The phosphatase inhibitor menadione (Vitamin K3) protects cells from EGFR inhibition by erlotinib and cetuximab

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Translational Statement:
This manuscript 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 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 demonstrates 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 currently in clinical development to prevent and treat the skin toxicity secondary to EGFR inhibitors. The clinical formulation has shown to be non-toxic in normal volunteers and result in minimal systemic absorption.
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ABSTRACT

Purpose: Skin toxicity is the main side effect of 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 skin keratinocyte cells and A431 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 non-toxic concentrations. EGFR activation by menadione was associated with reversible protein tyrosine phosphatase inhibition, which appeared to be mediated by ROS generation as exposure to antioxidants prevented both menadione-induced ROS generation and phosphatase inhibition. Short-term co-incubation of cells with non-toxic concentrations of menadione and the EGFR inhibitors erlotinib or cetuximab prevented EGFR dephosphorylation. Seventy two-hour co-incubation of cells with the highest non-toxic concentration of menadione and erlotinib provided a 4-fold cell growth inhibitory protection in HaCat human keratinocyte cells.

Conclusions: Menadione at non-toxic concentrations causes EGFR activation and prevents EGFR dephosphorylation by erlotinib and cetuximab. This effect appears to be mediated by ROS generation and secondary phosphatase inhibition. Mild oxidative
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stress in skin keratinocytes by topical menadione may protect the skin from the toxicity secondary to EGFR inhibitors without causing cytotoxicity.
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INTRODUCTION

EGFR inhibitors have become standard of therapy for several very common human malignancies, i.e. non-small cell lung cancer (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 dermatological 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 reductions, drug discontinuations, 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, consisting of the use of topical skin moisturizers, topical sunscreens, and topical and systemic anti-inflammatory agents and antibiotics (32-34).
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The use of topical steroids and immunosuppressant agents has not been clearly demonstrated 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 demonstrated 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 pre-emptive treatment compared to reactive treatment (38). Still, the incidence of grade 2 rash remained significant in the patients treated with these improved therapeutic strategies. Therefore, newer and more effective strategies addressing the mechanisms of the 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, non-ligand binding related activation of these receptors. Inhibition of protein tyrosine phosphatases induces intrinsic receptor phosphorylation/activation by shifting the balance towards 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
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should at least partially antagonize the effects of EGFR inhibitors and exert a protective effect by shifting the baseline intracellular EGFR status from de-phosphorylated 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 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 at the catalytic site of these enzymes and/or through generation of reactive oxygen species (ROS). (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 demonstrate that menadione causes in a concentration dependent manner EGFR activation, that it antagonizes the EGFR inhibitory effect and cell growth inhibitory effect of both erlotinib
Menadione abrogates EGFR inhibition by erlotinib and cetuximab in HaCat human skin keratinocytes and A431 human squamous cell carcinoma cells, and that such effect appears to be mediated by the generation of ROS and secondary inhibition of cellular phosphatases. Since these effects are substantial at non-toxic concentrations, our results demonstrate 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).
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MATERIALS AND METHODS

Chemicals and Antibodies. Menadione was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and dissolved in ethanol (100mM) as a stock solution. Erlotinib was obtained from OSI pharmaceuticals (Melville, NY), and dissolved in DMSO at a stock concentration of 10 mM. Cetuximab was a gift from Dr. Fan (M. D. 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 (Fremont, CA), 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 (Danvers, MA). 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 (Eppelheim, Germany). 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 (ATCC, Manassas, VA). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin, and maintained in a humidified incubator at 37°C with 5% CO2.
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**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 μM menadione at 37°C for 72 h. After exposure, cells survival was determined by a colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (54).

**Cell Cycle Analysis.** HaCaT cells were treated with 1 μM erlotinib alone, or plus 25-50 μM menadione at 37°C for 24 h. After treatment, cells were harvested by trypsinization, and fixed with 75% ethanol at -20°C overnight, and then incubated at room temperature for 3 h with 5 μg/ml propidium iodide and 5 μg/ml RNase I (Roache Molecular Biochemicals, Indianapolis, IN). Cell cycle distribution was measured by FACS analysis (BD Biosciences, San Joes, CA).

**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 h. Cells were then incubated with 10 μM H₂DCF-DA (2',7',-dichlorofluorescein diacetate (H₂DCF-DA) (Invitrogen, Carlsbad, CA) at 37°C for 30 min. and then harvested by trypsinization and washed three times with PBS. The intracellular level of ROS was analyzed by FACS Calibur analysis (55).

**Measurement of Protein Tyrosine Phosphatase (PTP) Activity.** Protein tyrosine phosphatase (PTP) activity was assayed using a universal tyrosine phosphatase assay
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kit from Takara Bio Inc. (Madison, WI) (56). In brief, cells were treated with various concentrations of menadione at 37°C for 1 h or with 50 μM 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 min. 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 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na3VO4, 1 mM PMFS, 1 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1% Triton X-100, and 1% SDS) at 0-4°C for 15 min. After centrifugation at 15,000 x g for 10 min at 0°C, the supernatants were collected and the protein concentration determined with a Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA). 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 polyclonal anti-EGFR and anti-p-EGFR (Tyr-1068) antibodies. The membrane was washed three times with TBST solution and then incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody diluted 1:1000 with TBST solution. The proteins were visualized by an enhancement
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chemiluminescence reaction system as directed by the manufacturer (Amersham, Arlington Heights, IL).

**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 μM erlotinib, the combination of both agents or to the same volume of medium as control at 37°C for 1 h. Cells were then washed twice with cold PBS solution, fixed with 4% paraformaldehyde in PBS solution at room temperature for 15 min, and treated with 1% Triton X-100 in PBS solution for 10 min. After blocking with 5% bovine serum albumin in PBS solution for 30 min, cells were incubated with monoclonal anti-EGFR antibody (Ab-5, Neomarkers) and polyclonal anti-p-EGFR (Tyr-1068, Cell Signaling Technology) antibodies (1:400) at room temperature for 1 h. The cells were then washed three 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 DAPI solution for 30 min 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 mM) 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.
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Data Analysis. Data are presented as mean ± S.D. of three independent experiments. Differences were analyzed by t test and considered to be statistically significant if the p value was <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 hour, 2 hours, 4 hours and 24 hours exposure were 148 +/- 28 μM, 75 +/- 5 μM, 53 +/- 14 μM, and 35 +/- 5 μM, respectively. Similar results were obtained in A431 cells. The ID$_{10}$ of menadione was above 50 μM when a 1 hr exposure exposure was used and around 25 μM when a 24-72 hour 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 hr 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
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by western blot analysis (Fig 1B and C). Exposure to 25 μM menadione caused phosphorylation of about 30% EGFR, and exposure to 100-500 μM menadione complete phosphorylation of EGFR. Increased EGFR phosphorylation was observed as early as 10 min, reached a plateau at 30 min, and persisted thereafter during the 120 min observation time. Exposure of A431 human epidermoid carcinoma cells to menadione also resulted in a similar dose and time dependent phosphorylation of EGFR (supplemental Fig. 1).

Since 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 1000 μM Vitamin K1, K2, and menadione at 37°C for 1 h. 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 (Supplemental Fig. 2).

ROS generation induced by menadione is associated with EGFR phosphorylation.

Reactive oxygen species (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 25-100 μM menadione for 1hr 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 to control cells (Fig 2A). Menadione at concentrations as low as 25 μM caused a 2-fold increase in ROS levels compared with
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control cells. A 4- to 6-fold increase in intracellular ROS levels was observed after exposure to 50 μM and 100 μM 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 μM menadione in the presence of 10 mM antioxidant N-acetylcysteine (NAC) and 5 mM reduced glutathione (GSH) to determine the effects of antioxidants on menadione-induced EGFR phosphorylation. As shown in Fig. 2C, co-treatment 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 (PTP) 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 μM menadione for 1 hr at 37°C or with 50 μM menadione for 5 to 60 min. 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 μM menadione, and by 80% in cells treated with 100-500 μM menadione (Fig 3A). Inhibition of PTP activity was observed as early as 5 min with exposure to 50 μM menadione,
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reaching approximately 50% inhibition by 1 h (Fig. 3B). Subsequently, we examined whether menadione-induced inhibition of PTP was reversible. HaCaT cells were treated with 50 μM menadione for 1 hr at 37°C, washed with 1xPBS and then incubated in menadione-free medium containing 10% fetal bovine serum. 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 h compared with control cells, and PTP activity significantly recovered after 2 h incubation in fresh medium (30% vs 50% inhibition, p<0.05), and recovered completely at 4-6 h. The recovery in PTP activity was associated with a progressive decrease in menadione-induced EGFR phosphorylation as shown by western blot analysis, i.e. 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 h 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 μM erlotinib alone, 50 μM menadione alone, or the combination of both for 1 h. Treated cells were then assessed for total and phosphorylated EGFR by immunofluorescence staining.
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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 confirming that erlotinib inhibits EGFR phosphorylation. Co-treatment with 50 μM 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 EGF caused EGFR activation that was inhibited by erlotinib (Fig 4B). In contrast, menadione caused concentration-dependent EGFR activation that was not affected by erlotinib at all menadione concentrations tested.

Previous studies have shown that inhibition of EGFR signaling and tumor cell growth by EGFR inhibitors is linked to blockade of cell-cycle progression and associated with the induction of p27 protein (59). We determined the effects of menadione on erlotinib-induced G1-phase arrest and p27 protein expression. HaCaT cells were treated with 1 μM erlotinib alone or with 25-50 μM menadione or with the same volume of medium as control for 24 h at 37°C. After treatment, cells were harvested and assessed for cell–cycle analysis and p27 expression. As shown in Fig. 4C (upper panel), the flow cytometric analysis showed that erlotinib treatment caused significant cell-cycle arrest at G1-phase compared with control cells (76% cells at G1-phase in erlotinib treated cells vs
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50% in control cells, $p<0.01$). The percentage of cells at G1-phase in cells co-treated with 1 μM erlotinib plus 25 or 50 μM menadione was significantly reduced to 67% and 52%, respectively ($p<0.05$ compared with erlotinib alone), respectively, indicating that co-treatment with menadione prevents erlotinib-induced G1-phase arrest. Immunoblot analysis showed that erlotinib induced the expression of p27, and co-treatment with menadione prevented erlotinib-induced p27 protein accumulation (Fig 4C, lower 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 μM menadione for 72 h. 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 non-toxic concentration of menadione when cells are exposed for 72 hours (ID 10: 25 μM). Co-treatment with 25 μM menadione resulted in a significant attenuation of erlotinib-induced cell growth inhibition (Fig 4D) ($p<0.05$). The IC50 in cells co-exposed to erlotinib and menadione was 4.7-fold higher than in cells treated with erlotinib alone (1.04 vs 0.22 μM). 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 μM erlotinib alone, 10 μg/ml cetuximab alone, or plus 10
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– 500 μM menadione for 1 hr. As a positive control, cells were treated with 100 ng/ml EGF for 10 min 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 (supplemental Figure 3).

In vivo abrogation of EGFR inhibition in mouse skin

We tested the ability of topical menadione in solution in ethanol (15 mM) applied twice daily to mouse skin to prevent EGFR inhibition in ICR mice treated with erlotinib for 5 consecutive days (100 mg/kg/day). 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 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 one experiment.
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DISCUSSION

Our studies clearly demonstrate that short term exposure to non-toxic 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 non-toxic concentration of menadione (ID10=25 μM) 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 to 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 demonstrate 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.
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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. 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 bloodstream 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 10-15% of non-small cell lung cancers 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...
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Clinical developmental strategy of topical menadione is focused on determining the minimal topical dose that reduces receptor activation just below the 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 (San Francisco, CA), which is currently being evaluated in clinical trials (www.clinicaltrials.gov). Studies in nude mice bearing A431 xenografts demonstrated that topical menadione lotion did not affect the antitumor effect of erlotinib and studies completed in healthy volunteers with different drug concentrations have demonstrated negligible systemic absorption and an acceptable toxicity profile (manuscript in preparation). A split face pilot study to demonstrate 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 (64).

The protective effect of menadione on human keratinocytes exposed to anti-EGFR agents appears 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
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activated phosphorylated state (45). These effects are non-specific 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 excessive toxicity and a narrow therapeutic index. In such 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 appeared 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 (supplemental Figure 3). 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 (70-71). The potential use of menadione and other
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more specific phosphatase inhibitors in preventing the skin toxicity of some of
these new irreversible and/or more toxic EGFR inhibitors is being evaluated.
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Menadione abrogates EGFR inhibition by erlotinib


Menadione abrogates EGFR inhibition by erlotinib

FIGURE LEGENDS

FIGURE 1. Menadione induces EGFR phosphorylation in HaCaT cells. (A), Cells were plated in the glass coverslips overnight to allow cell attachment, and then treated with various concentrations of menadione or with the same volume of medium containing 0.1% ethanol as a control at 37°C for 1 h. After treatment, cells were fixed with 4% paraformaldehyde for 15 min, and permeabilized with 1% tritonx100. EGFR and p-EGFR were probed by immunofluorescent analysis using monoclonal anti-EGFR antibody and polyclonal anti-p-EGFR antibody as described in Materials and Methods. Representative confocal microscopy images are presented; nuclei were stained with DAPI solution. (B), Cells were treated with various concentrations of menadione at 37°C for 1. After treatment, cells were harvested and cell lysates were prepared for immunoblot analysis. (C), Time course study of menadione-induced EGFR phosphorylation. Cells were treated with 50 μM menadione for the indicated time periods. At the time points indicated, cells were harvested and cell lysates were prepared for immunoblot analysis. The lower panels in (B) and (C) show the quantification of percentage of p-EGFR based on the EGFR amount. Data represent mean ± S.D. of three independent experiments. **, p<0.01 compared with 0 μM concentration or with exposure time 0.

FIGURE 2. Menadione induces ROS generation associated with EGFR phosphorylation in HaCaT Cells. Cells were treated with various concentrations of menadione at 37°C for 1 h. After treatment, cells were washed twice with PBS solution, and then incubated in medium contained 10 μM H2DCF-DA at 37°C for 30 min. After
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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 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 one. Each column represents the mean ± S.D. of three independent experiments. **, p<0.01 compared with 0 μM. (C), Effects of antioxidants NAC and GSH on menadione-induced EGFR phosphorylation. Cells were pretreated with 10 mM NAC or with 5 mM GSH at 37°C for 1 h, and co-treated with 50 μM or 100 μM menadione for the additional 1 h. Following treatment, cells were harvested and cell lysates were prepared for determination of EGFR phosphorylation by Immunoblot analysis.

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 h (A), or with 50 μM 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 hr which were given a value of one. Data represent mean ± S.D. of three independent experiments. *, p<0.05, and **, p<0.01 compared with 0 μM concentration or with exposure time 0 hr. (C), Reversibility of menadione-induced PTP inactivation and EGFR phosphorylation. Cells were treated with 50 μM menadione at 37°C for 1 h, or cells were treated with the same volume of medium as control. After treatment, cells were washed twice with PBS
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solution, and re-incubated in menadione-free fresh medium containing 10% fetal bovine serum at 37°C for the indicated time periods. At the indicated time points, cells were harvested and divided into two 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 ± S.D. of three independent experiments. *, p<0.05, **, p<0.01 compared with incubation time 0 hr in drug-free medium. (D), Percentage of p-EGFR/EGFR was assessed based on EGFR amount. Data are mean ± S.D. of three independent experiments. *, p<0.05, **, p<0.01 compared with incubation time 0 hr in drug-free medium. A representative immunoblot of EGFR phosphorylation at different incubation time points is shown in the upper panel.

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 μM menadione or with the same volume of medium containing 0.1% ethanol as control for 1 h at 37°C. Cells were then fixed with 4% paraformaldehyde for 15 min, and permeabilized with 1% tritonx100. 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 and mbut does not abrogate erlotinib-induced dephosphorylation. (C), Cells were treated with 0.5 μM erlotinib alone
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or with 25-50 μM menadione, or with the same volume of medium containing 0.1% ethanol as control for 24 h at 37°C. Cells were then harvested and divided into two aliquots for determination of cell-cycle distribution (upper panel), or for p27 expression analysis by immunoblot using an anti-p27 antibody; β-actin was used as sample loading control (lower panel). Each column represents mean ± S.D. of three independent experiments. **, p<0.01 comparing erlotinib with control, * p<0.05 comparing erlotinib with menadione 25-50 μM. (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 μM menadione at 37°C for 72 h. After treatment, cell viability was determined by MTT assay. Each point represents the mean ± S.D. of three independent experiments. *, p<0.05 compared with erlotinib alone.

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 μM erlotinib or 10 μg/ml cetuximab at 37°C for 1 h. Cells were stimulated with 100 ng/ml EGF at 37°C for 10 min 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 one of three 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
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daily x 5 days and areas of mouse skin were exposed to an ethanol solution of 15 mM 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 two experiments showing similar results.
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**Fig. 1**

(A) Control

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(B) Menadione (µM)

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(C) Exposure Time (min)

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Fig. 2

(A) Flow cytometry analysis of ROS levels in cells treated with different concentrations of menadione. Control and Menadione treatments at 25 μM, 50 μM, and 100 μM are shown.

(B) Bar graph showing relative ROS levels (fold induction) compared to control at 0, 25, 50, and 100 μM menadione concentrations. Significant differences are indicated by asterisks.

(C) Western blot analysis of EGFR and p-EGFR expression in cells treated with menadione at 0, 50, and 100 μM. Menadione concentrations are indicated at the bottom of the gel.
Fig. 3

(A) Relative PTP Activity

(B) Relative PTP Activity

(C) Incubation Time (h)

(D) Incubation Time (h)

Menadione Activity

PTP Activity

Incubation Time (h)
in Menadione-free medium

Relative PTP Activity

Relative PTP Activity

p-EGFR/EGFR (%)

p-EGFR/EGFR (%)

Incubation Time (h)
in Menadione-free medium

Con 0 1 2 4 6
Fig. 4
Fig. 5

(A) Erlotinib (2 μM) - + - + - + - + - + - +

EGF (100 ng/ml) - - + + - - - - - - - -

Menadione (μM) 10 10 50 50 100 100 500 500

(C) Cetuximab (10 μg/ml) - + - + - + - + - + - +

EGF (100 ng/ml) - - + + ---- ----

Menadione (μM) 10 10 50 50 100 100 500 500

180 kDa
Fig. 6

P-EGFR 180 kDa

Topical Ethanol + + - +
Topical Menadione - + - +
No Erlotinib Erlotinib Treatment Treatment

180 kDa
The phosphatase inhibitor menadione (Vitamin K3) protects cells from EGFR inhibition by erlotinib and cetuximab

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