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SUPPLEMENTARY MATERIAL

Table S1. Primers for *KRT5* and *KRT14* amplification.

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Detection of Metabolites of Fumaric Acid Esters in Human Urine: Implications for Their Mode of Action

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TO THE EDITOR

In the treatment of psoriasis, fumaric acid esters show good clinical efficacy combined with a favorable safety profile (Mrowietz *et al.*, 1999).

Fumaderm, registered in Germany, consists of dimethylfumarate (DMF) and three salts of monoethylfumarate (MEF), and it has been shown that only DMF is required for clinical effect (Nieboer *et al.*, 1990). It is not yet clear whether DMF itself represents the active compound *in vivo* because only its hydrolysis product monomethylfumarate (MMF) could be detected in the plasma of healthy humans after oral intake (Litjens *et al.*, 2004a).

DMF exerts pharmacodynamic effects in low concentrations *in vitro* but could not be detected *in vivo*. In contrast, MMF showed *in vitro* effects only at concen-

Abbreviations: DMF, dimethylfumarate; GS-DMS, S-(1,2-dimethoxycarbonylethyl)glutathione; GSH, glutathione; MEF, monoethylfumarate; MMF, monomethylfumarate; NAC-DMS, N-acetyl-S-(1,2-dimethoxycarbonylethyl)cysteine; NAC-MES, mixture of N-acetyl-S-(1-carboxy-2-ethoxycarbonylethyl)-cysteine and N-acetyl-S-(2-carboxy-1-ethoxycarbonylethyl)cysteine; NAC-MMS, mixture of N-acetyl-S-(1-carboxy-2-methoxycarbonylethyl)cysteine and N-acetyl-S-(2-carboxy-1-methoxycarbonylethyl)-cysteine

trations much higher than those detectable in plasma of humans after intake of one Fumaderm tablet (120 mg of DMF) (Zhu and Mrowietz, 2001; Treumer *et al.*, 2003; Litjens *et al.*, 2006; Gerdes *et al.*, 2007).

It has been shown *in vitro* that DMF as an α,β-unsaturated carboxylic acid ester reacts with glutathione (GSH) quickly and completely at physiological pH through a Michael-type addition reaction, leading to the formation of *S*-(1,2-dimethoxycarbonylethyl)glutathione (GS-DMS). *In vitro*, MMF reacts spontaneously with GSH to form a mixture of *S*-(1-carboxy-2-methoxycarbonylethyl)glutathione and *S*-(2-carboxy-1-methoxycarbonylethyl)glutathione, but much slower and to a smaller extent than DMF (Schmidt *et al.*, 2007). *In vivo* GSH-adducts conjugates often underlie a sequence of enzymatically catalyzed reactions to mercapturic acids, which can be excreted in the urine (Figure 1).

Here, we report the results of an *in vivo* study where urine samples of psoriasis patients were analyzed for mercapturic acids of MMF, MEF, and DMF (mixture of *N*-acetyl-*S*-(1-carboxy-2-methoxycarbonylethyl)cysteine and *N*-acetyl-*S*-(2-carboxy-1-methoxycarbonylethyl)cysteine (NAC-MMS), mixture of *N*-acetyl-*S*-(1-carboxy-2-ethoxycarbonylethyl)cysteine and *N*-acetyl-*S*-(2-carboxy-1-ethoxycarbonylethyl)cysteine (NAC-MES), and *N*-acetyl-*S*-(1,2-dimethoxycarbonylethyl)cysteine (NAC-DMS)) after oral intake of two tablets of Fumaderm (240 mg of DMF) under fasting conditions. The study was conducted according to the Declaration of Helsinki principles and written informed consent was obtained from all patients taking part in the study.

A liquid chromatography/mass spectrometry system was used to detect mercapturic acids. Using an ESI + ion source, fragmentation of NAC-MMS, NAC-MES, and NAC-DMS was observed characteristic for each substance. A representative mass spectrum of a NAC-DMS standard is shown in Figure 2a. All urine samples obtained before drug intake were free of analytes.

In the first part of this study, relatively high concentrations of NAC-DMS and NAC-MMS, but not of NAC-MES, were detected in the urine of three subjects 210–240 minutes after drug intake (NAC-MMS 4.1 ± 3.5 μM; NAC-DMS 5.8 ± 2.1 μM). Six hours after drug intake, concentrations increased to 33.1 ± 5.0 μM NAC-MMS and 54.4 ± 9.5 μM NAC-DMS. A representative mass spectrum of NAC-DMS detected in urine is shown in Figure 2b.

In the second part of our study, NAC-DMS and NAC-MMS were quantified in urine collected for 24 hours from four subjects. On average, 12.4 ± 3.3 mg of NAC-MMS and 5.4 ± 4.4 mg of NAC-DMS were detected.

The presence of NAC-MMS in the urine may result from nonenzymatic hydrolysis of one ester group of GS-DMS, its intermediate metabolites, and/or NAC-DMS (Figure 1) and therefore seems not to be the product of a reaction from MMF with GSH (data not shown).

The main result of our study, the detection of NAC-DMS in urine, substantiates that DMF treatment is GSH consumptive. The formation of GS-

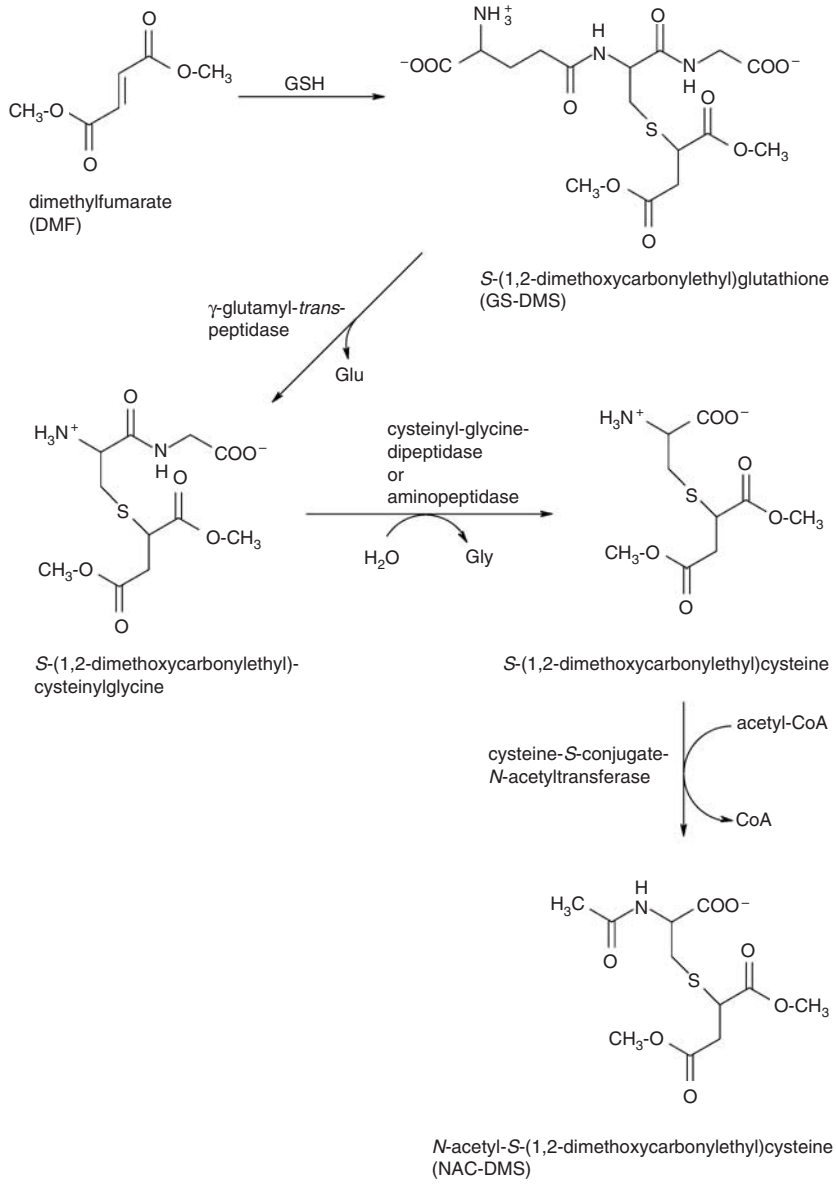

Figure 1. Metabolic pathway of a DMF-GSH-adduct. DMF is conjugated to GSH *in vivo* and resulting GS-DMS is metabolized to NAC-DMS followed by excretion in the urine.

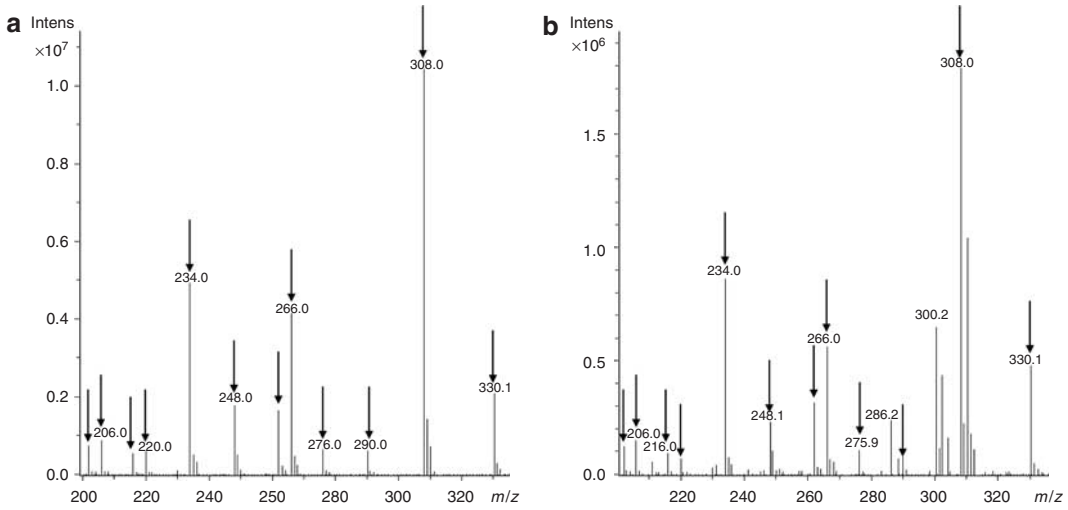

Figure 2. NAC-DMS was determined in the urine of psoriasis patients after oral intake of Fumaderm. (**a**) Representative mass spectrum of an NAC-DMS (*m/z* 308 $[M+H]^+$) standard and (**b**) NAC-DMS detected in urine of psoriasis patients after application of two tablets of Fumaderm; ion source: ESI+. Arrows mark characteristic ions for NAC-DMS resulting from fragmentation.

DMS may already occur in blood cells including immune cells of the portal vein blood, as DMF is not detectable in the plasma of peripheral venous blood after oral intake of Fumaderm, has a high tendency to penetrate into cells, and reacts very quickly and completely with GSH (Werdenberg *et al.*, 2003; Schmidt *et al.*, 2007). This is supported by DMF stability data, where it was concluded that blood cells are able to ''bind'' DMF (Litjens *et al.*, 2004b). A reaction of DMF with intracellular GSH in enterocytes seems to be negligible, as DMF penetrates fast through a CaCo-2 monolayer, and therefore cannot react with GSH to a greater extent (Rostami-Yazdi *et al.*, in preparation).

Taken together, our findings can explain the discrepancy between DMF being active *in vitro* and the lack of plasma levels *in vivo*. It is known that T cells promote inflammatory events in psoriatic skin, and treatment with Fumaderm leads to a reduction of T cells *in vivo*, which is at least in part due to apoptosis before the clinical effect becomes evident (Höxtermann *et al.*, 1998; Schimrigk *et al.*, 2006; Sabat *et al.*, 2007). *In vitro* DMF induces apoptosis in various cell types, including T cells (Zhu and Mrowietz, 2001; Treumer *et al.*, 2003).

This effect can be explained by DMF's ability to strongly deplete intracellular GSH, which correlates with induction of apoptosis (Held *et al.*, 1991; Nelson *et al.* 1999; Hollins *et al.*, 2006).

Another effect of fumaric acid ester therapy is that peripheral blood mononuclear cells of psoriasis patients produce lower levels of proinflammatory Th-1 cytokines (Litjens *et al.*, 2003). In addition, it has been shown *in vitro* that treatment of peripheral blood mononuclear cells with 5 μM DMF inhibits the production of proinflammatory cytokines (Stoof *et al.*, 2001).

These effects of DMF were linked to the fact that in GSH-depleted mice, a correlation with the induction of anti-inflammatory cytokines could be shown (Peterson *et al.*, 1998). Furthermore, decreased GSH levels of T cells were correlated with downregulation of Th1-cytokines (Hadzic *et al.*, 2005).

In summary, the postulated mode of action of fumaric acid esters is based on the ability of DMF to deplete intracellular GSH in immune cells followed by the generation of anti-inflammatory cytokines and/or induction of apoptosis.

To our knowledge, the results of our study, that a considerable part of DMF is not hydrolyzed after oral intake but enters circulation and reacts with GSH *in vivo*, are previously unreported.

CONFLICT OF INTEREST

U.M. served as a speaker and received honoraria from Biogen Idec and Allmiral-Hermal, the manufacturer and co-distributor of Fumaderm. All other authors declare no conflict of interest.

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Poly(ADP-Ribose) Polymerase Mediates Inflammation in a Mouse Model of Contact Hypersensitivity

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TO THE EDITOR

Contact hypersensitivity (CHS) is a form of a delayed type of hypersensitivity, a classic T-cell-mediated, clinically important phenomenon elicited by small molecular weight molecules (haptens) that bind to host proteins to form a complete allergen (Grabbe and Schwarz, 1998). The CHS reaction can be divided in to two phases: sensitization and elicitation. The elicitation phase is dominated by the production of proinflammatory cytokines and cellular infiltration by lymphocytes and granulocytes. Infiltration is accompanied by strong oxidative stress due to the formation of superoxide, nitric oxide, peroxynitrite, and further reactive species (Rowe *et al.*, 1997; Ross *et al.*, 1998).

The poly(ADP-ribose) polymerase (PARP) superfamily consists of 17 members (Ame *et al.*, 2004). A subgroup of these enzymes can be activated by DNA single-strand breaks and aberrant DNA forms (Ame *et al.*, 1999; Schreiber *et al.*, 2006). In tissues and cells, PARP-1 is responsible for most of the PARP activity due to its abundance and high catalytic activity. Activated PARP-1 uses NAD^+ as a substrate and synthesizes the formation of poly(ADP-ribose) polymers covalently attached to different acceptor proteins. The presence of poly(ADP-ribose) polymers may regulate the functions of the acceptor proteins (Schreiber *et al.*, 2006). Inhibition of PARP activity or knocking out the PARP-1 gene has been shown to suppress inflammatory reactions such as colitis, arthritis, and uveitis (Shall and De Murcia, 2000; Virag and Szabo, 2002; Cuzzocrea, 2005). Prevention of cellular dysfunction and inhibition of NF-κB activation have been proposed to be the mechanisms underlying the anti-inflammatory effects of PARP inhibition/knockout (Virag and Szabo, 2002).

Poly(ADP-ribose) polymerase plays a role in the regulation of the transcription of various inflammatory mediators such as cytokines, chemokines, inducible nitric oxide synthase, and matrix metalloproteinases (MMPs).

In our previous report, we have demonstrated peroxynitrite production, DNA breakage, and poly(ADP-ribose) formation during the elicitation phase of the CHS (Szabo *et al.*, 2001). Moreover, we have shown that peroxynitrite, superoxide, and hydrogen peroxide impair proliferation and viability of HaCaT keratinocytes (Szabo *et al.*, 2001). PARP inhibitors prevented necrotic cell death with a slight increase in apoptotic DNA fragmentation and also reduced cytokine-induced expression of IL-8 and ICAM-1 in HaCaT cells. (Szabo *et al.*, 2001). Kehe *et al.* (2008) reported similar findings in a model of sulfur mustard-induced cell death of HaCaT cells. They also found

Abbreviations: CHS, contact hypersensitivity; MMP, matrix metalloproteinase; MPO, myeloperoxidase; PARP, poly(ADP-ribose) polymerase; ROI, reactive oxygen intermediate; RNI, reactive nitrogen intermediate; TIMP, tissue inhibitor of metalloproteinases