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Effect of Immunosuppressants Tacrolimus and Mycophenolate Mofetil on the Keratinocyte UVB Response[†]

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Abstract

Non-melanoma skin cancer, derived from epidermal keratinocytes, is the most common malignancy in organ transplant recipients, causes serious morbidity and mortality, and is strongly associated with solar ultraviolet (UV) exposure. Preventing and treating skin cancer in these individuals has been extraordinarily challenging. Following organ transplantation, the immunosuppressants are used to prevent graft rejection. Until now, immunosuppression has been assumed to be the major factor leading to skin cancer in this setting. However, the mechanism of skin carcinogenesis in organ transplant recipients has not been understood to date; specifically, it remains unknown whether these cancers are immunosuppression-dependent or -independent. In particular, it remains poorly understood what is the mechanistic carcinogenic action of the newer generation of immunosuppressants including tacrolimus and mycophenolate mofetil (MMF). Here we show that tacrolimus and MMF impairs UVB-induced DNA damage repair and apoptosis in human epidermal keratinocytes. In addition, tacrolimus inhibits UVB-induced checkpoint signaling. However, MMF had no effect. Our findings have demonstrated that tacrolimus and MMF compromises proper UVB response in keratinocytes, suggesting an immunosuppression-independent mechanism in the tumor-promoting action of these immunosuppressants.

INTRODUCTION

Skin cancer is the most common cancer in the United States. Its major environmental risk factor is UVB radiation in sunlight, which causes DNA damage (1). Incomplete DNA repair, impaired checkpoint function, and deregulated cell survival and growth can lead to tumor initiation and promotion, which ultimately will accelerate the development of skin cancer (1, 2).

Organ transplant recipients are a population at particularly high risk for cancer, and skin cancers are their most common malignant conditions. Among skin cancers, squamous cell carcinoma (SCC) is the most common type among transplant recipients, occurring 65–250 times as frequently as in the general population (3). Many organ transplant recipients are given the immunosuppressants to suppress their immune system. However, a lifetime course of such treatment causes a dramatic increase in risk of skin cancer as a major adverse effect

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(3–5). The first immunosuppressant is cyclosporin A, a transforming medicine that substantially increases graft survival and lifespan of OTRs.

Due to the nephrotoxicity, newer immunosuppressants have been developed to replace cyclosporin A. These include tacrolimus (FK506, FK) and mycophenolate mofetil (MMF). Similar to cyclosporin A, Tacrolimus is an inhibitor for calcineurin, a serine/threonine phosphatase dephosphorylating family of transcription factor NFATs, which mediate an immune response (6). Tacrolimus is the current mainstream immunosuppressant widely used for preventing or treating graft rejection in organ transplantation patients, and for treating autoimmune diseases including myasthenia gravis, arthritis, and atopic dermatitis (7). Mycophenolate mofetil (MMF) has been used widely as a part of various combination regimens of immunosuppressive agents. It is an ester prodrug of the active immunosuppressant mycophenolic acid (MPA). MMF and/or MPA inhibit the proliferation of lymphocytes and the production of antibodies induced by a variety of mitogens and antigens (8). Large clinical trials have shown that MMF is effective in preventing acute rejection and improving graft and patient survival in combination with calcineurin inhibitors (cyclosporin A and tacrolimus) (9).

UVB radiation in sunlight is the major human skin carcinogen and is a profound factor in increased skin cancer risk in OTRs (5). The main mechanism in UVB-induced skin cancer is causing DNA damage. The predominant UVB-induced DNA photoproducts caused are cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidone dimers (6-4PP) (10, 11). In response to DNA damage, the cells activate a specific DNA repair mechanism, i.e., global genome nucleotide excision repair (GG-NER), which involves well-coordinated action of multiple proteins (12–15). In addition, the damaged cells activate the DNA damage response (DDR) signal-transduction pathway to coordinate cell-cycle transitions, DNA replication, DNA repair, and apoptosis. The checkpoint activation involves the phosphorylation of the Ser/Thr kinase checkpoint kinase-1 (Chk1) (16, 17) and 2 (Chk2) (18–21). Defects in the Chk1 or Chk2 pathways are known to increase cancer risk (22–27).

We have recently shown that, independent of immunosuppression, cyclosporin A suppresses the expression of the tumor suppressor PTEN and UVB-induced DNA damage repair and checkpoint activation in human keratinocytes, and promotes UVB-dependent skin tumorigenesis (28, 29). However, it remains unknown about the immunosuppression-independent action of tacrolimus and MMF on human epidermal keratinocytes. In this study we seek to determine the influence of tacrolimus and MMF on human epidermal keratinocytes on UVB-induced molecular and cellular responses critical for suppressing tumorigenesis.

MATERIALS AND METHODS

Cell culture and UVB treatment

HaCaT cells (kindly provided by Dr. Fusenig) were cultured in 60 mm dishes. For UVB treatment, the Stratalinker 2400 equipped with 312-nm UVB bulbs (Stratagene, La Jolla, CA) (UVC 0%, UVB 51%, and UVA 49%) was used. The UV exposure was performed in PBS after washing the cells with PBS twice to avoid the photosensitization effect of

components in culture medium on the cells. In all experiments, cells were preincubated with vehicle (Veh), Tacrolimus (FK506, FK, 0.1, 1 or 10 µg/ml, or MMF (0.1 µg/ml) for 1 week prior to UVB irradiation. These concentrations were selected based on the literature (30, 31) and our data on the toxicity of FK506 or MMF in human keratinocytes (data not shown). Cells were collected at predetermined time points for analysis of DNA repair, apoptosis, and checkpoint activation based on our recent work (32–34).

Western blotting

Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA). Equal amounts of protein were subjected to electrophoresis. Western blotting was performed as described previously (35, 36). Antibodies used included Chk1, Chk2, PARP, GAPDH (Santa Cruz), p-Chk1, and p-Chk2 (Cell Signaling Technology).

Determination of UVB-induced CPD/6-4PP level in genomic DNA by slot blot assay

Slot blot assays of CPD/6-4PP were performed as described previously (37). Briefly, cells were collected at different time points post-UVB, and DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The DNA concentration was calculated from the absorbance at 260 nm using NanoDrop 1000 (NanoDrop products, Wilmington, DE, USA). The CPD in DNA were quantified by slot-blot (Bio-Rad, Hercules, CA, USA) with a monoantibody (TDM-2 for CPD and 6-4PP, COSMO BIO Co., Tokyo, Japan) as described previously (38). The chemiluminescence was detected with a Carestream Imaging Station (Carestream, Rochester, NY, USA). For examining repair kinetics, the percentage of repair was calculated by comparing the optical density at the indicated time with that of the corresponding absorbance at time zero, when there was no opportunity for repair, and 100% of CPDs were present post-UVB.

Sub-G1 flow cytometric analysis

Sub-G1 flow cytometric analysis was performed to determine apoptosis as described previously (39). Briefly, cells were fixed and stained with propidium iodide, and the percentage of cells at the sub-G1 phase was quantified by flow cytometry.

Statistical analyses

Statistical analyses were performed using Prism 5 (GraphPad software, San Diego, CA). Data were expressed as the mean of three independent experiments and analyzed by Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

Results

FK506 or MMF inhibit UVB-induced DNA damage repair in HaCaT cells

To determine the effect of FK506 and MMF on UVB-induced DNA damage repair, we measured the percentage of CPDs and 64-PPs remaining at different time points post-UVB, and thereby determined the percentage of DNA repair, in vehicle, FK, or MMF-pretreated HaCaT cells for 1 week. FK506 significantly inhibited repair of CPDs (Fig. 1A–D) and this inhibitory effect was dose-dependent, but it had no effect on repair of 64-PPs (Fig. 1A–D).

Similarly, MMF significantly suppressed repair of CPDs (Fig. 1E–F) but not 64-PPs (Fig. 1E–F). Our findings demonstrated that in human epidermal non-tumorigenic HaCaT cells FK506 and MMF inhibit UVB-induced DNA repair of CPD, but not 64-PP at the time points examined.

FK506 or MMF suppresses UVB-induced apoptosis in HaCaT cells

It has been reported that FK506 inhibited proliferation and induced apoptosis of fibroblasts *in vitro* (40). Here we explored the effect of FK506 and MMF in HaCaT cells on apoptosis by flow cytometry. FK506 or MMF suppressed UVB-induced apoptosis dose-dependently (Fig. 2A–C). Cleaved PARP, a hallmark for apoptosis, was reduced by FK506 or MMF post-UVB irradiation (Fig. 2D–F). These findings suggested that chronic FK506 or MMF suppresses UVB-induced apoptosis.

FK506 or MMF inhibits UVB-induced apoptosis in A431 cells

In addition to non-tumorigenic HaCaT cells, we determined the effect of FK506 or MMF on UVB-induced apoptosis in human A431 skin cancer cells by flow cytometric analysis. Both FK (1 µg/ml) and MMF (0.1 µg/ml) significantly reduced UVB-induced apoptosis, as compared with vehicle control (Fig. 3A–C). These data further indicate that either FK506 or MMF suppressed apoptosis in both non-tumorigenic keratinocytes and skin cancer cells.

FK506 but not MMF inhibits UVB-induced checkpoint signaling in HaCaT cells

To determine the effect of FK506 or MMF on UVB-induced checkpoint signaling, we assessed the phosphorylation of Chk1 and Chk2 in vehicle, FK506 (1 µg/ml, 10µg/ml), or MMF (0.1µg/ml)-treated HacaT cells. UVB irradiation (20 mJ/cm²) increased Chk1 phosphorylation at serine 345 (p-Chk1) and Chk2 phosphorylation at threonine 68 (p-Chk2) at 1.5 and 6 hours (Fig. 4A–C). However, in HaCaT cells at 1.5 and 6 hours post-UVB irradiation (20mJ/cm²), FK506 significantly reduced UVB-induced phosphorylation of both Chk1 and Chk2 (Fig. 4A–B), whereas MMF had no effect (Fig. 4C). These data indicated that, following UVB irradiation, FK506 inhibited UVB-induced checkpoint signaling.

DISCUSSION

FK506 and MMF have been widely used to prevent graft rejection in organ transplantation. It is generally believed that the use of drugs that suppress cellular immune function is associated with an increased risk of cancer development. Here we have shown in epidermal keratinocytes *in vitro*, we found that FK506 and MMF impaired UVB-induced DNA damage repair and apoptosis. FK506 but not MMF reduced UVB-induced checkpoint signaling. These findings suggest that FK506 and MMF promote tumor initiation.

Recent evidence in mouse models have shown that topical FK506 on the skin with a mouse model revealed a marked tumor-promoting effect of topical FK506, associated with evidence that a significant decrease in CD4/CD8 ratio (41). In contrast in another report, it has been shown that topical application of FK506 inhibited TPA-induced tumor promotion, epidermal ornithine decarboxylase induction and skin inflammation caused by TPA in a

dose-related manner (42). It has been reported that immunosuppressant FK506 acts as a growth factor for VSMCs (43).

Here we have shown that in human epidermal HaCaT keratinocytes, FK506 and MMF inhibited UVB-induced CPD repair apoptosis, which supports their oncogenic effect in tumor initiation by increasing survival of unrepaired cells. Our findings suggest a cocarcinogenic effect of FK506 and MMF on skin carcinogenesis (41), which may be different from those studies by Jiang and colleagues (42), who reported that topical FK506 suppressed tumorigenesis in CD-1 mice treated with DMBA and TPA. FK506 was reported to up-regulate C/EBP family members, especially C/EBPbeta and CHOP, and overexpression of either C/EBPbeta or CHOP significantly attenuated TNF-alpha-triggered NF-kappaB activation (30). Recent studies have shown that JNK and ERK are involved in FK506-induced fibroblast apoptosis (40). However, the role of these signaling pathways in the carcinogenic effect of FK506 or MMF requires future investigation.

Inhibition of UVB-induced DNA repair by FK506 and MMF may be mediated through altering the key factors in this DNA repair machinery, including xeroderma pigmentosum proteins (12–15). It is possible that FK506 or MMF inhibits the expression or the activity of these factors (16, 17). Inhibition of checkpoint signaling by FK506 may be resulted from the impact of FK506 on the upstream pathways in the checkpoint activation such as the kinases ATR or ATM (18–21). Future investigation will elucidate the molecular mechanism for the influence of FK506 and MMF in keratinocytes.

In summary, our findings have demonstrated that tacrolimus and MMF inhibits UVB-induced DNA damage repair and apoptosis. Furthermore, tacrolimus reduces UVB-induced checkpoint signaling, whereas MMF has no effect. Our studies suggest an immunosuppression-independent carcinogenic role of the two newer immunosuppressants in skin cancer and may provide valuable knowledge for future investigation of the tumorigenicity of tacrolimus and MMF in animal models and human patients.

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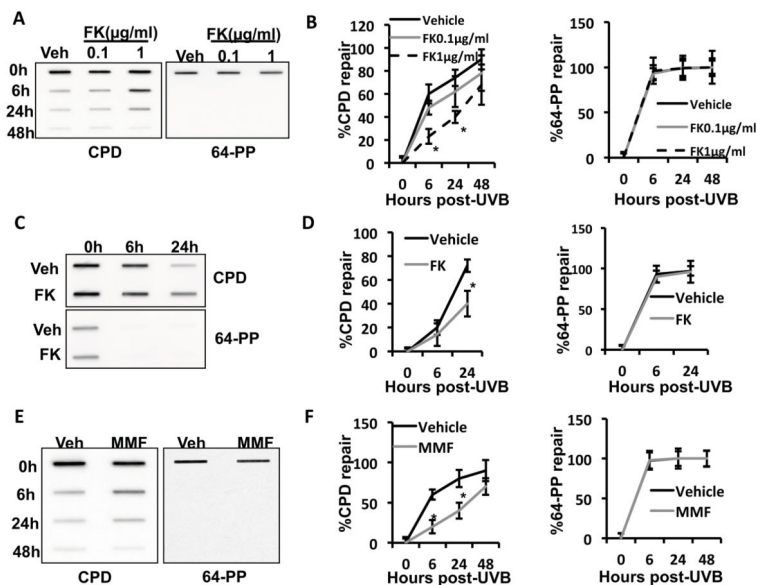


Figure 1. Chronic treatment with FK506 and MMF in HacaT cells inhibits global genome nucleotide excision repair

(A) Slot-blot analysis of percent repair of CPD and 64-PP at different time points post-UVB (20 mJ/cm^2) in HaCaT cells treated with vehicle (Veh) or FK506 ($0.1 \mu\text{g/ml}$ or $1 \mu\text{g/ml}$) for 1 week. (B) Quantification of percentage (%) of CPD and 64-PP repair in A. (C) Slot-blot analysis of percent repair of CPD and 64-PP at different time points post-UVB (20 mJ/cm^2) in HaCaT cells treated with vehicle or FK506 ($10 \mu\text{g/ml}$) for 1 week. (D) Quantification of repair percentage (%) of CPD and 64-PP repair in C. (E) Slot-blot analysis of percent of CPD and 64-PP repair at different time points post-UVB (20 mJ/cm^2) in HaCaT cells treated with vehicle (Veh) or MMF ($0.1 \mu\text{g/ml}$) for 1 week. (F) Quantification of percentage (%) of CPD and 64-PP repair in E.

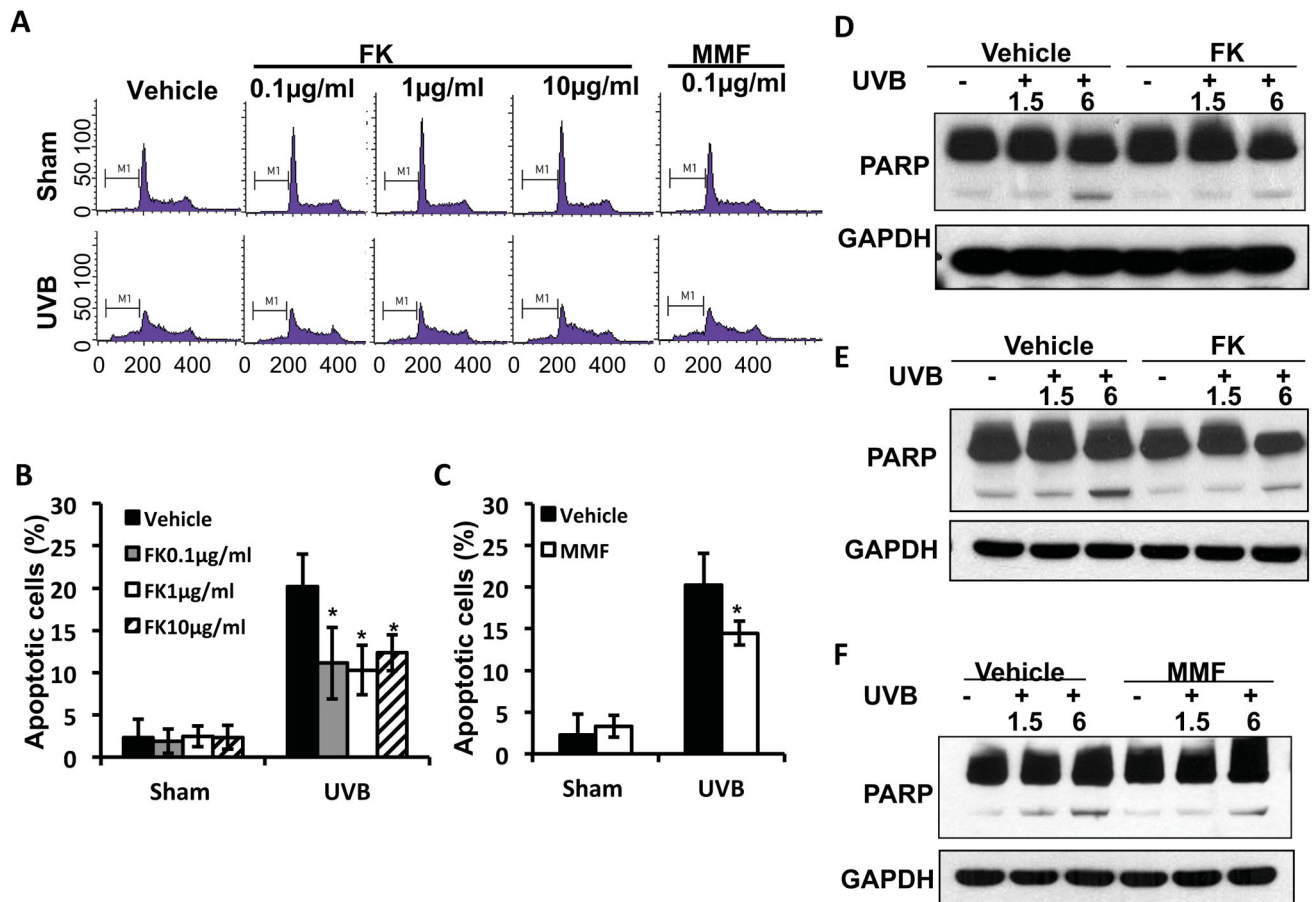


Figure 2. FK506 and MMF suppresses UVB-induced apoptosis in HaCaT cells

(A) Flow cytometric analysis of UVB (30mJ/cm²)-induced apoptosis in HaCaT cells treated with vehicle, FK506 (0.1, 1 or 10 μg/ml) or MMF (0.1 μg/ml) for 1 week. (B–C)

Quantitation of apoptotic cells (%) in A for FK506 treatment (B) and MMF treatment (C). *, $P < 0.05$, t -test, significant differences between FK506 (0.1, 1 or 10 μg/ml), MMF (0.1 μg/ml) and vehicle control. (D–F) Immunoblot analysis of PARP and GAPDH in HaCaT cells at 1.5 or 6 h post-UVB (20mJ/cm²) after treatment with vehicle, FK (1 μg/ml) (D), FK (10 μg/ml) (E), or MMF (0.1 μg/ml) (F) for 1 week.

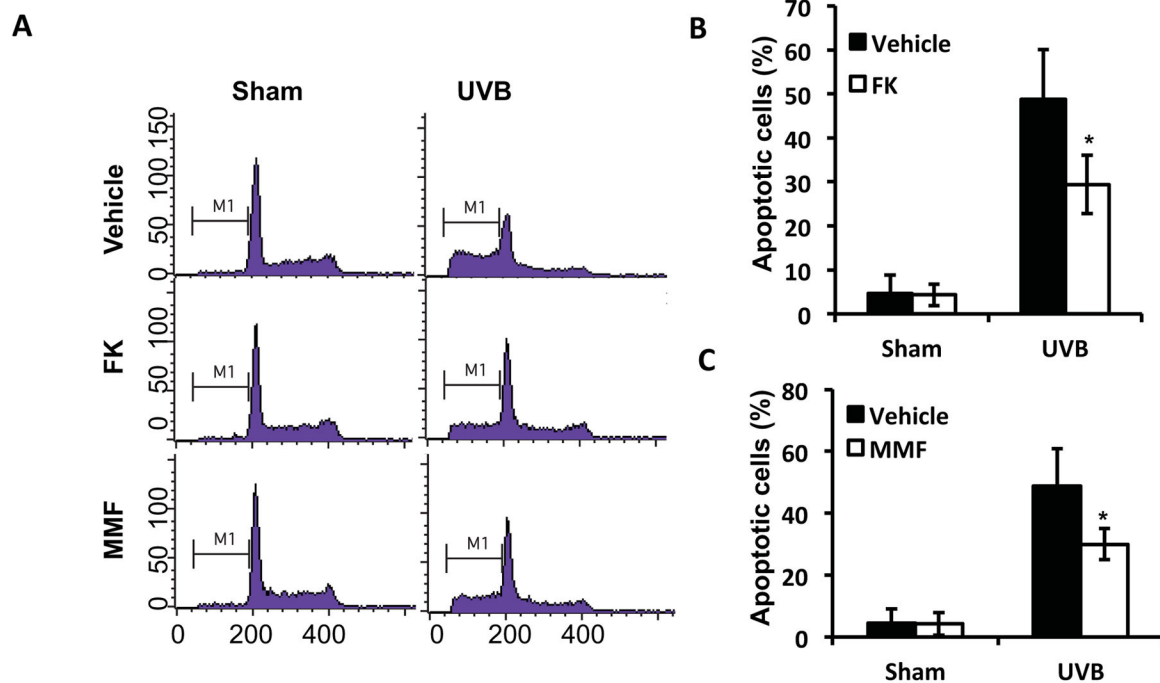


Figure 3. FK506 and MMF inhibits UVB-induced apoptosis in A431 cells

(A) Flow cytometric analysis of UVB (30 mJ/cm^2)-induced apoptosis in A431 cells pretreated with vehicle, FK506 ($1 \mu\text{g/ml}$), or MMF ($0.1 \mu\text{g/ml}$) for 1 week. (B–C) Quantitation of apoptotic cells (%) in A for FK506 ($1 \mu\text{g/ml}$) (B) and MMF treatment ($0.1 \mu\text{g/ml}$) (C). *, $P < 0.05$, t-test, significant differences between FK506 ($1 \mu\text{g/ml}$), MMF ($0.1 \mu\text{g/ml}$) and Vehicle control.

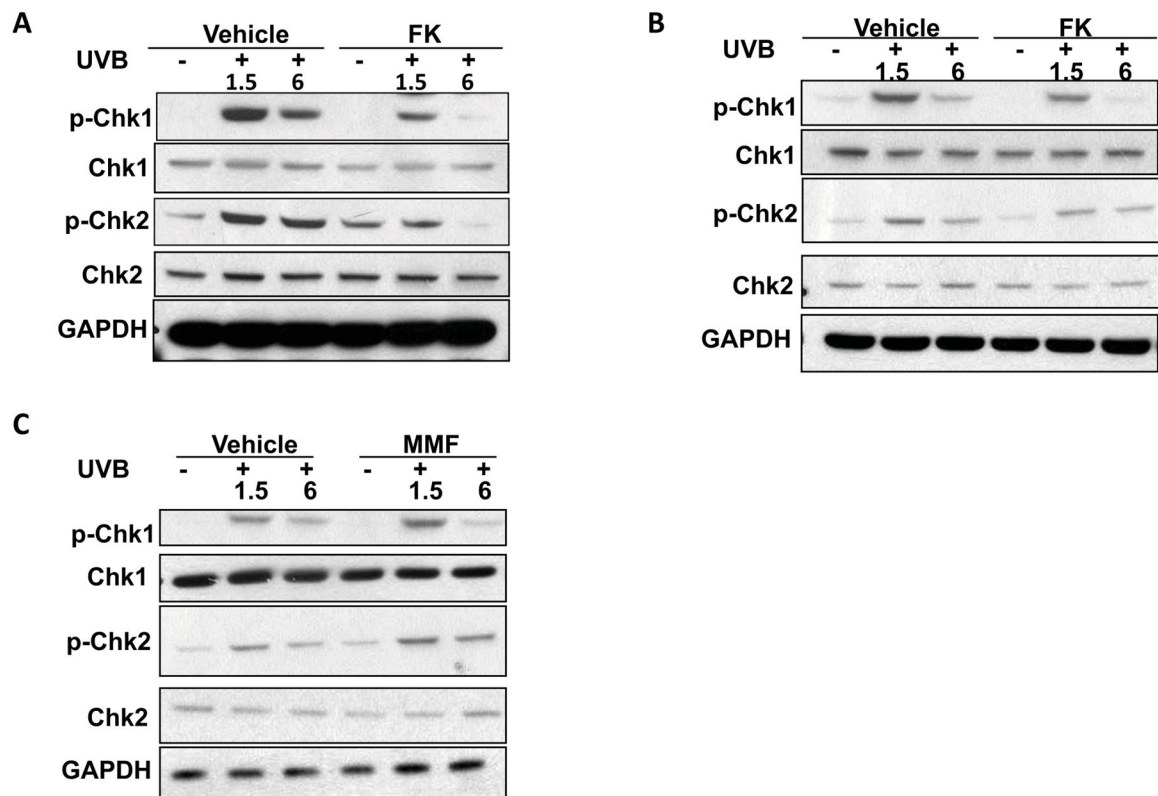


Figure 4. FK506 but not MMF inhibits UVB-induced checkpoint signaling in HaCaT cells
 (A–C) Immunoblot analysis of p-Chk1, Chk1, p-Chk2, Chk2, and GAPDH in HaCaT cells at 1.5 and 6 h post-UVB (20 mJ/cm²) prior to treatment with vehicle, FK506 (1 µg/ml) (A), FK506 (10 µg/ml) (B), or MMF (0.1 µg/ml) (C) for 1 week.