex vivo evaluation of ascorbate radical levels

The method [18] was used to evaluate the ascorbate radical levels (Asc[•]) and the protection against CCNU-induced damage by SQGD treatment. In brief, 200 mg liver samples and 100 µl plasma were homogenized in cold DMSO (10% w/v) and centrifuged at 4000 × g at 4°C for 10 min. Supernatants were transferred into Eppendorf tubes and immediately analyzed. The spin-adduct formed

between DMSO and generated Asc[•] radicals was recorded in real time.

Statistical analysis

The data were presented as means ± standard error (SE) of three independent experiments. Statistical analysis was performed with Statistica 8.0, *Stasoft, Inc.* Level of significance $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Oxidation-reduction processes involving oxygen, peroxides, hydroperoxides, etc., as well as many biochemical oxidation-reduction reactions in the body, form ROS that can exhaust the antioxidant protective system and lead to excessive damage to cellular membrane lipid structures, important enzymes, proteins and DNA. Redox processes are also involved in the metabolism of a number of drugs, which can also produce ROS and reactive nitrogen species (RNS). Redox processes also participate in the metabolism of a number of drugs that can also produce ROS and RNS. In order to protect biological molecules against drug toxicity, there is an increasing interest in the search for new natural antioxidants and combinations thereof for the purpose of disposing of ROS and RNS produced by drug therapy or radiation [19, 20].

In this study, a comprehensive assessment of SQGD was conducted against the potential for practical significance and application, the extent of diffusion and exclusion from different tissues and blood flow, reduction of oxidative stress levels, lipid peroxidation and protective properties against CCNU-induced toxicity. The results of the *ex vivo* blood clearance study of SQGD in mice are presented on Fig. 1.

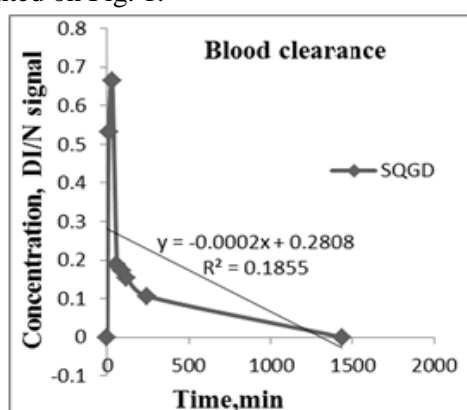


Fig.1 Investigation of blood clearance (a.u.) of SQGD in animals (n=6). Concentration was calculated by double integration of the registered EPR spectrum.

The maximum concentration of free SQGD (*arbitrary units*) in the blood serum was established 30 min after i.p. injection, and was almost

completely reduced after 240 min. Significantly, the bacterial fraction has a short half-life and is rapidly and permanently absorbed by the bloodstream. In accordance with our results, Mishra *et al.* [21] found rapid serum resorption of SQGD, as well as significantly improved expression of G-CSF in sera from mice treated with the antioxidant alone.

Based on data demonstrating the selective accumulation of TEMPO and spin-labeled synthetic agents [16, 22] in certain organs in animals, we investigated and compared the distribution and the pharmacokinetic profile of SQGD by the EPR method.

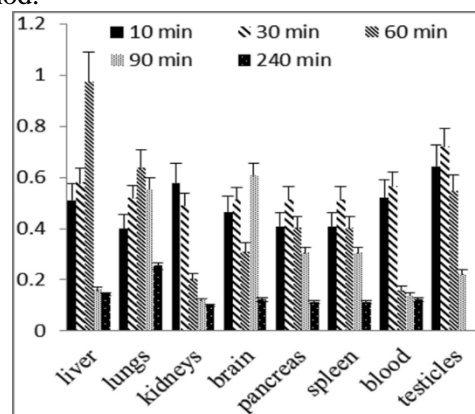


Fig.2 EPR measurements of tissue biodistribution of SQGD in organs (liver, spleen, lung, kidneys, pancreas, brain, testicles) and blood (n=6). Concentration was calculated by double integration and calculated g-value (a.u) of the registered EPR spectrum.

The results (Fig. 2) showed that SQGD accumulates selectively, mostly in liver and lungs (60 min), brain (90 min), followed by kidney, pancreas, spleen and blood. It is obvious that SQGD, as a fraction isolated from bacteria surviving in extreme conditions, has a high antioxidant activity due to the radical structure of o-semiquinone, as evidenced by our group [10,15]. A stable radical function is also a probable cause of selective accumulation in the liver and brain, rapid elimination in the blood and suggests a low total toxicity [21] of the metabolite. Other authors [23] commented on the accumulation of SQGD in the liver as therapeutically acceptable, because it is a common metabolic organ. Ascorbic acid undergoes long-lasting ROS modifications in the body, which leads to the formation of stable structures - ascorbate radicals ($\bullet\text{Asc}$) [18]. They are identified as a good endogenous marker to prove the generation of toxic reactive radicals in the body. To confirm the efficacy of SQGD in neutralizing oxidative toxicity generated by drug agents such as CCNU, ascorbate radical levels in liver and blood serum were evaluated. The results clearly demonstrate the immunosuppressive toxic effects of CCNU: 2 hours

after injection an almost four-fold increase of ascorbate radicals levels in liver was registered (mean 1.108 ± 0.093 vs mean 0.308 ± 0.06 , $*p < 0.005$), and serum (mean 0.769 ± 0.08 vs mean 0.179 ± 0.006 ; $*p < 0.05$) relative to the controls, (see Fig. 3).

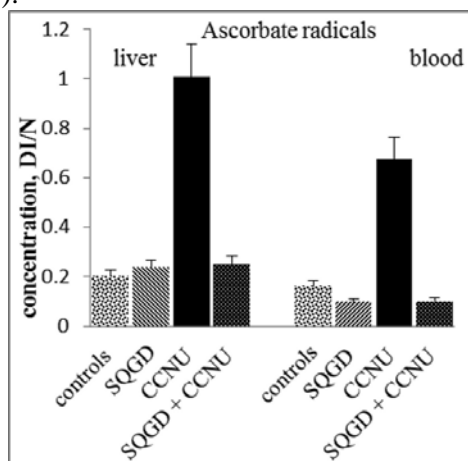


Fig.3 The levels of ascorbate radicals (a.u.) of mice treated with SQGD, CCNU and combinations in liver and blood serum 2h post injection. The values are presented as mean \pm SE.

In contrast, the oxidative status in mice treated with SQGD alone in the liver (0.291 ± 0.007 , $*p < 0.00001$) and in blood serum (0.0997 ± 0.0006 , $*p < 0.001$) was almost commensurate with that of the controls. The combination of SQGD + CCNU resulted in a 2.4-fold decrease in Asc• values in both studied organs ($**p < 0.00003$). It was reported the increased cellular antioxidant activity of SQGD in the liver and the ability of the metabolite to overcome oxidative disorders generated by toxic agents such as H_2O_2 [23]. Additional *ex vivo* studies [21] have shown that SQGD as a potent M-CSF inducer exerts immune-stimulatory effects and protects not only liver oxidative disorders but also similar in blood serum.

Ex vivo demonstration of short-living free radicals is accomplished by the use of suitable spin-accelerators that react with radical structures to form stable products (spin-adducts).

The latter are suitable for EPR analyses [24]. In the present study, PBN was used as a spin-trap dissolved in DMSO to investigate the lipid peroxidation levels (ROS products in aerobic conditions), simultaneously in liver and serum (Fig. 4).

The results revealed a statistical increase in ROS products in the liver (mean 1.683 ± 0.017 vs 0.697 ± 0.06 , $*p < 0.005$) and serum (1.844 ± 0.11 vs 0.821 ± 0.02 , $*p < 0.05$) in the CCNU treated groups, compared to the controls. The study showed that SQGD treatment inhibited lipid peroxidation ($\sim 58\%$, $p > 0.05$) in serum.

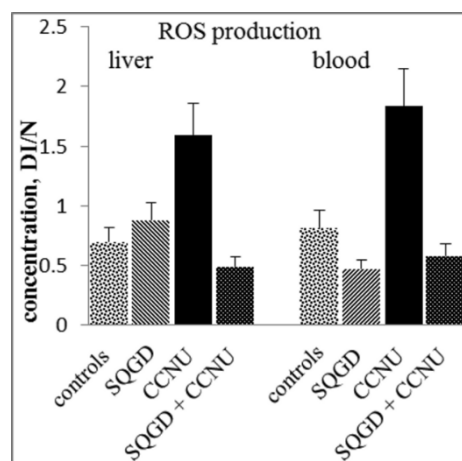


Fig.4 The ROS production levels (a.u.) of animals treated with SQGD, CCNU and combinations in liver and blood serum 2h post administration (12 groups; n= 6). The values are presented as mean \pm SE.

The combination of SQGD + CCNU (40 mg/kg +40 mg/kg) demonstrated a statistically comparable reduction in lipid peroxidation in both cases against animals treated only with CCNU. Similar results have been reported in our previous studies, in both agents at a ratio of 20mg/kg / 80mg/kg [15]. Inhibition of lipid peroxidation by SQGD in both liposomes and irradiated hepatic and brain homogenates is also reported by other groups [14]. It is speculated, that highly toxic CCNU *in vivo* generates $NO\bullet$ radicals, and contributes to the overproduction of $ONOO^-$ and $OH\bullet$ in tissues [3].

Therefore, SQGD, as a bacterial metabolite and antioxidant, possibly containing non-enzymatic moieties (tocopherols, phenolic acids, ascorbic acid, flavonoids), exhibits a better reducing effect on toxic oxidative processes initiated by CCNU. Probably, SQGD inhibits and completely neutralizes the overproduction of ROS and in particular $OH\bullet$ by a series of electron-donor reactions [25, 26] and thus significantly modulates the influence of free radicals on bio-molecules [14].

Finally, the present study demonstrates that SQGD alone or in combination provides a protective effect against induced oxidative damages and thus can be used in the future as an excellent inhibitor against drug/or radiation induced toxicity.

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СЕМИХИНОН ГЛЮКОЗИДЕН ДЕРИВАТ (SQGD), ИЗОЛИРАН ОТ *BACILLUS SP. INM-1*, ОСИГУРЯВАЩ АНТИОКСИДАНТНА ЗАЩИТА СРЕЩУ CCNU-ИНДУЦИРАНА ОКСИДАТИВНА ТОКСИЧНОСТ ПРИ МЪЖКИ МИШКИ

Я. Д. Карамалакова^{1*}, А. М. Желева¹, Р. Кумар², Г. Д. Николова¹, В. Г. Гаджева¹

¹Катедра "Химия и биохимия", Медицински факултет, Тракийски университет, ул. "Армейска" 11, 6000 Стара Загора, България

²Институт по ядрена медицина и природни науки, Организация за изследване и развитие на отбраната (DRDO), Бриг С. К. Масумдар Марг, Делхи-110054, Индия

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(Резюме)

Настоящото изследване е фокусирано върху оценката на семихинон глюкозидния дериват (SQGD), изолиран от радиоустойчивата бактерия *Bacillus sp. INM-1* при неутрализирането на CCNU-индуциран оксидативен стрес при здрави мишки. Мишките се разделят на четири групи, т.е. (I) нетретирани контроли; (II) мишки лекувани с SQGD (40 mg/ kg телесно тегло, интраперитонеално); (III) мишки, третирани с CCNU (40 mg/ kg телесно тегло, интраперитонеално); и (IV) мишки, третирани с SQGD (40 mg/kg телесно тегло, интраперитонеално), приложен 1 час преди прилагане на CCNU (40 mg/ kg телесно тегло, интраперитонеално). След провеждане на лечението са оценени нивата на аскорбатни радикали и продуцирането на ROS в чернодробни хомогенати и кръвен серум от третираните животни. Резултатът показва, че приложението на SQGD + CCNU значително намалява ($p < 0,05$) нивата на аскорбатни радикали и ROS продукти в черния дроб и серума при мишките в сравнение с групата, третирана единствено с CCNU. При третираните с SQGD здрави мишки е регистрирано намаляване на оксидативните нарушения, спрямо здравите контроли. Освен това максималната концентрация на SQGD (а.у.) в кръвния поток се установява 30 минути след интраперитонеално прилагане и е напълно редуцирана след 240 минути. Фармакокинетичният профил на SQGD показва значително селективно натрупване, максимално в черния дроб и белите дробове (60 минути), мозъка (90 минути), последвано от бъбреци, панкреас, далак, кръв и тестиси. По този начин може да се заключи, че третирането с SQGD самостоятелно и в комбинация SQGD + CCNU неутрализира оксидативната токсичност, причинена от лекарства, не само чрез намаляване на липидното перокисление, но и чрез подобряване на антиоксидантния статус на органите и кръвта. Това подчертава ефекта на SQGD като силен радикало-уловител и отличен природен протектор.