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Original paper

1,4-Naphthoquinones as inducers of oxidative damage and stress signaling in HaCaT human keratinocytes

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ABSTRACT

Selected biological effects of 1,4-naphthoquinone, menadione (2-methyl-1,4-naphthoquinone) and structurally related quinones from natural sources – the 5-hydroxy-naphthoquinones juglone, plumbagin and the 2-hydroxy-naphthoquinones lawsone and lapachol – were studied in human keratinocytes (HaCaT). 1,4-naphthoquinone and menadione as well as juglone and plumbagin were highly cytotoxic, strongly induced reactive oxygen species (ROS) formation and depleted cellular glutathione. Moreover, they induced oxidative DNA base damage and accumulation of DNA strand breaks, as demonstrated in an alkaline DNA unwinding assay. Neither lawsone nor lapachol (up to 100 μM) were active in any of these assays. Cytotoxic and oxidative action was paralleled by stimulation of stress signaling: all tested quinones except lawsone and lapachol strongly induced phosphorylation of the epidermal growth factor receptor (EGFR) and the related ErbB2 receptor tyrosine kinase. EGFR activation by plumbagin, juglone and menadione was attenuated by a superoxide dismutase mimetic, indicating that ROS-related mechanisms contribute to EGFR activation by these naphthoquinones.

Introduction

Naturally occurring naphthoquinones may exhibit significant cytotoxic potential and have been discussed as toxic ingredients of plants such as leadwort or walnut. Owing to their cytotoxicity, naphthoquinone derivatives have been investigated as model compounds for the development of anti-cancer drugs acting on target cells both by the generation of reactive metabolites and by directly interfering with cellular enzymes crucial for cell proliferation. Menadione (2-methyl-1,4-naphthoquinone, also termed vitamin K_3) has been classically used as a tool to generate oxidative stress in exposed cells and tissues, generating reactive oxygen species by redox cycling and depleting glutathione [1–3]. Moreover, it was demonstrated to stimulate signaling cascades downstream of receptor tyrosine kinases by directly interfering with protein tyrosine phosphatases (PTPases) that catalyze dephosphorylation and inactivation of receptor tyrosine kinases [4], such as PTP1B [5]. PTPases including PTP1B or the cell cycle-regulating cdc25 phosphatases were inhibited even more efficiently by synthetic naphthoquinone derivatives, such as NSC 95397 [6], followed by attenuation of cell proliferation and cell death [6,7]. As a consequence of PTPase inhibition, tyrosine phosphorylation and activity of receptor tyrosine kinases – including the epidermal growth factor receptor (EGFR[1] = ErbB1) and the related ErbB2 receptor tyrosine kinase [5,7] – is sustained in exposed cells.

This work aims at analyzing the cytotoxic and genotoxic activity as well as the potential of inducing oxidative stress of structurally related naphthoquinones from natural sources (Fig. 1A): plumbagin (an ingredient of leadwort, *plumbago* sp.), juglone (from various types of walnut, *juglans* sp.), lawsone (found in colorants made from henna, i.e. *lawsonia* sp.) and lapachol (found in lapacho tea) in comparison to 1,4-naphthoquinone and menadione. Moreover, signaling elicited in cells exposed to these naphthoquinones is assessed by testing for stimulation of ErbB2 and EGFR, which was previously identified as a molecular target of various stressful

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[1] *Abbreviations used:* EGFR, epidermal growth factor receptor; Erb, avian erythroblastosis virus oncogene; ERK, extracellular signal-regulated kinase; Fpg, formamidopyrimidine-DNA glycosylase; GSH, glutathione; GSSG, glutathione disulfide; HaCaT, human adult, low Ca^{2+}, high temperature (keratinocyte source and culture conditions for establishment of cell line); MnTBAP, manganese (III) tetrakis (4-benzoic acid) porphyrin; MQ, menadione; PTPase, protein tyrosine phosphatase; ROS, reactive oxygen species.

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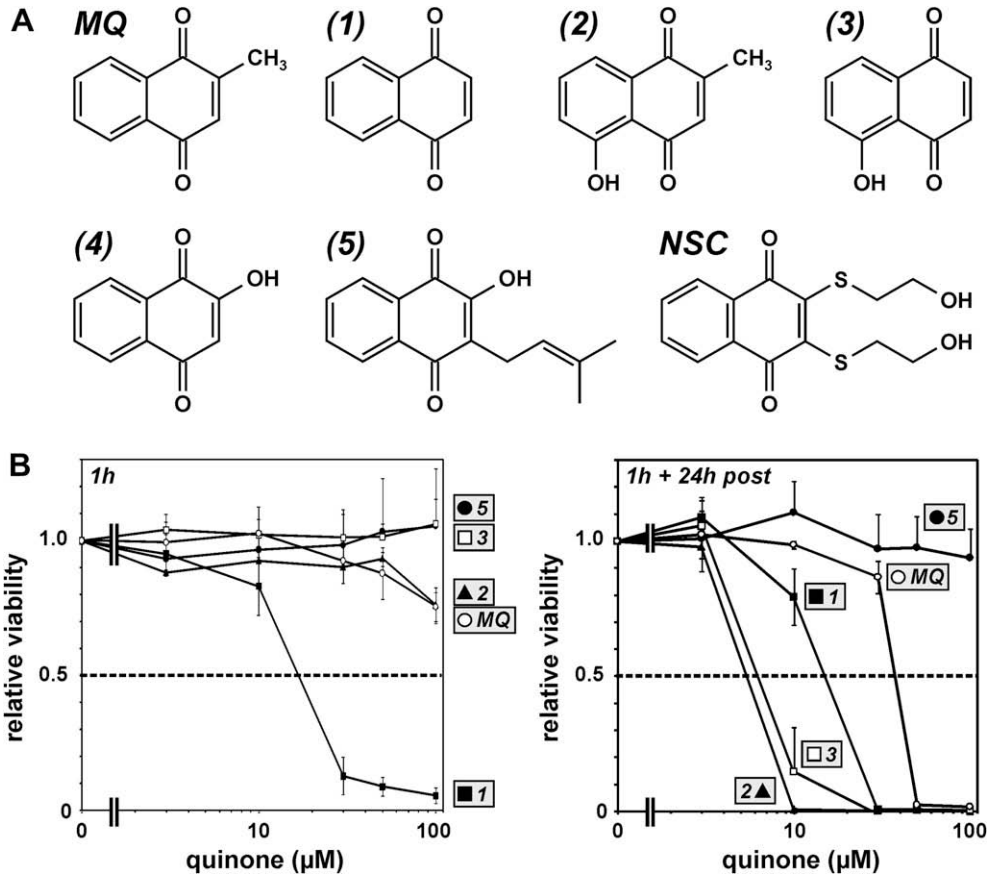

Fig. 1. Cytotoxicity of naphthoquinones. (A) Structures of the naphthoquinones employed or mentioned in this study: MQ – menadione; 1 – 1,4-naphthoquinone; 2 – plumbagin; 3 – juglone; 4 – lawsone; 5 – lapachol; NSC – NSC 95397 (2,3-bis-[2-hydroxyethylsulfanyl]-1,4-naphthoquinone). (B) HaCaT cells were exposed to naphthoquinones in serum-free media for 1 h. Viabilities of cells were determined directly following exposure (left) or after 24 h of postincubation in serum-free cell culture media (right). Data for lawsone were similar to those for lapachol and are not shown for reasons of clarity.

stimuli, integrating the action of diverse adverse agents to result in stimulation of cellular signaling pathways [4].

Owing to the fact that several of these quinones occur in cosmetics or colorants applied to skin (with henna dyes and walnut extracts being most famous examples), theses analyses were performed employing a human keratinocyte line, HaCaT cells.

Materials and methods

Cell culture

HaCaT human immortalized keratinocytes [8] were a kind gift from Prof. P. Boukamp, Heidelberg, Germany. Cells were held at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 and cultured in Dulbecco's modified Eagle's medium (DMEM; PAA, Pasching, Austria) supplemented with (final concentrations) 9% (v/v) fetal calf serum (PAA), 2 mM Glutamax (Gibco/Invitrogen, Karlsruhe, Germany) and penicillin/streptomycin (100 units/ml and 0.1 mg/ml, respectively; PAA). Cell viabilities were determined using standard procedures, i.e. by staining viable cells with neutral red and by using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to the corresponding blue formazan by viable cells.

Cells were grown to near confluence prior to exposure to naphthoquinones. All naphthoquinones were obtained from Sigma–Aldrich in the highest available purity and were kept frozen as aliquots of stock solutions (50–100 mM) in DMSO.

Analysis of the production of reactive oxygen species (ROS)

For ROS detection by fluorescence microscopy, HaCaT cells were grown to near confluence in 79 cm^2 cell culture dishes and incubated in the presence of naphthoquinones or DMSO (solvent control) dissolved in serum-free cell culture media for 1 h. Following exposure, media were removed, cells washed with PBS and hydroethidine (dihydroethidium; diluted in culture media at a final concentration of 20 μM from a stock solution in DMSO) added to cells, followed by incubation for 1 h. Cells were washed once with PBS and analyzed by fluorescence microscopy (excitation 490 nm/emission 560 nm). In cells treated with the manganese superoxide dismutase mimic, MnTBAP [Manganese (III) tetrakis (4-benzoic acid) porphyrin], the compound was present both during exposure to naphthoquinones and during posttreatment with hydroethidine. MnTBAP was from Sigma–Aldrich and was diluted into cell culture media from a stock solution in DMSO to be used at a final concentration of 300 μM. Relative fluorescence intensities were calculated after processing of fluorescence microscopy images by Scion Image software (Scion Corporation, Frederick, MD, USA), with control treatments (both with or without MnTBAP) arbitrarily set equal to 1.

For analysis of superoxide formation by detection of oxy-ethidium [9–11], cells were cultured as above and loaded with hydroethidine (20 μM) for 40 min, washed twice with PBS and exposed to naphthoquinones (or DMSO as solvent control) for 1 h. Following exposure, cells were washed once with PBS, then lysed on ice in 500 μl/dish of ice-cold methanol and collected into Eppendorf vials. Samples were stored at −80 °C. For further analysis, samples