

Active Transport of Contact Allergens and Steroid Hormones in Epidermal Keratinocytes is Mediated by Multidrug Resistance Related Proteins

Journal of Investigative Dermatology advance online publication, 9 July 2009; doi:10.1038/jid.2009.200

TO THE EDITOR

The phenomenon of multidrug resistance (MDR) is defined as the ability of a cell to show resistance to a wide variety of structurally and functionally unrelated molecules and is, in large part, related to membrane efflux transporters because its principal mechanism is the active transport of substrates out of the cell (Higgins, 2007). Multidrug transporters have broad specificity for a wide range of chemically unrelated agents (Higgins, 2007). One superfamily of active membrane transporters is the family of ATP-binding cassette (ABC) transport proteins that use the energy of ATP hydrolysis to transport large organic molecules either directly or, in case of many natural substrates, conjugated to acidic ligands, such as glutathione, glucuronate, or sulfate out of the cell (König *et al.*, 1999). Although most ABC transporters were discovered as drug transporters, they frequently transport a wide range of physiological substrates, including peptides, steroids, and inflammatory lipid mediators (Robbiani *et al.*, 2000; Borges-Walmsley *et al.*, 2003; Reid *et al.*, 2003). Therefore, it is further understood that these efflux pumps have, in addition to conferring resistance of tumor cells to various chemotherapeutic drugs, crucial physiological roles (Piddock, 2006). Several studies have been carried out to investigate the expression, regulation, and specific substrates of ABC efflux transport proteins, such as P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRP, gene name: ATP binding cassette C transporters, ABCC)

(Flens *et al.*, 1996; Ishikawa *et al.*, 2000). Earlier it has been shown that cells of the skin express a specific pattern of several efflux proteins (Slee-*man et al.*, 2000; Baron *et al.*, 2001; Colone *et al.*, 2008). Gene expression analysis of normal human epidermal keratinocytes (NHEK) showed constitutive expression of MRP1 (ABCC1) as well as MRP 3–7 (ABCC3–7), but was negative for MDR1 and MRP2 (ABCC2) (Baron *et al.*, 2001). With regard to the regulatory mechanisms of these transport proteins in skin cells, it was shown that the expression of different MRP family members can be significantly enhanced by IL-6-type cytokines. Furthermore, upregulation of MRP expression was found in lesional skin samples collected from patients with inflammatory skin disorders such as psoriasis and lichen planus (Dreuw *et al.*, 2005).

In this study, we provide evidence for the functional MRP-mediated efflux activity of NHEKs using an *in vitro* transport assay. Therefore, the efflux transport of various radiolabeled compounds, including eugenol (4-allyl-2-methoxyphenol), isoeugenol, estradiol 17 β -D-glucuronid (E217 β G), oestrone-3-sulfate, cyclosporine, and dexpantenol, was inhibited by the specific MRP inhibitors, such as indomethacin (Figure 1) (Draper *et al.*, 1997; Zhou *et al.*, 2008) or MK571 (Figure 2) (Skazik *et al.*, 2008; Zhou *et al.*, 2008), and the intracellular accumulation of these substrates was determined after 15, 30, and 60 minutes. Cells not treated with the inhibitor served as a control. Treatment with indomethacin

at a concentration of 1 mM decreased the efflux transport of [³H]E217 β G up to 6.5-fold and of [³H]oestrone-3-sulfate up to 3.6-fold in NHEKs in comparison with control transport measured in the absence of the inhibitor (Figure 1a and b). Treatment of NHEKs with MK571 decreased the efflux of [³H]E217 β G in a concentration-dependent manner at a concentration of 25 μ M up to 4.1-fold and at a concentration of 100 μ M up to 5.1-fold (Figure 2a). MK571 strongly reduced the efflux of [³H]oestrone-3-sulfate as well in a concentration-dependent manner, as the cells accumulated this steroid hormone up to 3.2-fold using 25 μ M of MK571 and up to 6.1-fold using 100 μ M of the inhibitor (Figure 2b) in comparison with control transport that is measured in the absence of the inhibitor. Indomethacin blocked the efflux transport of [³H]eugenol up to 1.6-fold compared with control cells without indomethacin treatment (Figure 1c). Treatment of cells with indomethacin increased intracellular concentration of [³H]isoeugenol only slightly up to 1.1-fold (Figure 1d). Treatment of NHEKs with MK571 at concentrations of 100 μ M and 25 μ M decreased efflux transport of [³H]eugenol and [³H]isoeugenol, respectively, to 1.6-fold after 60 minutes of incubation (Figure 2c and d). Time course experiments show stronger effects of both inhibitors on the accumulation of E217 β G, oestrone-3-sulfate, eugenol, and isoeugenol (Figures 1a–d and 2a–d) in the cell after 60 minutes compared with 15 or 30 minutes. This adds to the suggestion that these effects are due to the inhibition of efflux transport and not due to influx transport. Treatment of cells with indomethacin or MK571

Abbreviations: ABC, ATP-binding cassette; ABCC, ATP binding cassette C transporter; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; NHEK, normal human epidermal keratinocyte

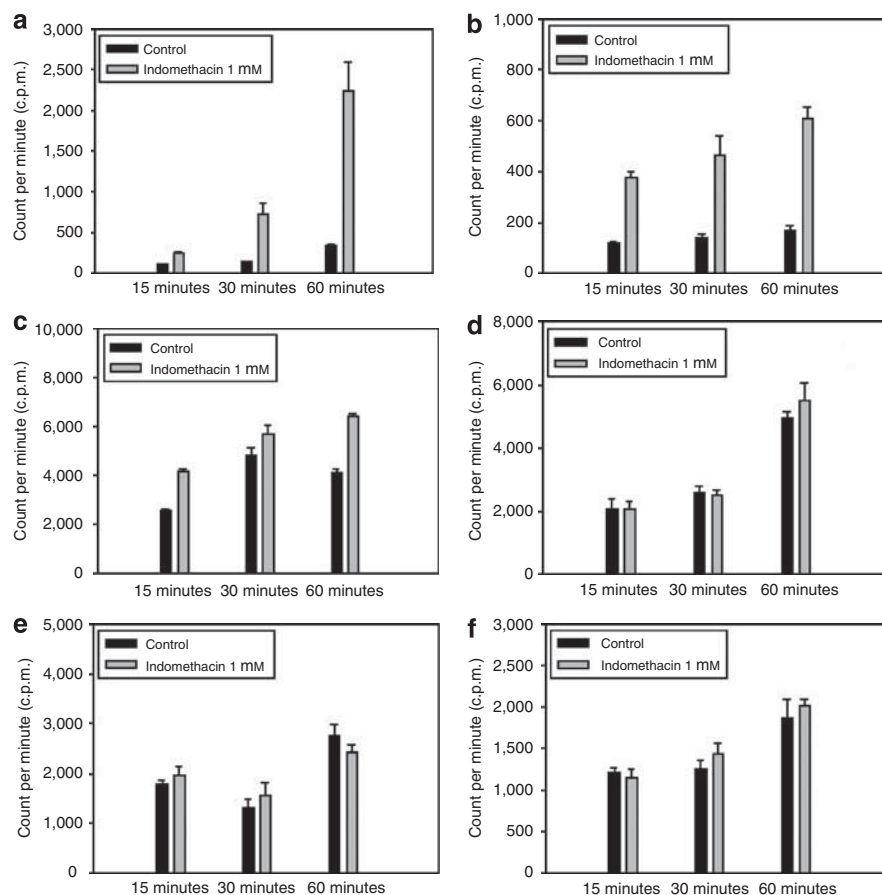


Figure 1. The inhibitory effect of indomethacin on MRP-mediated efflux of various substrates in NHEKs. Cells were pre-incubated with the inhibitor indomethacin (1 mM) for 15, 30, and 60 minutes at 37 °C. Control cells were treated with DMSO. Subsequently, the cells were exposed to various tritium-labeled substrates including (a) E217βG (3×10^{-10} mM ml⁻¹, 2 μCi ml⁻¹), (b) oestrone-3-sulfate (1.15×10^{-8} mM ml⁻¹, 2 μCi ml⁻¹), (c) eugenol (2×10^{-7} mM ml⁻¹, 2 μCi ml⁻¹), (d) isoeugenol (0.2 mM ml⁻¹, 2 μCi ml⁻¹), (e) cyclosporine (1.25×10^{-7} mM ml⁻¹, 2 μCi ml⁻¹), and (f) dexpanthenol (1×10^{-7} mM ml⁻¹, 2 μCi ml⁻¹) in the presence or the absence of the inhibitor. After incubation for 15, 30, or 60 minutes at 37 °C, the radioactive medium was removed and cells were washed. The cells were lysed by adding scintillation fluid. The cell-associated radioactivity was determined. The values are expressed as counts per minute (c.p.m.). Median values of three independent experiments in triplicate were used for statistical data analysis.

had only a minor effect on the transport of [³H]cyclosporine and no influence on the transport of [³H]dexpanthenol, because a marginal or a corresponding absence of alteration of intracellular accumulation could be detected compared with control cells without inhibitor treatment (Figures 1e, f, 2e and f).

The human MRP subfamily consists of at least nine members. Detailed *in vitro* transport measurements, using membrane vesicles purified from MRP1-transfected cells, have shown that MRP1 is a lipophilic anion transporter, capable of transporting sulfate or glucuronate conjugates, such as oestrone 3-sulfate or E217βG (Kruh *et al.*, 2001; Qian *et al.*, 2001). Membrane vesicles prepared from MRP3-transfected HEK293 cells also transport the prototypical MRP1 substrate E217βG,

but at a lower affinity. Both MRP1 and MRP3 are constitutively expressed in epidermal keratinocytes (Baron *et al.*, 2001), and active efflux transport of their ligands E217βG and oestrone 3-sulfate in NHEKs was shown in this study (Figures 1a, b, 2a and b).

In previous studies, we were able to show that the active uptake of E217βG and oestrone-sulfate in NHEKs is mediated by organic anion transporting proteins (Schiffer *et al.*, 2003). In addition this study demonstrated the MRP-mediated efflux activity of NHEK for sulfated estrogen (Figure 1b and Figure 2b) and E217βG (Figure 1a and Figure 2a), and thereby revealed, to our knowledge for the first time, the capability of human skin cells for the vectorial transport of compounds combining OATP-mediated influx and

MRP-mediated efflux transport. This direct interaction of structurally different influx and efflux transporters has been recently discovered in liver cells by Liu *et al.* (2006).

Fragrances are worldwide a major cause of allergic contact dermatitis, a delayed-type hypersensitivity reaction mediated by T lymphocytes. Studies by Sieben *et al.* (2001) shown that metabolic activation of eugenol using cytochrome P450 enzymes may be essential for positive T-cell responses to this fragrance. In our studies, we could show an MRP-mediated active transport of the small-molecular-weight contact allergens, eugenol and isoeugenol, in human skin cells (Figures 1c, d, and 2c and d), a novel mechanism that might have a significant role in the pathogenesis of contact allergy.

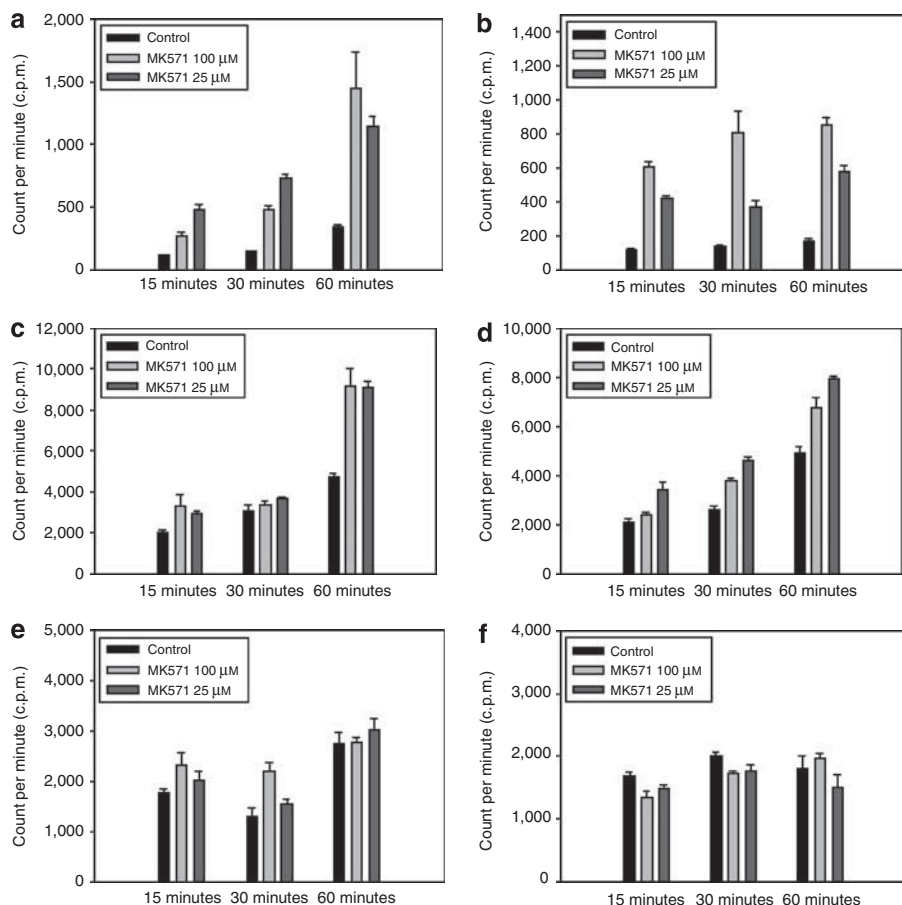


Figure 2. The inhibitory effect of MK571 on MRP-mediated efflux of various substrates in NHEKs. Cells were pre-incubated with the inhibitor MK571 (100 µM, 25 µM) for 15, 30, and 60 minutes at 37 °C. Control cells were treated with DMSO. Subsequently, the cells were exposed to various tritium-labeled substrates including (a) E217βG (3×10^{-10} mmol L⁻¹, 2 µCi mL⁻¹), (b) oestrone-3-sulfate (1.15×10^{-8} mmol L⁻¹, 2 µCi mL⁻¹), (c) eugenol (2×10^{-7} mmol L⁻¹, 2 µCi mL⁻¹), (d) isoeugenol (0.2 mmol L⁻¹, 2 µCi mL⁻¹), (e) cyclosporine (1.25×10^{-7} mmol L⁻¹, 2 µCi mL⁻¹), and (f) dexpanthenol (1×10^{-7} mmol L⁻¹, 2 µCi mL⁻¹) in the presence or the absence of the inhibitor. After incubation for 15, 30, or 60 minutes at 37 °C, the radioactive medium was removed and cells were washed. The cells were lysed by adding scintillation fluid. The cell-associated radioactivity was determined. The values are expressed as counts per minute (c.p.m.). Median values of three independent experiments in triplicate were used for statistical data analysis.

Cyclosporine is an immunosuppressive agents used in the setting of solid organ and hematopoietic stem cell transplantation, as well as inflammatory skin diseases. It has been shown earlier that cyclosporine A is a broad-spectrum MDR modulator, effectively impairing drug transport in cells overexpressing P-gp, MRP1, BCRP, and LRP at the clinically achievable concentrations. Cyclosporine modulates drug transport mediated by P-gp through competitive inhibition, a mechanism that is independent of that by which immunosuppression occurs. In addition, studies by Förster *et al.* (2008) using canine Madin-Darby cell line cells heterologously expressing MRP2 showed that cyclosporine is a strong inhibitor of MRP2-mediated transport of glutathione

methylfluorescein. Recently, it has been shown that verapamil-induced inhibition of P-gp and other efflux transporters led to a significant increase in cellular concentration and cytotoxic effects of cyclosporine in normal human renal epithelial cells (Anglicheau *et al.*, 2006). Our studies showed only a minor effect of MRP inhibitors on the efflux transport of cyclosporine in skin cells (Figures 1e and 2e). If cyclosporine itself is a weak MRP substrate or if transport activity is disturbed by conformational changes caused by ³H labeling of the compound needs to be addressed in further studies.

Pantothenic acid and its derivatives are beneficial in the maintenance of healthy skin and for cellular wound-healing processes. Grafe *et al.* (2003)

showed that human keratinocytes express the sodium-dependent multivitamin transporter mediating the influx transport and intracellular accumulation of pantothenic acid. Active efflux transport of [³H]dexpanthenol could not be detected in our study (Figures 1f and 2f), but further experiments using pantothenate as a substrate should be performed.

In addition to previous studies showing the mRNA and protein expression of efflux transport-associated proteins in human epidermal keratinocytes, we now provide data that strongly support the functional role of these transport proteins in the active efflux of compounds relevant for skin physiology, cell migration and proliferation, inflammation, and tumor suppression.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the START program of the Medical Faculty of the RWTH Aachen University.

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