

# Cellular growth inhibition by FK778 is linked to G1 arrest or S phase accumulation, dependent on the functional status of the retinoblastoma protein

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**Abstract.** The malononitrilamide FK778 is a novel immunosuppressive agent with antiproliferative activities. To gain insight into the molecular mechanism of FK778-mediated growth inhibition, we analyzed cells which differ in their p53 status and functionality of retinoblastoma protein (pRb). FK778 acted as a broad inhibitor of cell proliferation independent of the p53 or pRb status. However, the mechanism of FK778-mediated growth inhibition differed, leading either to cell cycle arrest in G1, or cell accumulation in S phase. This differential response was linked to the phosphorylation status of pRb. In addition, since FK778 was reported to exhibit antiviral activities, we analyzed the effect of FK778 on the growth stimulatory human papillomavirus (HPV)-16 and -18 E7 genes. Although growth of HPV-positive cells was strongly inhibited by FK778, we did not observe significant effects on viral E7 expression, indicating that the antiproliferative effect is not linked to an antiviral activity of FK778.

## Introduction

The malononitrilamide (MNA) FK778 is a novel immunosuppressive agent (1). It is a synthetic drug analogue of A77 1726, the active metabolite of leflunomide which is a widely used agent in clinical treatments of rheumatoid arthritis (2). FK778 exerts beneficial effects in different experimental transplan-

tation models, including suppression of acute and chronic allograft and xenograft rejection (1,3). In addition, clinical trials proved that FK778 is safe, well tolerated and reduces the incidence of acute rejection in human kidney transplant recipients (4).

At the biochemical level, MNAs can interfere with *de novo* pyrimidine synthesis by blocking the rate-limiting enzyme dihydroorotate dehydrogenase (DHODH) (5). This results in intracellular pyrimidine depletion and consequently, attenuation of DNA and RNA synthesis. In addition, FK778 exerts pleiotropic effects which include inhibition of cytomegalovirus replication (6) and signaling (7), downregulation of TGF $\beta$  expression (8), or modulation of endothelial adhesion molecules (9). Similar to other MNAs, FK778 can exhibit antiproliferative effects (1). This antiproliferative activity of FK778 may not only be important for FK778-induced immunosuppression (attenuation of proliferation of immune cells) but also for the reported ability of FK778 to prevent proliferation-associated allograft complications, such as chronic allograft vasculopathy (10), chronic allograft nephropathy (11,12), or obliterative airway disease (13).

Little is known about the critical cellular responses which mediate the antiproliferative response of FK778-treated cells. To gain more mechanistic insights into this process, we investigated the response to FK778 in cell lines differing in the functionality of the crucial growth regulators p53 and retinoblastoma protein (pRb). Upregulation of p53 expression can induce cell cycle arrest in the G1 phase in response to various stimuli, including DNA damage (14) or ribonucleotide depletion (15). The hypophosphorylated form of pRb protein blocks G1/S progression by sequestering cellular proteins, such as members of the E2F family. Upon phosphorylation by cyclin D/cyclin-dependent kinase (CDK) 4, cyclin D/CDK6, and cyclin E/CDK2 complexes, this pRb protein-binding function is abrogated, resulting in G1/S progression (16). Here, we found that FK778 was a strong growth-inhibitor in all cell lines analyzed, irrespective of their p53 or pRb status. However, cell cycle analyses revealed that the underlying mechanism of growth inhibition was different, resulting either in G1 arrest or S phase accumulation, dependent on the functional pRb status.

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## Materials and methods

**Cell culture and reagents.** The following cell lines were analyzed: HPV18-positive HeLa cervical carcinoma cells, HPV16-positive CaSki and SiHa cervical carcinoma cells, H1299 lung cancers cells, U2OS osteosarcoma cells, HCT116 colon cancer cells and their derivatives HCT116 p21<sup>-/-</sup> (17), RKO colon carcinoma cells, and Huh7 hepatoma cells. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM), except for HCT116 and HCT116 p21<sup>-/-</sup> which were grown in McCoy's medium, supplemented with 10% fetal calf serum.

Cells were treated with FK778 (30  $\mu$ g/ml) (Astellas Pharma, Munich, Germany), either with or without uridine (100  $\mu$ M) (Sigma, Taufkirchen, Germany), for the indicated time points. Control cells were treated with DMSO solvent. Cell culture medium was exchanged after 48 h.

**Protein analyses.** Protein extracts were prepared 24–48 h after treatment of cells, following previously described protocols (18). For Western blot analyses, 30  $\mu$ g of protein extract were separated by SDS-PAGE, transferred to an Immobilon-P membrane (Milipore, Eschborn, Germany), and analysed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany). The following antibodies were used: anti-18E7 antibody 18E7C (19), anti-16E7 (NM2) antibody (kind gift from Dr Martin Müller), anti-tubulin antibody CP06 (Calbiochem, Schwalbach, Germany), anti-p21<sup>WAF-1</sup> antibody OP64 (Calbiochem), anti-p53 antibody DO-1 (BD Pharmingen, San Diego, CA, USA), anti-cyclin E antibody HE-12 (Santa Cruz, Sanata Cruz, CA, USA), anti-cyclin A (H-432) antibody (Santa Cruz), anti-cyclin D1 antibody DSC6 (Cell Signaling, Danvers, MA, USA), anti-Rb (4H1) antibody (Cell Signaling), and anti-Phospho-Rb (Ser 807/811) antibody (Cell Signaling).

**Cell count and cell cycle analyses.** For cell count analyses, cell suspensions were diluted in CASYton (Innovatis AG, Reutlingen, Germany). Total cells per ml were determined using a CASY TTC cell counter (Innovatis AG).

For cell cycle analyses, cells were trypsinized, washed in ice-cold phosphate-buffered saline (PBS), and fixed in 80% cold ethanol overnight at 4°C. Subsequently, cells were pelleted, resuspended in PBS containing 1 mg/ml RNase A (Roche Diagnostics, Mannheim, Germany) and 2.1  $\mu$ g/ml propidium iodide (Sigma-Aldrich), and incubated for 30 min at 37°C. Cell cycle analyses were performed using a FACSCalibur (BD Biosciences, Heidelberg, Germany) with CellQuest Pro software provided by the manufacturer. Quantitation of the percentage of cells in individual cell cycle phases was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA), applying the Watson (Pragmatic) model.

## Results

**FK778 is an efficient inhibitor of cell proliferation, irrespective of the p53 or pRb status.** A panel of different cell lines with varying p53 and pRb status was treated with FK778, and cells were investigated for proliferative capacities. Analyzed cells included: (i) HCT116 colon cancer cells, in which both p53- and pRb-associated pathways are intact, (ii)

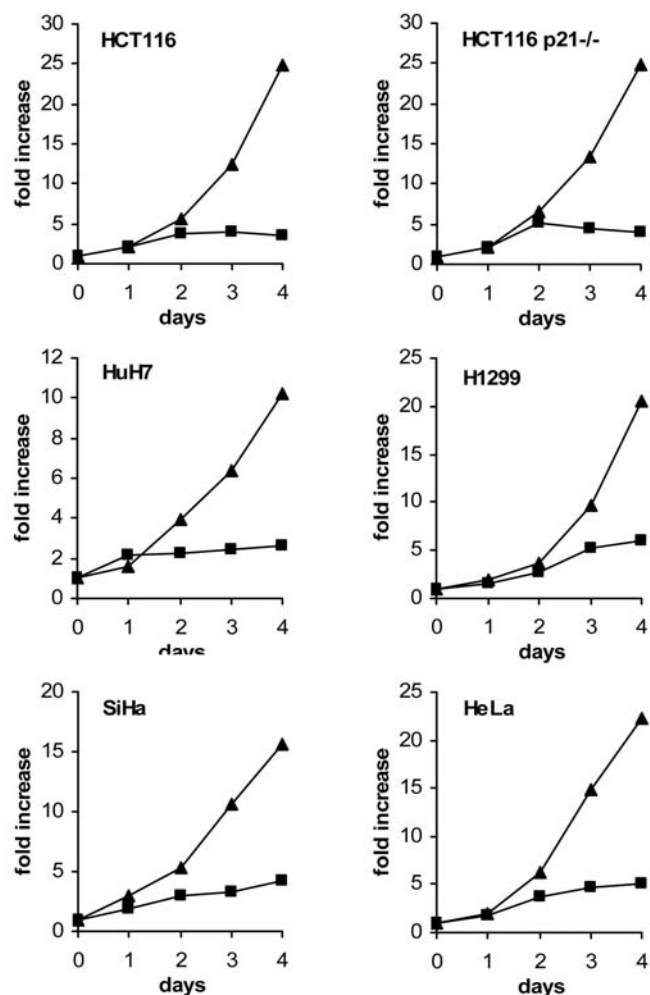


Figure 1. Growth inhibition of cells with differing p53 and pRb status by FK778. Cells were treated with 30  $\mu$ g FK778 (rectangles) or control-treated with DMSO (triangles), for up to 4 days. Cell numbers were determined by CASY counter analysis. Indicated is the fold increase in cell numbers at the indicated time points, initial cell numbers (time point 0) were set at 1.0.

HCT116 p21<sup>-/-</sup> cells which differ from the parental HCT116 cells by deletion of the p21 gene (17) encoding a broad inhibitor of CDKs, (iii) Huh7 liver cancer cells which express mutant p53 and wild-type pRb protein (20), (iv) H1299 lung cancer cells which contain a deletion of the p53 gene and express wild-type pRb protein (21), and (v) human papillomavirus (HPV)-type 16 positive SiHa or HPV18-positive HeLa cells, in which both the p53 and pRb pathways are impaired because of the activities of the HPV E6 and E7 oncogenes (22).

Over a period of four days, cell count analyses revealed that the growth of all cell lines was efficiently blocked by FK778 treatment, irrespective of their p53 or pRb status (Fig. 1). Morphological inspections, chromatin staining with diamidino-2-phenylindole (DAPI), or terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays did not provide any evidence for apoptosis induction upon FK778 treatment (data not shown).

**Differential cell cycle responses of FK778-treated cells.** Fluorescence-activated cell sorting (FACS) analyses were performed in order to obtain insight into the mechanism of FK778-mediated growth inhibition. HCT116 cells exhibited

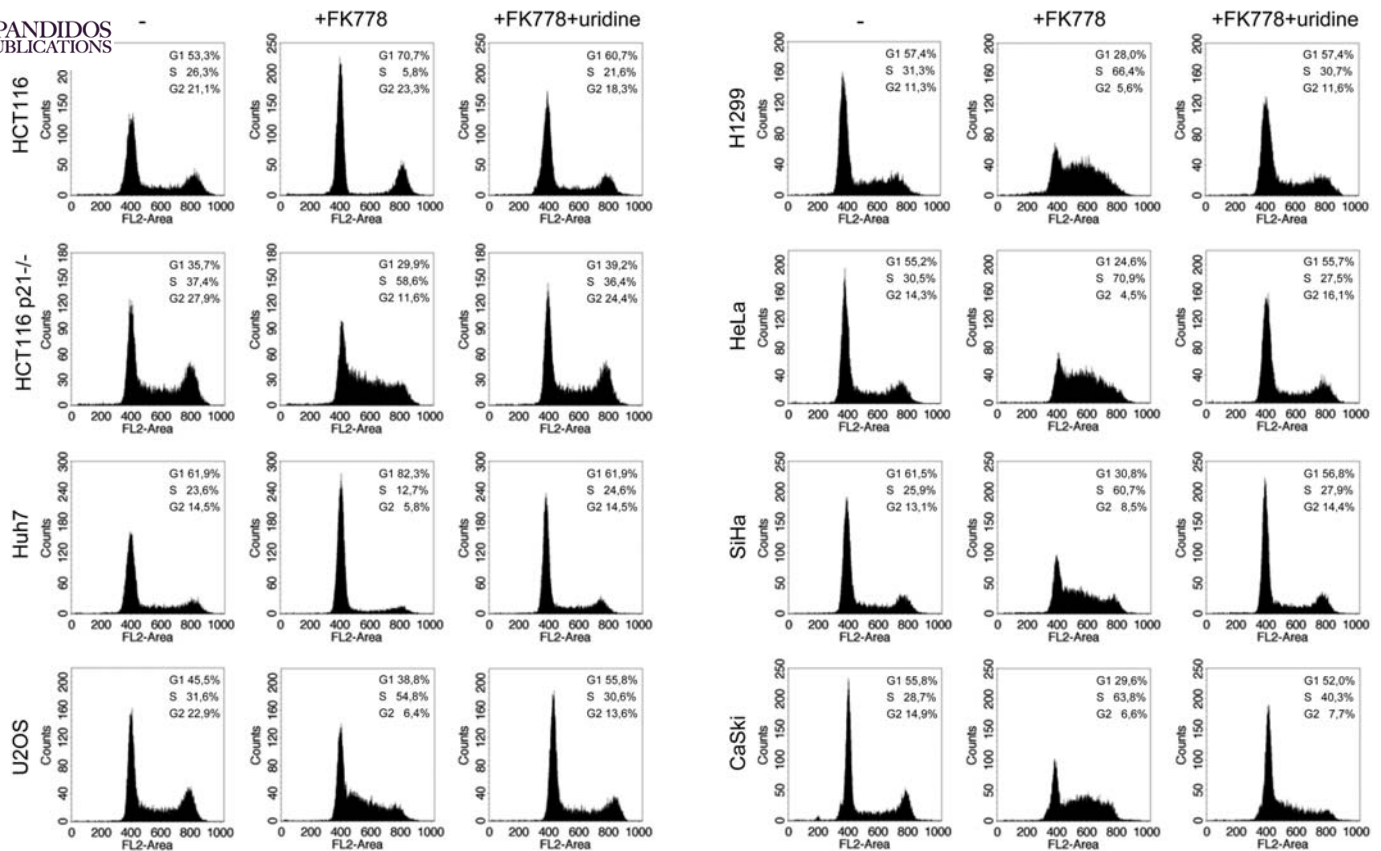
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Figure 2. Cell cycle analysis of cells with differing p53 and pRb status, following FK778 treatment. Cells were either control-treated with DMSO solvent, treated with 30  $\mu\text{g}/\text{ml}$  FK778, or treated with 30  $\mu\text{g}/\text{ml}$  FK778 plus 100  $\mu\text{M}$  uridine. Cell cycle distribution was determined by FACS analysis and percentages of cells in G1, S, or G2 phases are indicated. All measurements were repeated at least three times in independent experiments and yielded consistent results.

a profound arrest in the G1 phase of the cell cycle following FK778 treatment, as revealed by a substantial increase in G1 phase and decrease in S phase populations (Fig. 2). This effect was reversible by adding uridine (Fig. 2) which supplies cells with pyrimidine via salvage pathways (1).

FK778 treatment did not induce G1-arrest in HCT116 p21<sup>-/-</sup> cells, indicating that the G1 arrest of the parental HCT116 cells is mediated by p21. Rather, HCT116 p21<sup>-/-</sup> cells treated with FK778 accumulated in the S phase (Fig. 2). This effect was also reversible by addition of uridine.

Similar to HCT116 cells, Huh-7 cells (Fig. 2) or RKO colon cancer cells (data not shown) also exhibited efficient G1 arrest upon FK778 treatment.

In contrast, U2OS osteosarcoma cells which express wild-type p53 but exhibit pRb dysregulation (23), H1299 lung cancer cells, and all three investigated HPV-positive cervical cancer cell lines, HeLa, SiHa, and CaSki, accumulated in the S-phase, following FK778 treatment.

Taken together, these results indicate that FK778 induces either G1 arrest or S phase accumulation, depending on the cellular background.

*Differential modulation of growth-regulatory factors by FK778.* To further explore the differential cellular responses towards FK778, the expression of a panel of growth-regulatory factors was analyzed upon FK778 treatment.

In HCT116 cells, exhibiting G1 arrest (Fig. 2), treatment with FK778 induced a clear reduction in phosphorylated pRb

protein, at maintained overall pRb levels (Fig. 3A). This indicates that FK778 increases the amount of hypophosphorylated pRb which acts as an inhibitor of G1/S progression (16), in line with the data from the cell cycle analyses of HCT116 cells (Fig. 2). Addition of uridine reverted the effect of FK778 on pRb phosphorylation (Fig. 3A).

On the contrary, no reduction in phosphorylated pRb levels was observed in p21-deficient HCT116 p21<sup>-/-</sup> cells. This indicates that the increase in hypophosphorylated pRb in the parental HCT116 cells is caused by p21.

Since p21 is transcriptionally activated by p53, we next analyzed the effect of FK778 on p53 and p21 expression. The levels of p53 increased both in parental HCT116 cells and the HCT116 p21<sup>-/-</sup> variants (Fig. 3A). This was linked to a clear increase in p21 protein levels in HCT116 cells whereas p21 expression was absent in HCT116 p21<sup>-/-</sup> cells.

As observed for HCT116 cells, other cells exhibiting G1 arrest upon FK778 treatment (Huh7, RKO) also showed a reduction in phosphorylated pRb protein, at maintained overall pRb levels, indicating an increase in hypophosphorylated pRb concentrations (Fig. 3A). As in HCT116 cells, this was linked to an increase of p53 and p21 levels in RKO cells. In contrast, Huh7 cells, which express mutant p53, did not increase p53 amounts in response to FK778, and p21 protein levels stayed below the detection limit. Thus, in the latter cells, the increase in hypophosphorylated pRb does not appear to be linked to activation of the p53/p21 cascade. Since cyclins mediate phosphorylation of pRb (16), we



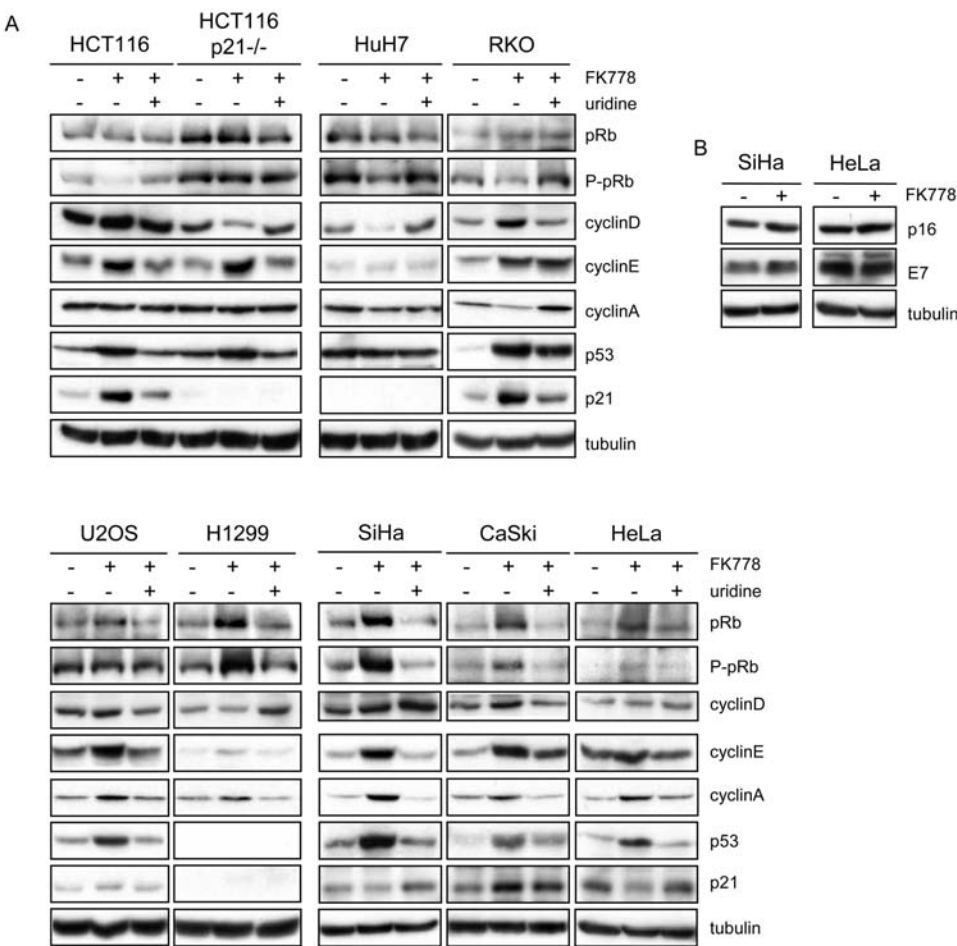


Figure 3. Expression of growth-regulatory proteins following FK778 treatment. (A) Cells were either control-treated with DMSO, treated with 30 μg/ml FK778, or treated with 30 μg/ml FK778 plus 100 μM uridine, and subsequently analyzed by immunoblotting. pRb, detection of total pRb protein; P-pRb, detection of phosphorylated pRb; tubulin, loading control. (B) FK778 does not modulate HPV16 and HPV18 E7 expression. Analysis of HPV E7 and cellular p16 protein expression upon FK778 treatment. Western blot analysis of HPV16-positive SiHa and HPV18-positive HeLa cells, either control-treated with DMSO or treated with 30 μg FK778. Tubulin, loading control. All analyses were repeated at least three times in independent experiments and yielded consistent results.

included analyses of cyclin D, E, and A expression. As shown in Fig. 3A, treatment of Huh7 cells with FK778 resulted in a clear reduction in cyclin D expression levels, providing a possible explanation for the reduction of phosphorylated pRb.

In contrast to G1-arrested cells, all cell lines responding to FK778 treatment with S phase accumulation did not exhibit a reduction in phosphorylated pRb. Rather, H1299, SiHa, CaSki, and HeLa cells showed an increase in phosphorylated pRb concentrations (Fig. 3A). This effect was associated with increased expression of different cyclins.

Taken together, these results demonstrate that the cell cycle phase associated with FK778-induced growth arrest can differ in a cell-specific manner and is ultimately linked to the phosphorylation status of pRb.

*FK778 does not exert antiviral effects on HPV E7 expression.* FK778 and/or its parental compound, leflunomide, exert antiviral effects against different viruses associated with transplantation complications (1). The effect of FK778 on HPV activity is unknown. In light of the growth-inhibitory effects of FK778 on both HPV16- and 18-positive cells and the fact that the chromosomally integrated viral E7 gene is a crucial activator of cellular proliferation (22), we investigated whether

FK778 may modulate E7 expression during growth inhibition of HPV-positive cancer cells.

Immunoblot analyses of HPV16-positive SiHa and HPV18-positive HeLa cells showed that FK778 treatment did not alter E7 concentrations (Fig. 3B). Likewise, p16 levels, representing a surrogate HPV marker which correlates with expression of HPV E7 (24), were not affected (Fig. 3B). Thus, these experiments do not provide evidence that FK778 blocks growth of HPV-positive cells by exerting antiviral activities.

Discussion

The results of this study showed that FK778 acts as a broad and efficient inhibitor of cell proliferation. However, the underlying antiproliferative mechanisms differ depending on the cellular context, and are intimately linked to the phosphorylation status of pRb. Specifically, cells which contain a functional p53/p21 cascade or which are able to prevent phosphorylation of pRb by p53/p21-independent pathways, arrest in the G1 phase of the cell cycle. On the contrary, cells in which the p53/p21 cascade is disturbed or in which pRb function is impaired, proceed through the G1/S phase checkpoint and accumulate in the S phase of the cell cycle. Both cellular responses are likely to be



the depletion of pyrimidines via FK778-mediated of DHODH, since they were reversible by adding uridine which supplies cells with pyrimidine via salvage pathways (1).

Comparative analyses of HCT116 and HCT116 p21<sup>-/-</sup> cells indicate that the p21 gene is a critical mediator of FK778-induced G1 arrest. The p21 protein acts as a broad inhibitor of G1 cyclin/CDK complexes, thereby inhibiting pRb phosphorylation and G1/S progression (25). Expression of the p21 gene is transcriptionally activated by the p53 protein (26) which itself can act as a sensor of intracellular ribonucleotide depletion (15). Thus, these findings are consistent with a model in which the p53/p21 cascade is induced upon FK778-mediated ribonucleotide depletion, ultimately resulting in pRb hypophosphorylation and G1 arrest. Notably, the results in Huh7 cells suggest that cells can alternatively prevent phosphorylation of pRb and induce G1 arrest, in response to FK778 treatment, via pathways which are independent of the p53/p21 cascade. These cells express mutant p53 and their p21 protein levels stayed below the detection limit. Since cyclin D concentrations were markedly reduced upon FK778 treatment in Huh7 cells, it is conceivable that the reduction of phosphorylated pRb in these cells could be a consequence of decreased activity of cyclin D/CDK complexes.

In contrast, FK778 treatment of cells with impaired pRb function led to S phase accumulation. This was observed in HCT116 p21<sup>-/-</sup> cells in which pRb is dysregulated because of the lack of p21. The p21 null status of HCT116 p21<sup>-/-</sup> cells also provides an explanation for their substantially higher overall pRb levels when compared with parental HCT116 cells, since p21 mediates proteolytic degradation of pRb (27). Accumulation in S phase was also observed for HPV-positive cancer cells in which pRb activity is blocked by the viral E7 oncoprotein (22). The amounts of phosphorylated pRb increased in HPV-positive cells following FK778 treatment, which was associated with increased expression of cyclin A and/or E. Similarly, H1299 cells which are p53-negative and do not express detectable p21 levels, showed increased amounts of hyperphosphorylated pRb linked to increased cyclin A and D expression. Conceivably, increased expression of cyclins provide a pathway to increase pRb phosphorylation. Finally, U2OS cells, which due to an unknown defect are unable to generate active, hypophosphorylated pRb (23), also accumulated in the S phase of the cell cycle, following FK778 treatment.

FK778, as well as other MNAs, exert antiviral effects, in addition to their immunosuppressive activities. Affected viruses include CMV (7,28), herpes simplex virus-1 (29), different polyomaviruses (1,30), and human immunodeficiency virus-1 (31). In view of the fact that infections with these virus types represent important complications in transplantation medicine, the reported antiviral activities of MNAs are suggested to provide important additional benefit for transplant recipients (1).

HPV16- and 18-associated cancers, such as anogenital and oropharyngeal carcinomas, represent clinically relevant complications in transplant recipients (32). Since the proliferation of HPV-positive cancer cells is critically dependent on the activities of the HPV E7 oncogene which overrides normal G1/S control (22), we tested possible effects of FK778 on E7

expression. We did not observe a significant effect of FK778 on E7 protein expression in cervical cancer cells at conditions where cell proliferation is markedly inhibited. In addition, p16 levels, which correlate with HPV E7 activities (24), were not affected in HPV-positive cancer cells by FK778 treatment. Thus, our investigations do not provide evidence for an antiviral effect of FK778 on HPV E7 expression.

Taken together, the results of this study indicate at least three possible antiproliferative pathways which are induced with FK778 treatment. First, in cells containing functional p53 and pRb pathways (HCT116, RKO), depletion of ribonucleotides through DHODH inhibition will activate p53 and, consequently, stimulate p21 expression. In turn, the amounts of hypophosphorylated pRb will be increased by p21-mediated inhibition of CDKs, ultimately leading to G1 arrest. This scenario is also compatible with the growth-inhibitory effects reported for MNA-treated primary cells which also arrested in the G1 phase of the cell cycle (33). Second, the response of Huh7 cells indicate that some cells with an impaired p53/p21 cascade can alternatively reduce pRb phosphorylation by decreasing expression of cyclins, such as cyclin D, which will also result in G1 arrest. Third, cells which are unable to decrease pRb phosphorylation will pass the G1/S phase and accumulate in the S phase due to the depletion of ribonucleotide precursors. In this group, the dysregulation of pRb can be caused by different mechanisms which are not mutually exclusive: (i) the antagonistic activity of the viral E7 oncoprotein (HPV-positive cancer cells), (ii) increased expression of cyclins leading to pRb phosphorylation and inactivation (H1299, HPV-positive cancer cells), or (iii) the inability to accumulate hypophosphorylated pRb (U2OS).

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