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

Roman Perez-Soler1, Yiyu Zou2, Tianhong Li3, and Yi He Ling2

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.

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 that occurs in about two-thirds of the patients, the most common manifestation being an inflammatory follicular rash in the face and, less frequently, in the torso and extremities (9, 10). The pathophysiology of this new dermatologic entity has not been fully elucidated, but the leading hypothesis is that the keratinocytes of the basal layer of the epidermis react to EGFR inhibition by secreting cytokines that trigger an inflammatory response that eventually causes loss of skin barrier protection and secondary skin infections involving mainly the hair follicles (11–20).

The cutaneous toxicity is almost never lethal but is clinically relevant because it causes discomfort (21, 22) that may lead to dose interruption, dose reduction, drug discontinuation, or poor compliance in a significant number of patients (5, 23). Interestingly, the incidence and severity of the skin toxicity have been consistently found to be associated with increased tumor response rate and longer survival when analyzed in the context of well-controlled trials (24–29). The reasons for such association are unclear and several hypotheses, attributing either a predictive or prognostic role to the skin toxicity, have been proposed but none of them has been proved (12, 13, 30, 31).

The treatment of the skin toxicity secondary to EGFR inhibitors has until recently been mostly empirical,
OF2

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 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, a synthetic prodrug 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 in 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 Co. and dissolved in ethanol (100 mmol/L) as a stock solution. Erlotinib was obtained from OSI pharmaceuticals Co. and dissolved in ethanol (100 mmol/L) as a stock solution. 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
Menadione Abrogates EGFR Inhibition by Erlotinib

(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 10 μg/mL propidium iodide and 5 μg/mL RNase A (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′-dichlorofluorescein 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 microliters 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, 1 mmol/L DTT, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 0.1% Triton X-100, and 1% SDS] at 0 to 4°C for 15 minutes. After centrifugation at 15,000 × g for 10 minutes at 0°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 TBST solution and incubated at 4°C overnight with monoclonal EGFR and anti–p-EGFR (Tyr-1068) antibodies. The membrane was washed 3 times with TBST solution and then incubated at room temperature for 1 hour with horseradish peroxidase–conjugated secondary antibody diluted 1:4,000 with TBST solution. The proteins were visualized by an enhancement 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 ID_{50} 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 ID_{50} 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 previously 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 as 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 to 500 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. S1).

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 H$_2$DCF-DA fluorescence curves when compared with control cells (Fig. 2A). Menadione at concentrations as low as 25 μmol/L caused a 2-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-acetyl-cystein (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 to 500 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 μmol/L erlotinib alone, 50 μmol/L menadione alone, or the combination of both for 1 hour. Treated cells were assessed for total and phosphorylated EGFR by immunofluorescence staining using the monoclonal anti-EGFR antibody and polyclonal anti–p-EGFR antibody as described above. Confocal microscopy 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, 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 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 10 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 phosphorylation. Treatment of cells with 100 ng/mL H2DCF-DA 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.

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
either erlotinib or cetuximab. Menadione treatment of cell-cycle arrest at G1 and subsequent inhibition required high concentrations (Supplementary Fig. S3). Secondary to cetuximab but not erlotinib and such effect could also prevent cetuximab-induced EGFR dephosphorylation in A431 cells. 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 combined indicate that menadione prevents erlotinib-induced cell-cycle arrest at G1 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

- **Figure 3.** Effect of menadione on protein tyrosine phosphatase activity in Hela T 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 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 0 μmol/L concentration or with exposure time 0. C, reversibility of menadione-induced PTP inactivation and EGFR phosphorylation. Cells were treated with 50 μmol/L menadione at 37°C for 1 hour, or 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; ∗∗, P < 0.01 compared with incubation time 0 hour in drug-free medium. D, percentage of p-EGFR/EGFR 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.
when compared with animals not treated with erlotinib. Skin treated with topical menadione showed EGFR activation of the same magnitude in both animals treated and not treated with erlotinib. Therefore, topical menadione was able to abrogate the EGFR-inhibitory activity of erlotinib. The experiment was repeated 2 times and gave similar results. Results presented in Fig. 6 are representative of 1 experiment.

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$_{50} =$ 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,
Menadione Abrogates EGFR Inhibition by Erlotinib

Figure 5. Effect of menadione on erlotinib- and cetuximab-induced inhibition of EGFR phosphorylation in A431 cells. A and B, cells were treated with various concentrations of menadione in the absence or in the presence of 2 μmol/L erlotinib or 10 μg/mL cetuximab at 37°C for 1 hour. Cells were stimulated with 100 ng/mL EGF at 37°C for 10 minutes as a positive control. Following treatment, cells were harvested and cell lysates were prepared for determination of levels of EGFR and p-EGFR by immunoblot analysis using the corresponding antibodies. The immunoblot shown corresponds to 1 of 3 reproducible experiments.

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.

were able to show that topical menadione can abrogate the EGFR inhibitory effect of erlotinib when applied topically 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 5 weeks and progressively improves with time in most 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 toxicity, 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 toxicity may be a predictor of efficacy because a similar inflammatory response may occur at the tumor site and/or the skin inflammation may result in the release into the blood stream of therapeutic plasma levels of cytokines with potential 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 mechanism for such association is currently unknown, dose reductions and adjustments to a tolerable level of toxicity have become a sort of “reasonable compromise” in standard 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 overexpression 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 menadione 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 therefore 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 Therapeutics, which is currently being evaluated in clinical trials (www.clinicaltrials.gov). Studies in nude mice bearing
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 (NCT01094444). 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 here apply also to serine–threonine phosphatases (52, 68, 69).

On the other hand, the lack of specificity of menadione may result in excess 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. S3). These 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.

Disclosure of Potential Conflicts of Interest

Y.H. Ling and R. Perez-Soler, with the Albert Einstein College of Medicine, are inventors and are entitled to share patent royalties with Talon Therapeutics, Inc. The other authors disclosed no potential conflicts of interest.

Grant Support

The work was supported by NIH CA 113360 and a grant from Talon Therapeutics, Inc. to R. Perez-Soler.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 25, 2011; revised August 2, 2011; accepted September 2, 2011; published OnlineFirst September 13, 2011.

References

Menadione Abrogates EGFR Inhibition by Erlotinib


Clinical Cancer Research

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

Roman Perez-Soler, Yiyu Zou, Tianhong Li, et al.

Clin Cancer Res  Published OnlineFirst September 13, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-0545

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/09/13/1078-0432.CCR-11-0545.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.