Inhibition of Cell Cycle Progression by the Novel Cyclophilin Ligand Sanglifehrin A Is Mediated through the NFκB-dependent Activation of p53

Ling-Hua Zhang, Hong-Duk Youn, and Jun O. Liu‡

From the Department of Pharmacology and Molecular Sciences and Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Center for Cancer Research, Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Sanglifehrin A belongs to a novel family of immunophilin-binding ligands. Sanglifehrin A is similar to cyclosporin A in that it binds to cyclophilins. Unlike cyclosporin A, however, the cyclophilin-sanglifehrin A complex has no effect on the calcium-dependent protein phosphatase calcineurin. It has been previously shown that sanglifehrin A specifically blocks T cell proliferation in response to interleukin 2 by inhibiting the appearance of cell cycle kinase activity cyclinE-Cdk2. How sanglifehrin A treatment leads to the cell cycle blockade has remained unknown. We report that sanglifehrin A is capable of activating the tumor suppressor gene p53 at the transcription level, leading to up-regulation of p21 that then binds and inhibits the cyclinE-Cdk2 complex. Further analysis of different elements in the p53 promoter showed that sanglifehrin A activates p53 transcription primarily through the activation of the transcription factor NFκB by activating IκB kinase in a manner that is similar to several genotoxic agents. Unlike other genotoxic drugs, sanglifehrin A does not cause DNA damage, making it a unique natural product that is capable of activating the NFκB signaling pathway without affecting DNA.

The immunosuppressive drugs cyclosporin A, FK506, and rapamycin constitute a unique family of natural products that work by an unusual mechanism (1–8). They serve as natural dimerizers that bring together two proteins, suppressing the function of both proteins as a consequence. Thus, CsA is known to bind to the cyclophilin family of proteins (9) while FK506 and rapamycin are known to bind to the FKBP family of proteins (10, 11). Each of the immunophilin-drug complexes specifically interacts with and inhibits the function of their ultimate target. The CsA-cyclophilin and the FKBP-FK506 complexes specifically inhibit the protein phosphatase calcineurin (12, 13) while the FKBP-rapamycin complex specifically inhibits the function of the protein known as FRAP/RAFT/TOR (14–18). Inhibition of the phosphatase activity of calcineurin prevents the dephosphorylation of a critical transcription factor, NFAT, thereby blocking the transcription of a number of cytokine genes (5, 8). The binding of PKB-rapamycin to RRAP/RAFT/TOR interferes with the function of TOR, leading to a blockade of cell cycle at the G1 phase of the cell cycle.

Sanglifehrin A (SFA) is a new member of the immunophilin ligand superfamily. It was discovered through a screen for novel cyclophilin ligands that block T cell activation (19, 20). Similar to CsA, SFA binds to cyclophilin with high affinity. Unlike CsA, however, the cyclophilin-SFA complex has no effect on the phosphatase activity of calcineurin (21). Although SFA was shown to inhibit mouse and human mixed lymphocyte reactions (19), we and others have recently found that SFA does not affect T cell receptor-mediated signal transduction pathways leading to the production of cytokines such as IL-2 (21, 22). Instead, SFA inhibits IL-2-dependent T cell proliferation, similar to rapamycin (23, 24). Moreover, SFA blocks the cell cycle progression of T lymphocyte in response to IL-2 at the G1 phase of the cell cycle, an effect also exhibited by rapamycin (22). Unlike rapamycin, however, the activation of the p70S6k activity was unaffected by SFA (22). These results indicate that SFA has a novel mechanism of action that is distinct from the other known immunophilin ligands, CsA, FK506, and rapamycin.

Further studies revealed that SFA inhibited the hyperphosphorylation of Rb and abrogated the appearance of the G1 cyclin-dependent kinase cyclinE-Cdk2 upon IL-2 stimulation (22). However, how SFA inhibits cell cycle progression has remained unknown. We report here that the cell cycle effect of SFA is not specific to T cells. Using the tumor cell line HCT 116 as a model system, we show that SFA stimulates the transcription of p53 and consequently p21, leading to a significant increase of p21 that is likely to be responsible for the G1 cell cycle blockade. The role of p53 as a mediator of SFA is underscored by the demonstration that p53 null mouse embryo fibroblasts gain significant, albeit not complete, resistance to SFA. Furthermore, we demonstrate that the NFκB binding site in the p53 promoter is required for the SFA-induced transcriptional activation of p53. SFA was found to activate NFκB through the activation of the IκB kinase activity. Analysis of the integrity of the genomic DNA ruled out the possibility that SFA activates NFκB via DNA damage, making SFA a unique inducer of NFκB and p53.

EXPERIMENTAL PROCEDURES

Reagents—SFA was kindly provided by Dr. Richard Sedrani from Novartis Pharma Inc. (Basel, Switzerland). Doxorubicin, cycloheximide, ribonuclease A (RNase, type III-A), ATP, and propidium iodide were purchased from Sigma. [3H]thymidine, [γ-32P]ATP and ECL reagents were obtained from PerkinElmer Life Sciences. Titan™ One
Tube RT-PCR System was from Roche Molecular Biochemicals. Purified polyclonal rabbit antibody to IKB (c-21), IcB (c-20), IKKa (H-744), actin, mouse monoclonal antibody to p21 (P-5), p53 (Pab 240), and protein GA/SA-Sepharose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Protein A—A human p53 luciferase reporter comprised of the 2.4-kilobase or 356-bp p53 promoter region were generated by excising the Sma I site of pGEM basic lucerase reporter vector from Promega after making each end blunt. pG313Luc was provided by Dr. Lazano (32). Mutations of the 5 or 4X356Luc sites in the 356-bp luciferase reporter were generated by polymerase chain reaction-mediated mutagenesis and confirmed by sequencing. NFkB lucerase reporter construct was previously described (22). Expression vectors for mutant IcB, dominant negative IKKa, and GST-IcB were kindly provided by Dr. Warner Greene (Univ. of California San Francisco).

Cell Culture—Wild-type and knockout mouse embryos fibroblasts (MEFs) were prepared from day 13.5 mouse embryos as previously described (26). Human colon cancer cell line HCT 116 (p53+/+ or p53−/−, ATCC CCL247) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively), and 6 mM HEPS. The HCT 116 cell line was purchased from American Type Culture Collection (Manassas, VA) and grown in a humidified incubator at 37°C in 5% CO2.

Fluorescence-activated Cell Sorter Analysis—Following treatment of cells with test agents, the 200 × g cell pellet was fixed in 2 ml of cold absolute ethanol at 4°C for 1 h and then washed twice with cold PBS. The cells were resuspended in 1.76 ml of PBS solution to which 100 μl of RNase (1 mg/ml in PBS) and 40 μl of propidium iodide (2.5 mg/ml in PBS) were added. The cell suspension was incubated in the dark for 15 min and kept at 4°C until analyses. The propidium iodide fluorescence of individual nuclei was determined using a Becton-Dickson FACScan (emission at 675 nm with excitation at 488 nm). Cell cycle distribution was analyzed with CellQuestTM v3.1 acquisition software and the ModFit LT v2.0 program.

Protein Extraction and Immunoblot—For whole cell lysate preparation, 1 × 107 cells were harvested and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 4 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 1% Nonidet P-40, and 0.1% Triton X-100). After incubation at 4°C for 15 min, the whole lysate was separated by microcentrifugation at full speed for 15 min at 4°C. The supernatants were collected and microcentrifuged at 10,000 × g for 20 min. The pellets were then mixed with 50 μl of recombinant glutathione-S-transferase (GST)-IcBα and incubated for 30 min on a rotator at 4°C and then microcentrifuged at full speed for 30 min. The supernatants were collected as nuclear protein.

For subcellular fractionation 1 ml of cytoplasmic buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.5% Triton X-100) was mixed with 50 μl of recombinant GST-IcBα and incubated for 30 min on a rotator at 4°C and then microcentrifuged at full speed for 30 min. The supernatants were collected as cytoplasmic fraction.

The protein concentration in each sample was determined with Bradford assay. Equal amounts of protein were denatured by heating to 95°C in Laemmli sample buffer and were resolved by 12% SDS-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene difluoride membranes. The membranes were probed with indicated antibodies and detected using an ECL system per manufacturer’s instruction.

Immunoprecipitation—For whole cell lysate (1 × 107 cells) were harvested and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 4 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 1% Nonidet P-40, and 0.1% Triton X-100). After incubation at 4°C for 15 min, the whole lysate was separated by microcentrifugation at full speed for 10 min at 4°C. Nuclear and cytoplasmic extracts were prepared as follows. Cells were lysed with cytoplasmic buffer (20 mM HEPES, pH 7.6, 20% glycerol, 0.1% Nonidet P-40, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.7 μg/ml pepstatin A) and then centrifuged at 300 × g for 4 min. The supernatants were collected as cytoplasmic fraction. The pellet was resuspended in nuclear buffer (cytoplasmic buffer+0.5 mM NaCl) and incubated for 30 min on a rotator at 4°C and then microcentrifuged at full speed for 30 min. The supernatants were collected as nuclear protein.

The protein concentration in each sample was determined with Bradford assay. Equal amounts of protein were denatured by heating to 95°C in Laemmli sample buffer and were resolved by 12% SDS-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene difluoride membranes. The membranes were probed with indicated antibodies and detected using an ECL system per manufacturer’s instruction.

Immunoprecipitation—Whole cell lysate (200 μg of protein in 0.5 ml lysis buffer) was mixed with 4 μg of polyclonal rabbit anti-IKKa antibodies (H-744) and incubated at 4°C on a rotator for 4 h. Protein-G/SA-Sepharose beads (50 μl) were added and the incubation was continued for an additional 1 h. Immunoprecipitates were isolated by centrifugation and washed twice with 500 μl of ice-cold lysis buffer and once with 500 μl of ice-cold kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). Pellets were then mixed with 500 μl of kinase buffer containing 1 μg of recombinant-IkappaB (IkappaBα, GST-IkappaBα) (1–62 and 1–250 μg ATP, and 5 μl [γ-32P]ATP (specific activity 3,000 Ci/mmol) on ice, followed by incubation at 30°C for 15 min. The reaction was terminated by the addition of 25 μl of 3× Laemmli’s sample buffer and boiling in water for 5 min. The 32P-labeled GST-IkappaBα was resolved by 12% SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—To determine whether SFA is specific for T lymphocyte, we examined several transformed cell lines. As shown in Fig. 1, the human colon cancer cell line HCT 116 is sensitive to SFA. Like CTLL2, the transformed cell lines. As shown in Fig. 1, the human colon cancer cell line HCT 116 is sensitive to SFA. Like CTLL2, the transformed cell lines. As shown in Fig. 1, the human colon cancer cell line HCT 116 is sensitive to SFA. Like CTLL2, the transformed cell lines.

RESULTS

Cell Cycle Arrest at G1/S Phase Is General—We have previously shown that SFA induces IL-2-dependent T cell proliferation at the G1 phase of the cell cycle (22). To determine whether SFA is specific for T lymphocyte, we examined several transformed cell lines. As shown in Fig. 1, the human colon cancer cell line HCT 116 is sensitive to SFA. Like CTLL2, the cell cycle progression of HCT 116 is also blocked in the G1 phase of the cell cycle. To simplify subsequent studies of the cell cycle effect of SFA, we decided to use HCT 116 as a model system.

SFA Induces p53 mRNA Synthesis—We have shown that SFA blocks the appearance of growth factor-dependent cyclinE-Cdk2 activity, likely explaining the G1 effect of SFA (22). This inhibition is not due to down-regulation of either CyclinE or Cdk2 proteins as determined by Western blot analysis (22). We further examined the expression of the p21 family of CyclinE-Cdk2 inhibitor proteins. It was found that SFA treatment of HCT 116 cells led to a significant increase in the level of p21 protein expression (Fig. 2A). When p21 mRNA was examined, it was also induced dramatically by SFA (Fig. 3A). As p21 transcription is known to be regulated by p53, we checked whether p53 expression is affected by SFA.

We first examined the effect of SFA on p53 at the protein
Indeed, p53 protein is significantly induced by SFA (Fig. 2A). Given that p53 is known to be regulated at multiple levels, including protein stability and mRNA synthesis, we determined whether SFA stabilized p53 protein. We treated HCT 116 cells with SFA for 3 h to induce p53. Cycloheximide was then added into the cell culture to block de novo protein synthesis for an additional 1–8 h. The SFA-induced p53 was degraded over time upon treatment with cycloheximide even in the continued presence of SFA (Fig. 2B). This result clearly indicated that the increase in p53 in the presence of SFA is not due to stabilization of p53 protein. When p53 mRNA synthesis was examined by RT-PCR, it was found to be significantly induced by SFA (Fig. 3A), indicating that SFA activates p53 primarily at the mRNA level either by induction of its transcription or by stabilization of its mRNA or both.

**SFA Activates p53 Promoter**—To determine whether SFA affected p53 transcription, we examined its effect on a luciferase reporter gene under the control of the p53 promoter. Indeed, SFA activated the p53-luciferase reporter gene in a dose-dependent manner (Fig. 3B). As a control, we included a p21 reporter, pG13py.luc, which was activated by SFA as expected. In addition to the full-length p53 reporter gene, we also examined another reporter gene containing a 356-bp fragment of the p53 promoter with several important transcription factor binding sites responsive to DNA-damaging agents (29–31). Similar to the full-length p53 reporter, the minimal p53 reporter, p53Luc (356 bp), is induced by SFA in a dose-dependent manner (Fig. 3B), suggesting that the most important SFA-responsive elements lie within this 356-bp fragment.

**NFκB Is Required for the Activation of p53 Transcription by SFA**—Several transcription factor binding sites within this 356-bp fragment have been previously shown to be important for p53 transcription, including a HoxA5 and an NFκB binding site (29, 32). To determine whether either of the transcription factor binding sites is important for the activation of the p53

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**Fig. 1.** SFA blocks HCT116 cell cycle progression at G1-to-S transition. HCT116 cells were starved in 0.1% serum for 2 days before they were stimulated with 10% serum in the presence or absence of 500 nM SFA for an additional 12 h. Cells were harvested and subjected to FACScan analysis of DNA content upon staining with propidium iodide. The numbers of cells in the G0/G1, S, G2, and M phases of the cell cycle (expressed as a percentage of the total cell population in each case) are indicated.

**Fig. 2.** SFA induces p53 and p21 proteins. A, HCT116 cells were treated with 500 nM SFA for various times as indicated under low serum conditions (2% fetal calf serum). Cell lysates were prepared, subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-p53 monoclonal antibody (Pab1801), anti-p21 antibody (N-20), or anti-actin antibody. B, HCT116 cells were treated with 500 nM SFA for 0, 1, 3, or 6 h. For a subset of cell cultures, treatment with cycloheximide (CHX, 25 μg/ml) ensued for an additional 1–8 h upon incubation with SFA for 3 h. Cells were lysed immediately, and cell lysates were subjected to Western blot analysis.
Sanglifehrin A Activates NFκB and p53

FIG. 3. SFA induces p53 and p21 at the level of transcription. A, p53 and p21 mRNA levels in HCT116 cells were determined by RT-PCR after cells were treated with SFA at indicated concentrations for 3 h. B, HCT116 cells were transiently transfected with one of the following luciferase reporter constructs (1 μg): pG13.pyLuc, p53Luc (2.4 kilobase) or p53Luc (356 bp). Transfected cells were allowed to recover for 24 h. They were then incubated in the presence or absence of SFA for 12 h before they were harvested for the determination of luciferase activity.

promoter by SFA, we mutated both sites and constructed the corresponding mutant reporter genes (Fig. 4A). Mutation of the HoxA5 binding site had negligible effect on the response to SFA-mediated activation (Fig. 4B). In contrast, mutation of the NFκB binding site completely abolished the response of the reporter gene to SFA. These data strongly suggest that the NFκB binding element in the p53 promoter is essential for the activation of p53 transcription by SFA.

The signal transduction pathways leading to NFκB activation have been largely elucidated. A number of stimuli have been shown to converge on the upstream kinases, IKKs, to activate NFκB. The IKKs phosphorylate the cytoplasmic-anchoring protein IκB, which leads to the degradation of IκB and nuclear translocation of NFκB. To determine which step in the upstream NFκB activation pathway SFA affects, we repeated the p53 reporter gene assay in the presence of a dominant negative IKKα and a mutant IκBα that is incapable of being phosphorylated by IKKs. We thus cotransfected HCT116 cells with the p53Luc reporter gene along with the dominant negative IKKα (dnIKKα) and the mutant IκBα (mIκBα) and examined the activation of the p53Luc reporter gene by SFA. Both mIκBα and dnIKKα abolished the activation of the p53Luc reporter by SFA, suggesting that SFA activated NFκB at a step upstream of IKKα (Fig. 4C).

To confirm that SFA is capable of activating NFκB, we examined the effect of SFA on an NFκB luciferase reporter gene comprising of multimerized NFκB binding sites. SFA stimulated the NFκB reporter by about 4-fold, consistent with its stimulation of the p53Luc reporter (Fig. 5A). Like the activation of the p53Luc reporter gene, the activation of the NFκB reporter gene by SFA is also inhibited by both dominant negative IKKα and the mutant form of IκBα (Fig. 5A). We next determined whether SFA induced IκBβ degradation and IKK activation. Of the two isoforms of IκBα examined, SFA induced IκBβ degradation in a dose-dependent manner, while IκBβ appeared to be less responsive, with appreciable degradation observed only at the highest dosage of SFA used (Fig. 5B). When we checked the IKK activity by immunoprecipitation–kinase assay, SFA was found to activate IKK activity in a dose-dependent manner (Fig. 5C). At the highest dose used, SFA induced a similar amount of IKK activity as 20 ng/ml of TNFα. It can thus be concluded that SFA activates NFκB at a step upstream of IKK.

P53 Is an Important Mediator of Inhibition of Cell Proliferation by SFA—We have shown that SFA induces p53 transcription, which appeared to be sufficient to cause cell cycle arrest at the G1 phase through the induction of p21. It is not clear, however, whether p53 is in fact necessary for the cell cycle blockade caused by SFA. To address this question, we took advantage of the availability of MEFs that are deficient in p53. Similar to T cells and HCT 116 cells, the proliferation of wild type MEFs is sensitive to SFA (Fig. 6). In contrast, p53−/− MEFs exhibited significant resistance to SFA. It is noteworthy, however, that the resistance of p53 null MEFs to SFA is not complete, indicating that there exist proteins that play a redundant role with p53.

The Effects of SFA Is Not Mediated through DNA Damage—The ability of SFA to activate p53 via the NFκB signaling pathway is reminiscent of several cytotoxic drugs or carcinogens that act by causing DNA damage (31, 33). For example, it has been previously reported that the anticancer drug daunomycin exhibited similar effects on cells through activation of NFκB and p53 (33). We have extended those observations by demonstrating that activation of p53 and NFκB reporter genes by the DNA damaging agent doxorubicin is sensitive to inhibition by both dominant negative IKKα and mutant IκBα, similar to SFA (Figs. 4C and 5A). These observations raised the possibility that SFA may manifest its cell cycle effect through DNA damage. To test this possibility, we turned to the so-called Comet assay that allows for quantitative determination of DNA damage at individual cell level (27, 28). As expected, doxorubicin caused extensive DNA damage to HCT 116 cells (Fig. 7). However, SFA had little effect on the integrity of chromosomal DNA at concentrations that are sufficient to cause cell cycle arrest. These results clearly distinguish SFA from other known cytotoxic DNA damaging drugs and indicate that SFA is a unique agent that interacts with immunophilins on the one hand and activates NFκB and p53 on the other without causing DNA damage.

DISCUSSION

SFA was discovered as a novel immunosuppressive agent (19, 20). It was recently shown that SFA blocks IL-2-dependent T cell proliferation at the G1 phase of the cell cycle, similar to...
the immunosuppressive drug rapamycin (21, 22, 24, 34). The site of action of SFA, however, is different from that of rapamycin, as it has no effect on p70s6k activity (21, 22). In this study, we found that the cell cycle effect of SFA is not confined to lymphocytes; it is equally effective at inhibiting the proliferation of several cancer cell lines, including HCT 116. Using HCT 116 as a model system, we have uncovered a pathway that appears to mediate the cell cycle effect of SFA. This pathway includes the IκB kinases that phosphorylate IκB, leading to activation of NFκB. The activated NFκB then induces the transcriptional activation of p53, which in turn activates the transcription of p21. The signaling cascade from IκB kinase activation to p21 expression accounts for the inhibition of the critical G1 kinase CyclinE-Cdk2 (22). These findings shed new light on the mechanism of action of SFA and further distinguish SFA from rapamycin or CsA in their modes of action.

As a cellular gate keeper for growth and division, p53 plays an essential role in sensing various stress signals and serves as a focal point of signal integration to decide whether cells will undergo growth arrest or apoptosis (35–37). A multitude of stress signals are known to activate p53, including ionizing irradiation, UV, hypoxia, nucleotide deprivation, and chemotherapeutic agents. While most chemotherapeutic drugs are known to activate p53 through DNA damage, other small molecules capable of activating p53 have been reported. Among those is the fumagillin family of angiogenesis inhibitors that...
block endothelial cell cycle progression by activating p53 (38, 39). The ability of SFA to induce p53 in a variety of cells suggests that it may have potential as an inhibitor of angiogenesis in addition to its immunosuppressive activity.

We have shown that SFA is capable of activating p53 transcription, though it remains to be determined whether SFA also affects the stability of p53 mRNA. Several transcription factors have been identified that appear to play a role in the activation of the p53 promoter, among which are NFκB and HoxA5 (29, 32). Of these two transcription factors, NFκB has been shown to mediate the transcriptional activation of p53 in response to DNA damage, though the extent to which NFκB contribute to p53 transcription has remained somewhat controversial (30, 33). It was reported that an NFκB-binding element overlaps with a minimal p53 promoter that responds to chemotherapeutic agents (30). But NFκB was reported to play an insignificant role in the activation of p53, as evidenced by the lack of activation of a p53 reporter by expressed p65 and the lack of effect by N-acetyl cysteine on genotoxic stress-induced p53 activation. In contrast, both benzo[a]pyrene, a carcinogen, and daunomycin, an anticancer drug, have been shown to induce p53 transcription via activation of NFκB (31, 33). Our

FIG. 6. Mouse embryo fibroblasts deficient in p53 gain significant resistance to SFA. MEFs were incubated in the presence or absence of varying concentrations of SFA for 20 h and pulsed with [3H]thymidine (1 μCi) for additional 4 h. Cells were harvested, and the amounts of incorporated tritium were determined by scintillation counting. Data represent the mean ± S.D. of three experimental determinations.

FIG. 7. SFA does not cause damage to genomic DNA. HCT116 cells were treated with test drugs at 37 °C for 1 h. The extent of DNA damage was assessed by COMET assay. Fifty nucleotides per sample were scored visually for comet tail size based on arbitrary scale of 0–4, ranging from no damage to extensive damage of DNA. Individual nucleotide scores were added to give the final COMET scores. Data represent the mean ± S.D. of three experimental determinations.
observations that mutation of the NFκB site within the p53 promoter led to complete abrogation of reporter gene activation by SFA and that dominant negative form of IKKα and mutant IκBα inhibited activation of p53 reporter gene corroborate with the conclusion that NFκB can play an essential role in the transcriptional activation by certain reagents.

Although activation of p53 seems sufficient for the SFA-mediated cell cycle arrest at the G1 phase, it is not absolutely necessary (Fig. 6). When MEFs deficient in p53 were tested for their sensitivity to SFA, they were much less sensitive to SFA than wild type MEFs (Fig. 6), suggesting that p53 is indeed necessary (Fig. 6). When MEFs deficient in their sensitivity to SFA, they were much less sensitive to SFA than wild type MEFs (Fig. 6), suggesting that p53 is indeed necessary (Fig. 6).

And NFκB, has no effect on the integrity of genomic DNA, distinguishing SFA, which may unravel an entry point for pharmacological activation of the NFκB pathway and p53.

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Ling-Hua Zhang, Hong-Duk Youn and Jun O. Liu

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