The Phosphatase Inhibitor Menadione (Vitamin K3) Protects Cells from EGFR Inhibition by Erlotinib and Cetuximab

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Abstract

Purpose: Skin toxicity is the main side effect of epidermal growth factor receptor (EGFR) inhibitors, often leading to dose reduction or discontinuation. We hypothesized that phosphatase inhibition in the skin keratinocytes may prevent receptor dephosphorylation caused by EGFR inhibitors and be used as a new potential strategy for the prevention or treatment of this side effect.

Experimental Design: Menadione (Vitamin K3) was used as the prototype compound to test our hypothesis. HaCat human skin keratinocyte cells and A431 human squamous carcinoma cells were used. EGFR inhibition was measured by Western blotting and immunofluorescence. Phosphatase inhibition and reactive oxygen species (ROS) generation were measured by standard ELISA and fluorescence assays.

Results: Menadione caused significant and reversible EGFR activation in a dose-dependent manner starting at nontoxic concentrations. EGFR activation by menadione was associated with reversible protein tyrosine phosphatase inhibition, which seemed to be mediated by ROS generation as exposure to antioxidants prevented both menadione-induced ROS generation and phosphatase inhibition. Short-term coincubation of cells with nontoxic concentrations of menadione and the EGFR inhibitors erlotinib or cetuximab prevented EGFR dephosphorylation. Seventy-two-hour coincubation of cells with the highest nontoxic concentration of menadione and erlotinib provided for a fourfold cell growth inhibitory protection in HaCat human keratinocyte cells.

Conclusions: Menadione at nontoxic concentrations causes EGFR activation and prevents EGFR dephosphorylation by erlotinib and cetuximab. This effect seems to be mediated by ROS generation and secondary phosphatase inhibition. Mild oxidative stress in skin keratinocytes by topical menadione may protect the skin from the toxicity secondary to EGFR inhibitors without causing cytotoxicity. Clin Cancer Res; 17(21): 6766–77. ©2011 AACR.

Introduction

Epidermal growth factor receptor (EGFR) inhibitors have become standard of therapy for several very common human malignancies, that is, non–small cell lung cancer (NSCLC; refs. 1, 2), colorectal cancer (3, 4), pancreatic cancer (5), and head and neck cancer (6–8). The main side effect of these agents is a cutaneous toxicity starting at nontoxic concentrations. EGFR activation by menadione was associated with reversible protein tyrosine phosphatase inhibition, which seemed to be mediated by ROS generation as exposure to antioxidants prevented both menadione-induced ROS generation and phosphatase inhibition. Short-term coincubation of cells with nontoxic concentrations of menadione and the EGFR inhibitors erlotinib or cetuximab prevented EGFR dephosphorylation. Seventy-two-hour coincubation of cells with the highest nontoxic concentration of menadione and erlotinib provided for a fourfold cell growth inhibitory protection in HaCat human keratinocyte cells.

Conclusions: Menadione at nontoxic concentrations causes EGFR activation and prevents EGFR dephosphorylation by erlotinib and cetuximab. This effect seems to be mediated by ROS generation and secondary phosphatase inhibition. Mild oxidative stress in skin keratinocytes by topical menadione may protect the skin from the toxicity secondary to EGFR inhibitors without causing cytotoxicity. Clin Cancer Res; 17(21): 6766–77. ©2011 AACR.
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Translational Relevance

This article reports a summary of the preclinical experiments that provide the scientific rationale for the clinical development of topical menadione for the treatment and prevention of the skin toxicity secondary to epidermal growth factor receptor (EGFR) inhibitors. This work was conducted to test the novel general hypothesis that phosphatase inhibitors can be used to restore kinase activity in the presence of different kinase inhibitors. The work presented here shows that at subtoxic concentrations, menadione protects human skin keratinocytes from the EGFR inhibitory effects of erlotinib and cetuximab. This work led to the development by Talon Therapeutics of a menadione lotion, which is currently in clinical development, to prevent and treat the skin toxicity secondary to EGFR inhibitors.

Menadione was purchased from Sigma-Aldrich Chemical Co. and dissolved in ethanol (100 mmol/L) as a stock solution. Erlotinib was obtained from OSI pharmaceuticals and dissolved in Dulbecco’s modified Eagle’s medium at a stock concentration of 10 mmol/L. Cetuximab was a gift from Dr. Fan (MD Anderson Cancer Center, Houston, TX). All drugs were diluted to the indicated concentrations with RPMI-1640 medium. Monoclonal anti-EGFR antibody consisting of the use of topical skin moisturizers, topical sunscreens, and topical and systemic anti-inflammatory agents and antibiotics (32–34). The use of topical steroids and immunosuppressants agents has not been clearly shown to be of clinical value. Two randomized phase II studies of oral tetracyclines as preventive agents based on their mild anti-inflammatory effects (35) have shown a reduction in the incidence of grade 2 skin toxicity without an impact on its overall incidence (36, 37), and a third randomized phase II trial using a combination of topical and oral agents showed also a significant reduction in the incidence of grade 2 skin toxicity when used as preemptive treatment compared with reactive treatment (38). Still, the incidence of grade 2 rash remained significant in the patients treated with the improved therapeutic strategies. Therefore, newer and more effective strategies addressing the mechanisms of skin toxicity are needed to provide a more consistent symptomatic relief to a larger proportion, if not all patients, and avoid drug reductions and discontinuations that may compromise efficacy.

Protein tyrosine phosphatases are a group of enzymes that regulate the phosphorylation/activation of the intracytoplasmic domain of membrane-bound receptors (39). They play a role in modulating the intrinsic, nonligand binding-related activation of these receptors. Inhibition of protein tyrosine phosphatases induces intrinsic receptor phosphorylation/activation by shifting the balance toward the phosphorylated state (40). As a result, inhibitors of these enzymes have been explored to restore deficient signaling in a number of diseases, mainly insulin resistance in diabetic patients (41, 42).

Because the skin toxicity is the consequence of a series of events initiated by EGFR signal inhibition in the skin, local restoration of EGFR signaling without affecting the therapeutic EGFR signal inhibition at the tumor site should be a rational approach to its management. We have hypothesized that protein tyrosine phosphatase inhibitors should at least partially antagonize the effects of EGFR inhibitors and exert a protective effect by shifting the baseline intracellular EGFR status from dephosphorylated to phosphorylated, the net result being maintenance of EGFR signaling above the threshold that triggers cellular damage (40, 43, 44). If this hypothesis was proven to be true, the topical use of phosphatase inhibitors that are not absorbed systemically would represent a new mechanistic strategy to treat the skin toxicity secondary to EGFR inhibitors by effectively maintaining a differentially higher level of EGFR activation in the keratinocytes of the epidermis basal layer than in the tumor tissue when patients are exposed to therapeutic doses of these agents.

Menadione, a synthetic product of vitamin K, is a quinone that has been shown to cause several effects, among them DNA strand breaks and to inhibit protein tyrosine phosphatases, probably by directly alkylating the thiol group at the catalytic site of these enzymes and/or through generation of reactive oxygen species (ROS; refs. 45–47). Menadione has been shown to phosphorylate EGFR in rat hepatocytes (48), it has been used in the clinic as a topical treatment of vascular disorders in the skin to enhance vascular regeneration with an acceptable toxicity profile, although topical side effects have been observed in some patients (49, 50). We chose menadione as the prototype compound to test our hypothesis in anticipation of its potential clinical development.

In this work, we present the biochemical and molecular evidence that provides the scientific basis for developing menadione as a potentially useful topical agent to treat and prevent the skin toxicity secondary to EGFR inhibitors. We show that menadione causes in a concentration-dependent manner EGFR activation and that it antagonizes the EGFR inhibitory effect and cell growth inhibitory effect of both erlotinib and cetuximab in HaCat human skin keratinocytes and A431 human squamous cell carcinoma cells, and that such effect seems to be mediated by the generation of ROS and secondary inhibition of cellular phosphatases. Because these effects are substantial at nontoxic concentrations, our results show that there is a therapeutic window for topical menadione in the management of EGFR inhibitor–induced skin toxicity. The studies presented here were in part presented at the American Society of Clinical Oncology Annual Meetings in 2006 and 2007 (51, 52).

Materials and Methods

Chemicals and antibodies

Menadione was purchased from Sigma-Aldrich Chemical Co. and dissolved in ethanol (100 mmol/L) as a stock solution. Erlotinib was obtained from OSI pharmaceuticals and dissolved in Dulbecco’s modified Eagle’s medium at a stock concentration of 10 mmol/L. Cetuximab was a gift from Dr. Fan (MD Anderson Cancer Center, Houston, TX). All drugs were diluted to the indicated concentrations with RPMI-1640 medium. Monoclonal anti-EGFR antibody
(H11) was purchased from NeoMarkers, and polyclonal anti-p-EGFR (Tyr1064) antibody, anti-erbB-2, p-erbB2, erbB-3, and p-erbB3, and anti-p27 antibodies were purchased from Cell Signaling Technology. Remaining chemicals were purchased from Sigma-Aldrich Chemical CO.

**Cell lines and cell culture**

We did an extensive literature search to identify cell lines representative of human skin keratinocytes and found that HaCaT cells are the best characterized (53). HaCaT cells were obtained from the Cell Line Service. We also used human epidermoid carcinoma wt EGFR A431 cells to corroborate the results obtained with HaCaT cells. A431 cells were purchased from American Type Culture Collection. All cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and maintained in a humidified incubator at 37°C with 5% CO2.

**Cell viability assay**

Exponentially growing cells were plated in 96-well plates overnight to allow cell attachment and then exposed to varying concentrations of menadione, erlotinib, or erlotinib plus 25 μmol/L menadione at 37°C for 72 hours. After exposure, cell survival was determined by a colorimetric assay based on the reduction of MTT as previously described (54).

**Cell-cycle analysis**

HaCaT cells were treated with 1 μmol/L erlotinib alone or plus 25 to 50 μmol/L menadione at 37°C for 24 hours. After treatment, cells were harvested by trypsinization and fixed with 75% ethanol at −20°C overnight, and then incubated at room temperature for 3 hours with 5 μg/mL propidium iodide and 5 μg/mL RNase I (Roche Molecular Biochemicals). Cell-cycle distribution was measured by fluorescence-activated cell sorting (FACS) analysis (BD Biosciences).

**Measurement of intracellular ROS levels**

HaCaT cells were plated in 6-well plates and treated with the indicated concentrations of menadione at 37°C for 1 hour. Cells were then incubated with 10 μmol/L H2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate; Invitrogen) at 37°C for 30 minutes and then harvested by trypsinization and washed 3 times with PBS. The intracellular level of ROS was analyzed by FACS Calibur analysis (55).

**Measurement of protein tyrosine phosphatase activity**

Protein tyrosine phosphatase (PTP) activity was assayed using a universal tyrosine phosphatase assay kit from Takara Bio Inc. (56). In brief, cells were treated with various concentrations of menadione at 37°C for 1 hour or with 50 μmol/L menadione at 37°C for the indicated time. Following treatment, cells were harvested by trypsinization and lysed. Fifty microfilters of cell lysate (1 μg of protein) were added into 96-well plates coated with PTP substrate and incubated at 37°C for 60 minutes. After incubation, cell lysates were removed from plates, and PTP activity was determined according to manufacturer’s instructions. PTP activity was calculated and expressed relative to that observed in control cells.

**Immunoblot analysis**

Cells were incubated with the indicated concentrations of menadione and then scraped from their culture plates, washed twice with cold PBS solution, and suspended in lysis buffer [50 mmol/L Tris.HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L PMFS, 7 mmol/L iPTT, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 1% Triton X-100, and 1% SDS] at 0°C for 15 minutes. After centrifugation at 15,000 × g for 10 minutes at 4°C, the supernatants were collected and the protein concentration determined with a Bio-Rad protein DC assay kit (Bio-Rad). Cell lysates (30 μg protein) were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBS solution and incubated at 4°C overnight with polyclonal anti-EGFR and anti-p-EGFR (Tyr-1068) antibodies. The membrane was washed 3 times with TBS solution and then incubated at room temperature for 1 hour with horseradish peroxidase–conjugated secondary antibody (1:1,000 with TBS solution). The proteins were visualized by an enhanced chemiluminescence reaction system as directed by the manufacturer (Amersham).

**Immunofluorescence staining**

Cells were plated on glass coverslips in 6-well plates overnight to allow cell attachment and then exposed to the indicated concentrations of menadione, 1 μmol/L erlotinib, the combination of both agents, or to the same volume of medium as control at 37°C for 1 hour. Cells were then washed twice with cold PBS solution, fixed with 4% paraformaldehyde in PBS solution at room temperature for 15 minutes, and treated with 1% Triton X-100 in PBS solution for 10 minutes. After blocking with 5% bovine serum albumin in PBS solution for 30 minutes, cells were incubated with monoclonal anti-EGFR antibody (Ab-5, Neo-markers) and polyclonal anti–p-EGFR (Tyr-1068; Cell Signaling Technology) antibodies (1:400) at room temperature for 1 hour. The cells were then washed 3 times with PBS solution and incubated with Alexa Fluor 488 anti-mouse and Rhodamine Red anti-rabbit secondary antibodies (1:500), and 1 μg/mL 4′, 6-diamidino-2-phenylindole (DAPI) solution for 30 minutes in a dark room. The immunofluorescence signals were visualized with a Zeiss confocal laser scanning microscope.

**In vivo abrogation of EGFR inhibition by erlotinib in mouse skin**

ICR mice (groups of 5) were treated with erlotinib 100 mg/kg by oral administration for 5 consecutive days. Non-treated animals were used as controls. Topical menadione in solution in ethanol (15 mmol/L) was applied twice daily (days 1–5) to a surface of mouse skin of about 2 cm².
Ethanol alone was applied to control animals. Two hours after the last topical menadione treatment, the skin was resected, the protein extracted, and assayed for p-EGFR by Western blot analysis using polyclonal anti–p-EGFR (Try1068) antibody.

Data analysis
Data are presented as mean ± SD of 3 independent experiments. Differences were analyzed by t test and considered to be statistically significant if the P value was less than 0.05.

Results

Menadione induces EGFR phosphorylation in HaCaT and A431 cells
We initially determined the cell growth inhibitory activity of menadione in HaCaT cells by MTT assay using different exposure times to select the concentration range for our experiments. The ID50 of menadione using 1, 2, 4, and 24 hours exposure were 148 ± 28 μmol/L, 75 ± 5 μmol/L, 53 ± 14 μmol/L, and 35 ± 5 μmol/L, respectively. Similar results were obtained in A431 cells. The ID10 of menadione was above 50 μmol/L when a 1 hour exposure was used and around 25 μmol/L when a 24 to 72 hours of exposure was used.

We sought to extend the previous reported data indicating that menadione induces EGFR phosphorylation in rat liver epithelial cells to human skin keratinocyte cells (48). HaCaT cells were exposed to different concentrations of menadione or with the same volume of medium control for 1 hour at 37°C and then EGFR phosphorylation was detected by immunohistochemical staining with monoclonal anti-EGFR antibody and polyclonal anti–p-EGFR antibody as described in Materials and Methods. No signal of phosphorylated EGFR was observed in control cells, however, a dose-dependent increase in phosphorylated EGFR was clearly observed in cells exposed to menadione (Fig. 1A). Dose- and time-dependent increases in EGFR phosphorylation were quantified by Western blot analysis (Fig. 1B and C). Exposure to 25 μmol/L menadione caused phosphorylation of about 30% EGFR, and exposure to 100 μmol/L menadione complete phosphorylation of EGFR. Increased EGFR phosphorylation was observed as early as 10 minutes, reached a plateau at 30 minutes, and persisted thereafter during the 120 minutes observation time. Exposure of A431 human epidermoid carcinoma cells to menadione also resulted in a similar dose- and time-dependent phosphorylation of EGFR (Supplementary Fig. S5).

Because menadione is a synthetic member of the vitamin K family, we investigated whether other vitamin K family members could also cause EGFR phosphorylation. A431 cells were treated with 50 to 1,000 μmol/L vitamin K1, K2, and menadione at 37°C for 1 hour. In contrast to menadione, vitamins K1 was about 10-fold less potent as an inducer of EGFR phosphorylation and vitamin K2 was inactive up to the highest concentration tested (Supplementary Fig. S2).

**ROS generation induced by menadione is associated with EGFR phosphorylation**
ROS have been reported to play a critical role in the regulation of cell survival, proliferation, and apoptosis (57) and to cause phosphorylation of ErbB family proteins (58). We examined whether menadione-induced EGFR phosphorylation was associated with intracellular ROS generation. Treatment of HaCaT cells with 25 to 100 μmol/L menadione for 1 hour at 37°C caused a concentration-dependent increase in intracellular ROS levels, as shown by a shift to the right of the H2DCF-DA fluorescence curves when compared with control cells (Fig. 2A). Menadione at concentrations as low as 25 μmol/L caused a 2- to fold increase in ROS levels compared with control cells. A 4- to 6-fold increase in intracellular ROS levels was observed after exposure to 50 and 100 μmol/L menadione, respectively (Fig. 2B). To further investigate the dependence of EGFR phosphorylation on intracellular ROS generation, as suggested by others (55), we treated cells with 0, 50, and 100 μmol/L menadione in the presence of 10 mmol/L antioxidant N-acetylcysteine (NAC) and 5 mmol/L reduced glutathione (GSH) to determine the effects of antioxidants on menadione-induced EGFR phosphorylation. As shown in Fig. 2C, pretreatment with either NAC or GSH led to the complete abrogation of menadione-induced EGFR phosphorylation, suggesting that it is mediated by ROS generation.

**Effect of menadione on protein tyrosine phosphatase activity**
The phosphorylation and dephosphorylation of EGFR at different tyrosine residues play a crucial role in the regulation of EGFR function and its downstream signaling pathways and is regulated by receptor type protein tyrosine phosphatase-kappa (40, 44). There is evidence that ROS signaling may be involved in the inactivation of phosphatases through oxidation of the cysteine residues at the active enzymatic site (45). Thus, we examined whether menadione-induced EGFR phosphorylation correlated with inhibition of cellular phosphatase activity. HaCaT cells were treated with 10 to 500 μmol/L menadione for 1 hour at 37°C or with 50 μmol/L menadione for 5 to 60 minutes. Treatment with menadione caused a marked concentration- and time-dependent reduction of PTP activity (Fig. 3). PTP activity was reduced by about 30% in cells treated with 25 μmol/L menadione and by 80% in cells treated with 100 μmol/L menadione (Fig. 3A). Inhibition of PTP activity was observed as early as 5 minutes with exposure to 50 μmol/L menadione, reaching approximately 50% inhibition by 1 hour (Fig. 3B). Subsequently, we examined whether menadione-induced inhibition of PTP was reversible. HaCaT cells were treated with 50 μmol/L menadione for 1 hour at 37°C, washed with 1 × PBS, and then incubated in menadione-free medium containing 10% FBS. Cells were assayed for PTP activity and EGFR phosphorylation at different time points. As shown in Fig. 3C, PTP activity was reduced by approximately 50% at 0 hour compared with control cells, and PTP activity significantly recovered after
2 hours incubation in fresh medium (30% vs. 50% inhibition, \( P < 0.05 \)), and recovered completely at 4 to 6 hours. The recovery in PTP activity was associated with a progressive decrease in menadione-induced EGFR phosphorylation as shown by Western blot analysis, that is, EGFR phosphorylation was highest at time 0 and gradually declined thereafter, the level of EGFR phosphorylation reaching the level of control cells after a 6-hour incubation in fresh medium. These data suggest that menadione-induced inhibition of phosphatase activity and EGFR phosphorylation are reversible and inversely related.

**Effect of menadione on erlotinib-induced EGFR dephosphorylation, G1-phase arrest, and cell growth inhibition in HaCaT cells**

Erlotinib, a small molecule inhibitor of the EGFR tyrosine kinase, is indicated as second line/third line and maintenance treatment for advanced NSCLC (1, 2) and as frontline therapy in patients with pancreatic cancer (5). We evaluated whether menadione could prevent erlotinib-induced inhibition of EGFR phosphorylation and cell growth inhibition. HaCaT cells were treated with 1 \( \mu \)mol/L erlotinib alone, 50 \( \mu \)mol/L menadione alone, or the combination of both for 1 hour. Treated cells were then assessed for total and phosphorylated EGFR by immunofluorescence staining using the monoclonal anti-EGFR antibody and polyclonal anti–phos-EGFR antibody as described above. Confocal fluorescence images showed that EGFR was localized on the cell membrane with similar intensity in both samples, suggesting that neither erlotinib nor menadione caused alteration in EGFR expression and subcellular localization (Fig. 4A). However, the signal of phosphorylated EGFR (p-EGFR) with red fluorescence was very faint in cells treated with erlotinib as compared with that in control cells,
Menadione Abrogates EGFR Inhibition by Erlotinib

Menadione induces ROS generation associated with EGFR phosphorylation in HaCaT cells. Cells were treated with various concentrations of menadione at 37°C for 1 hour. After treatment, cells were washed twice with PBS solution and then incubated in medium containing 10 μmol/L H2DCF-DA at 37°C for 30 minutes. After incubation, cells were harvested by trypsinization, and intracellular levels of ROS were measured by FACS analysis as described in Materials and Methods. A, representative fluorescence histograms show that menadione induces ROS generation in a concentration-dependent manner. B, relative ROS levels in cells after treatment with various concentrations of menadione were calculated in relation to control, which was given a value of 1. Each column represents the mean ± SD of 3 independent experiments. **P < 0.01 compared with 0 μmol/L. C, effects of antioxidants NAC and GSH on menadione-induced EGFR phosphorylation. Cells were pretreated with 50 or 100 μmol/L NAC or with 5 μmol/L GSH at 37°C for 1 hour, and cotreated with 50 or 100 μmol/L menadione for the additional 1 hour. Following treatment, cells were harvested and cell lysates were prepared for determination of EGFR phosphorylation by immunoblot analysis.

confirming that erlotinib inhibits EGFR phosphorylation. Cotreatment with 50 μmol/L menadione resulted in an increase in p-EGFR signals in both erlotinib-treated and control cells, thus suggesting that menadione prevents erlotinib-induced inhibition of EGFR phosphorylation. We then sought to confirm by Western blot analysis the protective effect of menadione against erlotinib-induced inhibition of EGFR phosphorylation. Treatment of cells with 100 ng/mL of epidermal growth factor (EGF) caused EGFR activation that was not affected by erlotinib at all concentrations of erlotinib in the presence or absence of 25 μmol/L menadione was significantly reduced to 67% and 52% (P < 0.05 compared with erlotinib alone), respectively, indicating that cotreatment with menadione prevents erlotinib-induced G1-phase arrest. Immunoblot analysis showed that erlotinib induced the expression of p27, and cotreatment with menadione prevented erlotinib-induced p27 protein accumulation (Fig. 4C, bottom panel).

Finally, we investigated whether menadione could prevent erlotinib-induced cell growth inhibition. Cells were plated on 96-well plates and treated with various concentrations of erlotinib in the presence or absence of 25 μmol/L menadione for 72 hours. We used in these experiments a continuous drug exposure time (72 hours) as required to observe cell growth inhibition from erlotinib alone and the highest nontoxic concentration of menadione when cells are exposed for 72 hours (ID 10: 25 μmol/L). Cotreatment with 25 μmol/L menadione resulted in a significant attenuation of erlotinib-induced cell growth inhibition (Fig. 4D; P < 0.05). The IC50 in cells coexposed to erlotinib and menadione was 4.7-fold higher than in cells treated with erlotinib alone (1.04 vs. 0.22 μmol/L). All these data
combined indicate that menadione prevents erlotinib-induced cell-cycle arrest at G_{1} and subsequent inhibition of cell growth.

**Effect of menadione on erlotinib- and cetuximab-induced EGFR dephosphorylation in A431 cells**

Cetuximab is a monoclonal antibody that binds to the extracellular EGF-binding domain of EGFR, thus inhibiting EGFR signaling (6). We investigated whether menadione could also prevent cetuximab-induced EGFR dephosphorylation. Human A431 cells were treated with 2 μmol/L erlotinib alone, 10 μg/mL cetuximab alone, or plus 10 to 500 μmol/L menadione for 1 hour. As a positive control, cells were treated with 100 ng/mL EGF for 10 minutes to induce EGFR phosphorylation. As shown in Fig. 5, 100 ng/mL EGF strongly activated EGFR and EGF-induced EGFR phosphorylation was effectively blocked by addition of either erlotinib or cetuximab. Menadione treatment completely prevented EGFR dephosphorylation by either erlotinib or cetuximab at all concentrations tested.

In another set of experiments, we investigated whether increasing concentrations of EGF could prevent EGFR inhibition by erlotinib and cetuximab. In contrast with menadione, which abrogates EGFR-induced inhibition by both agents, EGF was only able to abrogate EGFR inhibition secondary to cetuximab but not erlotinib and such effect required high concentrations (Supplementary Fig. S3).

**In vivo abrogation of EGFR inhibition in mouse skin**

We tested the ability of topical menadione in solution in ethanol (15 mmol/L) applied twice daily to mouse skin to prevent EGFR inhibition in ICR mice treated with erlotinib for 5 consecutive days (100 mg/kg/d). We used Western blot to determine p-EGFR expression in the skin. Our results confirmed the observations of the *in vitro* experiments. Skin of animals treated with erlotinib showed EGFR inhibition.

Figure 3. Effect of menadione on protein tyrosine phosphatase activity in HaCaT cells. Cells were treated with various concentrations of menadione at 37°C for 1 hour (A), or with 50 μmol/L menadione at 37°C for the indicated time periods (B). After treatment, cells were harvested and cell lysates were prepared for the determination of PTP activity by using a universal tyrosine phosphatase assay kit as described in Materials and Methods. The relative PTP activity was calculated in relation to control or time 0, which were given a value of 1. Data represent mean ± SD of 3 independent experiments. P < 0.05 and **P < 0.01 compared with control or with time 0. (C) Reversibility of menadione-induced PTP inactivation and EGFR phosphorylation. Cells were treated with 50 μmol/L menadione for 1 hour. As a positive control, cells were treated with the same volume of medium as control. After treatment, cells were washed twice with PBS solution, and reincubated in menadione-free fresh medium containing 10% FBS at 37°C for the indicated time periods. At the indicated time points, cells were harvested and divided into 2 aliquots for determination of PTP activity and EGFR phosphorylation. The relative PTP activity in menadione-treated cells after incubation in drug-free medium at different time points was calculated in relation to control (control = 1). Each column represents mean ± SD of 3 independent experiments. **P < 0.05 and ***P < 0.01 compared with incubation time 0 hour in drug-free medium. D, percentage of p-EGFR/EGFR was assessed on the basis of EGFR amount. Data are mean ± SD of 3 independent experiments. **P < 0.05, ***P < 0.01 compared with incubation time 0 in drug-free medium. A representative immunoblot of EGFR phosphorylation at different incubation time points is shown in the top panel.
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Figure 4. Effect of menadione on erlotinib-induced EGFR dephosphorylation, G1-phase arrest, p27 expression, and cell growth inhibition in HaCaT cells. A, cells were treated with 50 μmol/L menadione or with the same volume of medium containing 0.1% ethanol as control for 1 hour at 37°C. Cells were then fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 1% Triton X-100. EGFR and p-EGFR were detected by immunofluorescence analysis using monoclonal anti-EGFR and polyclonal anti-p-EGFR antibodies as described above. Representative confocal fluorescence images show that erlotinib inhibits EGFR activation, and menadione prevents erlotinib-induced EGFR dephosphorylation. B, immunoblot analysis shows that menadione induces EGFR phosphorylation in a concentration-dependent manner that is not affected by erlotinib. C, cells were treated with 0.5 μmol/L erlotinib alone or with 25 to 50 μmol/L menadione, or with the same volume of medium containing 0.1% ethanol as control for 24 hours at 37°C. Cells were then harvested and divided into 2 aliquots for determination of cell-cycle distribution (top) and for p27 expression analysis by immunoblot using an anti-p27 antibody. β-Actin was used as sample loading control (bottom). Each column represents mean ± SD of 3 independent experiments. ∗, P < 0.01 comparing erlotinib with control; ∗∗, P < 0.05 comparing erlotinib with menadione 25 to 50 μmol/L. D, effect of menadione on erlotinib-induced cell growth inhibition. Cells were treated with various concentrations of erlotinib in the absence of or in the presence of 25 μmol/L menadione at 37°C for 72 hours. After treatment, cell viability was determined by MTT assay. Each point represents the mean ± SD of 3 independent experiments. ∗, P < 0.05 compared with erlotinib alone.

Discussion

Our studies clearly show that short-term exposure to nontoxic concentrations of menadione causes EGFR activation and antagonizes the EGFR inhibitory effect of both erlotinib and cetuximab in HaCat human skin normal keratinocytes and A431 squamous carcinoma cells. When used as a continuous exposure for 72 hours, the highest nontoxic concentration of menadione (ID_{10} = 25 μmol/L) protects from the growth inhibitory effects of erlotinib by about 4-fold. These results provide the biochemical evidence of a therapeutic window for the use of topical menadione as a novel therapeutic and preventive strategy for the skin toxicity secondary to EGFR inhibitors. Compared with other members of the vitamin K family, menadione is more potent than vitamin K1 in causing these effects whereas vitamin K2 is inactive.

The logical next step in the preclinical development of topical menadione as a novel strategy to prevent and treat the skin toxicity secondary to EGFR inhibitors would have been to test such strategy in an animal model. Unfortunately, there is no reliable in vivo model of skin toxicity secondary to the treatment with EGFR inhibitors (60). However,
we were able to show that topical menadione can abrogate
the EGFR inhibitory effect of erlotinib when applied topo-
ically to the skin of mice treated with erlotinib.

The course and intensity of the skin toxicity secondary to
EGFR inhibitors are dose dependent but also related to
personal susceptibility (21). The skin toxicity peaks at 6
weeks and progressively improves with time in many cases
(61), thus indicating that the emergence of mechanisms of
biological adaptation is common. Several hypotheses have
been proposed to explain the relationship between the
incidence and intensity of skin toxicity with favorable
outcome. The skin toxicity may be a predictor of efficacy
because EGFR inhibition in the skin, and hence skin tox-
icity, is basically determined by the skin drug levels, which
accurately mirror the drug levels and EGFR inhibition at the
tumor site, and in the end, both determined largely by the
serum drug levels and/or AUC. Alternatively, the skin tox-

icity may be a predictor of efficacy because a similar inflam-
mmatory response may occur at the tumor site and/or the skin
when inflammation may result in the release into the blood
stream of therapeutic plasma levels of cytokines with poten-
tial antitumor activity (12, 13). Finally, skin toxicity may be
determined by individual susceptibility factors such as
EGFR polymorphisms (30) and type of skin (31) but mostly
by the baseline immunocompetence of the host, which may
be a prognostic factor independently of the therapy given.

Because of the demonstrated relationship between the
incidence and severity of skin toxicity secondary to EGFR
inhibitors and survival and because the biological mech-
anism for such association is currently unknown, dose
reductions and adjustments to a tolerable level of toxicity
have become a sort of “reasonable compromise” in stan-
dard practice (10, 21). Dose reduction strategies may have a
negative impact on efficacy by decreasing the tumor drug
levels, particularly in tumors that are driven by overexpres-
sion of wild-type EGFR, which include all current approved
indications, except the 10% to 15% of NSCLC that carry
EGFR mutations (62). Topical strategies are devoid of such
risk provided that the systemic absorption of the agents is
negligible. However, topical strategies can cause topical
toxicity as patients treated with EGFR inhibitors are very
sensitive to topical medications (9, 63). Therefore, the
current clinical developmental strategy of topical menadi-
one is focused on determining the lowest topical dose that
keeps receptor activation just above the reduced activation
threshold that triggers a severe inflammatory response and
confirming that systemic absorption is negligible and there-
fore there is no risk of decreased antitumor efficacy (46, 64).

The results presented here led to the development of a
topical formulation of menadione lotion by Talon Thera-
puetic, which is currently being evaluated in clinical trials
(www.clinicaltrials.gov). Studies in nude mice bearing

Figure 6. Menadione abrogates erlotinib-induced inhibition of p-EGFR
expression in mouse skin tissue. Mice were treated with oral 100 mg/kg
erlotinib daily × 5 days and areas of mouse skin were exposed to an
ethanol solution of 15 mmol/L menadione every 12 hours or ethanol alone
(solvent). Two hours after the last topical application of menadione, mice
were sacrificed and skin tissue harvested. The expression of p-EGFR was
detected by immunoblot using polyclonal anti-p-EGFR (Try1068)
antibody. Data presented are representative of 2 experiments showing
similar results.
A431 xenografts showed that topical menadione lotion did not affect the antitumor effect of erlotinib and studies completed in healthy volunteers with different drug concentrations have shown negligible systemic absorption and an acceptable toxicity profile (manuscript in preparation). A split face pilot study to show proof-of-principle is in progress (NCT00656786). In this study, patients treated with an EGFR inhibitor (such as erlotinib, cetuximab, or panitumumab) are randomized to different concentrations of menadione lotion applied twice daily to half their face and a vehicle lotion to the other half on a double blind randomized design. A randomized phase II study against standard of care in patients with cetuximab-induced skin rash is being planned (NCT01094444). In parallel with this effort, other investigators have engaged in testing topical vitamin K1 for the same indication and preliminary encouraging results have been reported (65).

The protective effect of menadione on human keratinocytes exposed to anti-EGFR agents seems to be mediated by oxidative stress (ROS generation), which in turn leads to phosphatase inhibition and shifts the state of the intracellular receptor to the activated phosphorylated state (45). These effects are nonspecific for the phosphatase involved in EGFR dephosphorylation. Therefore, topical menadione may be effective also in antagonizing the effects of other kinase inhibitors that also cause skin toxicity and are approved for different indications such as sorafenib (66) and MEK inhibitors (67). Preliminary studies in our laboratory suggest that the effects of menadione described above apply also to serine–threonine phosphatases (52, 68, 69). On the other hand, the lack of specificity of menadione may result in excessive toxicity and a narrow therapeutic index. In such a case, the clinical success of this strategy may require the identification and selection of inhibitors with preferential selectivity for the phosphatase that dephosphorylates EGFR (44).

In our studies, menadione seemed to be equally effective in antagonizing the EGFR inhibitory effects of both the EGFR tyrosine kinase inhibitor erlotinib and cetuximab, a monoclonal antibody that competes with EGF for the extracellular ligand binding domain of EGFR (6). In contrast, exogenous EGF, which is a competitor of cetuximab for EGFR was only effective in antagonizing the EGFR inhibitory effects of cetuximab, and still requiring very high concentrations (Supplementary Fig. S1). The concentrations may be difficult to be achieved in the basal skin keratinocytes as EGF is a large peptide. These observations suggest that topical menadione should be superior to topical EGF in preventing the skin toxicity of EGFR inhibitors. Finally, erlotinib is a reversible EGFR inhibitor. Irreversible EGFR tyrosine kinase inhibitors, other anti-EGFR antibodies, and combinations of both, with higher incidence and severity of skin toxicity, are now in clinical development (70–72). The potential use of menadione and other more specific phosphatase inhibitors in preventing the skin toxicity of some of these new irreversible and/or more toxic EGFR inhibitors is being evaluated.

References

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Retraction: The Phosphatase Inhibitor Menadione (Vitamin K3) Protects Cells from EGFR Inhibition by Erlotinib and Cetuximab

The authors wish to retract the article titled “The Phosphatase Inhibitor Menadione (Vitamin K3) Protects Cells from EGFR Inhibition by Erlotinib and Cetuximab,” which was published in the November 1, 2011, issue of Clinical Cancer Research (1).

After an unidentified concerned reader made allegations of image mishandling in several figures, the authors requested that their medical school conduct an investigation as per its established protocol. The investigators concluded that there was enhancement of bands in some of the questioned figures and that the changes were subtle and seen only on special analysis. One of the coauthors, who since has returned to his home country, accepted full responsibility for the changes. He never mentioned the enhancements to any of the authors before manuscript submission. In no case would the conclusions from the experiments have been different if no alterations had been made. Independent repetition of key experiments by another senior investigator showed reproducibility of the major findings. In view of the reproducibility of the original data by a third party, the authors continue to stand by the conclusions of the article. However, because the submission did not meet the standards for manuscript submission to Clinical Cancer Research as described in the Instructions to Authors, the authors voluntarily retract this article. The authors apologize to the readers for the inconvenience that this oversight may have caused.

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Reference

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