Toxicology Letters 233 (2015) 1–7

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

A guanine-ethylthioethyl-glutathione adduct as a major DNA lesion in the skin and in organs of mice exposed to sulfur mustard

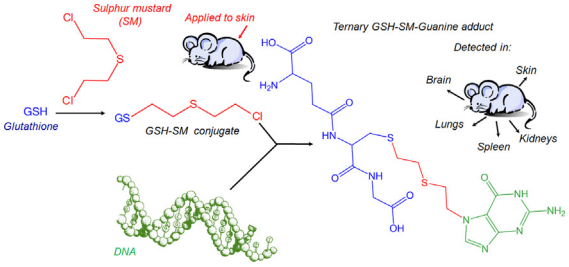
Mohamed Batal [a,b,d], Silvestre Rebelo-Moreira [a,b], Nadège Hamon [a,b], Pierre-Alain Bayle [a,c], Stéphane Mouret [d], Cécile Cléry-Barraud [d], Isabelle Boudry [d], Thierry Douki [a,b,*]

[a] Univ. Grenoble Alpes, INAC, LCIB, LAN, F-38000 Grenoble, France
[b] CEA, INAC, SCIB, LAN, F-38000 Grenoble, France
[c] CEA, INAC, SCIB, LRM, F-38000 Grenoble, France
[d] Département de Toxicologie et Risques Chimiques, Unité de Brûlure Chimique, Institut de Recherche Biomédicale des Armées, Antenne de La Tronche, BP87, F-38702 La Tronche Cedex, France

HIGHLIGHTS

- We isolated a ternary adduct involving DNA, glutathione and sulfur mustard.
- A HPLC-mass spectrometry assay was developed for the quantification of this adduct.
- The ternary adduct was detected in the skin and internal organs of SM-exposed mice.
- The ternary adduct was present for two weeks after SM exposure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 December 2014
Received in revised form 30 December 2014
Accepted 2 January 2015
Available online 3 January 2015

Keywords:
Sulfur mustard
DNA adducts
Glutathione
Bifunctional alkylating agent
Skin toxicity

ABSTRACT

Sulfur mustard (SM) is an old chemical warfare but it remains a threat to both militaries and civilians. SM mainly targets skin, eyes and lungs and diffuses to internal organs. At the molecular level, SM is able to damage DNA through the formation of monoadducts and biadduct. Glutathione (GSH) is another critical target of SM in cells since it is part of the detoxification mechanism against alkylating agents. In the present work, we investigated whether SM could form covalent bonds simultaneously with a DNA base and the sulfhydryl group of GSH. The expected guanine adduct, S-[2-(N7-guanyl)-ethylthioethyl]-glutathione (N7Gua-ETE-GSH), was synthesized and detected in several tissues of SKH-1 mice exposed to 60 mg/kg of SM in the dorsal-lumbar region. N7Gua-ETE-GSH was detected in all organs studied, except in the liver. The tissue exhibiting the highest levels of N7Gua-ETE-GSH was skin, followed by brain, lungs, kidneys and spleen. N7Gua-ETE-GSH was detected in skin, brain and lungs as long as two weeks after exposure. The persistence was less in other organs. The observation of the formation of N7Gua-ETE-GSH *in vivo* confirms the variety of damages induced by SM in DNA. It also provides another example of the formation of DNA adducts involving glutathione following *in vivo* exposure to bifunctional alkylating compounds.

* Corresponding author at: INAC/SCIB, CEA-Grenoble, 17 Rue des Martyrs, 38054 Grenoble Cedex 9, France. Tel.: +33 4 38 78 31 91; fax: +33 4 38 78 50 90.
E-mail address: thierry.douki@cea.fr (T. Douki).

http://dx.doi.org/10.1016/j.toxlet.2015.01.001
0378-4274/

1. Introduction

Sulfur mustard (SM) is a warfare agent used for the first time in World War I and more recently in the Iran–Iraq conflict. SM is also a concern with regard to terrorism since it could be easily synthesized and used against the general population (Smith et al., 1995; Wattana and Bey, 2009). The major targets of SM in humans are the eyes, lungs and skin. In the latter organ, SM leads to the formation of large blisters and is thus classified as a vesicant agent. These wounds heal poorly and therapeutic strategies remain to be improved. A better understanding of the patho-physiological process is thus necessary. SM is a powerful alkylating agent which, after transient cyclization into an episulfonium ion in water, covalently binds to proteins, RNA and DNA. DNA alkylation is a critical step in SM-induced toxicity since it triggers massive cell death by either apoptosis or necrosis and thus leads to tissue degradation (Papirmeister et al., 1985; Lodhi et al., 2001; Kehe et al., 2009; Shakarjian et al., 2010). Moreover, formation of DNA adducts is likely to be involved in the previously reported SM-induced carcinogenicity in humans (Easton et al., 1988; Emadi et al., 2008). Several adducts have been characterized (Brookes and Lawley, 1960, 1961, 1963; Fidder et al., 1994), namely N7-hydroxyethylthioethyl-guanine (HETE-N7Gua), N3-hydroxyethylthioethyl-adenine (HETE-N3Ade) and the intra- and inter-strand crosslink between two guanines bis(N7-guanyl)-ethylthioethyl (N7Gua-ETE-N7Gua). These three types of SM adducts were recently detected *in vivo* following cutaneous exposure of mice, both in skin (Batal et al., 2013b; Yue et al., 2014) and in internal organs (Batal et al., 2014).

Glutathione (GSH) is another major target of SM in exposed tissues. In addition to being the most important low molecular weight antioxidant synthesized in cells, GSH plays a key role in the detoxification of many xenobiotics through the formation of conjugates catalyzed by glutathione-*S*-transferases (Forman et al., 2009). Formation of GSH-conjugates with SM is well documented. It can occur either spontaneously or be mediated by glutathione-*S*-transferases (Kinsey and Grant, 1947; Davison et al., 1961; Black et al., 1992a,b; Abel et al., 2011). The reactivity of SM toward GSH is so large that a phenomenon of GSH depletion was observed both in *in vitro* (Ray et al., 1995; Gross et al., 1997; Amir et al., 1998) and *in vivo* models exposed to SM (Gautam and Vijayaraghavan, 2007; Jafari, 2007; Vijayaraghavan et al., 2008; Pohanka et al., 2013). This depletion is followed by a strong oxidative stress associated with damage to proteins (Pohanka et al., 2011), lipids (Kumar et al., 2001; Jafari, 2007) and DNA (Pal et al., 2009; Tewari-Singh et al., 2012). Consequences of this oxidative stress have even been reported in humans (Sharma et al., 2009), showing the patho-physiological relevance of the reactivity of SM toward GSH.

In the present work, we investigated whether the bifunctional SM molecule could generate an adduct exhibiting a covalent bond with a DNA base and the sulfhydryl group of GSH, as reported for 1,2-dibromoethane (Ozawa and Guengerich, 1983; Inskeep and Guengerich, 1984; Inskeep et al., 1986; Koga et al., 1986) and 1,2,3,4-diepoxybutane (Cho and Guengerich, 2012a). We isolated the expected guanine adduct S-[2-(N7-guanyl)ethylthioethyl]-glutathione (N7Gua-ETE-GSH) from SM-treated isolated DNA. With an authentic standard in hand, we could then set-up an HPLC-mass spectrometry assay for the quantification of N7Gua-ETE-GSH in DNA. The latter technique was applied to the skin and several internal organs of mice cutaneously exposed to SM.

2. Material and methods

2.1. Chemicals and enzymes

Reduced glutathione and other chemicals were purchased from Sigma–Aldrich (Saint Quentin Falavier, France). HPLC-grade acetonitrile was from Merck (Darmstadt, Germany). SM (95% pure) was obtained from the French Single National Small-Scale Facility (DGA Maîtrise NRBC, Vert-Le-Petit, France). Ribonuclease T1, ribonuclease A, phosphodiesterase II, deoxyribonuclease II, alkaline phosphatase and nuclease P1 were obtained from Sigma. Protease was purchased from Qiagen and phosphodiesterase I from Worthington (Lakewood, NJ, USA).

2.2. Synthesis of N7Gua-ETE-GSH

A solution of calf thymus DNA (1 mg/mL) and reduced glutathione (200 mM) in a Na_2HPO_4 buffer (50 mM, pH 7) was incubated with 100 mM of SM during 4 h at room temperature. DNA was then precipitated in presence of absolute ethanol (2.5 volumes) and NaCl 4 M (0.1 volume). Several washings with ethanol 70% (v/v) were carried out to eliminate possible traces of SM. DNA was suspended in deionized water and hydrolyzed as previously reported (Batal et al., 2013a). N7Gua-ETE-GSH was isolated using a preparative HPLC system consisting of a L6200 intelligent pump from Merck, a 7125 injector valve from Rheodyne (San Jose, CA, USA), a C18-reverse phase column (4.0 × 250 mm I.D, 5 μm particle size, Uptisphere ODB, Interchim, Montluçon, France), a Merck L4000 detector (Munich, Germany) and a FC 204 fraction collector from Gilson (Middleton, WI, USA). A gradient of ammonium formate (pH 6.3) and methanol at a flow rate of 1 mL/min was used. Fractions of 2 mL were collected from which 20 μL were analyzed by HPLC-MS/MS (see below). The fractions of interest were pooled and further injected on the HPLC system until a pure solution was obtained. Its concentration in N7Gua-ETE-GSH was determined by HPLC with diode array UV detection as previously reported for HETE-N7Gua (Batal et al., 2013a).

2.3. Animal study

Male, euthymic and hairless SKH-1 mice (Crl: SKH1-*hr*) of 4–6 weeks of age were obtained from Charles River Laboratories (L'Arbresle, France). The animals were housed and acclimatized for 7 days before experiment in a controlled environment (21 ± 1 °C and 12 h light/dark cycle) with food and water *ad libitum*. Study protocols were approved by the Ethical Committee of the Institut de Recherche Biomédicale des Armées. Exposure to 60 mg/kg of SM diluted in dichloromethane was performed on the dorsal-lumbar region of the animal centered on the body axis on a circular zone of 0.28 cm^2. Five or 6 animals were used per exposed group and 4 animals per control group. Before exposure, animals were anesthetized by an intraperitoneal injection of a mixture of diazepam (3.75 mg/kg) and ketamine hydrochloride (33.30 mg/kg). For pain relief, buprenorphin (0.05 mg/kg) was delivered by a subcutaneous injection during the first 3 days. SM, diluted in 2 μL of dichloromethane, was applied topically on a marked circular zone of 0.28 cm^2 on the dorsal-lumbar region of the animal centered on the body axis (Dorandeu et al., 2011). Four hours after SM exposure, the exposed skin site was gently wiped with a fresh solution of 0.8% sodium hypochlorite, using natural sponges, to perform skin decontamination as recommended by Suchard (2011). Control animals were treated following the same protocol, by replacing SM solution by pure dichloromethane. The mice were euthanized by injection of an overdose of sodium pentobarbital (*ca.* 100 mg/kg, i.p.) 6 h, 1, 3, 7, 14 and 21 days after the beginning of the exposure. Brain, lungs, kidneys, spleen and liver were collected and frozen in liquid nitrogen. Skin biopsies were taken. All samples were stored at −80 °C until use.

2.4. HPLC-MS/MS quantification

DNA extraction from skin biopsies was achieved as performed earlier for UV-irradiated human skin biopsies (Mouret et al., 2006).