Administration of Menadione, Vitamin K3, Ameliorates Off-Target Effects on Corneal Epithelial Wound Healing Due to Receptor Tyrosine Kinase Inhibition

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PURPOSE. The antiangiogenic receptor tyrosine kinase inhibitor (RTKi), 3-[(4-bromo-2,6-difluorophenyl)methoxy]-5-[[[[4-(1-pyrrolidinyl) butyl] amino] carbonyl]amino]-4-isothiazole-carboxamide hydrochloride, targets VEGFR2 (half maximal inhibitory concentration [IC_{50}] = 11 nM); however, off-target inhibition of epidermal growth factor receptor (EGFR) occurs at higher concentrations. ($IC_{50} = 5.8$ μM). This study was designed to determine the effect of topical RTKi treatment on EGF-mediated corneal epithelial wound healing and to develop new strategies to minimize off-target EGFR inhibition.

METHODS. In vitro corneal epithelial wound healing was measured in response to EGF using a transformed human cell line (hTCEpi cells). In vivo corneal wound healing was assessed using a murine model. In these complementary assays, wound healing was measured in the presence of varying RTKi concentrations. Immunoblot analysis was used to examine EGFR and VEGFR2 phosphorylation and the kinetics of EGFR degradation. An Alamar Blue assay measured VEGFR2-mediated cell biology.

RESULTS. Receptor tyrosine kinase inhibitor exposure caused dose-dependent inhibition of EGFR-mediated corneal epithelial wound healing in vitro and in vivo. Nanomolar concentrations of menadione, a vitamin K3 analog, when coadministered with the RTKi, slowed EGFR degradation and ameliorated the inhibitory effects on epithelial wound healing both in vitro and in vivo. Menadione did not alter the RTKi's IC_{50} against VEGFR2 phosphorylation or its inhibition of VEGF-induced retinal endothelial cell proliferation.

CONCLUSIONS. An antiangiogenic RTKi exhibited off-target effects on the corneal epithelium that can be minimized by menadione without deleteriously affecting its on-target VEGFR2 blockade. These data indicate that menadione has potential as a topical supplement for individuals suffering from perturbations in corneal epithelial homeostasis, especially as an untoward side effect of kinase inhibitors.

Keywords: corneal epithelium, EGFR, kinase inhibitor, VEGFR, vitamin K3

Receptor tyrosine kinases play fundamental roles in developmental biology, tissue homeostasis, and cancer biology. For most receptor tyrosine kinases, ligand binding induces dimerization with another receptor, activation of the kinase domain, and phosphorylation of tyrosine residues on the intracellular domain of the receptor partner. These phospho-tyrosines serve as docking sites for downstream signaling proteins (effectors) that on activation modulate intracellular biochemical pathways to alter cell biology.

A number of small molecule inhibitors of the receptor tyrosine kinase have been developed to antagonize receptor activity and modulate receptor-specific biology. Receptor tyrosine kinase inhibitors (RTKi) have become promising treatments for pathologic processes, such as tumor-associated angiogenesis (sorafenib [Nexavar])[1] and cancer cell progression (gefitinib [Iressa]).[2] Despite the best efforts in designing receptor-specific molecules, some cross-reactivity is inevitable with other "off-target" receptors, albeit usually with a lower potency. This cross-reactivity can result in undesirable side effects by inhibiting the biological function(s) associated with one or more of these off-target receptors.

The antiangiogenic receptor tyrosine kinase inhibitor (RTKi), 3-[(4-bromo-2,6-difluorophenyl)methoxy]-5-[[[[4-(1-pyrrolidinyl) butyl] amino] carbonyl]amino]-4-isothiazolecarboxamide hydrochloride, was originally designed to inhibit the VEGF receptor 2 (VEGFR2) (half maximal inhibitory concentration [IC_{50}] = 11 nM) with the goal of inhibiting VEGFR2-mediated angiogenesis in various cancers.[3] In addition to VEGFR2, this RTKi also inhibits other proangiogenic receptor tyrosine kinases, such as FGF receptors 1 to 3 (FGFR1–3), Tie-2, and ephrin receptor B4 (EphB4).[3] This compound attenuated the blood supply of lung cancers and reduce tumor size in animal studies,[3] but it did not demonstrate sufficient efficacy for treating advanced non–small-cell lung cancer.[4]

Currently, this RTKi is being explored as a topical ocular treatment for neovascular eye diseases, such as neovascular or

wet AMD. The neovascular form of AMD, although less common than the atrophic or dry type, affects approximately 2 million Americans and is responsible for 90% of the blindness caused by the disease.[5] Current therapies approved by the Food and Drug Administration for neovascular AMD are humanized biologic molecules that bind VEGF-A and include an aptamer (pegaptanib/Macugen; Eyetech, New York City, NY, USA, and Pfizer, New York City, NY, USA), an antibody fragment (ranibizumab/Lucentis; Genentech, South San Francisco, CA, USA, and Roche, Basel, Switzerland), and fusion protein consisting of a human IgG Fc portion and Fab portions consisting of VEGFR1- and VEGFR2-binding domains (aflibercept/Eylea; Regeneron, Tarrytown, NY, USA). All of these approved therapies are administered by intravitreal injection ad must be administered at relatively frequent intervals (e.g., 1–3 months) to maintain benefit for most patients. Topical administration of a safe and efficacious anti-VEGFR RTKi is predicted to minimize the need for intraocular treatment, increase long-term compliance, and reduce patient and physician treatment burdens associated with frequent intravitreal injections.

Along with its potent inhibition of proangiogenic kinases, this RTKi also blocks epidermal growth factor receptor (EGFR) activity, albeit at an approximately 500-fold higher concentration as compared to its VEGFR2 IC_{50}.[3] The EGFR is critical for the homeostasis and pathophysiology of the corneal epithelium.[6-12] Epidermal growth factor receptor activation is necessary and sufficient for corneal epithelial migration, proliferation, and differentiation.[8,12] Epidermal growth factor receptor is the primary mediator of wound healing during in vitro experiments with immortalized human corneal epithelial cells,[11] as well as for other species, including rodents, rabbits, dogs, horses, and primates[12-16]; EGFR signaling also promotes corneal wound healing in patients with diabetes mellitus.[17,18]

Coupled with these findings are the reported ocular surface side effects following systemic dosing of anti-EGFR cancer therapies. Patients with various types of cancer report persistent adverse corneal changes while patients were undergoing therapy with the anti-EGFR compound erlotinib (Tarceva/OSI-774),[19,20] including corneal epithelial defects, ulceration, keratitis, as well as perforation necessitating penetrating keratoplasty. Similar corneal and conjunctival events have been reported during the development of another anti-EGFR RTKi, gefitinib.[21] The systemic use of the approved anti-EGFR monoclonal antibodies, cetuximab and panitumumab, resulted in similar adverse events to the cornea.[20,22-24] Damage to the corneal epithelium is not only painful, but as the first anatomical barrier against external agents, such as small particles, viruses, and bacteria, the eye is susceptible to infection when the epithelium is compromised, where more severe infections may lead to transient and even permanent vision loss.

Given its potential to treat AMD, we examined whether this antiangiogenic RTKi affected corneal epithelial wound healing. We found that the RTKi inhibits both in vitro and in vivo wound healing in concentrations far above those required for its antiangiogenic effect that is mediated by VEGFR2 blockade. However, when corneal epithelial cells are treated with menadione (vitamin K3), the untoward effects of the RTKi are markedly ameliorated in both test systems. Menadione slows the degradation of the EGF:EGFR complex, suggesting the protective mechanism of action for menadione is likely through disrupting membrane trafficking, at least in part. Importantly, menadione had no effect on the RTKi's ability to block VEGFR2 phosphorylation or inhibit VEGF-induced proliferation of retinal endothelial cells. These data demonstrate a potential new strategy to overcome the off-target effects of dual specificity kinase inhibitors applied through topical administration.

MATERIALS AND METHODS

Cell Culture

Human telomerase-immortalized corneal epithelial cells (hTCE-pi) were obtained from Geron Corp. (Menlo Park, CA, USA)[25] and maintained in growth media (Defined Keratinocyte Media with growth supplement; Invitrogen, Grand Island, NY, USA) containing 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in 5% CO_2.

Primary human retinal microvascular endothelial cells were from Cell Systems (Kirkland, WA, USA) and maintained in CSC complete medium (Cell Systems), according to the manufacturer's directions. Cells were maintained at 37°C in 5% CO_2.

Chemicals

Chemicals used were 3-[(4-bromo-2,6-difluorophenyl)methoxy]-5-[[[[4-(1-pyrrolidinyl) butyl] amino] carbonyl]amino]-4-isothiazolecarboxamide hydrochloride (RTKi) (PanOptica, Inc., Bernardsville, NJ, USA), and menadione crystalline (Sigma-Aldrich Corp., St. Louis, MO, USA), AG1478 (Cayman Chemical, Ann Arbor, MI, USA).

Cell Lysate Preparation and Immunoblotting

Cell lysates were generated and immunoblots were performed as described previously.[26] Proteins were detected using antibodies against EGFR (SC-03; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-EGFR pY1068 (Tyr1068; Cell Signaling, Danvers, MA, USA), α-Tubulin (Sigma-Aldrich Corp.), VEGFR2 (Cell Signaling), Phospho-VEGFR2 (Cell Signaling), horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Pierce; Rockford, IL, USA). Detected proteins were visualized by enhanced chemiluminescence using a Fotodyne imaging system (Hartland, WI, USA).

In Vitro Wound Healing Assays

Silicone elastomer (Sylgard 184 Elastomer; Dow Corning, Corning, NY, USA) used to create 2-mm plugs that were placed onto a 6-well tissue culture dish, spaced 2 mm apart. Cells were plated and grown in complete media. Plugs were removed and nonadherent cells were removed by washing with PBS.[11] Serum-free media with or without ligands (EGF, VEGF), and menadione crystalline and with or without inhibitor at various concentrations (AG1478, RTKi) was added. The area was photographed using Nikon Eclipse Ti microscope with a ×4 objective using NIS-Elements AR Acquisition software (Nikon Instruments, Inc., Melville, NY, USA). The uncovered area was quantified using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Cell Treatment With Menadione

Menadione was prepared in 100% ethanol at a concentration of 92.9 mM with subsequent dilutions in serum-free media. Cells were pretreated with the indicated concentration of menadione for 4 hours at 37°C in 5% CO_2.

In Vivo Murine Corneal Epithelial Wounding

Adult female C57BL6/J mice (8–10 weeks, Jackson Laboratory, Bar Harbor, ME, USA) were pretreated with PBS, RTKi,

AG1478, menadione, or a combination of the mentioned, one drop per day in one eye 48 hours before wounding. The mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) (Butler Schein, Dublin, OH, USA). The central epithelium was demarcated with a 1.5-mm-diameter biopsy punch and removed with an Algerbrush II (Alger Company, Inc., Lago Vista, TX, USA) with a 0.5-mm burr taking care not to disrupt the basement membrane.[11,27] Following wounding, new eye drops with RTKi (0.0, 1.0, 10, 20, 30, and 50 µM in PBS) were administered. At each time point (0, 16, 24, 40 hours) the corneal wounds were visualized using sterile fluorescein sodium ophthalmic strips USP (Fluorets; Chauvin Laboratory, Aubenas, France) damped with sterile PBS. Wounds were photographed at ×3 magnification with a stereoscopic zoom microscope (SMZ1000, Nikon, Tokyo, Japan) equipped with a digital sight DS-Fi2 camera (Nikon). The wound areas were measured using Image J software. All treatment of animals was in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC#12046).

Statistical Analysis

Data were analyzed using GraphPad Software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using a Student's t-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.00001$.

RESULTS

The antiangiogenic RTKi evaluated in this study was specifically designed to selectively target the ATP binding domain of VEGFR2. It antagonizes VEGFR2 kinase activity with an IC_{50} of 11 nM and potently blocks other proangiogenic kinases, such as FGF receptors and Tie-2. However, similar to other small molecule kinase inhibitors, it also inhibits numerous other kinases, such as EGFR, but at much higher concentrations (EGFR $IC_{50} = 5.8$ µM).[3] If RTKi were to be used topically to inhibit angiogenesis in the eye, it would need to be applied to the cornea at concentrations in excess of 1 mM so as to be effective in the posterior segment of the eye. Thus, we asked the question: Does RTKi affect EGFR-mediated corneal epithelial wound healing and homeostasis?

We performed a series of in vitro wound experiments (described in Materials and Methods and Refs. 11, 27), that monitored the ligand- and/or inhibitor-dependent movement of immortalized human corneal epithelial (hTCEpi) cells into a 2-mm diameter acellular area.[25] This process is clearly EGF dependent (compare vehicle-treated cells [Fig. 1A1] with those containing 10 ng/mL or 50 ng/mL EGF [Figs. 1A5, 1A9]). Receptor tyrosine kinase inhibitor salt diluted in PBS was used to treat the cells. Receptor tyrosine kinase inhibitor treatment caused a dose-dependent inhibition of EGFR-mediated corneal epithelial wound healing, as measured with in vitro assays (Figs. 1A5–12). The RTKi was less potent, but equally efficacious as the selective EGFR inhibitor, AG1478 (Figs. 1A2–4). The addition of VEGF provided no increase in wound healing over vehicle controls, indicating that VEGFR signaling is not involved in this process. The RTKi decreased wound healing in cells treated with VEGF (Figs. 1A13–1A16), but based on previous data, we believe this is due to inhibition of autocrine EGF secretion.[11] These experiments indicate the RTKi's IC_{50} is approximately 10 µM for EGFR-mediated in vitro wound healing.

A kinetic analysis of in vitro wound healing (Fig. 1C, Supplementary Fig. S1) indicates that either RTKi or AG1478 alone can inhibit wound healing over the course of 48 hours. The addition of EGF promotes wound healing in the presence of RTKi, but not AG1478. This observation is consistent with AG1478 being a more robust inhibitor of the EGFR than RTKi.

We next wanted to know if the RTKi also inhibited corneal epithelial cell biology in vivo (Fig. 2). Using a murine model, a 1.5-mm wound was made to the corneal epithelium followed by topical administration of varying RTKi concentrations. The wound was imaged and its size calculated at 0, 16, 24, and 40 hours after wounding; the RTKi was readministered at these times as well. Consistent with our in vitro findings, we observed that the RTKi slowed the kinetics of corneal wound healing in a dose-dependent manner (Note time course in Fig. 2D). At 16 and 24 hours after wounding, there were statistically significant differences in the wound size with 30 µM and 50 µM RTKi, as compared with the vehicle control (Figs. 2B, 2C). At 16 hours, this inhibition was comparable to what was observed with the EGFR specific inhibitor 1 µM AG1478 (Fig. 2B).

Given these off-target effects of the RTKi, albeit at concentrations much higher than pharmacologic concentrations used to inhibit angiogenesis, we were interested in developing a strategy to minimize the effects of corneal epithelial wound healing and homeostasis. We wanted to develop an approach that would mitigate the inhibition of EGFR activity, but not interfere with the RTKi's on-target anti-VEGFR2 activity. Vitamin K3 emerged as a candidate, as it has been reported to enhance the activity of EGFR in nonocular tissues, potentially through inhibiting phosphatase activity.[28–31] We hypothesized that sustaining EGFR activity could overcome the reduced EGFR activity induced by RTKi exposure. These experiments were performed using the more stable, synthetic analog of vitamin K3, menadione.[32]

We tested this hypothesis by examining the kinetics of EGFR phosphorylation in the absence and presence of two different concentrations of menadione (Fig. 3). If our hypothesis was correct and menadione was a phosphatase inhibitor, we predicted menadione would induce either a higher basal EGFR phosphorylation, or sustained EGFR phosphorylation. Human telomerase-immortalized corneal epithelial cells were pretreated with 0, 0.3, or 3 µM menadione for 4 hours followed by EGF treatment for 0 to 180 minutes. Cell lysates then were immunoblotted for phosphorylated EGFR (phosphotyrosine 1068–pY1068), total EGFR, as well as α-tubulin as a loading control. We did not observe an increase in the basal EGFR phosphorylation or an increase in the duration of receptor phosphorylation. However, we did observe slowing of the kinetics of EGFR degradation, suggesting that lysosomal degradation of the receptor is inhibited. This observation is supported by previous reports that menadione inhibits the membrane trafficking of the transferrin receptor, by blocking its recycling.[33,34]

We next determined if menadione pretreatment would moderate the effects of the RTKi on in vitro wound healing (Fig. 4). Human telomerase-immortalized corneal epithelial cells were pretreated for 4 hours with 0, 0.3, or 3 µM menadione before RTKi and EGF treatment. Menadione ameliorated, in part, the inhibitory effects of the RTKi. This protective effect was most obvious with 0.3 µM menadione pretreatment (compare panels 4A6 with 4A10). At 3.0 µM menadione, some cytotoxicity that inhibited even the basal rates of wound healing was noted.

To determine if menadione had a similar protective effect during in vivo corneal epithelial wound healing, we used a drug administration regimen that would reflect what would occur in patients. Consequently, the RTKi was administered

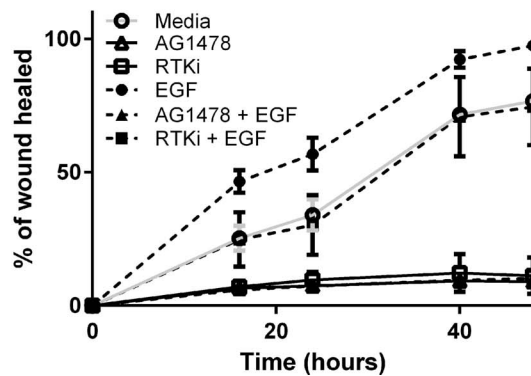## A. *In Vitro* Wound Healing
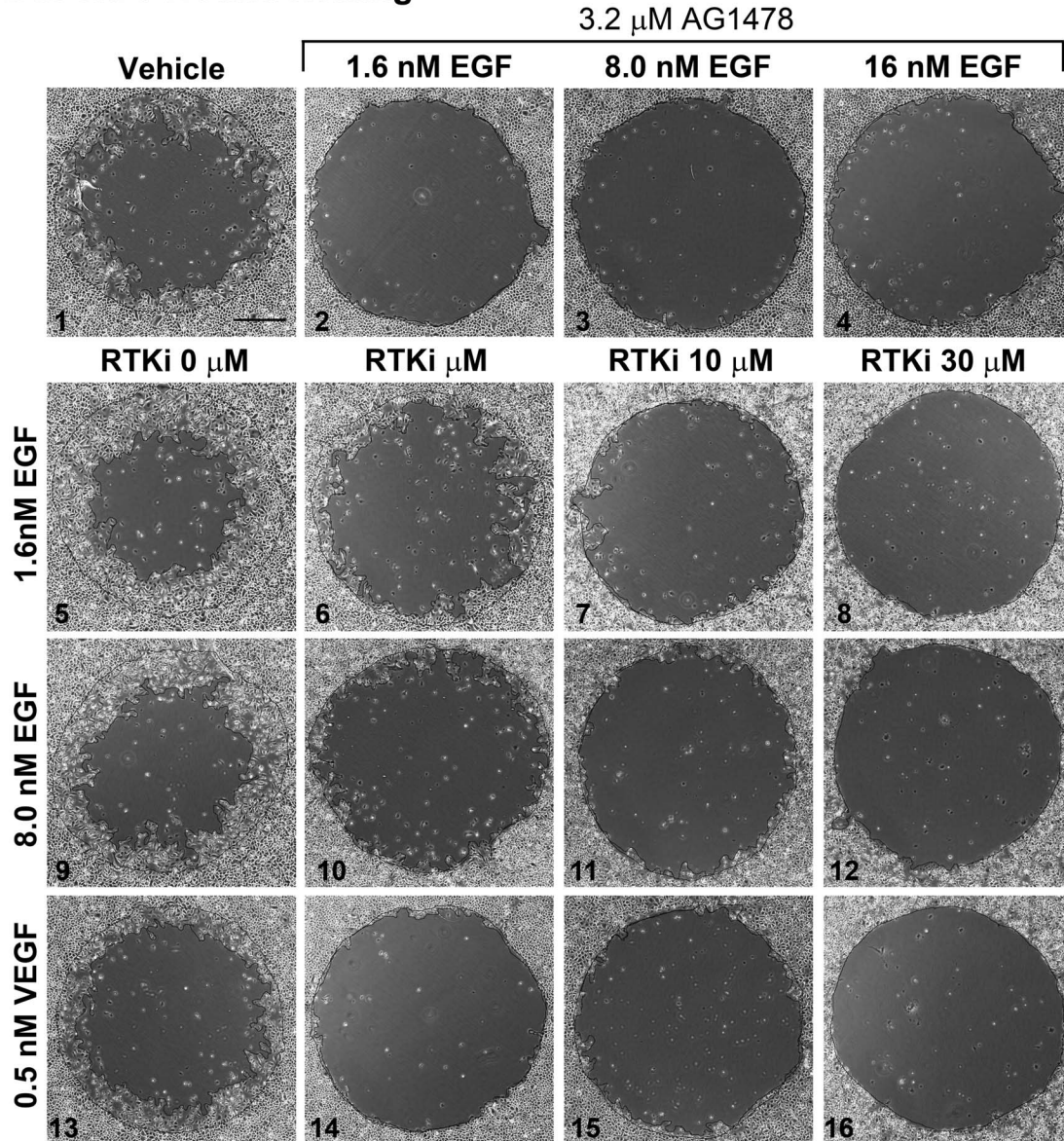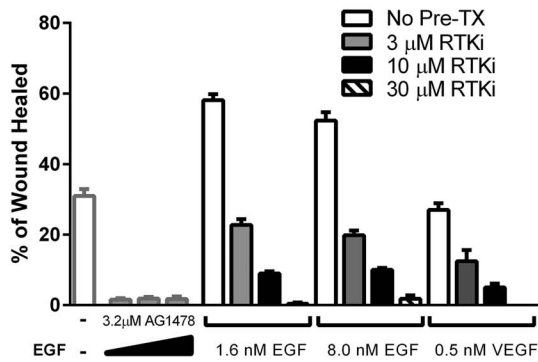
FIGURE 1. Receptor tyrosine kinase inhibitor inhibits EGFR-mediated in vitro wound healing. Human telomerase-immortalized corneal epithelial cells were plated on tissue culture dishes with 2-mm-diameter silicone plugs that, when removed, created an acellular area to monitor wound healing. Cells were pretreated for 30 minutes with the indicated concentrations of RTKi or AG1478, followed by 16 hours with the addition of the indicated concentration of EGF or VEGF. (**A**) Representative micrographs are shown and were used to quantify the in vitro wound healing response. Photographs were used to trace, measure, and quantify the area of the initial wound (*outer circle*) and the remaining wound (*inner circle*). *Scale*

bar: 500 μm. (**B**) Graphical representation of the quantification of multiple dose response experiments. (**C**) Graphical representation of a time course of in vitro wound healing with media alone, 1.6 nM EGF, 3.2 μM AG1478, 3.0 μM RTKi, 1.6 nM EGF and 3.2 μM AG1478, or 1.6 nM EGF and 3.0 μM RTKi. Representative micrographs are shown in Supplementary Data Figure S1. Data are plotted as the average ± SEM. from three experiments. Data were analyzed using an unpaired Student's *t*-test. *$P < 0.05$; **$P < 0.01$.

topically in mice 24 and 48 hours before wounding. Using this RTKi pretreatment regimen, reduced wound healing was observed with much lower RTKi concentrations as compared to initiating RTKi dosing at the time of wounding (data not shown). To investigate the potential protective effects of menadione, 10 μM RTKi pretreatment was used (Fig. 5), 10 μM RTKi reduced wound healing by 59.4% at 16 hours after wounding, as compared with vehicle-treated controls. Eyes cotreated with the RTKi and 0.3 μM menadione showed a statistically significant improvement in reduced wound healing (73.2%; $P = 0.0355$). Similar improvements were seen after 24 hours as well (69.1% with 10 μM RTKi versus 82.4% with 10 μM RTKi and 0.3 μM menadione; $P = 0.0132$). Treatment with 0.3 μM menadione was statistically indistinguishable from vehicle alone. Together, these data indicate that menadione treatment can ameliorate the off-target effects of this antiangiogenic RTKi on corneal epithelial wound healing.

Finally, we wanted to know if menadione affected ability of the RTKi to inhibit its intended target, VEGFR2 (Fig. 6). First, we examined whether or not menadione affected RTKi's inhibition of VEGFR2 phosphorylation. Human retinal endothelial cells were pretreated with 0, 0.3, or 3.0 μM menadione for 4 hours, followed by the addition of varying concentrations (0–1.0 μM) of RTKi for 30 minutes, and then treatment with VEGF. Menadione had no effect on the dose-dependent inhibition of kinase activity as measured by ligand-dependent VEGFR2 phosphorylation (Fig. 6A). Second, to determine if menadione interfered with VEGFR2-mediated signaling, we examined RTKi-mediated inhibition of VEGF-induced human retinal endothelial cell proliferation, in the absence and presence of menadione. Cells were pretreated with menadione for 4 hours and then the media was supplemented with the

indicated doses of VEGF for 16 hours. Cell viability was measured using an Alamar Blue Assay (Fig. 6B). In the presence of 0.3 μM menadione, the IC$_{50}$ of RTKi was indistinguishable from the 0-μM menadione control. Higher concentrations of menadione (3.0 μM) reduced the maximal cell growth in response to VEGF alone, but did not significantly alter the RTKi IC$_{50}$.

DISCUSSION

The RTKi investigated in this study was originally designed to inhibit VEGFR2 receptor kinase activity, but it also inhibits EGFR kinase activity at 500-fold higher concentrations. Our data indicate that due to EGFR inhibition, the RTKi causes a dose-dependent inhibition of corneal epithelial wound healing in both in vitro and in vivo assays (Figs. 1, 2). Therefore, topical ocular application of RTKi could disrupt corneal epithelial homeostasis and/or wound healing.

In both in vitro and in vivo assays, we observed that menadione treatment attenuates the untoward, off-target effects of the RTKi (Figs. 4, 5). Vitamin K3 and its synthetic analog, menadione, have been reported to potentiate EGFR signaling in nonocular tissues by inhibiting phosphatases that negatively regulate receptor activity.[28,29,31] We did not observe any significant differences between the effects of menadione treatment on basal levels of EGFR phosphorylation or the duration of EGFR phosphorylation versus control cells (Fig. 3). These findings suggest that menadione does not inhibit phosphatases in transformed human corneal epithelial cells. Instead, we observed that the kinetics of EGFR degradation were slowed, indicating menadione may disrupt receptor
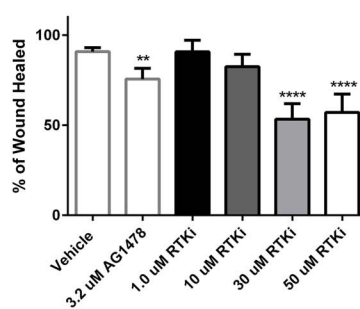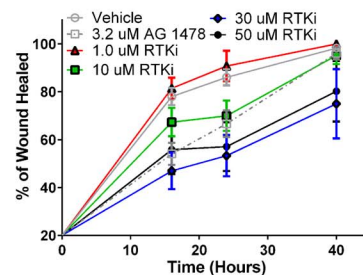
A. *In vivo* Wound Healing
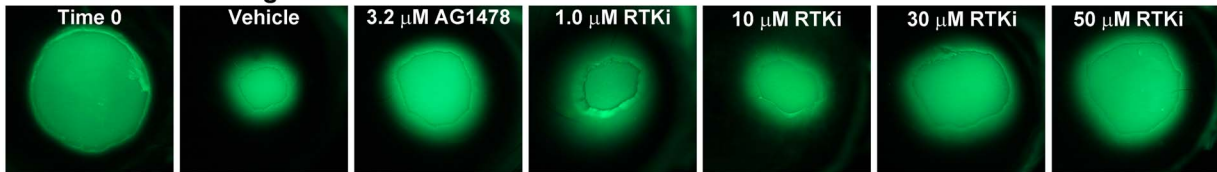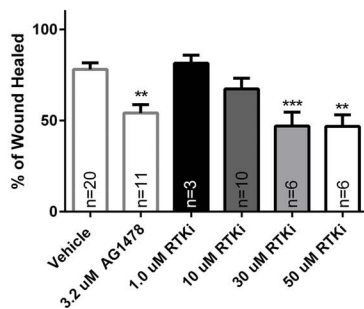
FIGURE 2. Receptor tyrosine kinase inhibitor slows corneal epithelial wound healing in mice. Epithelial wounds (1.5 mm in diameter) were made on the corneas of 8-week-old C57Bl6 mice. Following wounding, PBS, an EGFR-inhibitor (AG1478–1 μM), or the indicated concentrations of RTKi were topically administered to the wounded area. The epithelial wounds were visualized after fluorescein staining using a fluorescent dissecting microscope. (**A**) Representative images at the time of the initial wound (0 hour), 16 hours, 20 hours, or 40 hours after wounding. (**B, C**) Graphical representation of the percentage of wound healing at 16 and 24 hours after wounding, respectively. (**D**) The percentage of wound healed as a function of time. For (**B–D**), data are plotted as the average ± SEM from 3 to 20 experiments. Data were analyzed using an unpaired Student's *t*-test. *$P < 0.05$; **$P < 0.01$.
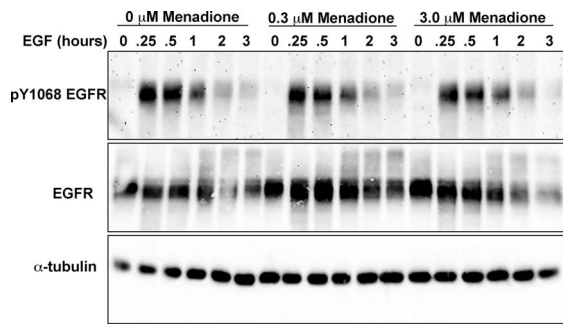
Figure 3. Menadione treatment slows the kinetics of EGF-mediated EGFR degradation. Human telomerase-immortalized corneal epithelial cells were pretreated with menadione (0, 0.3, or 3.0 μM) for 4 hours. Cells were then incubated with EGF (50 ng/mL) for the indicated periods of time (0–3 hours). Cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted for phosphorylated EGFR (pY1068) (*top*), total EGFR (*middle*), or α-tubulin (*bottom*). Shown is a representative image from an experiment repeated three times.

trafficking. This observation is consistent with reports that the oxidative stress induced by menadione interferes with endocytic trafficking.[33–35] Although previous studies examined the effects of menadione on the transferrin receptor, the intracellular pathways are shared by the EGFR.

It is unclear as to why we did not observe a change in the kinetics of EGFR phosphorylation with menadione treatment in the hTCEpi cells. One explanation is there is a different complement of phosphatases in hTCEpi cells versus the previously tested cell lines, or that our phospho-specific antibodies are not sensitive enough to pick up these subtle differences. Notably, controversy exists in the literature regarding which phosphatases menadione/vitamin K3 inhibit. It has been linked to phosphatases that target Cdc25,[36] p34cdc2,[37] and the EGFR.[30,38] Alternatively, menadione may disrupt lysosomal trafficking of the EGF:EGFR complex and the delayed kinetics of degradation have been misinterpreted as prolonged phosphorylation. Thus, the following possible actions by which menadione may enhance EGFR-mediated responses must be considered: inhibiting the EGFR dephosphorylation, inhibiting the dephosphorylation of another effector downstream of the EGFR, or slowing the rate of EGFR degradation and prolonging the duration of activity. Our data support the third model, but we are not willing to completely discount contributions from the other two mechanisms.

Regardless of the exact mechanism(s) of action of menadione, it is apparent that enhancing EGFR activity could be beneficial for corneal epithelial homeostasis and wound healing. The work presented here demonstrates that coadministration of menadione with the RTKi can mitigate the off-target effects; however, as a preventive therapy, menadione could be administered between RTKi doses. Menadione has a relatively short half-life (~30 minutes)[39]; systemic accumulation is not predicted from topical administration to the eye. A similar dosing regimen of menadione may be useful to locally overcome the corneal epithelial side effects of systemically delivered EGFR inhibitors (i.e., Erbitux and Iressa),[21,22] as proposed for menadione improving the dermatologic side-effect profile of EGFR inhibitors.[38] Finally, menadione alone may be useful for accelerating re-epithelialization of the cornea following wounding from surgery or trauma. Our laboratory and others[11,40,41] have shown that tear fluid contains sufficient levels of EGF such that there is substantial receptor occupancy. This limits the magnitude of response yielded from the addition of more growth factor. Menadione treatment may

A. *In Vitro* Wound Healing
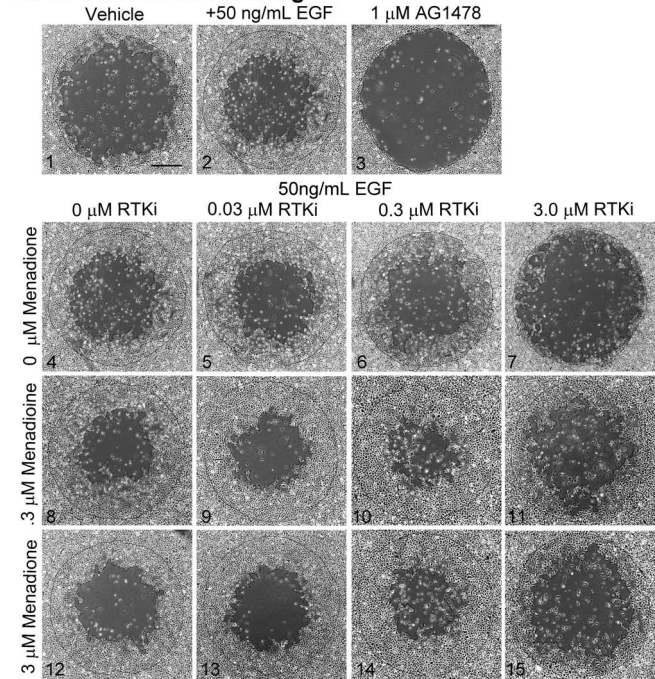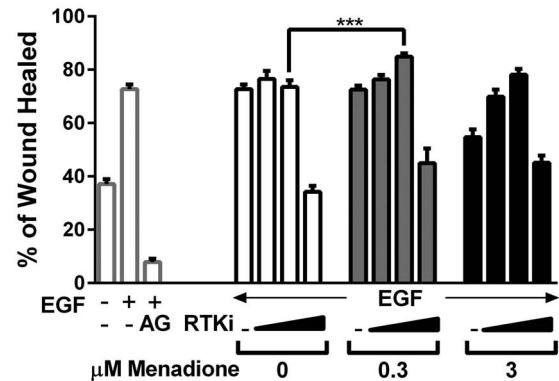

B. Quantification

Figure 4. Vitamin K3 increases in vitro wound healing. Human telomerase-immortalized corneal epithelial cells were plated for an in vitro wound healing assay as described in Figure 1. Before removing the silicone plug, cells were treated with the indicated concentrations of menadione for 4 hours and supplemented with the varying concentrations of RTKi for 30 minutes. Once the silicone plug was removed, the cells were incubated with the indicated concentrations of menadione, RTKi, and EGF. (**A**) Representative micrographs are shown and were used to quantify the in vitro wound healing response. Photographs were used to trace, measure, and quantify the area of the initial wound (*outer circle*) and the remaining wound (*inner circle*). *Scale bar*: 500 μm. (**B**) Graphical representation of multiple experiments. Data are plotted as the average ± SEM from three to four experiments. Data were analyzed using an unpaired Student's *t*-test. *$P < 0.05$; **$P < 0.01$.

bypass such limitations by prolonging EGFR signaling to produce an overall greater response.

The therapeutic study of menadione should proceed with caution. A recent study by Halilovic et al.[42] indicates that menadione can induce toxicity in corneal endothelial cells. At high levels of menadione (25 μM), endothelial cells exhibited DNA damage and associated mitochondrial dysfunction; similar toxicity has been observed in the lens at even higher menadione concentrations (200 μM).[43] This finding is consistent with our observation of cytotoxicity in immortalized
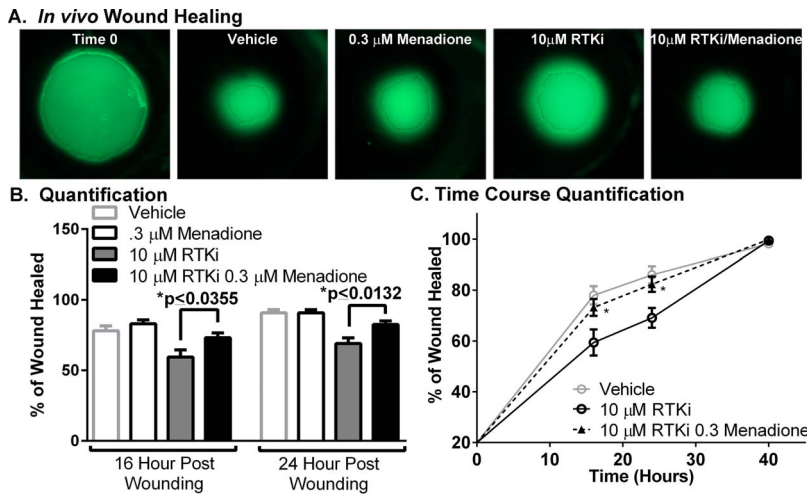
Figure 5. Vitamin K3 increases in vivo corneal epithelial wound healing. Eight-week-old C57Bl6 mice were subjected to 1.5-mm-diameter corneal epithelial wounds. Forty-eight hours before wounding, mice were topically administered with vehicle, 0.3 μM menadione, 10 μM RTKi, or 10 μM RTKi with 0.3 μM menadione. The epithelial wounds were visualized after fluorescein staining using a fluorescent dissecting microscope. (**A**) Representative images of the epithelial wounds at various times after staining. (**B, C**) Quantification of wound healing at 16 hours and 24 hours after wounding, respectively. (**D**) Kinetics of wound healing over the course of 40 hours. Data were analyzed using an unpaired Student's *t*-test. *$P < 0.05$; **$P < 0.01$.

corneal epithelial cells with 3.0 μM menadione for 20 hours. Together, our study and the previous report indicate the dose and duration of menadione need to be considered when designing potential therapeutic applications.
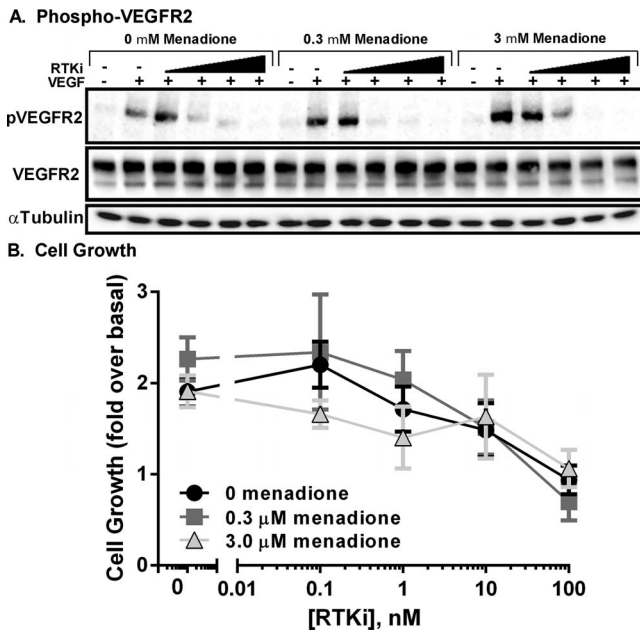

Figure 6. Vitamin K3 does not affect RTKi inhibition of VEGF. (**A**) Human retinal endothelial cells were treated with 0, 0.3, or 3 μM menadione for 4 hours, followed by 30 minutes of treatment with varying concentrations of RTKi (1 nM, 10 nM, 100 nM, or 1μM) in menadione. Finally, cells were stimulated with 10 ng/mL VEGF. Cell lysates were prepared resolved by 7.5% SDS-PAGE, transferred to nitrocellulose an immunoblotted for phosphorylated VEGFR2 (*top*), total VEGFR2 (*middle*), or α-tubulin (*bottom*) as a loading control. (**B**) Human retinal endothelial cells were pretreated with menadione for 4 hours then the indicated concentration of RTKi for 30 minutes, and supplemented with 10 ng/mL VEGF overnight. Viable cells were quantified by Alamar Blue Assay (Thermo Fisher). Data are plated as the fold growth relative to cells treated with no VEGF or RTKi and are shown as the average ± SEM.

CONCLUSIONS

The RTKi evaluated in this study potently targets VEGFR2 (IC_{50} = 11 nM); however, off-target inhibition of EGFR occurs at substantially higher concentrations than those needed for antiangiogenic activity. Because of this off-target EGFR inhibition, the RTKi induces dose-dependent reductions in corneal epithelial wound healing, both in vitro and in vivo. These untoward effects can be minimized by the addition of nanomolar concentrations of menadione without any deleterious effect on the RTKi's intended antiangiogenic activity. Menadione is a potential topical treatment for individuals suffering from perturbations in corneal epithelial homeostasis that arise as a side effect of EGFR inhibition.

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