

## Antitumor Efficacy and Molecular Mechanism of TLK58747, a Novel DNA-Alkylating Prodrug

HUA XU, ZHUO WANG, JOHN C. DONALDSON, HONG YAO, SIQUN ZHOU, ANDREW B. KELSON, WENLI MA, KEVIN T. WEBER, EDGARDO LABORDE, MINGSHAN CHENG, LIDIA SAMBUCETTI and JAMES G. KECK

Telik, Inc., Palo Alto, CA 94304, U.S.A.

**Abstract.** *Background: DNA-damaging agents are widely used for the treatment of human malignancies. Agents containing the multifunctional alkylating moiety tetrakis(2-chloroethyl)phosphorodiamidic acid are currently under development as cancer therapeutics. Materials and Methods: TLK58747, a phosphorodiamidate-based prodrug, was tested in vivo for antitumor efficacy and safety. The in vitro responses of tumor cells to TLK58747 were examined by cytotoxicity assays, cell cycle analysis, immunoblots and microscopy. Results: TLK58747 was efficacious in xenograft models of human breast, pancreas, and prostate cancer, as well as in leukemia and glioma. It caused less bone marrow suppression in rats than did cyclophosphamide. In vitro, TLK58747 inhibited the growth of a wide variety of cancer cells and activated the DNA damage-response pathway, leading to G<sub>2</sub>/M cell cycle arrest and subsequent premature senescence or apoptosis. Conclusion: TLK58747 is a promising new alkylating agent with broad antitumor activity and superior safety that warrants further development.*

Since their discovery and subsequent introduction into the clinic, alkylating agents such as cyclophosphamide (CPA), carmustine (BCNU) and melphalan have demonstrated efficacy in the treatment of leukemias, lymphomas, brain tumors and other malignancies (1-5). These alkylating agents remain widely used for cancer treatment and form the basis of many drug combination therapies, especially those for lymphoproliferative disorders (5). Due to the development of drug resistance, there is a continued medical need for new alkylating agents with superior efficacy and safety profiles (5-7). This need is exemplified by the recent FDA approval of bendamustine hydrochloride (Treanda®), a unique alkylating agent that is non-cross-resistant with other

alkylators, for the treatment of patients with chronic lymphocytic leukemia (CLL) (2, 7-8).

2-(Alkylsulfonyl)ethyl *N,N,N,N*-tetrakis(2-chloroethyl)phosphorodiamidates are a class of alkylating agents being explored as cancer therapeutics (9, 10). They are prodrugs since the active phosphorodiamidic acid moiety is inherently unstable and cannot be isolated as such for use as a drug. They do not produce acrolein as a byproduct, which is responsible for the urothelial toxicity of cyclophosphamide. The high chlorine content of the active moiety greatly increases the lipophilicity, which likely facilitates its transfer across cellular membranes. TLK286 (TELCYTA™), a conjugate of a glutathione analog and the *N,N,N,N*-tetrakis(2-chloroethyl)phosphorodiamidic acid, is an example of this class of compounds. The cleavage or activation of TLK286 is accelerated by glutathione-S-transferase (GST) P1-1, which is often overexpressed in tumor cells (11-13). TLK286 has demonstrated antitumor activity and was well tolerated with generally mild and reversible non-hematological toxicities in clinical trials of non-small cell lung cancer (NSCLC) and ovarian cancer (11, 14).

TLK58747 was identified through a program designed to develop novel, broad-spectrum, intravenously and orally efficacious alkylating agents with an improved safety profile. It can deliver the phosphorodiamidic acid moiety upon a  $\beta$ -elimination reaction occurring at physiological pH. Since not all tumor cells express a high level of GST P1-1, TLK58747 may have a broader antitumor spectrum than TLK286. It was also selected for its improved antitumor efficacy. In this article, we report the *in vivo* activity and safety profile of TLK58747, as well as results regarding its mechanism of action in tumor cells.

### Materials and Methods

*Chemical nature of TLK58747.* TLK58747 is the *N,N*-diethylaminoethylsulfonyl ester of *N,N,N,N*-tetrakis(2-chloroethyl)phosphorodiamidic acid (Figure 1). The *N,N*-diethylamino moiety in TLK58747 enables the formation of water-soluble salts, thus facilitating the development of parenteral as well as oral formulations of the compound.

Correspondence to: James G. Keck, 3165 Porter Drive, Palo Alto, CA 94304, U.S.A. Tel: +1 6508457810, Fax: +1 6508457800, e-mail: jkeck@telik.com

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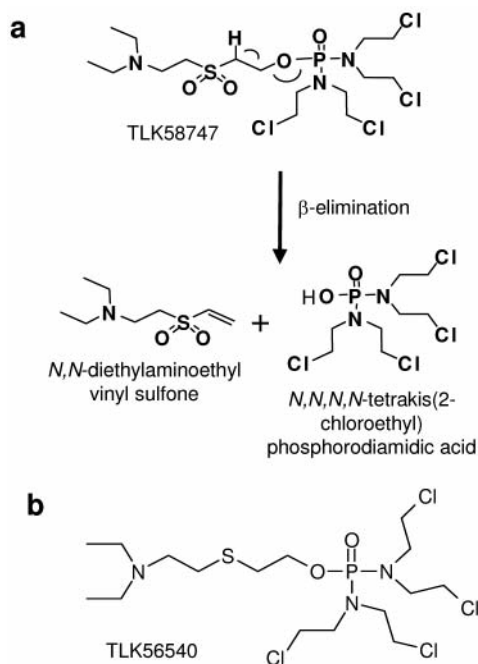


Figure 1. a, Chemical structure of TLK58747 and its cleavage products, *N,N,N,N*-tetrakis(2-chloroethyl)phosphorodiamidic acid and *N,N*-diethylaminoethyl vinyl sulfone. b, Structure of TLK56540, the non-cleavable analog of TLK58747.

**Cell lines and reagents.** The following human cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA): DLD-1, MIA PaCa-2, U-87MG, PC-3, and LNCap. MX-1, A549, OVCAR3, and HL-60 cell lines were obtained from the National Cancer Institute (Frederick, MD, USA). All cells were maintained according to the suppliers' instructions. OVCAR3 derivatives resistant to carboplatin (OVCAR3R) and to paclitaxel (OVCAR-TaxolR) were established in house by exposing the parental line to gradually increasing concentrations of the drugs (Xu *et al.*, unpublished data).

TLK58747 and related compounds were synthesized at Telik (Palo Alto, CA, USA). Cisplatin and melphalan were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and kept frozen until use.

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): anti-H2AX, anti-PARP, anti-Chk1, anti-phospho-H2AX (Ser139), anti-phospho-cdc2 (Tyr15), anti-phospho-Chk1 (Ser345), and anti-phospho-Chk2 (Thr68). Anti-Chk2 (hCDS1) clone DCS-270 and anti-β-actin clone AC-15 were from Sigma. Anti-cdc2 (POH-1) and anti-phospho-Rb (Ser780) were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-Rb (LM95.1) antibody was obtained from Calbiochem (San Diego, CA, USA).

**Cell growth inhibition assays.** Briefly, exponentially growing cells were seeded in 96-well plates for 4-16 hours. The diluted drugs (approximately 200-0.1 μM for TLK58747, 150-0.01 μM for cisplatin, 150-0.1 μM for carboplatin, 50-0.1 μM for melphalan,

10,000-0.1 nM for doxorubicine and 1,000-0.1 nM for paclitaxel) and DMSO were then added to the cells in triplicates and incubated for 2 or 3 doubling times (3-5 days depending on the cell line). The extent of cell growth, as measured by the amount of the ATP present in the cells, was determined using the CellTiter-Glo Luminescent Cell Viability Assay Kit from Promega (Madison, WI, USA) following the manufacturer's instructions.

**Cell cycle analysis.** Exponentially growing cells were seeded overnight at low densities such that on the day of harvesting the cells would be approximately 80% confluent. The cells were treated with DMSO or the test compounds at concentrations of 6 to 40 μM for 1-3 days. Cells were collected, fixed in 75% ethanol and stored at -20°C until analysis. DNA content was assessed using a Cellular DNA Flow Cytometric Analysis Kit from Roche Diagnostics (Indianapolis, IN, USA). Flow cytometry analysis was conducted using a FACScalibur instrument and CELLQuest software (BD Biosciences, San Jose, CA, USA).

**Caspase-3 activity assay.** OVCAR3 cells were treated with the test compounds or DMSO as described for cell cycle analysis. Cells were harvested by trypsinization, and live cells were counted following Trypan Blue staining (Invitrogen, Carlsbad, CA, USA). Cells were lysed in a hypotonic buffer (Clontech, Mountain View, CA, USA) at 50 μl per 10<sup>6</sup> live cells. The caspase-3 activity in each lysate was assessed using an ApoAlert Caspase-3 Fluorescent Assay Kit (Clontech). Fluorescence was detected with a FLUOstar OPTIMA plate reader (BMG LABTECH, Durham, NC, USA).

**SA-β-galactosidase staining.** Staining for senescence-associated β-galactosidase (SA-β-gal) was performed as described by Dimri *et al.* (15). Briefly, subconfluent A549 cells were treated with DMSO or TLK58747 at different concentrations (50-6.25 μM) for 6 days or TLK58747 at 40 μM for 2-8 days in a dish and then fixed with 2% formaldehyde and 0.2% glutaldehyde for 5 minutes. After washing with PBS, the cells were incubated overnight at 37°C with the staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, and 40 mM citric acid/sodium phosphate, pH 6.0). Cells were observed under an Olympus IX51 microscope, and representative fields were photographed using an Olympus DP70 camera and DP Controller software.

**Immunoblot analysis.** Following drug treatment, cells were harvested and total lysates were prepared by sonicating the cells in the lysis buffer (150 mM NaCl, 20 mM Tris.Cl, pH 7.5, 5 mM EDTA, 1% NP-40, 5 mM NaF, 2 mM PMSF, and 22.5 μl Complete Mini EDTA-Free Protease Inhibitor Cocktail from Roche Diagnostics). Protein concentrations in the lysates were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). For Western blots, equal amounts (10-30 μg) of each lysate were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were sometimes divided in order to probe for proteins of differing molecular weights. After blocking the non-specific protein binding sites, the blots were incubated sequentially with the primary antibodies and Alexa 680 (Molecular Probes, Carlsbad, CA, USA) or IRDye 800 (Rockland, Gilbertsville, PA, USA) labeled secondary antibodies. The blots were imaged using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

**Mouse tumor xenograft models.** MX-1 and MIA PaCa-2 human tumor xenografts were developed from fresh tumor tissues. PC-3 and HL-60 xenografts were derived from cultured tumor cells. Briefly, freshly prepared tumor tissues (20-30 mg per animal for the MX-1 and MIA PaCa-2 models) or cells ( $5 \times 10^6$  and  $10 \times 10^6$  cells per animal for PC-3 and HL-60 xenografts, respectively) were inoculated subcutaneously at the right or left lateral flank or into the mammary fat pad (for MX-1) of Hsd or NCr athymic nu/nu mice (Harlan, Indianapolis, Indiana, and Taconic, Hudson, New York, USA). When tumors reached approximately 100 mg each in size, mice were randomized into 2-5 treatment groups, depending on the experiment, of 10 animals each. TLK58747 or the vehicle control was administered in a volume of 5 ml/kg for intravenous and 10 ml/kg for intraperitoneal or oral administration once daily for 5 days. Body weight and tumor measurements were recorded twice weekly. Tumor burden was estimated from caliper measurements. Partial and complete regression, morbidity and mortality were also recorded. Animals were sacrificed at the end of the experiment by CO<sub>2</sub> inhalation and tumors were harvested and weighed. Results were analyzed by *t*-test or ANOVA using GraphPad Prism. A *p*-value of less than 0.05 was considered statistically significant.

The efficacy study of TLK58747 against human D54/luc glioma was carried out by MIR Preclinical Services (Ann Arbor, Michigan, USA). Each female NCr nu/nu mouse (Taconic) was inoculated subcutaneously with  $3 \times 10^6$  tumor cells. The mice were randomly divided into 4 groups of 10 animals each. Treatment began on day 16 post-transplantation. TLK58747 was dosed at 80 mg/kg by *i.v.* injection, once daily, 5 days per week for 2 weeks. Body weight and tumor measurements were recorded twice weekly. Tumor burden was estimated from caliper measurements.

**Bone marrow suppression studies.** Normal male CD (Sprague-Dawley) IGS rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were weighed and randomly assigned to different treatment groups of 10 animals each. Animals were given daily intraperitoneal administration of TLK58747, CPA or vehicle for 5 consecutive days. Blood samples (150-200  $\mu$ l from each rat) were collected from the saphenous vein prior to treatment and every other day thereafter until day 14. Hematological analysis was performed immediately after sample collection using a CELL-DYN 3700CS system (Abbott Diagnostics, Abbott Park, Illinois, USA). Animals were also monitored daily for appearance and twice weekly for body weight.

**Toxicity studies.** Normal CD (Sprague-Dawley) IGS rats or Swiss Webster mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were randomly divided into groups of 3-6 animals each and given a single dose of the vehicle or TLK58747 (200-1,000 mg/kg orally or 40-100 mg/kg by injection). Animals were observed for any obvious behavioral changes (*e.g.* convulsion, palpebral closure or piloerection). To observe for toxicity following multiple administrations, animals were dosed once daily for 5 consecutive days and observed for 30 minutes after dosing. All treated animals were observed twice daily for the subsequent 14 days for moribundity, mortality or overt signs of toxicity, such as changes in appearance (fur, eyes, and body position), somatomotor function, and general behavior. Body weights were measured prior to treatment and at least twice per week thereafter. Rats were euthanized on day 15 post-dosing and mice were euthanized on day 3 post-dosing by CO<sub>2</sub> inhalation. The necropsies were performed

Table I. Cytotoxicity of TLK58747 against established human cancer cell lines.

Cell line (tumor type)	IC <sub>50</sub> ( $\mu$ M)
MX-1 (breast)	21 $\pm$ 8.1
DLD-1 (colon)	9.0 $\pm$ 1.5
MIA PaCa-2 (pancreatic)	23 $\pm$ 10
A549 (lung)	7.0 $\pm$ 0.9
OVCAR3 (ovarian)	2.3 $\pm$ 0.1
U-87MG (glioma)	6.6 $\pm$ 1.0
PC-3 (prostate)	22 $\pm$ 0.7
HL-60 (AML)	2.2 $\pm$ 0.5
K562 (CML)	14.4 $\pm$ 1.0
LNCap (prostate)	78 $\pm$ 3.3

immediately after the sacrifice. A necropsy also was performed if the animal died during the study. The tissues and internal organs (heart, lung, kidney, liver and spleen) were examined grossly for changes in color, shape, size, or consistency. Careful notes were made of any macroscopically visible abnormalities.

**Stability study.** TLK58747 was dissolved at 50  $\mu$ M in PBS buffer at pH 7.4 and incubated at 37°C. Samples were taken at various time points (0.5-2.5 hours) for analysis by mass spectrometry. The chemical lifetimes were determined by manually measuring the integrated area of the molecular ion peak in the MS chromatogram ((M+H)<sup>+</sup>, (M-H)<sup>-</sup> or (M+Na)<sup>+</sup>) and plotting it as a function of time. The resulting curve was then fitted numerically to an exponential function based on the assumption that degradation of the compound was a first-order process.

## Results

**Inhibition of cancer cell growth in vitro.** TLK58747 inhibited the growth of multiple human cancer cell lines *in vitro*, including those derived from breast (MX-1), lung (A549), colorectal (DLD-1), ovary (OVCAR3), pancreas (MIA PaCa 2), and prostate (PC-3, LNCap) carcinomas, as well as glioma (U-87MG) and promyelocytic leukemia (HL-60). As reported in Table I, TLK58747 was cytotoxic to all tumor cell lines tested, producing 50% inhibition of cell growth (IC<sub>50</sub>) at concentrations from 2 to 78  $\mu$ M.

The development of drug resistance has limited the utility of cancer chemotherapeutics and is an increasingly important clinical problem (5, 16-19). Resistance may extend to multiple cancer drugs in a phenomenon known as cross-resistance. The cytotoxicity of TLK58747 was tested in OVCAR3 cell line derivatives that were adapted for resistance to carboplatin or paclitaxel and in P388ADR, a multidrug-resistant leukemia cell line that was originally selected for resistance to adriamycin but also showed cross-resistance to etoposide, paclitaxel, colchicine, mitoxantrone, actinomycin D and several other agents (20, 21). As shown in Table II, P388ADR cells exhibited more than 200-fold resistance to

Table II. Low cross-resistance of TLK58747 with standard cancer chemotherapeutics.

Drug	IC <sub>50</sub> (μM):		Resistance index
	Parent line	Resistant line	
Doxorubicin resistance (P388 & P388ADR lines):			
Doxorubicin	0.0017±0.0002	0.36±0.08	207
TLK58747	0.40±0.07	0.80±0.05	2.0
Cisplatin	0.099±0.004	0.36±0.10	3.6
Melphalan	0.20±0.01	0.35±0.02	1.8
Carboplatin resistance (OVCAR3 & OVCAR3R lines):			
Carboplatin	3.33±1.15	24.4±7.1	7.3
TLK58747	1.41±0.48	2.84±0.68	2.0
Cisplatin	0.61±0.17	4.80±1.31	7.8
Melphalan	1.11±0.09	2.08±0.40	1.9
Paclitaxel resistance (OVCAR3 & OVCAR-TaxolR lines):			
Paclitaxel	0.0005±0.0002	0.013±0.004	26
TLK58747	1.37±0.57	1.86±0.36	1.4
Cisplatin	0.41±0.07	1.54±0.32	3.8
Melphalan	1.13±0.08	1.94±0.45	1.7

adriamycin, but only 2-fold resistance to TLK58747 compared with the parental line. Similar low cross-resistance was observed in cancer cell line derivatives resistant to carboplatin or paclitaxel. In comparison, melphalan showed 2-fold cross-resistance to these 3 drugs, whereas cisplatin was completely cross-resistant to carboplatin and 4-fold cross-resistant to adriamycin or paclitaxel (Table II).

*Induction of G<sub>2</sub>/M cell cycle arrest and apoptosis or premature senescence in solid tumor cells.* We examined the cell cycle effects of TLK58747. In both p53<sup>+</sup> A549 (Figure 2a) and p53<sup>-</sup> OVCAR3 (Figure 2b) cell lines, TLK58747 induced a transient block in the S phase followed by G<sub>2</sub>/M arrest. This profile was also observed in other solid tumor cell lines (data not shown). For comparison, melphalan and 4-hydroperoxycyclophosphamide caused similar changes in cell cycle (Figure 2 and data not shown). Cisplatin had a cell cycle effect similar to that of TLK58747 in A549 cells but induced apoptosis without apparent cell cycle arrest in OVCAR3 cells (Figure 2). Interestingly, TLK58747 triggered rapid apoptosis without obvious cell cycle block in the HL-60 human promyelocytic leukemia cell line (data not shown), consistent with the observed sensitivity of leukemic cells to alkylating agents (1, 2, 22-24).

We investigated the fate of the cells following TLK58747 treatment. In A549 cell line, exposure to TLK58747 produced a flat, enlarged morphology and positive staining for SA-β-galactosidase, a marker for cellular senescence (15). The induction of premature senescence appeared to be

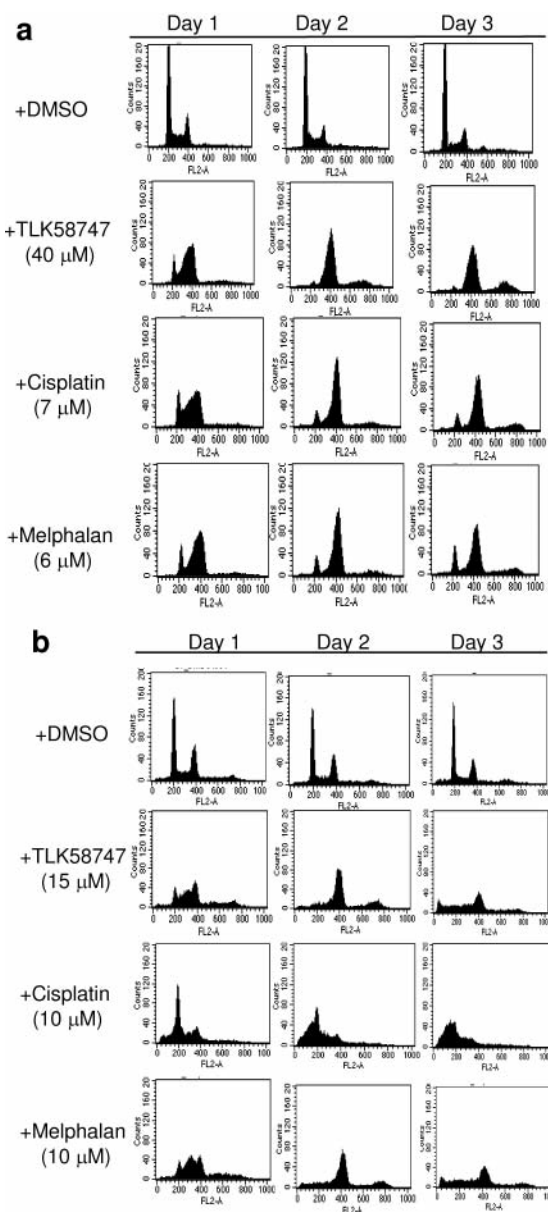


Figure 2. Induction of G<sub>2</sub>/M cell cycle arrest in solid tumor cells by TLK58747. A549 (a) or OVCAR3 (b) cells were treated with the indicated agents for various times and then harvested for cellular DNA content analysis by FACS following PI staining. Shifts in the G<sub>2</sub>/M peaks in drug-treated cells were due to increased cell size.

time and dose dependent (Figure 3a), with significantly detectable SA-β-galactosidase activity by day 4.

In p53<sup>-</sup> human cancer cell lines such as OVCAR3 and DLD-1, treatment with TLK58747 led to apparent cell death, as suggested by the appearance of the sub-G1 population in the cell cycle analysis (Figure 2b and data not shown). To confirm the induction of apoptosis, OVCAR3 cells were treated with TLK58747 and then harvested for examination

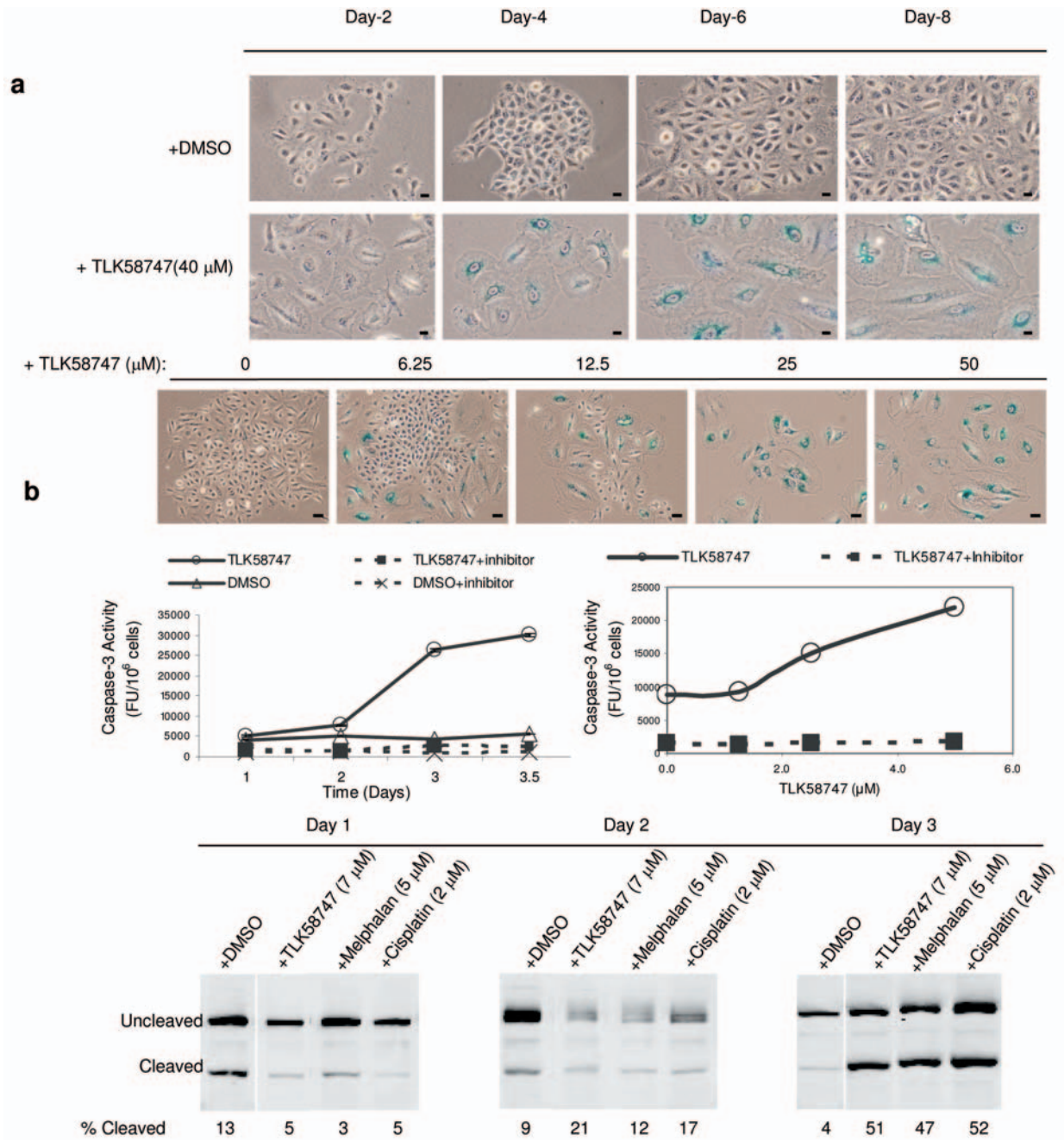


Figure 3. Induction of premature senescence or apoptosis in tumor cells by TLK58747. a, A549 cells were treated with TLK58747 for different lengths of time at 40 μM (upper panel) or for 6 days at different concentrations (lower panel). The cells were then fixed and stained for the activity of SA-β-galactosidase. The plates were observed under a microscope (×200 magnification, upper panel; ×100 magnification, lower panel) and representative fields were photographed. The scale bars show 20 μm and 50 μm in the upper and lower panels, respectively. b, Induction of apoptosis by TLK58747 in OVCAR3 cells. OVCAR3 cells were treated with TLK58747 at 7 μM for different lengths of time or for 3.5 days at different concentrations. Cells were then harvested for caspase-3 activity assay (top graphs) or Western blot analysis for PARP cleavage (bottom panel).

of caspase-3 activity, a marker of apoptosis (25). Dose- and time-dependent activation of caspase-3 was observed (Figure 3b, upper graphs). Apoptosis was further confirmed by increased poly(ADP-ribose) polymerase (PARP) cleavage,

another apoptosis marker (26). Increase in PARP cleavage followed a time course that correlated well with the induction of caspase-3 activity, with slight increase by day 2 and significant (approximately 10-fold) increase on day 3

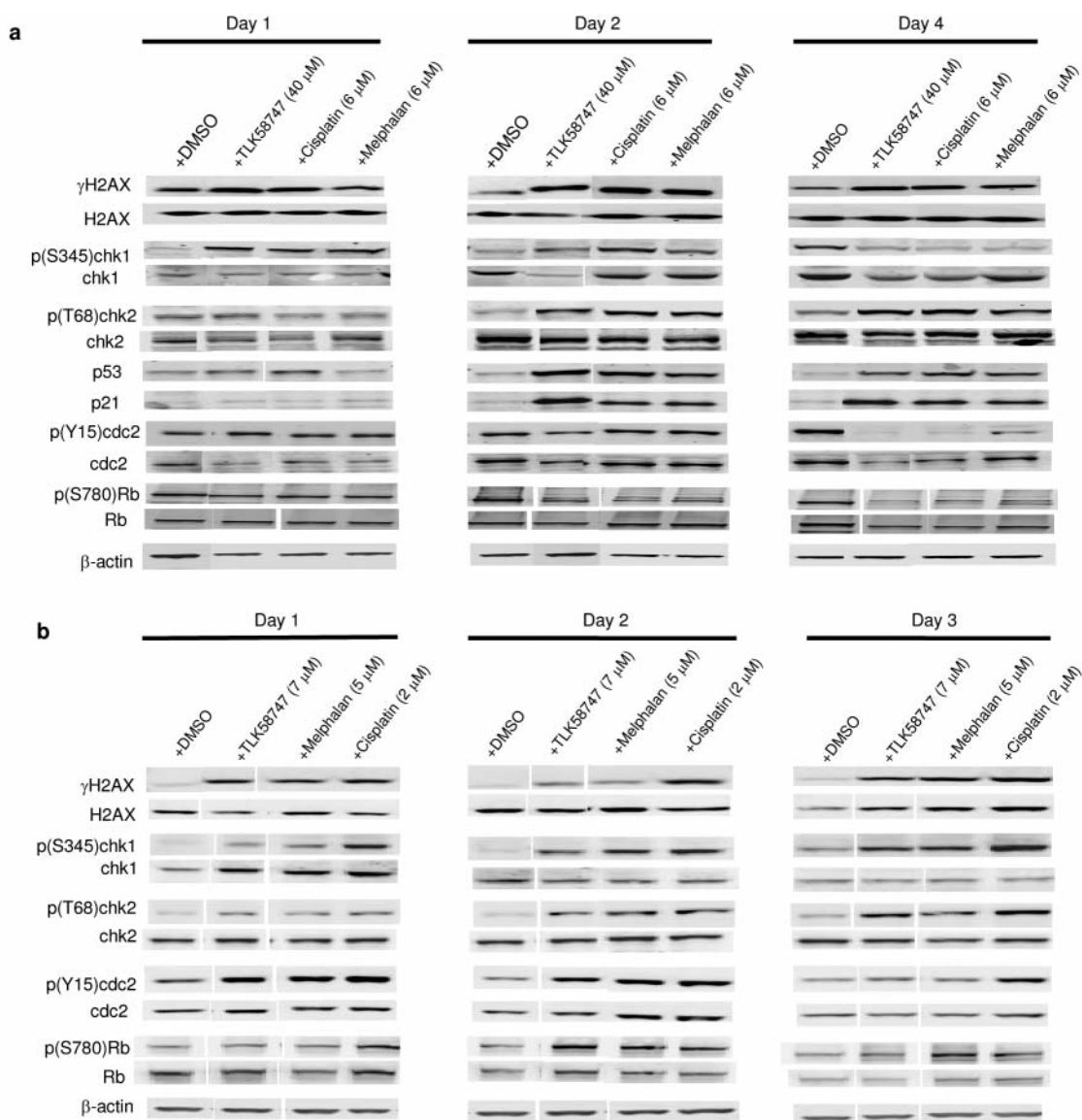


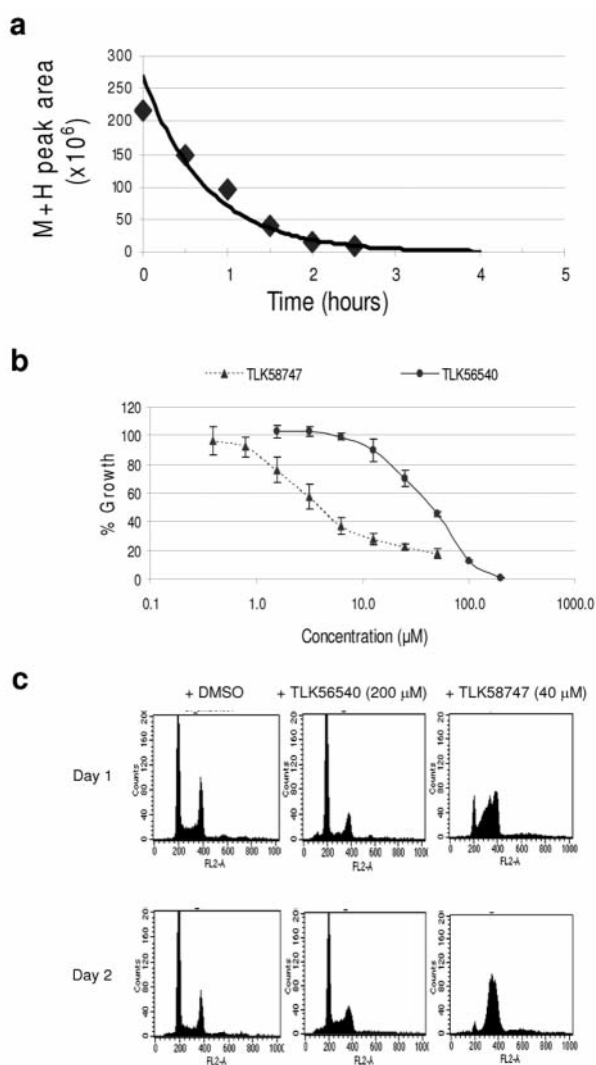
Figure 4. Activation of the DNA damage response pathway by TLK58747 in A549 cells (a) and OVCAR3 cells (b). The cells were treated with the indicated agents at approximately equipotent concentrations for different lengths of time. Total cell lysates were prepared and subjected to Western blot analysis using various antibodies as indicated. Beta-actin was used as the loading control. The blots were imaged using the Odyssey Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

(Figure 3b, lower panel). Melphalan and cisplatin also induced apoptosis in this cell line (Figure 3b).

**Activation of the DNA damage response pathway.** As shown in Figure 4a, treatment of A549 cells with TLK58747 led to changes in cell cycle regulatory proteins that were consistent with G<sub>2</sub> arrest, including reduced Rb phosphorylation and increased p53 and p21 protein levels (27-29). Cdc2 kinase, which is the gatekeeper of the G<sub>2</sub>/M transition, was initially inactivated post-translationally *via* increased phosphorylation

on Tyr-15, a site known to be the kinase negative regulatory site (30, 31), and later down-regulated at the protein level. Furthermore, Western blot analysis also revealed a significant increase in the phosphorylation of DNA damage response proteins such as H2AX, chk1, and chk2, indicating the activation of the G<sub>2</sub>/M checkpoint.

As shown in Figure 4b, Western blots showed an increased phosphorylation of cdc2 on Tyr-15 after TLK58747 treatment in OVCAR3 cells, consistent with the observed G<sub>2</sub>/M arrest. Treatment with TLK58747 in this cell line also



**Figure 5. Activation of TLK58747 by cleavage.** *a*, Stability of TLK58747 at physiological pH. TLK58747 was dissolved at 50  $\mu\text{M}$  in phosphate-buffered saline (pH 7.4) and incubated at 37°C. Samples were taken at different time points for analysis by mass spectrometry. The integrated area of the molecular ion peak in the MS chromatogram ( $(M+H)^+$ ,  $(M-H)^-$  or  $(M+Na)^+$ ) was plotted as a function of time. *b*, Cytotoxicity assay. TLK58747 and its noncleavable analog TLK56540 were tested in A549 cells. Cell growth after compound treatment was measured by using the CellTiter-Glo assay. The graph shows the average and standard deviations. *c*, Cell cycle analysis. A549 cells were treated with the indicated compounds and then harvested for cellular DNA content analysis by FACS following PI staining.

appeared to activate the DNA damage response pathway as shown by increased phosphorylation of H2AX, chk2 and chk1. Unlike what was observed in A549 cells, however, the level of phospho-Rb was increased in OVCAR3 cells, suggesting the involvement of a signaling pathway that mediates the induction of pro-apoptotic proteins (32).

*TLK58747 as a prodrug.* TLK58747 is cleaved under physiological conditions to produce a vinyl sulfone and *N,N,N,N*-tetrakis(2-chloroethyl)phosphordiamidic acid (Figure 1a). It has a half-life of approximately 30 minutes in PBS (pH 7.4) at 37°C (Figure 5a). To further confirm that TLK58747 is a prodrug, we compared TLK58747 with its sulfide analog (TLK56540) that cannot readily undergo cleavage by  $\beta$ -elimination (Figure 1b). In cytotoxicity assays, TLK56540 was more than 10-fold less active than TLK58747, with an  $\text{IC}_{50}$  of 52  $\mu\text{M}$  for TLK56540, compared to an  $\text{IC}_{50}$  of 5  $\mu\text{M}$  for TLK58747 in A549 cells (Figure 5b). Additionally, the weak cytotoxicity of TLK56540 was not associated with an obvious effect on cell cycle (Figure 5c).

*In vivo activity.* The *in vivo* antitumor efficacy of TLK58747 was examined in multiple human cancer xenograft models. When administered by oral gavage once a day for 5 days (*qdx5*), TLK58747 strongly inhibited the growth of MX-1 human mammary carcinoma xenografts implanted in the mammary fat pad in Hsh nu athymic mice (Figure 6a). Complete tumor regression was observed in 10 out of 10 animals treated with 300 mg/kg, and in 4 out of 10 animals treated with 200 mg/kg, while significant tumor growth inhibition (92%) was observed with 100 mg/kg of TLK58747. TLK58747 was also effective against other tumor types. Oral administration of TLK58747 significantly inhibited the growth of MIA PaCa-2 human pancreatic carcinoma (Figure 6b) and HL-60 human promyelocytic leukemia (Figure 6c, left graph), by 86% and 78%, respectively, when given at a dose of 300 mg/kg, *qdx5*. It also demonstrated significant activity against subcutaneous xenografts of human PC-3 prostate carcinoma and D54/luc glioma (data not shown). Importantly, at the oral doses tested, TLK58747 caused no mortality and produced no significant body weight loss (Figure 6c, right graph, and data not shown).

The activity of TLK58747 was also tested parenterally. In the MX-1 model, TLK58747 produced complete tumor regression when given by intraperitoneal injection at 100 mg/kg (Figure 6d, left graph). This compared favorably to TLK286, which produced 27% tumor growth inhibition at an equimolar dose (Figure 6d, right graph). Similar results were obtained for TLK58747 following intravenous injection. At 80 mg/kg of TLK58747, 8 out of 10 animals showed no detectable tumor mass 2 weeks after treatment while no significant toxicity was observed (data not shown).

*Toxicity studies.* When given orally in a single dose, TLK58747 caused no significant behavioral changes or body weight loss in mice at 1,000 mg/kg, which was the highest dose tested. In rats, TLK58747 was safe at 500 mg/kg, but lethality (3/6) was observed at 1,500 mg/kg. In repeated dosing by intravenous injection, TLK58747 was safe with no significant behavioral changes or body weight loss at 80 mg/kg, *qdx5* and 60 mg/kg, *qdx5*, in mice and rats, respectively (data not shown).

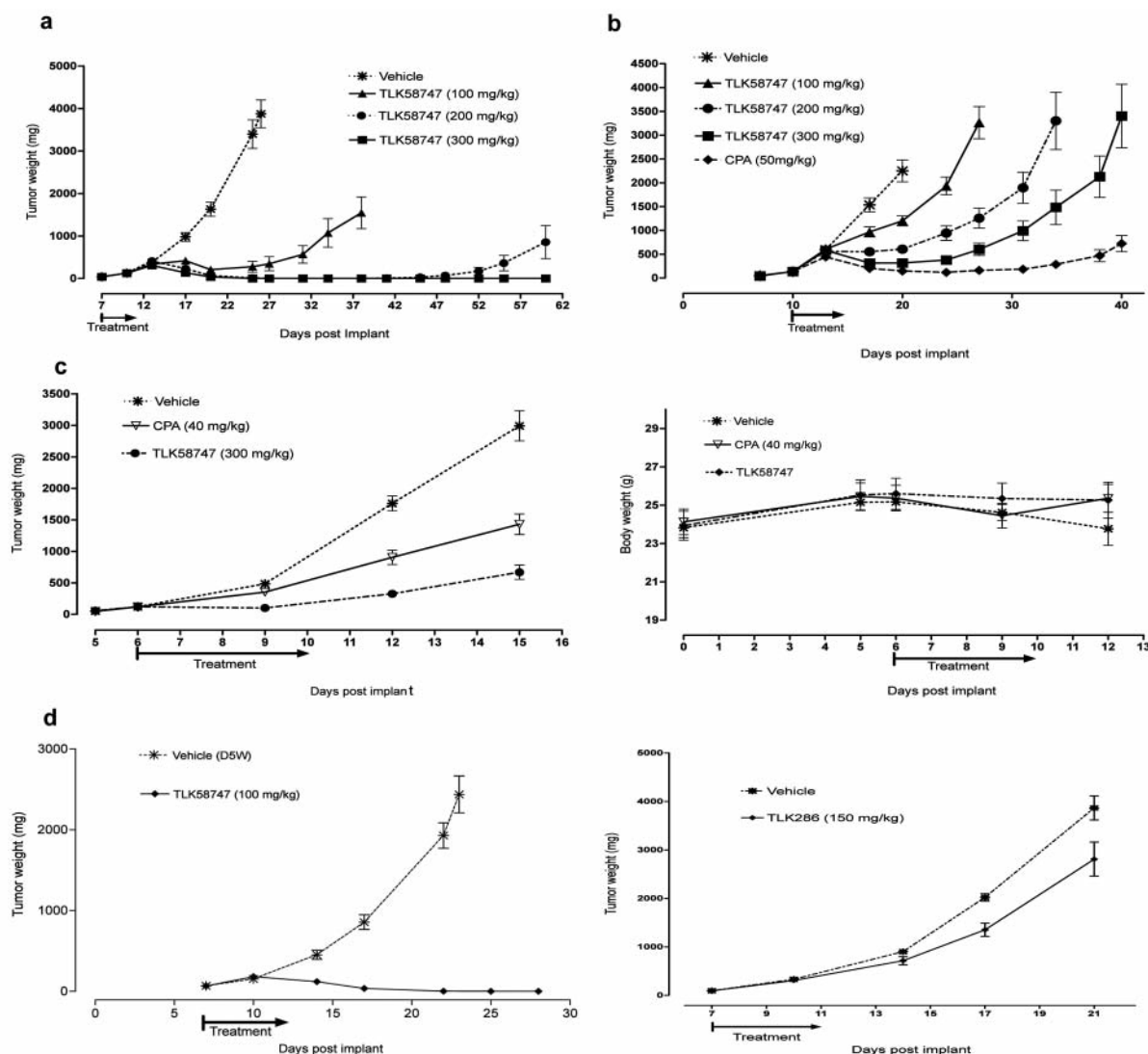


Figure 6. *In vivo* antitumor efficacy of TLK58747. Nude mice bearing MX-1 (a, d), Mia PaCa-2 (b), or HL-60 (c) subcutaneous tumor xenografts were treated with TLK58747 either orally (a-c) or by intravenous injection (d), qdX5. Intravenous dosing of MX-1-bearing nude mice with TLK286 is shown in Figure 6d, right graph. CPA was used as the positive control. Body weight for TLK58747- and CPA-treated animals is shown in Figure 6c, right graph. Tumor and body weight were measured twice weekly. Tumor growth was analyzed and graphed using GraphPad Prism. Tumor growth inhibition resulting from TLK58747 treatment was statistically significant in all cases.

Bone marrow toxicity is a common dose-limiting side-effect of most alkylating agents (33). We therefore tested the effect of TLK58747 on various blood lineages in rats and compared it with CPA. Intraperitoneal administration of TLK58747 at 100 mg/kg caused neutropenia (Figure 7a). However, TLK58747 had no effect on red blood cells (Figure 7b) and platelets (Figure 7c), in sharp contrast to CPA. In the lymphocyte lineage, the effects of TLK58747 and CPA were similar, causing a reduction in the lymphocyte count that gradually recovered (Figure 7d). These data indicate that TLK58747 has the potential to be bone-marrow sparing compared to alkylators such as CPA.

## Discussion

We report here the identification and characterization of TLK58747, a novel alkylating agent. Structurally, this compound is a substituted sulfonyl ethyl phosphorodiamidate which releases an alkylating moiety upon cleavage at physiological pH. TLK58747 was optimized for increased efficacy and a broad spectrum of antitumor activity. It was further selected based on its physicochemical properties, which facilitate its delivery both orally and parenterally.

TLK58747 may offer several advantages over alkylating agents such as cyclophosphamide and ifosfamide. Because



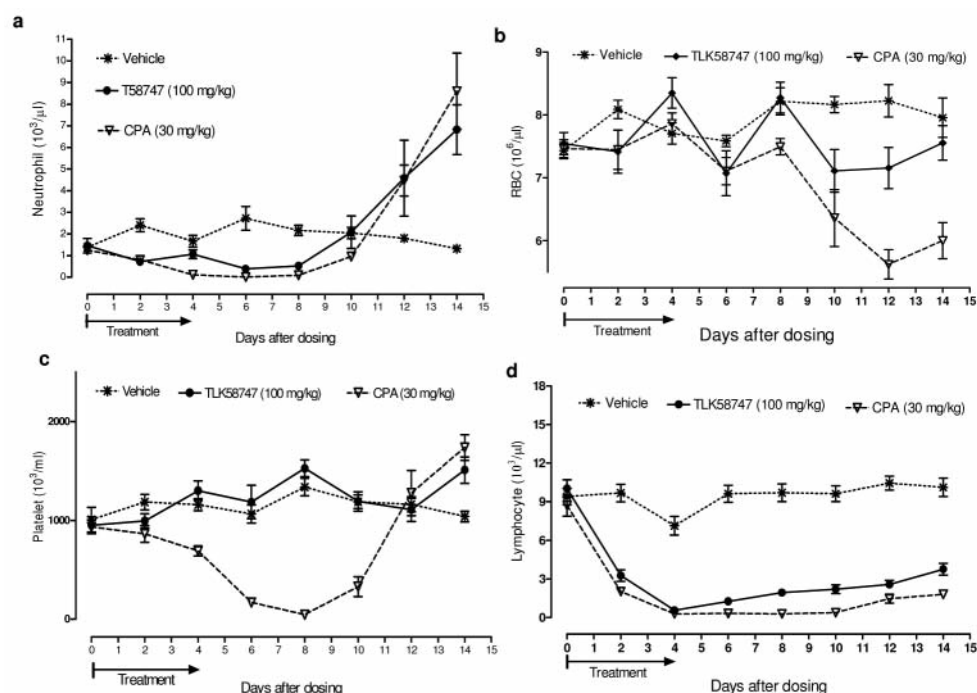


Figure 7. Mild hematological toxicity of TLK58747 *in vivo*. Normal rats were treated by intraperitoneal injection with TLK58747 or cyclophosphamide as indicated. Peripheral blood samples were collected at various time points and analyzed using the CELL-DYN 3700 system. The means and standard errors for the blood cell counts were calculated and graphed using GraphPad Prism. a, Neutrophils; b, red blood cells; c, platelets; d, lymphocytes.

of its structure, TLK58747 does not produce acrolein, a byproduct of cyclophosphamide that is responsible for serious hemorrhagic cystitis in the clinic (34). Another important feature of TLK58747 is its mild hematological toxicity, with little or no effect on red blood cells and platelets in animals. This is similar to what has been reported for TLK286, another prodrug which releases the same active moiety upon activation (35). This characteristic of TLK58747 could offer significant benefits over DNA-damaging agents such as cyclophosphamide, chlorambucil and bendamustine since hematological toxicity is often dose-limiting for such agents and requires a dose reduction or adjunct therapy (36-39).

Exposure of multiple human solid tumor cell lines to TLK58747 induced  $G_2/M$  arrest leading to premature senescence or apoptosis in a time- and dose-dependent manner. Increased levels of  $\gamma\text{H2AX}$ , phospho-chk1 and phospho-chk2 were observed, indicating the activation of the DNA damage response pathway (40, 41). Consistent with the activation of the  $G_2/M$  cell cycle checkpoint, cdc2 was increasingly phosphorylated on the negative regulatory site (30, 31).

The induction of premature senescence or apoptosis by chemotherapeutics may be related to the p53 status of the tumor cells (42). In the case of TLK58747, treatment of p53<sup>+</sup> A549 cells led to the induction of premature

senescence. Western blot analysis showed increased expression of p53 and p21 and decreased levels of phospho-Rb and cdc2. In contrast, apoptosis was observed in p53<sup>-</sup> OVCAR3 cells which had an increased level of phospho-Rb. It has been reported that DNA damage can result in transient or permanent  $G_2$  cell cycle arrest *via* p53-independent or p53-dependent mechanisms (43). It is also known that Rb can suppress the expression of pro-apoptotic genes (32). Based on our data, we propose a model in which TLK58747 induces transient  $G_2$  arrest through the activation of a p53-independent DNA damage-response pathway involving ATM/ATR, chk1 and chk2. In the presence of p53, the expression of p21 and other cdk inhibitors is elevated, which in turn leads to increased Rb suppressor activity and down-regulation of cdc2 transcription. These changes then lead to persistent  $G_2$  arrest, which ultimately activates the senescence program. In the absence of p53, Rb phosphorylation is increased *via* the activation of chk1, chk2 or other kinases involved in DNA damage response. The reduced Rb activity allows increased transcription of downstream target genes, such as Abl and E2F, which positively regulate the expression of pro-apoptotic proteins including Bax and Puma.

In summary, we have identified TLK58747 as a novel anticancer prodrug. It inhibited tumor cell growth *via*  $G_2/M$

cell cycle arrest mediated by the DNA damage-response pathway, leading to subsequent cell death or premature senescence. TLK58747 showed significant efficacy both orally and parenterally in xenograft models of human leukemia, as well as glioma and other solid tumors, including pancreatic cancer, that are known to be insensitive or resistant to other alkylating agents. TLK58747 had low cross-resistance with standard chemotherapeutics *in vitro* and thus may be suitable for combination therapies. TLK58747 also had reduced hematological toxicity *in vivo*, suggesting that it has the potential to be well tolerated.

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