

ATR–Chk1 Pathway Inhibition Promotes Apoptosis after UV Treatment in Primary Human Keratinocytes: Potential Basis for the UV Protective Effects of Caffeine

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New approaches to prevent and reverse UV damage are needed to combat rising sunlight-induced skin cancer rates. Mouse studies have shown that oral or topical caffeine promotes elimination of UV-damaged keratinocytes through apoptosis and markedly inhibits subsequent skin cancer development. This potentially important therapeutic effect has not been studied in human skin cells. Here, we use primary human keratinocytes to examine which of several caffeine effects mediates this process. In these cells, caffeine more than doubled apoptosis after 75 mJ cm⁻² of ultraviolet light B (UVB). Selectively targeting two of caffeine's known effects did not alter UVB-induced apoptosis: inhibition of ataxia-telangiectasia mutated and augmentation of cyclic AMP levels. In contrast, siRNA against ataxia-telangiectasia and Rad3-related (ATR) doubled apoptosis after UV through a p53-independent mechanism. Caffeine did not further augment apoptosis after UVB in cells in which ATR had been specifically depleted, suggesting that a key target of caffeine in this effect is ATR. Inhibition of a central ATR target, checkpoint kinase 1 (Chk1), through siRNA or a new and highly specific inhibitor (PF610666) also augmented UVB-induced apoptosis. These data suggest that a relevant target of caffeine is the ATR–Chk1 pathway and that inhibiting ATR or Chk1 might have promise in preventing or reversing UV damage.

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INTRODUCTION

Nonmelanoma skin cancer is the most common form of cancer in humans, with an annual incidence of over one million new cases in the United States. Epidemiological and

molecular studies suggest that exposure to UV light is the most important etiological factor (Brash *et al.*, 2008). With an increase in recreational sun exposure, the use of UV tanning beds, and deterioration of the ozone layer, the incidence of nonmelanoma skin cancer is expected to increase. There is thus a need to identify and develop new approaches to target UV-induced skin cancers.

Much of the mutagenic and carcinogenic effects of UV radiation are a consequence of DNA damage in the form of cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts (Brash *et al.*, 2008). UV exposure activates diverse cellular responses in human cells, including cell cycle arrest, DNA repair, and apoptosis. To ameliorate the effects of UV radiation, cells rely on an intricate network of signal transduction pathways known as cell cycle checkpoints. Cell cycle checkpoints are biochemical surveillance pathways that arrest cell cycle progression pending the completion of essential events and/or the repair of damaged DNA (Sancar *et al.*, 2004). By coordinating cell cycle progression with DNA repair, cell cycle checkpoints help to maintain genetic stability and prevent carcinogenesis (Abraham, 2001). However, recent studies have suggested that cell cycle checkpoints that are operational in cancer cells may reduce the effectiveness of many chemotherapeutic agents that function by inducing DNA damage (Zhou and Bartek, 2004). Indeed, chemical or genetic inhibition of cell cycle

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Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related; Chk1, checkpoint kinase 1; HKC, human keratinocytes; PARP, poly(adenosine diphosphate-ribose) polymerase; PBS, phosphate-buffered saline; UVB, ultraviolet light B

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checkpoint function sensitizes cancer cells to a wide variety of DNA-damaging agents, including UV (Nghiem *et al.*, 2001), ionizing radiation (Syljuasen *et al.*, 2004), alkylating agents (Bunch and Eastman, 1996), and topoisomerase inhibitors (Shao *et al.*, 1997).

In humans, consumption of coffee or tea is associated with lower incidences of nonmelanoma skin cancers in several epidemiological studies (Jacobsen *et al.*, 1986; Hakim *et al.*, 2000; Abel *et al.*, 2007; Rees *et al.*, 2007). In a recent study of 93,676 Caucasian women, each additional cup of caffeinated coffee ingested was associated with a 5% decreased risk of developing nonmelanoma skin cancer, whereas drinking decaffeinated coffee showed no such benefit (Abel *et al.*, 2007). These studies imply that caffeine, one of the shared constituents of coffee and tea, might inhibit skin carcinogenesis in humans. To examine this possibility, the inhibitory effect of these beverages on skin carcinogenesis has been investigated in mice. Indeed, oral administration of regular tea but not decaffeinated tea was found to protect mice from UV carcinogenesis, which led to subsequent direct tests of caffeine's effects on this process (Huang *et al.*, 1997; Lou *et al.*, 1999). Hairless mice treated with UV twice weekly for 20 weeks had normal-appearing skin, but were at a higher risk for developing subsequent papillomas, keratoacanthomas, and squamous cell carcinomas (Lou *et al.*, 1999; Lu *et al.*, 2002b). This animal model resembles humans that are chronically exposed to UV early in life and have an increased risk of developing skin cancer later in life. Using this model, Conney and colleagues (Lou *et al.*, 1999; Lu *et al.*, 2002b) have shown that caffeine, when administered orally or topically, inhibits tumor formation in these high-risk mice.

One potential mechanism for the inhibitory effect of caffeine is the induction of apoptosis in UV-damaged keratinocytes. Importantly, even long after completion of UV exposure, topical application of caffeine selectively induced apoptosis in nonmalignant tumors and squamous cell carcinomas without affecting neighboring "normal" non-tumor mouse skin (Lu *et al.*, 2002b). Moreover, the effect of caffeine does not require p53, as p53 mutant patches of keratinocytes were selectively killed upon topical application of caffeine (Lu *et al.*, 2005b). Although caffeine is a known inhibitor of cAMP phosphodiesterase (Butcher and Sutherland, 1962), the inhibition of ataxia-telangiectasia mutated (ATM) kinase and in particular ataxia-telangiectasia and Rad3-related (ATR) kinase (Blasina *et al.*, 1999; Sarkaria *et al.*, 1999) might account for the observed effects of caffeine in the hairless mouse model (Lu *et al.*, 2008).

The response to UV in human keratinocytes (HKC) is significantly different from that of other cell types that are more easily grown and more widely used in UV studies (D'Errico *et al.*, 2003, 2005) as detailed in the Discussion. Therefore, it is important to investigate the response to UV in HKC, the relevant cellular target for UV-induced skin cancers. In addition, it is important to use the physiologically relevant wavelengths of UV (ultraviolet light B (UVB), 290–320 nm), which penetrate the atmosphere and are the major carcinogenic wavelengths of the light spectrum. The goals of this study were to test the effect of caffeine on

UVB-irradiated HKC and to determine the target of caffeine in this response.

We found that caffeine indeed augments UVB-induced apoptosis in primary cultures of HKC. We show that the relevant mechanism of caffeine is not likely to be its inhibitory effect on cAMP phosphodiesterase because a cell-permeable analog of cAMP had no effect on UVB-induced apoptosis. Instead, we found that the ATR-checkpoint kinase 1 (Chk1) pathway is the probable target of caffeine in this response because inhibition of ATR or Chk1 each phenocopied the effect of caffeine and augmented UVB-induced apoptosis in HKC. Consistent with the observed effects of caffeine in the mouse, we found that replication checkpoint inhibition in primary HKC augmented cell death in a p53-independent manner. To our knowledge, these are the first data to show that primary HKC are sensitized to apoptosis by ATR pathway inhibition and also the most detailed dissection of the mechanism by which caffeine eliminates UV-damaged cells.

RESULTS

Caffeine augments UVB-induced apoptosis in HKC

Treatment of primary HKC with 75 mJ cm^{-2} of UVB induced an apoptotic phenotype within 8 hours, including cleavage of caspase 3 (Figure 1a and Figure S1a). Additionally, UVB induced an increase in poly(adenosine diphosphate-ribose) polymerase (PARP) cleavage, a response that is dependent on caspase activation and the induction of apoptosis. Consistent with the activation of caspase 3, propidium iodide (Figure 1b and Figure S1b) and annexin V (Figure S1c) staining of UVB-treated HKC showed an increase in the percentage of cells undergoing apoptosis. Taken together, activation of the caspase cascade and an increase in the percentage of cells with fragmented DNA suggests that UVB induces apoptosis in HKC.

To investigate the effect of caffeine on the response of HKC to UVB, cells were treated with 2 mM caffeine 30 minutes before irradiation with UVB. Treatment with caffeine augmented UVB-induced cleavage of caspase 3 and PARP (Figure 1a). Using flow cytometry to quantitate apoptotic cells, the proportion of cells undergoing apoptosis increased from 10% in the UVB-treated keratinocytes to 24% in cells treated with caffeine (Figure 1b). Although the absolute number of cells undergoing UVB-induced apoptosis varied depending on the growth characteristics of the HKC culture (in particular, the passage number), we observed a reproducible two- to threefold increase in apoptosis when cells were pretreated with caffeine (Figure 1c).

To better understand the mechanism of the observed caffeine effect, we investigated the contribution of several known mechanisms of action for caffeine. Caffeine is an inhibitor of cAMP phosphodiesterase ($\text{IC}_{50} = 3 \text{ mM}$) (Butcher and Sutherland, 1962) and thus effectively increases levels of cAMP in cells. To determine if elevated levels of intracellular cAMP could phenocopy the observed effects of caffeine, HKC were pretreated with varying doses of dibutyryl cAMP, a cell-permeable analog of cAMP. Whereas caffeine augmented UVB-induced apoptosis in HKC, doses of dibutyryl cAMP

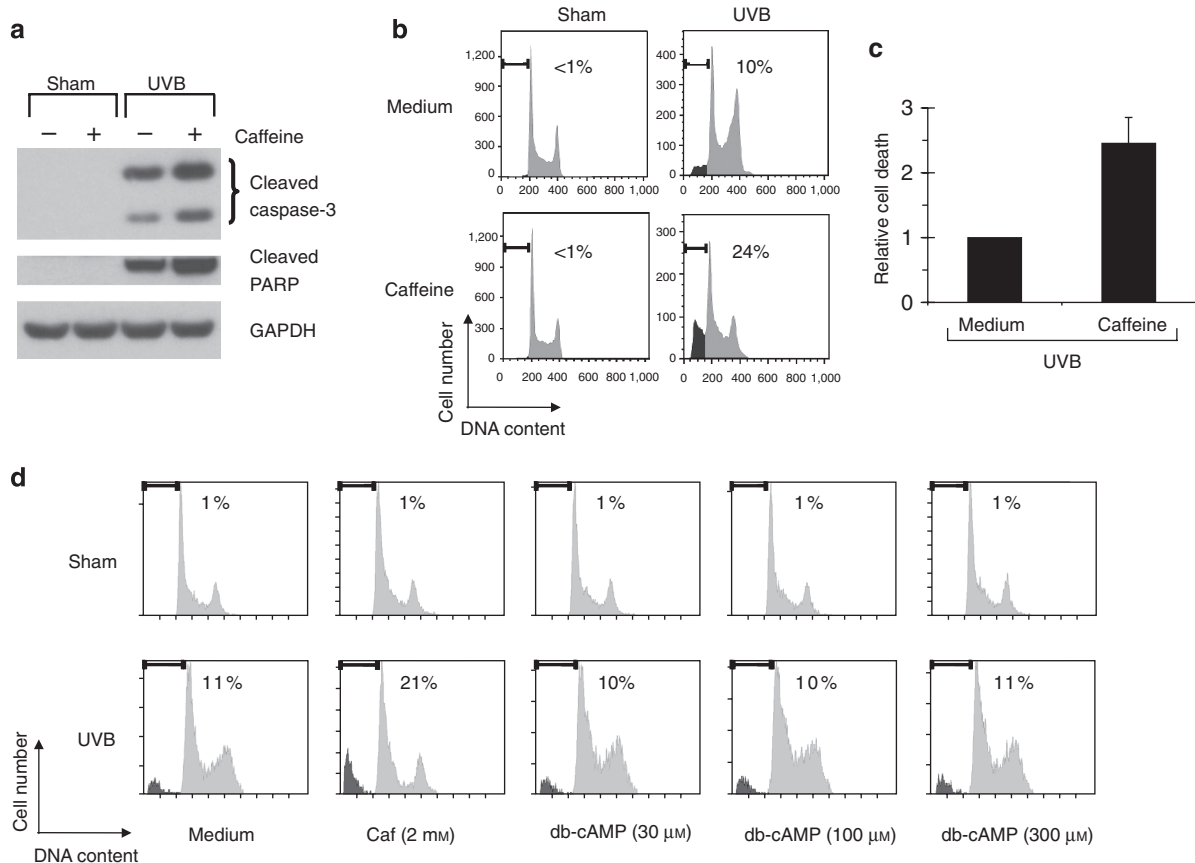


Figure 1. Caffeine augments UVB-induced apoptosis in human keratinocytes (HKC). (a–c) HKC were treated with vehicle (medium) or 2 mM of caffeine 30 minutes before 75 mJ cm⁻² of UVB irradiation. (a) Western blots using the indicated antibodies. Cells were harvested 8 hours after UVB irradiation. (b) Percentage of sub-2N DNA content measured by flow cytometry. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide. (c) Relative cell death was calculated by comparing percentage of sub-2N DNA content in caffeine/UVB-treated cells with that in medium/UVB-treated cells in each experiment. Average of relative cell death is shown (n = 4). Error bar, standard error of the mean. (d) Percentage of sub-2N DNA content measured by flow cytometry. HKC were treated with caffeine (Caf) or dibutyryl cyclic AMP (db-cAMP) at the indicated doses 30 minutes before 75 mJ cm⁻² of UVB irradiation. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide.

up to 300 μM had no effect (Figure 1d). It is highly likely that the effect of caffeine on apoptosis is not dependent on cAMP because processes that are cAMP dependent can typically be mimicked by significantly lower concentrations of dibutyryl cAMP (typically <100 μM) (Wang *et al.*, 2006b). These data suggest that caffeine augments UVB-induced apoptosis in HKC through a separate mechanism than cAMP augmentation through phosphodiesterase inhibition.

ATR siRNA mimics caffeine: inhibition of Chk1 phosphorylation and augmentation of apoptosis after UV treatment

To investigate whether caffeine inhibits ATR function in HKC following UVB treatment, we examined the phosphorylation of the ATR-dependent serine 345 residue of Chk1 in the presence and absence of caffeine. Indeed, treatment with 2 mM caffeine inhibited UVB-induced Chk1 phosphorylation at serine 345 (Figure 2a), suggesting that caffeine inhibits ATR under these conditions. To investigate a possible role for ATR in the response to UVB in HKC, we used siRNA to inhibit ATR expression. Keratinocytes were electroporated with siRNA against ATR or a nontargeting/scrambled control and

treated with UVB 48 hours later. siRNA against ATR reduced the abundance of ATR protein by >90% and interfered with ATR signaling as judged by marked attenuation of UVB-induced Chk1 phosphorylation (Figure 2b). Knockdown of ATR phenocopied the effects of caffeine in HKC and augmented the UVB-induced increase in caspase 3 and PARP cleavage (Figure 2b). To determine if the observed caffeine effect was through ATR inhibition, we treated ATR siRNA-electroporated HKC with medium or 2 mM caffeine as shown in Figure 2c. UVB treatment of HKC in which ATR had been inhibited by siRNA induced 50% apoptosis. However, there was no additive effect of caffeine on ATR-inhibited HKC (50% without caffeine, 48% with caffeine). The modest difference observed from 42% with caffeine alone to 48% with caffeine plus ATR-siRNA can be attributed to the more effective inhibition of ATR signaling through siRNA as compared with chemical inhibition by 2 mM caffeine (compare p-Chk1-Ser345 in Figure 2a and b at 1 hour time point). The lack of an additive effect of caffeine treatment on ATR siRNA-treated HKC suggests that the majority of the caffeine effect under these conditions is through ATR

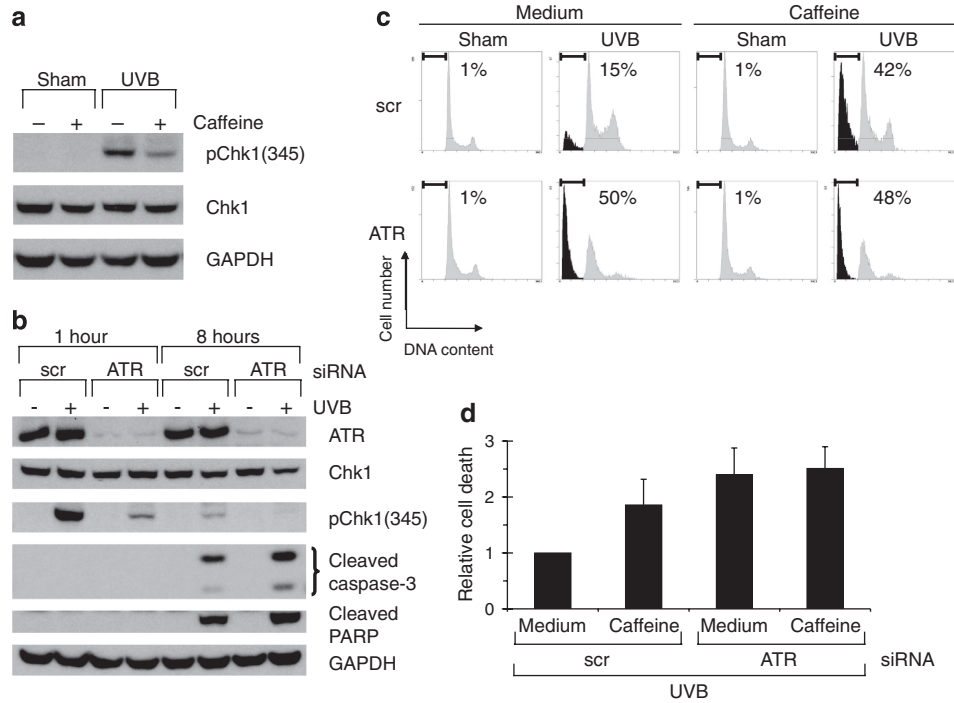


Figure 2. ATR siRNA mimics caffeine: inhibition of Chk1 phosphorylation and augmentation of apoptosis after UV treatment. (a) Western blots using the indicated antibodies. Human keratinocytes (HKC) were treated with vehicle (medium) or 2 mM of caffeine 30 minutes before 75 mJ cm⁻² of UVB irradiation. Cells were harvested 2 hours after UVB irradiation. (b-d) HKC were electroporated with siRNA against ATR or a nontargeting/scrambled control (scr). Forty-eight hours later, cells were exposed to 75 mJ cm⁻² of UVB. (b) Western blots using the indicated antibodies. Cells were harvested 1 or 8 hours after UVB irradiation as indicated. (c) Percentage of sub-2N DNA content measured by flow cytometry. Cells were treated with vehicle (medium) or 2 mM of caffeine 30 minutes before UVB irradiation. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide. (d) Relative cell death was calculated by comparing percentage of sub-2N DNA content in scr + caffeine/UVB-, ATR + medium/UVB-, or ATR + caffeine/UVB-treated cells with that in scr + medium/UVB-treated cells in each experiment. Average of relative cell death is shown (n=3). Error bar, standard error of the mean.

inhibition. Taken together, these data suggest that ATR is involved in the response to UVB in HKC and is a relevant target of caffeine.

ATM inhibition does not augment UVB-induced apoptosis

Caffeine inhibits not only ATR, but also ATM even more potently (IC₅₀ for ATM=0.2 mM, IC₅₀ for ATR=1.1 mM) (Sarkaria *et al.*, 1999). The fact that caffeine addition did not further increase UVB-induced apoptosis in HKC depleted of ATR by siRNA suggests that ATM is not involved in this UV-apoptosis pathway (Figure 2c and d). In addition, we depleted cells of ATM and/or ATR using siRNA to further investigate a possible role for ATM in this pathway. Knockdown of each protein kinase was monitored by western blot analysis (Figure 3a). Consistent with the findings in Figure 2, ATR knockdown once again potentiated the apoptotic effect of UVB (Figure 3). By targeting ATM with siRNA, protein expression was suppressed by roughly 70%, and the induction by UVB of active ATM (phospho-Serine 1981 ATM) was inhibited by roughly 50% (Figure S2). siRNA against ATM did not affect damage-induced phosphorylation of Thr-68 on Chk2 (a known target of ATM), but ATR is known to compensate for ATM in this process (Helt *et al.*, 2005; Wang *et al.*, 2006a). Indeed, double knockdown of ATM and ATR showed a marked suppression of Chk2

phosphorylation, suggesting that ATM function was significantly inhibited by siRNA in these cells (Figure S2). In no case, however, did the addition of siRNA against ATM affect UVB-induced apoptosis (Figure 3b and c). Taken together, these data suggest that ATM is not the relevant target of caffeine for this process in HKC.

Genetic and chemical inhibition of Chk1 augments UVB-induced apoptosis

In response to DNA damage, ATR mediates much of its checkpoint signaling through its downstream effector kinase Chk1. To investigate whether or not Chk1 inhibition would potentiate UVB-induced apoptosis in HKC, we abrogated Chk1 function both genetically and chemically. Electroporation of HKC with siRNA directed against Chk1 reduced protein expression by nearly 90% (Figure 4a). HKC with reduced expression of Chk1 were significantly sensitized to apoptosis induced by UVB (Figure 4a); however, Chk1 inhibition by siRNA did not augment UVB-induced apoptosis to the same degree as caffeine or ATR siRNA. We observed a 60% increase in cell death when comparing siRNA against Chk1 to scrambled siRNA controls.

Chemical inhibition of Chk1 has typically been carried out with UCN-01, originally identified as an inhibitor of protein kinase C (Wang *et al.*, 1996; Busby *et al.*, 2000). UCN-01 has

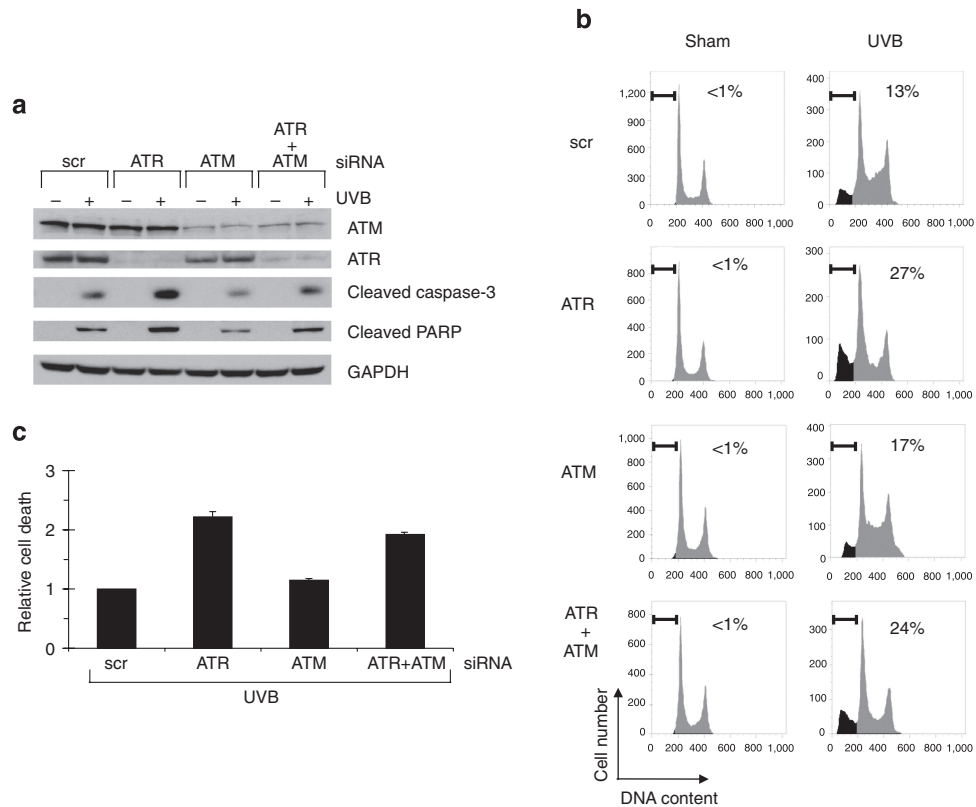


Figure 3. ATM inhibition does not augment UVB-induced apoptosis. HKC were electroporated with siRNA against ATR and/or ATM, or a nontargeting/scrambled control (scr). Forty-eight hours later, cells were exposed to 75 mJ cm^{-2} of UVB. (a) Western blots using the indicated antibodies. Cells were harvested 8 hours after UVB irradiation. (b) Percentage of sub-2N DNA content measured by flow cytometry. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide. (c) Relative cell death was calculated by comparing percentage of sub-2N DNA content in ATR/UVB-, ATM/UVB-, or ATR + ATM/UVB-treated cells with that in scr/UVB-treated cells in each experiment. Average of relative cell death is shown ($n = 3$). Error bar, standard error of the mean.

been shown to have inhibitory effects toward several protein kinases, including Chk1, protein kinase C, and cyclin-dependent kinases (CDKs). Thus a major effort in the field is to generate more selective Chk1 inhibitors. Here, we describe the characteristics and first use of a potent, highly selective Chk1 inhibitor, PF610666 (Figure 4b). The docking pose of PF610666 with the Chk1 enzyme (Figure 4b) was obtained by modifying the Chk1 cocrystal structure (Chen *et al.*, 2000) with PF279192, which differs only by a halogen atom that does not contact the active site (a chloride in PF610666 and a bromide in PF279192). The compound binds to the ATP-binding pocket through hydrogen bonds with Cys87 and Glu85 in the hinge region (Figure 4b, white asterisk on left). The phenylbutanamide tail (Figure 4b, white asterisk on right) is immediately adjacent to the key catalytic residue Asp148. In this manner, PF610666 prevents Chk1 from phosphorylating its protein substrates. The inhibitory effect of PF610666 was tested against a panel of human kinases and shown to be highly selective against Chk1 ($\text{IC}_{50} = 7 \text{ nM}$; Table 1). Effective concentration 50 (EC_{50}) of PF610666 for cellular activity measured as the ability to override the camptothecin-induced checkpoint was 70 nM .

To complement our genetic studies, we treated HKC with varying doses of PF610666. Treatment of HKC with PF610666 augmented UVB-induced PARP cleavage at 8 hours (Figure 4c) and increased the percentage of cells with sub-2N DNA content at 24 hours (Figure 4d). Taken together, genetic and chemical inhibition of Chk1 could each phenocopy the effect of caffeine and of ATR inhibition, providing further support for the importance of this pathway in the UV response and in the effect of caffeine on this response.

ATR-induced apoptosis is largely p53 independent in HKC

p53 is frequently mutated in nonmelanoma skin cancers, and therapies that induce apoptosis independent of p53 are of great interest. To investigate the contribution of p53 in the response of HKC to UVB, we inhibited p53 expression using siRNA. HKC were electroporated with siRNA against p53 and/or ATR, and then UV-irradiated 48 hours later (Figure 5a). Treatment of HKC with 75 mJ cm^{-2} UVB strongly induced p53 protein levels at 8 hours. Knockdown of p53 not only blocked this induction, but also suppressed p53 protein levels below the pre-UVB treatment baseline (Figure 5a). As in the previous experiments, knockdown of ATR markedly augmented the number of HKC that underwent UVB-induced

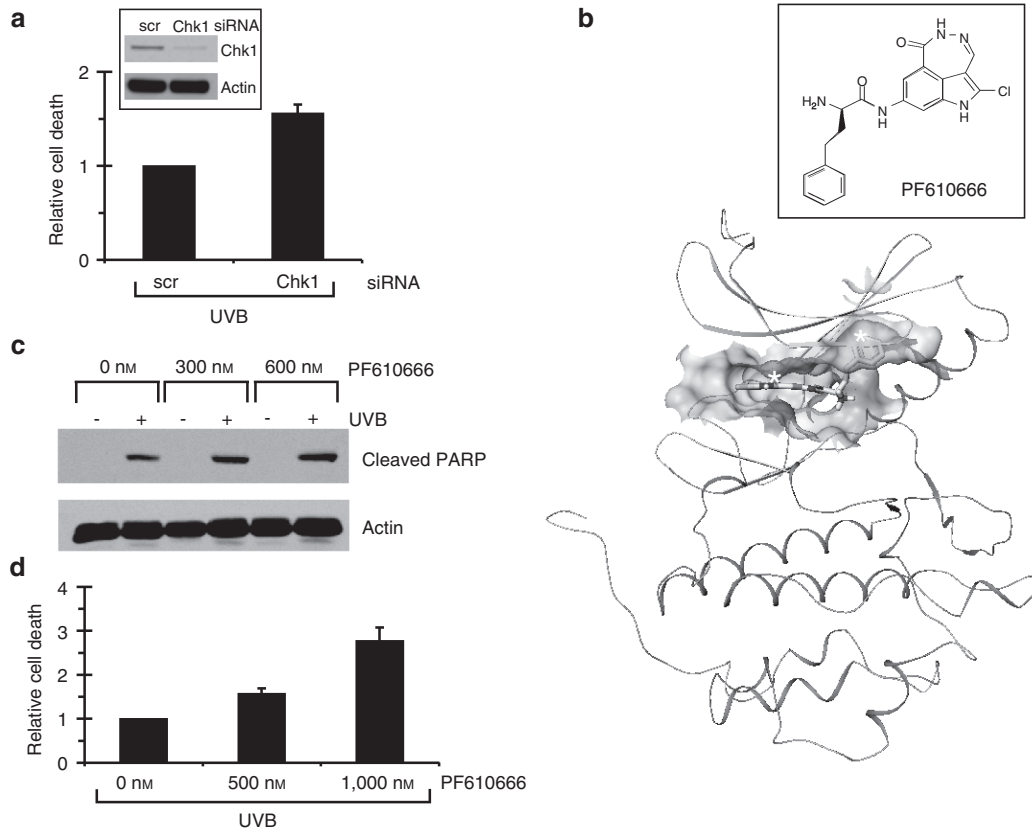


Figure 4. Genetic and chemical inhibition of Chk1 augments UVB-induced apoptosis. (a) HKC were electroporated with siRNA against Chk1 or a nontargeting/scrambled control (scr). Cells were exposed to 75 mJ cm^{-2} of UVB 48 hours after transfection. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide. Percentage of sub-2N DNA content was measured by flow cytometry. Relative cell death was calculated by comparing percentage of sub-2N DNA content in Chk1/UVB-treated cells with that in scr/UVB-treated cells in each experiment. Average of relative cell death is shown ($n=2$). Error bar, standard error of the mean. Western blots using the indicated antibodies are shown in inset. Cells were harvested 48 hours after transfection. (b) Front view of the docked pose of PF610666 into Chk1. See text for description of how the compound binds in the kinase catalytic domain. Chemical structure of PF610666 is shown in inset. (c) Western blots using the indicated antibodies. HKC were treated with PF610666 at the indicated doses 30 minutes before 75 mJ cm^{-2} of UVB irradiation. Cells were harvested 8 hours after UVB irradiation. (d) Relative cell death was calculated by comparing the percentage of cells with sub-2N DNA content in each experiment among cells that received UVB plus no PF610666 with those that received UVB plus 500 or 1,000 nM PF610666. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide. Percentage of sub-2N DNA content was measured by flow cytometry. Average of relative cell death is shown ($n=3$). Error bar, standard error of the mean.

apoptosis (Figure 5b and c). Blocking p53 protein induction, however, had no effect on UVB-induced apoptosis in HKC and only modestly reduced the ability of ATR inhibition to augment apoptosis.

DISCUSSION

In this study, we have used the relevant primary human cell type to examine the mechanism by which caffeine affects the response to UV. We found that genetic or chemical inhibition of the ATR–Chk1 pathway augmented UVB-induced apoptosis in a largely p53-independent manner in HKC. In contrast, other known and plausible targets of caffeine were not involved in the UV response, including ATM and the regulation of cAMP levels. Our finding that cAMP levels were not involved in UV signaling in HKC agrees well with a previous study that concluded that augmenting cAMP does not mimic the effects of caffeine on lowering UV-induced carcinogenesis in mouse skin (Zajdela and Latarjet, 1978).

The effects of caffeine on skin cancer are of interest due to human epidemiological studies linking coffee and tea consumption with lower rates of nonmelanoma skin cancer (Jacobsen *et al.*, 1986; Hakim *et al.*, 2000; Abel *et al.*, 2007; Rees *et al.*, 2007), as well as animal studies showing that caffeine inhibits tumor formation in mice chronically treated with UV (Zajdela and Latarjet, 1978; Huang *et al.*, 1997; Lu *et al.*, 2002b). We have shown previously that topical caffeine application to mouse skin after a single dose of UVB augmented the number of apoptotic keratinocytes as evaluated by sunburn cell formation and other markers of programmed cell death (Lu *et al.*, 2002a; Koo *et al.*, 2007). These findings suggest that topical application of caffeine to mouse skin after UV irradiation augments the deletion of DNA-damaged keratinocytes, and may provide protection from UV-induced skin cancer development.

Nonmelanoma skin cancers arise through the transformation of keratinocytes induced by UV. Thus, it is important to

Table 1. IC50 values of PF610666 and UCN-01 for selected kinases

Kinase analyzed	IC50 (nM)	
	PF610666	UCN-01
CHK1	7	11
CHK2	>1,000	1,040
CDK1	>1,000	31
CDK2	>1,000	30
PKC	>1,000	7
RAF	>1,000	620
JNK	>1,000	ND
MEK1	>1,000	ND
PLK3	>1,000	ND
mTOR	>1,000	ND

CDK, cyclin-dependent kinase; CHK, checkpoint kinase; JNK, c-Jun N-terminal kinase; MEK1, mitogen activated protein kinase/extracellular signal-regulated kinase kinase-1; mTOR, mammalian target of rapamycin; ND, not determined; PKC, protein kinase C; PLK3, polo-like kinase 3.

The concentration of the indicated compound at which the maximum *in vitro* activity of the indicated kinase has been inhibited to 50% (IC50) is shown in nM. IC50 values for PF610666 were determined as described in "Materials and Methods". IC50 values for UCN-01 were obtained from previous studies (Kawakami *et al.*, 1996; Busby *et al.*, 2000). "IC50 > 1,000 nM" means that the kinase inhibition was less than 50% at 1,000 nM.

The fraction of maximum *in vitro* kinase activity that was inhibited for each of the following kinases in the presence of 1 μ M PF610666 is listed below ("100%" means kinase activity was entirely blocked by 1 μ M of the compound): 100% for Chk1, 38% for Chk2, 14% for Raf, and less than 10% for each of these kinases: CDK1, CDK2, PKC, JNK, MEK1, PLK3, and mTOR.

investigate the cellular responses to UVB in the relevant cell type that gives rise to UV-induced skin cancers. A recent study comparing primary HKC and dermal fibroblasts showed that keratinocytes are far more susceptible to UVB-induced apoptosis than fibroblasts; yet they are more UVB-resistant than fibroblasts in terms of their proliferative ability as measured by colony survival assays (D'Errico *et al.*, 2003). Moreover, keratinocytes have a greater ability (through robust global genome repair) for UV-DNA repair than fibroblasts, as might be expected from cells that are "professional" UV-responsive cells (D'Errico *et al.*, 2005). Indeed, we found that caffeine markedly augmented UVB-induced apoptosis in HKC (Figure 1). This finding suggests that the promotion of UVB-apoptosis by caffeine described in mouse keratinocytes also occurs in human primary keratinocytes.

Caffeine concentration is an important issue when comparing studies in mice and humans. Oral administration of caffeine as drinking fluid (0.4 mg ml⁻¹) inhibits UV-induced skin carcinogenesis in mice (Huang *et al.*, 1997). The plasma and epidermal concentrations of caffeine in mice receiving the cancer-preventive dose of caffeine in drinking water (0.4 mg ml⁻¹, 2 mM) is 16 μ M and 16 nmol g⁻¹ tissue, respectively, on average in a 24-hour period (Conney *et al.*, 2007). The observed plasma concentration of caffeine in mice is

consistent with the plasma concentration in humans (3.2 mg l⁻¹, 16 μ M) drinking 2-5 cups of coffee per day (de Leon *et al.*, 2003). Recently, Conney and colleagues (Lu *et al.*, 2008) showed that caffeine administered orally (0.4 mg ml⁻¹, 2 mM) with drinking water markedly inhibited the UVB-induced phosphorylation of Chk1 on Ser345 in the epidermis of mice. As Ser345 phosphorylation of Chk1 has been shown to be highly dependent on ATR function, this means that, *in vivo* in the mouse, plasma concentrations of 16 μ M appear to be sufficient to inhibit ATR function in keratinocytes. Our *in vitro* studies using keratinocytes are similar to other studies using cells in culture in which effective ATR inhibition requires caffeine doses closer to those required in cell-free *in vitro* kinase assays where the IC50 against ATR is 1.1 mM for caffeine (Sarkaria *et al.*, 1999). These differences in the ability of caffeine to inhibit ATR function *in vitro* versus *in vivo* have yet to be explained but may be related to the nonphysiological conditions that are required to initiate ATR activity *in vitro* (Sarkaria *et al.*, 1999).

We also investigated the involvement of Chk1, which is phosphorylated by ATR in response to UV. These experiments were carried out because, if ATR is the relevant target of caffeine, its effects would most likely be mediated by Chk1 and also because previously unknown specific Chk1 inhibitors are on the horizon for human therapy. UV irradiation induced phosphorylation of Chk1 maximally at 1 hour, and the phosphorylation signal was nearly lost by 8 hours (Figure 2b and Figure S1a). Similar Chk1 phosphorylation kinetics have been observed in a recent study and attributed to PPM1D, a serine/threonine phosphatase, whose expression is transcriptionally induced shortly after UV irradiation, and which dephosphorylates phospho-Chk1 at later time points (8 hours or later post-UV) (Lu *et al.*, 2005a).

Augmentation of UVB-induced apoptosis by siRNA against Chk1 (Figure 4a) was not as robust as that by siRNA against ATR (Figure 2d). A possible explanation is that incomplete Chk1 knockdown by siRNA allowed sufficient Chk1 function to partially activate the checkpoint. Another possibility is that ATR targets other than Chk1 also play a role in UV-induced apoptosis, and ATR knockdown suppresses their function as well as the function of Chk1. Similarly, Chk1 knockdown was not as strong as 1 μ M PF610666 (Figure 4d) in augmenting UVB-induced apoptosis, and this may also be due to more effective inhibition of Chk1 by PF610666 or by this compound inhibiting kinases in addition to Chk1 (Chk2 and other kinases are partially inhibited *in vitro* at this concentration of PF610666; Table 1).

Pharmacological inhibition of Chk1 has been extensively used to study the contribution of Chk1 in the cellular responses to DNA damage. UCN-01, originally identified as an inhibitor of protein kinase C, has been shown to be a potent Chk1 inhibitor (Wang *et al.*, 1996; Busby *et al.*, 2000). Studies have shown that UCN-01 abrogates Chk1-dependent cellular responses and sensitizes cancer cells to several DNA-damaging agents, including mitomycin C (Akinaga *et al.*, 1993), cisplatin (Bunch and Eastman, 1996), camptothecin (Shao *et al.*, 1997), and 5-fluorouracil (Hsueh *et al.*, 1998). Owing to the known off-target inhibitory effects (that is,

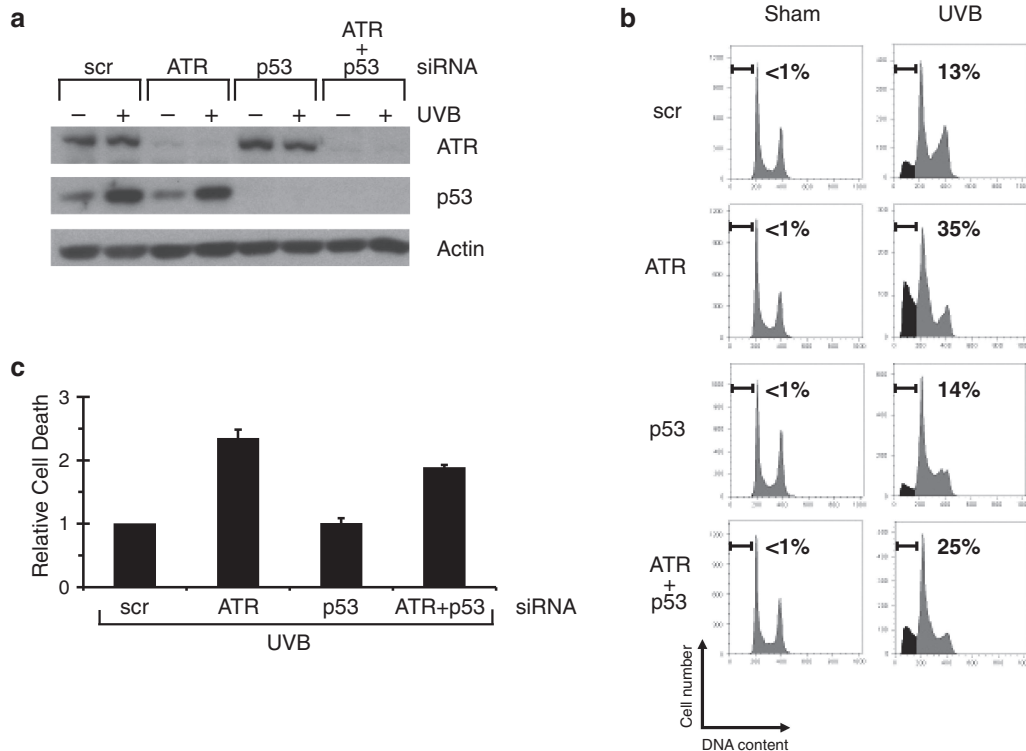


Figure 5. ATR-induced apoptosis is largely p53 independent in human keratinocytes (HKC). HKC were electroporated with siRNA against ATR and/or p53, or a nontargeting/scrambled control (scr). Forty-eight hours later, cells were exposed to 75 mJ cm⁻² of UVB. (a) Western blots using the indicated antibodies. Cells were harvested 8 hours after UVB irradiation. (b) Percentage of sub-2N DNA content measured by flow cytometry. Cells were harvested 24 hours after UVB irradiation and stained with propidium iodide. (c) Relative cell death was calculated by comparing percentage of sub-2N DNA content in ATR/UVB-, p53/UVB-, or ATR + p53/UVB-treated cells with that in scr/UVB-treated cells in each experiment. Average of relative cell death is shown (n=3). Error bar, standard error of the mean.

protein kinase C and CDKs) of UCN-01 (Kawakami *et al.*, 1996; Busby *et al.*, 2000) and an interest in therapeutic applications for Chk1 inhibition, previously unknown inhibitors of this kinase have been developed. Here, we show that a newly developed compound that is far more selective than UCN-01 for Chk1 inhibition (Table 1) potently augmented UVB-induced apoptosis (Figure 4c and d). The ability of this compound to potentiate the effects of DNA damage has led to the development of its sister compound as a cancer therapeutic (Blasina *et al.*, 2008).

Previous studies in human cancer cell lines (Nghiem *et al.*, 2001) and mouse skin (Lu *et al.*, 2004, 2005b) have shown that p53 disruption sensitizes cells to inhibition of ATR. In contrast, we found that p53 knockdown did not augment UVB-induced apoptosis by ATR inhibition in HKC (Figure 5b and c). This discrepancy might be explained by one or more of the following possibilities: (i) apoptosis-signaling mechanisms may differ between these cell types; (ii) differences in methodology—p53 expression itself was suppressed in this study, whereas p53 function was disrupted through overexpression of MDM2 or E6 in the human cancer cell line study. What is clear and in agreement between these studies is that the majority of the apoptosis augmentation by ATR inhibition is p53 independent.

Normal human skin carries clonal patches of p53-mutated keratinocytes in as much as 4% of the epidermis representing

thousands of such clones per person (Jonason *et al.*, 1996). Sun exposure increases the frequency and size of the p53-mutant clones in skin. These findings suggest that p53-mutant clones might be resistant to UV-induced apoptosis. There is thus a need for a p53-independent therapy against UV-damaged keratinocytes. Topical applications of caffeine to mice with multiple patches of p53-mutant skin cells enhanced the elimination of these cells in the absence of further UVB irradiation (Lu *et al.*, 2005b). Here, we have shown that inhibition of the ATR-Chk1 pathway by caffeine or a selective Chk1 inhibitor promotes culling of DNA-damaged human primary keratinocytes regardless of p53 status. These are the most detailed studies yet to determine the mechanism by which caffeine augments UV apoptosis. These data suggest that topical application of caffeine or another ATR-Chk1 pathway inhibitor, perhaps in a sunscreen or after-sun preparation, could be investigated as an approach to minimize or reverse the effects of UV damage in human skin.

MATERIALS AND METHODS

Cell lines and culture conditions

Normal HKC were isolated from adult abdominal epidermis harvested from two female patients (age 37 and 66) as described previously (Kitano and Okada, 1983; Normand and Karasek, 1995; Boswell *et al.*, 2007) in a protocol approved by the Massachusetts General Hospital Institutional Review Board. Written, informed patient consent was

obtained in adherence to the Helsinki Guidelines. Briefly, fresh specimens were washed in phosphate-buffered saline (PBS) and disinfected with 70% ethanol. After removing adipose and dermal tissue, the remaining skin was digested in dispase solution (Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) containing 10 mM HEPES, 0.075% sodium bicarbonate, and 50 $\mu\text{g ml}^{-1}$ gentamicin mixed 1:1 with Dispase II solution (Roche, Indianapolis, IN)) at 4°C for 18 hours. The epidermis was then peeled off and trypsinized to isolate the keratinocytes, which were plated in Defined Keratinocyte-SFM (serum-free medium) (Invitrogen) on collagen I (Inamed, Fremont, CA)-coated plates and maintained at 37°C in a humidified atmosphere of 5% CO₂. The stock culture was aliquoted, frozen, and thawed as needed. Experiments were conducted on logarithmically growing HKC between passages 2 and 5.

Chemicals

Caffeine (Sigma, St Louis, MO) was dissolved in PBS to a final concentration of 100 mM. Thirty minutes before UVB treatment, the appropriate volume of caffeine was added to culture medium to a final concentration of 2 mM. N⁶,2'-O-Dibutyryladenine 3',5'-cyclic monophosphate sodium salt (dibutyryl cAMP) (Sigma) was dissolved in purified water to a final concentration of 100 mM. Thirty minutes before UVB treatment, the appropriate volume of dibutyryl cAMP was added to culture medium. PF610666 was synthesized by Pfizer (San Diego, CA). PF610666 was dissolved in DMSO and stored at room temperature. Thirty minutes before UVB treatment, the appropriate volume of PF610666 or vehicle (DMSO) was added to culture medium.

PF610666 inhibitor potency and kinase selectivity

The inhibitory potency of PF610666 against the human, recombinant Chk1 kinase domain was determined in a biochemical activity-based assay. Chk1 activity was measured by a pyruvate kinase-lactate dehydrogenase coupled, continuous spectrophotometric assay where the phosphorylation of a Chk1 peptide substrate (Syntide-2, PLARTLS-VAGLPGKK) was coupled to the oxidation of NADH, and the corresponding change in absorbance intensity was measured at 340 nm using a SpectraMax plate reader (Molecular Devices Corp, Sunnyvale, CA). The assay was performed in a 96-well plate for 20 minutes at 30°C in 0.1 ml of assay buffer containing 50 mM Tris pH 7.5, 0.4 M NaCl, 4 mM PEP, 0.15 mM NADH, 28 U of lactate dehydrogenase per ml, 16 U of pyruvate kinase per ml, 3 mM DTT, 0.125 mM Syntide-2, 0.15 mM ATP, and 25 mM magnesium chloride. Assays were initiated with 1 nM of Chk1 kinase domain. The inhibition of Chk1 activity was determined by measuring initial velocities in the presence of varying concentrations of PF610666 (Blasina *et al.*, 2008). The data were analyzed using Enzyme Kinetic and Excel software and fit to a kinetic model for competitive inhibition to obtain a K_i value. The kinase selectivity of PF610666 was evaluated by screening the compound at 1 or 10 μM against a panel of about 100 protein kinases. These kinase selectivity screens were performed in-house at Pfizer or by the kinase screening laboratories at Upstate/Millipore and the Division of Signal Transduction Therapy, University of Dundee. Effective concentration 50 (EC₅₀) was defined as the concentration of a Chk1 inhibitor that overcame a camptothecin-induced S-phase checkpoint and caused mitosis (as measured by histone H3 phosphorylation at Ser10 in dot blot assay) in 50% of the cell population as compared with a nocodazole-only treated positive control.

RNA interference

SMARTpool siRNAs were synthesized by Dharmacon (Lafayette, CO) against human ATM, p53, and Chk1. A siRNA duplex against human p53 was also synthesized using the following target sequence: AAGACTCCAGTGGTAATCTAC. ATR knockdown was accomplished using a siRNA duplex against the following target sequence: AACCTCCGTGATGTTGCTTGA. A siCONTROL nontargeting siRNA (Dharmacon) was used as a nonspecific control. Cells were electroporated by Nucleofector I Device (Amaxa, Gaithersburg, MD) using human dermal fibroblast nucleofector kit and program T-24. Cells were harvested by trypsinization, resuspended in nucleofector solution at approximately 1–4 $\times 10^6$ cells per 100 μl of solution, transfected with 140 picomoles of siRNA, and split to the appropriate number of collagen I-coated plates. Cells were allowed to recover for 48 hours before treatment. Knockdown of targeted gene products was monitored by western immunoblot analysis.

UV irradiation

Before treatment with UVB, culture medium (containing caffeine, dibutyryl cAMP, or PF610666 as indicated) was removed and reserved. Cultures were washed once with warm PBS and then placed uncovered under a panel of four UVB bulbs (RPR-3000, Southern New England Ultraviolet, Branford, CT), emitting radiation centered around 311 nm. A Kodacel filter (K6808, Eastman Kodak, Rochester, NY) was used to eliminate any ultraviolet light C (<290 nm). Cells were exposed to 75 mJ cm^{-2} of UVB. UV dose was monitored with a Photolight IL1400A radiometer equipped with a SEL240/UVB detector (International Light Technologies, Peabody, MA). Following irradiation, the reserved medium was replaced, and the cultures were incubated for the indicated periods of time. Sham-treated cultures were handled exactly the same way, except that they were not exposed to UVB.

Western immunoblot analyses

Cells were harvested by trypsinization, washed once in PBS, and resuspended in RIPA (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate) supplemented with Complete Protease Inhibitor Cocktail (Roche). After clarifying the extract by centrifugation, protein concentration was determined using the Bradford Assay Reagent (Bio-Rad, Hercules, CA). Samples containing equal amounts of protein were mixed with 4 \times NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) containing 5% β -mercaptoethanol, boiled, and separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and probed with antibodies against Chk1 (G-4), actin (I-19) (both from Santa Cruz Biotechnology, Santa Cruz, CA); Chk2 (Ab-1) (NeoMarkers, Fremont, CA); p53 (1C12), phospho-Chk1 (Ser345) (133D3), phospho-Chk2 (Thr68), phospho-ATM (Ser1981) (10H11.E12), cleaved PARP (Asp214) (19F4), cleaved caspase-3 (Asp175) (5A1) (all from Cell Signaling Technology, Danvers, MA); GAPDH (ab9485) (Abcam, Cambridge, MA); ATM (2C1) (GeneTex, San Antonio, TX), and an ATR rabbit polyclonal antibody generated using a peptide spanning amino acids 1–20 of human ATR (Nghiem *et al.*, 2001).

Flow cytometry

For measuring sub-2N DNA content, cells were trypsinized and harvested 24 hours post-UV irradiation and fixed with 70% ethanol for 1 hour or overnight at 4°C. Cells were washed once with PBS,

treated with 1 mg ml⁻¹ ribonuclease A (Sigma) for 30 minutes at 37°C, and stained with 50 µg ml⁻¹ propidium iodide (Sigma). For measuring annexin V-positive cells, cells were stained with annexin V-FITC (Assay Designs, Ann Arbor, MI) following the manufacturer's protocol. Cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA) or Cytomics FC 500 (Beckman Coulter, Fullerton, CA) flow cytometer. The acquired data were analyzed using the FlowJo 6.3.4 software package (Tree Star Inc., Ashland, OR) or CXP Analysis 2.2 (Beckman Coulter).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Time course of UVB-induced apoptosis in human keratinocytes.

Figure S2. ATR/ATM-dependent signaling following UVB treatment.

REFERENCES

- Abel EL, Hendrix SO, McNeeley SG, Johnson KC, Rosenberg CA, Mossavar-Rahmani Y *et al.* (2007) Daily coffee consumption and prevalence of nonmelanoma skin cancer in Caucasian women. *Eur J Cancer Prev* 16:446-52
- Abraham RT (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* 15:2177-96
- Akinaga S, Nomura K, Gomi K, Okabe M (1993) Enhancement of antitumor activity of mitomycin C *in vitro* and *in vivo* by UCN-01, a selective inhibitor of protein kinase C. *Cancer Chemother Pharmacol* 32:183-9
- Blasina A, Hallin J, Chen E, Arango ME, Kraynov E, Register J *et al.* (2008) Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1. *Mol Cancer Ther* 7:2394-404
- Blasina A, Price BD, Turenne GA, McGowan CH (1999) Caffeine inhibits the checkpoint kinase ATM. *Curr Biol* 9:1135-8
- Boswell SA, Ongusaha PP, Nghiem P, Lee SW (2007) The protective role of a small GTPase RhoE against UVB-induced DNA damage in keratinocytes. *J Biol Chem* 282:4850-8
- Brash DE, Heffernan T, Nghiem P (2008) Carcinogenesis: ultraviolet radiation. In: *Fitzpatrick's Dermatology in General Medicine*. Wolff K, Goldsmith LA, Katz SL, Gilchrist BA, Paller AS, Leffell DJ, (eds). 7th edn, vol. 1. McGraw-Hill: New York, pp 999-1006
- Bunch RT, Eastman A (1996) Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G2-checkpoint inhibitor. *Clin Cancer Res* 2:791-7
- Busby EC, Leistritz DF, Abraham RT, Karnitz LM, Sarkaria JN (2000) The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. *Cancer Res* 60:2108-12
- Butcher RW, Sutherland EW (1962) Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine. *J Biol Chem* 237:1244-50
- Chen P, Luo C, Deng Y, Ryan K, Register J, Margosiak S *et al.* (2000) The 1.7 Å crystal structure of human cell cycle checkpoint kinase Chk1: implications for Chk1 regulation. *Cell* 100:681-92
- Conney AH, Zhou S, Lee MJ, Xie JG, Yang CS, Lou YR *et al.* (2007) Stimulatory effect of oral administration of tea, coffee or caffeine on UVB-induced apoptosis in the epidermis of SKH-1 mice. *Toxicol Appl Pharmacol* 224:209-13
- D'Errico M, Teson M, Calcagnile A, Nardo T, De Luca N, Lazzari C *et al.* (2005) Differential role of transcription-coupled repair in UVB-induced response of human fibroblasts and keratinocytes. *Cancer Res* 65:432-8
- D'Errico M, Teson M, Calcagnile A, Proietti De Santis L, Nikaido O, Botta E *et al.* (2003) Apoptosis and efficient repair of DNA damage protect human keratinocytes against UVB. *Cell Death Differ* 10:754-6
- de Leon J, Diaz FJ, Rogers T, Browne D, Dinsmore L, Ghosheh OH *et al.* (2003) A pilot study of plasma caffeine concentrations in a US sample of smoker and nonsmoker volunteers. *Prog Neuropsychopharmacol Biol Psychiatry* 27:165-71
- Hakim IA, Harris RB, Weisgerber UM (2000) Tea intake and squamous cell carcinoma of the skin: influence of type of tea beverages. *Cancer Epidemiol Biomarkers Prev* 9:727-31
- Helt CE, Cliby WA, Keng PC, Bambara RA, O'Reilly MA (2005) Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. *J Biol Chem* 280:1186-92
- Hsueh CT, Kelsen D, Schwartz GK (1998) UCN-01 suppresses thymidylate synthase gene expression and enhances 5-fluorouracil-induced apoptosis in a sequence-dependent manner. *Clin Cancer Res* 4:2201-6
- Huang MT, Xie JG, Wang ZY, Ho CT, Lou YR, Wang CX *et al.* (1997) Effects of tea, decaffeinated tea, and caffeine on UVB light-induced complete carcinogenesis in SKH-1 mice: demonstration of caffeine as a biologically important constituent of tea. *Cancer Res* 57:2623-9
- Jacobsen BK, Bjelke E, Kvale G, Heuch I (1986) Coffee drinking, mortality, and cancer incidence: results from a Norwegian prospective study. *J Natl Cancer Inst* 76:823-31
- Jonason AS, Kunala S, Price GJ, Restifo RJ, Spinelli HM, Persing JA *et al.* (1996) Frequent clones of p53-mutated keratinocytes in normal human skin. *Proc Natl Acad Sci USA* 93:14025-9
- Kawakami K, Futami H, Takahara J, Yamaguchi K (1996) UCN-01, 7-hydroxyl-staurosporine, inhibits kinase activity of cyclin-dependent kinases and reduces the phosphorylation of the retinoblastoma susceptibility gene product in A549 human lung cancer cell line. *Biochem Biophys Res Commun* 219:778-83
- Kitano Y, Okada N (1983) Separation of the epidermal sheet by dispase. *Br J Dermatol* 108:555-60
- Koo SW, Hirakawa S, Fujii S, Kawasumi M, Nghiem P (2007) Protection from photodamage by topical application of caffeine after ultraviolet irradiation. *Br J Dermatol* 156:957-64
- Lou YR, Lu YP, Xie JG, Huang MT, Conney AH (1999) Effects of oral administration of tea, decaffeinated tea, and caffeine on the formation and growth of tumors in high-risk SKH-1 mice previously treated with ultraviolet B light. *Nutr Cancer* 33:146-53
- Lu X, Nannenga B, Donehower LA (2005a) PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. *Genes Dev* 19:1162-74
- Lu YP, Lou YR, Li XH, Xie JG, Lin Y, Shih WJ *et al.* (2002a) Stimulatory effect of topical application of caffeine on UVB-induced apoptosis in mouse skin. *Oncol Res* 13:61-70
- Lu YP, Lou YR, Liao J, Xie JG, Peng QY, Yang CS *et al.* (2005b) Administration of green tea or caffeine enhances the disappearance of UVB-induced patches of mutant p53 positive epidermal cells in SKH-1 mice. *Carcinogenesis* 26:1465-72
- Lu YP, Lou YR, Peng QY, Xie JG, Conney AH (2004) Stimulatory effect of topical application of caffeine on UVB-induced apoptosis in the epidermis of p53 and Bax knockout mice. *Cancer Res* 64:5020-7
- Lu YP, Lou YR, Peng QY, Xie JG, Nghiem P, Conney AH (2008) Effect of caffeine on the ATR/Chk1 pathway in the epidermis of UVB-irradiated mice. *Cancer Res* 68:2523-9
- Lu YP, Lou YR, Xie JG, Peng QY, Liao J, Yang CS *et al.* (2002b) Topical applications of caffeine or (-)-epigallocatechin gallate (EGCG) inhibit carcinogenesis and selectively increase apoptosis in UVB-induced skin tumors in mice. *Proc Natl Acad Sci USA* 99:12455-60
- Nghiem P, Park PK, Kim Y, Vaziri C, Schreiber SL (2001) ATR inhibition selectively sensitizes G1 checkpoint-deficient cells to lethal premature chromatin condensation. *Proc Natl Acad Sci USA* 98:9092-7

- Normand J, Karasek MA (1995) A method for the isolation and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin. *In Vitro Cell Dev Biol Anim* 31:447-55
- Rees JR, Stukel TA, Perry AE, Zens MS, Spencer SK, Karagas MR (2007) Tea consumption and basal cell and squamous cell skin cancer: results of a case-control study. *J Am Acad Dermatol* 56:781-5
- Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 73:39-85
- Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM *et al.* (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* 59:4375-82
- Shao RG, Cao CX, Shimizu T, O'Connor PM, Kohn KW, Pommier Y (1997) Abrogation of an S-phase checkpoint and potentiation of camptothecin cytotoxicity by 7-hydroxystaurosporine (UCN-01) in human cancer cell lines, possibly influenced by p53 function. *Cancer Res* 57:4029-35
- Syljuasen RG, Sorensen CS, Nylandsted J, Lukas C, Lukas J, Bartek J (2004) Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing radiation. *Cancer Res* 64:9035-9040
- Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA, O'Connor PM (1996) UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst* 88:956-65
- Wang XQ, Redpath JL, Fan ST, Stanbridge EJ (2006a) ATR dependent activation of Chk2. *J Cell Physiol* 208:613-9
- Wang Y, Kim PK, Peng X, Loughran P, Vodovotz Y, Zhang B *et al.* (2006b) Cyclic AMP and cyclic GMP suppress TNF α -induced hepatocyte apoptosis by inhibiting FADD up-regulation via a protein kinase A-dependent pathway. *Apoptosis* 11:441-51
- Zajdela F, Latarjet R (1978) Ultraviolet light induction of skin carcinoma in the mouse; influence of cAMP modifying agents. *Bull Cancer* 65:305-13
- Zhou BB, Bartek J (2004) Targeting the checkpoint kinases: chemosensitization versus chemoprotection. *Nat Rev Cancer* 4:216-25