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ORIGINAL ARTICLE

Growth inhibition of hepatocellular carcinoma cells in vitro and in vivo by the 8-methoxy analog of WMC79

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Abstract HKH40A (RTA 502), an optimized 8-methoxy analog of the unsymmetrical bifunctional antitumor agent WMC79, was found to be potently active against liver cancer cell growth in vitro and in vivo. Studies on selected human hepatocellular carcinoma (HCC) cell lines with differing p53 status (HepG2, Hep3B, and PLC/PRF/5), revealed that drug-mediated growth inhibition was independent of p53 status. FACS analysis showed an accumulation of cells in S-phase within 24 h of treatment with 100 nM HKH40A. Subsequent incubation of cells, either in the presence of drug or without, caused cell cycle block at the S and G2/M checkpoints, which was consistent with the observed up-regulation of p21, cyclin A, cyclin B1, sustained phosphorylation of Cdk1, and down-regulation of Cdc6, Cdc7, and RRM2. This irreversible growth arrest eventually led to apoptosis. HKH40A completely suppressed growth of the rat transplantable HCC cell line (JM-1) in an orthotopic model in Fisher 344 rats in vivo, without evidence of toxicity. HKH40A may be a useful agent for new therapeutic strategies focusing on inhibition of HCC cell proliferation.

Keywords Anti-cancer activity · Hepatocellular carcinoma · Replication arrest · Cdc6 · Cdc7

Abbreviations

WMC79	5-nitro-2-(3-{4-[3-(6-oxo-6H-2,10b-diaza-aceanthrylen-5-ylamino)-propyl]-piperazin-1-yl}-propyl)-2-aza-phenalene-1,3-dione
HKH40A (RTA 502)	5-nitro-2-(3-{4-[3-(8-methoxy-6-oxo-6H-2,10b-diaza-aceanthrylen-5-ylamino)propyl]piperazin-1-yl}-propyl)-2-aza-phenalene-1,3-dione
wt	wild-type
mt	mutated
FBS	fetal bovine serum
TBS	Tris buffer saline
DMEM	Dulbecco's modified Eagle's medium
EMEM	Eagle's minimal essential medium
RRM2	ribonucleotide reductase M2
HCC	hepatocellular carcinoma

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We sadly report the death of our colleague Chris Michejda, during completion of this manuscript.

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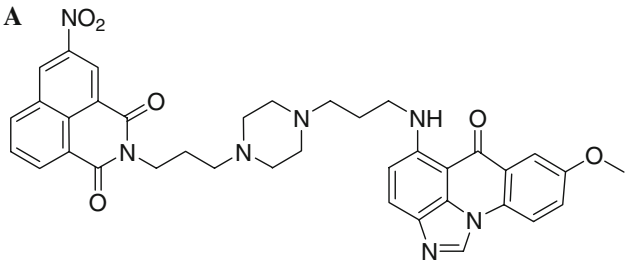
Introduction

HKH40A (RTA 502) or 5-nitro-2-(3-{4-[3-(8-methoxy-6-oxo-6H-2,10b-diaza-aceanthrylen-5-ylamino)propyl]piperazin-1-yl}-propyl)-2-aza-phenalene-1,3-dione (Fig. 1a), is a synthetic anticancer agent, consisting of an imidazoacridone linked to a 1,8-naphthalimide moiety via a 1,4-dipropylpiperazine linker, and which has demonstrated exceptional in vitro activity in the NCI-60 tumor cell line panel (IC_{50} about 1 nM in selected tumors) [1]. Chemically, this compound is an 8-methoxy analog of WMC79 (NSC 695942) which was designed and developed in our laboratory as a potential DNA-intercalating agent [2]. We expected that blocking position 8 of the imidazoacridone ring system of WMC79 with a methoxy group should preserve its DNA binding properties but render a metabolically more stable compound, with improved pharmacokinetic properties. Compounds from the bifunctional naphthalimide-imidazoacridone class bind to DNA but their anticancer activity is manifested only after the initial drug-DNA complex hijacks a protein that is involved in repair, transcription or replication. [2, 3].

A



B

Cell line	GI_{50} * (μmol/L)	TGI † (μmol/L)	LC_{50} ‡ (μmol/L)
HepG2 (p53 wt)	0.001	0.009	0.030
Hep3B (p53 null)	0.002	0.012	0.040
PLC/PRF/5 (p53 mt)	0.002	0.022	0.070
JM-1 (p53 mt)	0.004	0.020	0.050

Fig. 1 **a** Chemical structure of HKH40A. **b** Growth inhibitory activity of HKH40A against HCC cell lines in a MTT assay at 120 h. Note: The GI_{50}, TGI, and LC_{50} values were determined by interpolation from a plot of % growth (T/C) versus drug concentration from at least three independent determinations. *GI_{50} concentration of the drug resulting in inhibition of cell growth to 50% of controls. †TGI concentration of the drug resulting in total growth inhibition. ‡LC_{50} concentration of the drug required to reduce the initial cell number by 50%

Both, unsubstituted and the 8-methoxy compounds are very potent growth inhibitors of human colon cancer cell lines that express the wild type p53 tumor suppressor gene [4, 5]. Sensitive wt p53 cancer cell lines such as colon HCT116 and RKO are killed by a p53-dependent apoptotic cascade initiated by a rapid elevation of p53, which results in the activation of the FasL pathway, upregulation of the Bax/Bcl2 ratio and the resultant activation of the mitochondrial apoptosis pathway [5]. Growth of cancer cell lines with mutated p53 such as the colon tumor HT29 and pancreatic tumor ASPC1 or 10.05 is also inhibited by the drug, but as a result of a G2-M block and grossly dysregulated S-phase [6]. In contrast to WMC79 which showed about tenfold difference in cell killing potency between tumor cells with wild-type and mutated p53, its 8-methoxy analogs showed only two- to threefold difference in vitro [1]. This suggests that the optimized 8-methoxy analog maybe effective against broader spectrum of tumors than parent WMC79.

Clinically, hepatocellular carcinoma (HCC) is among the five leading causes of cancer death world-wide. A majority of patients (>80%) are diagnosed at an advanced stage when curative surgery is no longer possible [7]. There is an urgent need for new therapeutic strategies focusing on inhibition of HCC cell growth. We therefore evaluated the effects of HKH40A on a variety of human HCC cell lines, and on one rat line, the growth of which can be studied both in vitro and in vivo.

Materials and methods

Chemicals

All mammalian cell culture reagents were purchased from GIBCO-BRL (Grand Island, NY). Tissue culture materials were from Corning Incorporated (Acton, MA). Hoechst 53342 was from Molecular Probes, Inc. (Eugene, OR). Acridine Orange (AO), Crystal Violet, dimethyl sulphoxide (DMSO), ethylenediaminetetraaceticacid (EDTA), methanesulfonic acid, propidium iodide (PI), RNaseA, Sample Buffer Laemmli, sodium dodecyl sulfate (SDS), sodium chloride, Trizma-Base, and polyoxyethylene-sorbitan monolaurate(Tween 20) were from Sigma-Aldrich (St. Louis, MO).

Cell culture

Human HCC cell lines HepG2 (ATCC#: HB-8065, p53 wt), PLC/PRF/5 (ATCC #: CRL-8024, p53 mt) and Hep3B cells (ATCC#: HB-8064, p53 null) were purchased from the American Type Culture Collection (Rockville, MD).

Rat JM-1 HCC cell line was originally a gift of G. Michaelopoulos, then at University of Wisconsin. HepG2 and PLC/PRF/5 cells were grown in DMEM, Hep3B, and JM-1 cells were maintained in EMEM medium. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were grown at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells in exponential growth phase were used in all the described experiments.

Drug preparation

HKH40A was synthesized in our laboratory [1]. A stock solution (5 mmol/L) was prepared by dissolving its salt form in water and stored at 4°C. Prior to use, stocks were diluted to a final concentration of 500 μmol/L in distilled water and then used to prepare a 2 μmol/L working solution and its tenfold serial dilutions in appropriate tissue culture media.

Growth inhibition assays

Cell growth in the presence (T) or absence (C) of experimental agent was determined using the MTT -based, CellTiter96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to instructions provided by the supplier of the kit with small modifications as previously described [2]. Briefly, cells were seeded in six wells for each studied concentration into 96 flat well microtiter plates (100 μL of medium containing 1,000–1,500 cells per well) and were precultured for 1 day at 37°C. Time and dose dependent drug-activity was determined using several exposure durations to various concentrations of HKH40A ranging from 24 to 120 h. While the drug was added, assays were performed on extra reference plates to determine the cell population density at time 0 (T_0). The absorbance of the wells was determined at 544 nm by a FLUOstar/POLARstar Galaxy (BMG Labtechnologies GmbH) Microplate-Reader and the average value from six wells was used for calculations. Cellular responses were calculated from the data as described previously [8]: $100 \times [(T - T_0)/(C - T_0)]$ for $T > T_0$ and $100 \times [(T - T_0)/T_0]$ for $T < T_0$. Each experiment was repeated three times.

Colony formation assay

Colony formation assay was performed as previously described [9]. Briefly, exponentially growing Hep3B HCC cells were harvested, using trypsin–EDTA (0.25%), re-suspended in fresh culture medium and seeded into 6-well plates at a density of 250–300 cells/well. The following day they were treated in duplicate samples with increasing concentrations of HKH40A for 6 h, at which time the culture medium was replaced with drug-free medium. Cells were observed daily and fed weekly. After incubation for 14 days, they were washed with PBS, fixed by a series of solutions of methanol: PBS (v/v) of increasing methanol concentration (50, 70, and 100%)and then stained with 1% of crystal violet in methanol, rinsed with tap water and air dry. Colonies of greater than 30 cells were scored as survivors. The experiment was repeated twice.

BrdU cell proliferation assay

The effect of HKH40A on DNA synthesis was examined in terms of bromodeoxyuridine (BrdU) incorporation using a BrdU cell proliferation assay according to the manufacturer's protocol provided with the kit (Oncogene Research Products, Cambridge, MA). Briefly, cells were seeded in 96-well plate (10,000 cells/well) and allowed to attach for 24 h. Next, they were treated with 100 μM of the test drug for 72 h and then with BrdU for 24 h. The level of incorporated BrdU was detected immunochemically and then absorbance was measured using a spectrophotometric plate reader at dual wavelengths of 450–540 nm.

Fluorescence-activated cell sorting (FACS) analysis

Tumor cells in exponential phase of growth were seeded at a density of 0.5×10^6 cells in 25 cm^2 flasks, allowed to attach for 24 h, and then exposed to 100 nmol/L HKH40A. At the described intervals, drug-treated and control cells (attached and floating) were collected and prepared for cell cycle analysis according to standard procedures [5, 9]. Cell cycle analysis was done on a Beckman Coulter Epics XL-MCL flow cytometer (Fullerton, CA) with 10,000 events per collected sample.

Detection of active caspases 3 and 7

Caspase 3 and 7 activity was measured using a Magic Red™ Caspase Detection Kit (Immunochemistry Technologies, LLC, Bloomington, MN) according to instructions provided by the supplier of the kit. Briefly, non-treated and treated with 100 nmol/L HKH40A Hep3B cells were incubated in chamber slides for 96 h, labeled with MR-$(DEVD)_2$ (Magic Red-aspartylglutamylalanylaspartic acid), incubated for 2 h at 37°C and then for an additional 10 min with Hoechst dye. Cells were analyzed using fluorescence microscope. The red fluorescent MR product in apoptotic cells was observed at excitation 550 nm and a long pass >610 nm emission. Hoechst labeled cell nuclei were detected using a UV-filter with excitation at 365 nm and emission 480 nm.

Western blot analysis

Immunoblot analysis of cell protein lysates was done according to Santa Cruz Biotechnology, Inc. manufacturer's instructions as previously described [5]. Briefly, cells were lysed on ice for 30–60 min in radioimmunoprecipitation assay buffer (1× PBS, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added inhibitors (10 μg/mL phenylmethylsulfonyl fluoride, 50 μg/mL aprotinin, and 1 mmol/L sodium orthovanadate). Cell lysate was passed through a 21-gauge needle followed by centrifugation at 10,000×*g* for 10 min at 4°C. Protein concentration was determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Samples were mixed with 2× Laemmli buffer, denatured at 100°C for 3 min, and proteins were separated by electrophoresis (NuPAGE 4–12% Bis-Tris Gel, Invitrogen, Life Technologies, Carlsbad, CA). Separated proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and subjected to immunoblotting with various primary antibodies. Positive antibody reactions were visualized with a horseradish peroxidase–conjugated secondary antibody and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the protocol of the manufacturer. The membrane was then deprobed and reprobed with an anti-actin antibody to confirm that all samples contained similar amounts of proteins. TBS-0.05% Tween 20 was used as a wash buffer; 5% nonfat dry milk (Bio-Rad Laboratories) was dissolved in TBS-0.05% Tween 20 and was used as a blocking solution. The following antibodies were used in this study: mouse monoclonal anti-actin (Abcam Inc., Cambridge, MA), anti-p21 (Ab-1), anti-p53 (Ab-6) (Oncogene Research Products, Boston, MA), anti-cyclin A, anti-cyclin B1 (Biosource International, Camarillo, CA), anti-Cdc6 (Ab-1) (Lab Vision, Fremont, CA), anti-Cdc7 (MBL, Nagoya, Japan), polyclonal goat anti-R2 (I-15) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal rabbit anti-phospho-Cdc2 (Tyr15) (Cell Signaling Technology, Beverly, MA). All secondary antibodies (horse-radish peroxidase conjugates) and Cruz Marker molecular weight standards were from Santa Cruz Biotechnology.

In vivo anti-tumor effects

The chemically induced Fischer F344 rat HCC (hepatoma) cell line JM-1 [9] was cultured in EMEM + 10% FBS. Cells were harvested after trypsinization (0.2%) for 10 min at 37°C, washed once with 10 mL of ice cold PBS, and resuspended in cold PBS at 10^6 cells per mL. Normal Fischer F344 rats (2 months old with average weight of 250 g) were injected, with 1 mL of 10^6 JM-1 rat hepatoma cells in PBS, through their mesenteric vein. Three groups of rats (five per group) were subsequently injected intraperitoneally, every other day for a total of five injections, with 0.5 mL of 0.2 or 2 mg/kg body weight HKH40A or solvent control (1% DMSO), starting one day after JM-1 cell transplantation. Rats were sacrificed 3 weeks after cell transplantation and the numbers of liver tumor foci were counted.

In a second experimental series, three groups of rats (five per group) were treated intraperitoneally with 1 mg/kg body weight HKH40A, every other day for a total of five injections, starting on 1, 7, or 14 days after JM-1 cell transplantation. A control group of four rats was treated with solvent (1% DMSO). All rats were sacrificed 4 weeks after cell transplantation and the numbers of liver tumor foci were counted.

Results

Potent, p53-independent HCC cell growth inhibition in vitro by HKH40A

The effects of HKH40A on growth of three human HCC cell lines with different p53 status (HepG2, Hep3B and PCL-PRF5) and one rat HCC line (JM1) was assessed by the MTT assay (Fig. 1b). All cell lines were similarly sensitive to the agent, which significantly inhibited cell proliferation (GI_{50} and TGI) at low nanomolar concentrations. By contrast, >tenfold concentrations were required for the same cellular effects using camptothecin or doxorubicin (data not shown). Time course analysis showed that the cell growth inhibition effect was dose and time-dependent (Fig. 2a). Concentrations of 100 nmol/L and higher were sufficient to suppress the entire cell population after 120 h, and again this effect was independent of the p53 status. Activation of caspase 3 and 7 by the drug suggests that HKH40A activity is associated with induction of apoptosis (Fig. 2b). This activation of caspases 3 and 7 was blocked by approximately 70% by concomitant incubation of cells with HKH40A and the apoptosis inhibitor Z-VAD (data not shown).

Inhibition of clonogenicity by HKH40A

An important parameter for the efficacy of a chemotherapeutic drug is its long-term effect on cancer cell viability. Therefore, we used a clonogenic assay to test the effect of HKH40A on Hep3B cells. As shown in Fig. 2c, 6 h of treatment with the drug at concentrations of 10 nmol/L and higher, totally reduced colony formation. Additionally, we found that at concentrations of 100 nmol/L and higher HKH40A completely suppressed DNA synthesis, measured by the incorporation of bromodeoxyuridine (Fig. 2d).

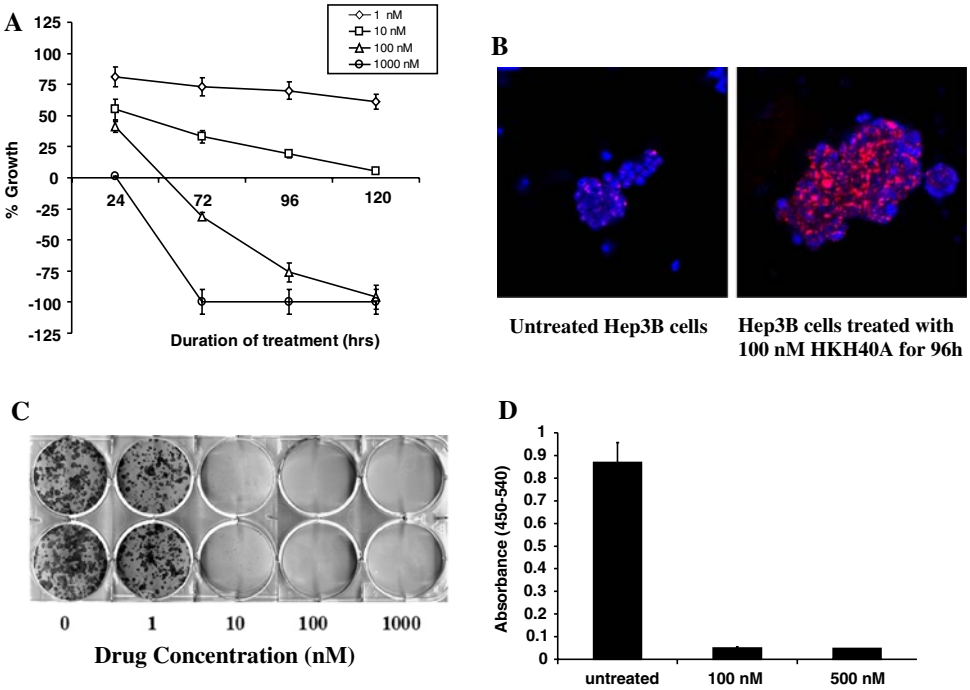


Fig. 2 **a** Time and dose dependent effects of HKH40A against Hep 3B cell growth**.** Cells were treated with various concentrations of the drug for increasing periods of time (from 24 to 120 h) before growth inhibitory activity was determined by the MTT assay. **b** Activation of caspases 3 and 7 in Hep 3B cells exposed to HKH40A. Caspase 3 and 7 activity is revealed by red staining in apoptotic tumor cells using Magic Red™ Caspase Detection Kit. **c** Inhibition of HCC colony formation after 6 h treatment with HKH40A. Fixed cells were stained with crystal violet. HKH40A at concentrations 10 nM and higher, completely inhibited colony formation in Hep 3B tumor cells. **d** Effect of HKH40A on DNA synthesis evaluated in terms of the level of incorporation of BrdU, measured immunocytochemically and by colorimetric assay. Treatment with 100 nM and higher concentration of HKH40A for 96 h totally suppressed DNA synthesis in Hep 3B cells lines. Data shown are representative of two independent experiments

HKH40A inhibited growth of JM-1 transplantable tumor cells in vivo

Since HKH40A was found to be a potent growth inhibitor of HCC cell lines in vitro, we tested its efficacy in vivo. The rat cell line JM-1 formed tumor foci, when transplanted in Fischer F344 rat liver [10]. We transplanted JM-1 cells in rat livers by injecting them through the rat mesenteric vein.

In the first set of experiments, two groups of rats (five per group) were treated with 0.2 or 2 mg/kg body weight of HKH40A, which was administered intraperitoneally every other day for five-times total. A third group was treated with the solvent control. Rats were sacrificed 3 weeks after cell transplantation, and the numbers of liver tumor foci were counted. The average numbers of tumor foci were found to decrease from 12, with solvent control, to 4 and 0.5, with 0.2 and 2 mg/kg body weight HKH40A, respectively (Fig. 3a). Both decreases were statistically significant ($P < 0.001$). Representative photographs of tumors in solvent- and HKH40A-treated livers are shown in Fig. 3c and d, respectively.

In the second set of experiments, treatment with 1 mg/kg body weight HKH40A was started on 1, 7, or 14 days after JM-1 cell transplantation. The frequency and route of drug administration was similar to the first in vivo experiment. A control group received treatment with the solvent. Rats were sacrificed 4 weeks after cell transplantation and the numbers of tumor foci were counted. HKH40A was found to inhibit in vivo tumor growth, when treatment started on 1 or 7 days after cell transplantation. However, no inhibition was observed when treatment started after 14 days (Fig. 3b), which was similar to the treated solvent control. Figure 3e shows the histology of a representative tumor from HKH40A treated and solvent control treated rat. The HKH40A treated tumors were fewer and smaller, although with similar cellular appearance.

HKH40A caused rapid growth arrest in S phase and subsequent cell death

FACS analysis showed that regardless of p53 status, 100 nmol/L of HKH40A after 24 h caused a marked decrease in G1 phase cell population (from 56.1 to 46.1% for HepG2, from 59.7 to 33.3% for PLC/PRF/5, and from 59.5 to 28.5% for Hep3B), and accumulation of cells in S-phase (from 19.3, 18.1, and 16.4% for untreated cells to 30.6,

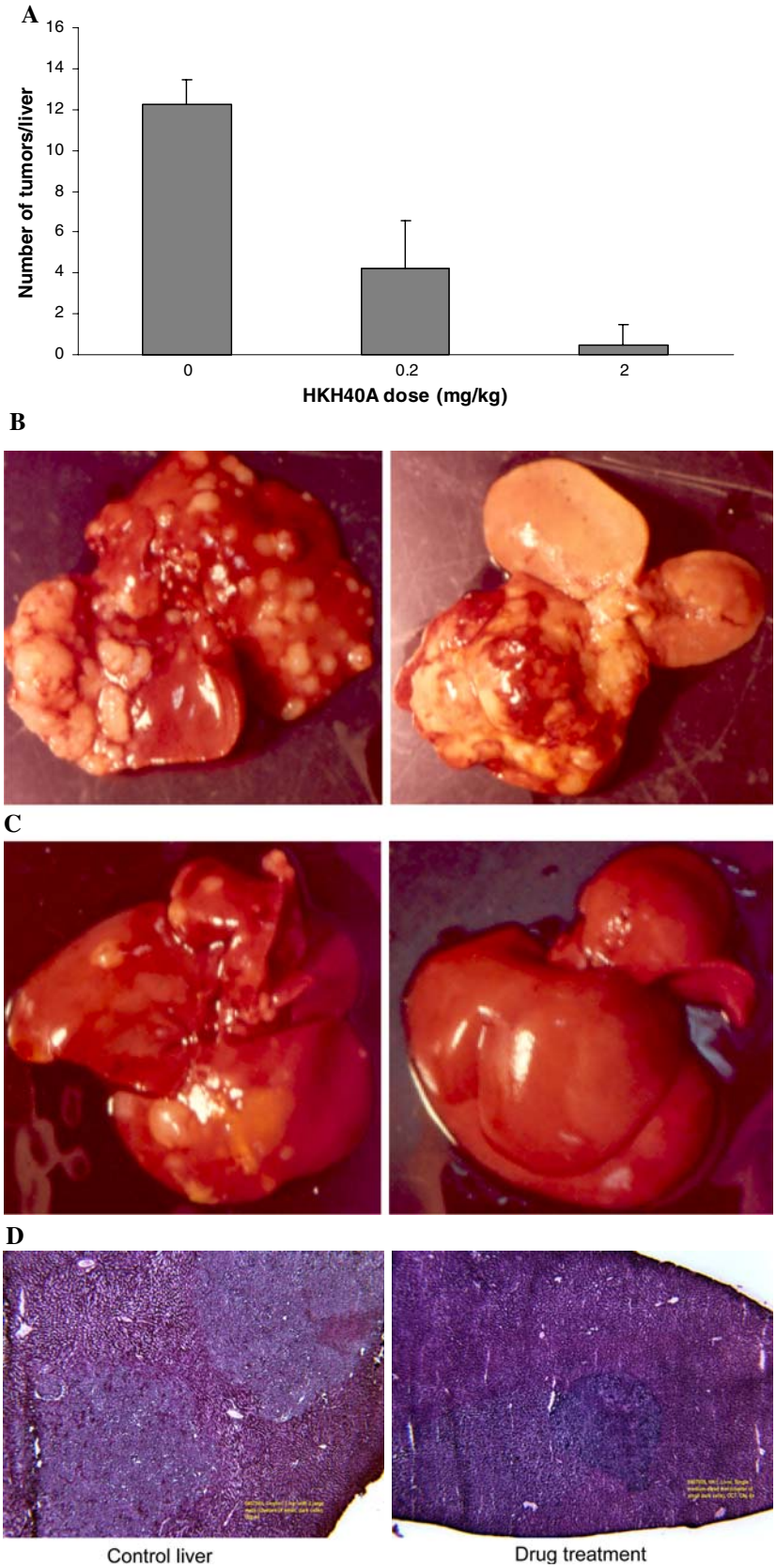


Fig. 3 In vivo activity of HKH40A (RTA 502) against transplantable HCC cell growth. JM-1 transplantable tumor growth in livers of rats. **a** Number of tumors formed per rat treated with 0, 0.2, or 2 mg/kg of HKH40A. **b** Number of tumors formed per rat treated with 1 mg/kg HKH40A starting at 1, 7, or 14 days after tumor cell transplantation. **c** Livers from untreated animals (DMSO solvent). **d** Livers from rats treated IP with HKH40A at dose 1 mg/kg of body weight every other day-5 times, starting two days after administration of JM-1 cells. Rats were sacrificed 2 week after tumor cell transplantation and the number of liver tumors was counted. **e** Pathology sections stained with hematoxylin and eosin from HKH40A treated and control treated tumors, showing smaller tumors in treated rats

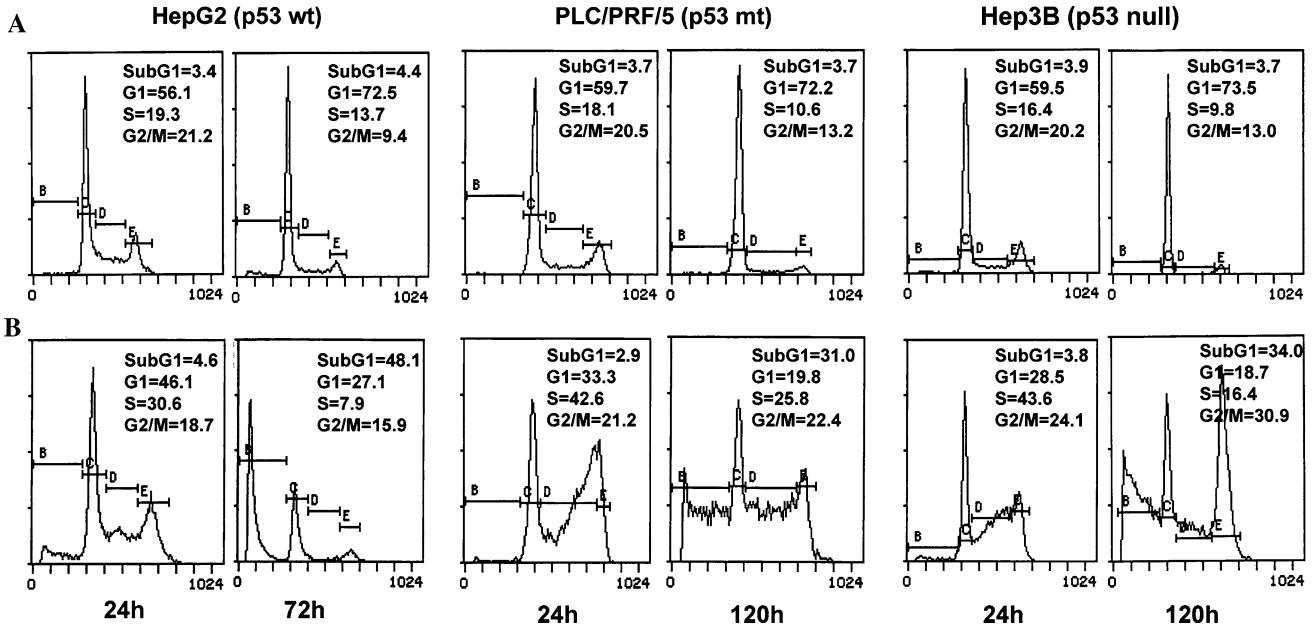


Fig. 4 Cell cycle analysis of selected liver tumor cells. Untreated cells (**a**) and cells exposed to100 nM HKH40A (**b**) were fixed with 70% ethanol, stained with propidium iodide and analyzed using a fluorescence-activated cell sorter (FACS). Graphs are from a single experiment that has been repeated twice with similar results

42.6, and 43.6% for HepG2, PLC/PRF/5, and Hep3B treated cells, respectively) (Fig. 4). Prolonged treatment led to cell death, with HepG2 cells carrying wild type p53 showing a much faster response to the drug than PLC/PRF/5 and Hep3B cells. After 72 h, almost 50% of HepG2 cells were dead and almost 100% by 120 h. In contrast, cells with mutated p53 (PLC/PRF/5) or null p53 (Hep3B) showed little change in distribution between of 24 and 72 h, and a significant population of dead cells was seen only after 120 h. In Fig. 4, we therefore show the 72 h time-point for HepG2 and the 120 h time points for PLC/PRF/5 and Hep3B cells. Our BrdU incorporation experiment showed that at concentrations of 100 nM and higher, DNA synthesis was totally suppressed. Because this concentration corresponds to the steady-state drug plasma levels achievable in rodents, we chose 100 nM for FACS analysis as the concentration most relevant to in vivo conditions. Two time points are presented, that show typical effects of the drug, accumulation of cells in S phase at 24 h, and significant cell death at 72 h for HepG2 cells or 120 h for PLC/PRF/5 and Hep3B cells. We performed FACS analysis for cells treated with HKH40A 10 nM, which resulted in a double block in G1 and G2/M and depletion of S phase, but did not trigger any cell death, even after several days.

Continuous long-term exposure to HKH40A also resulted in a significant increase of G2/M cell population. FACS analysis did not distinguish between G2 and M arrest. However, nuclear staining of cells treated with HKH40A showed an intact nuclear envelope and lack of chromatin condensation, while tubulin staining showed lack of mitotic spindle formation (Fig. 5), suggesting that the cells did not enter mitosis. Decreased amounts of selected proteins involved in DNA replication (Cdc6, Cdc7, and RRM2), as well as up-regulation of p21, cyclin A, cyclin B1, and sustained phosphorylation of Cdk1 on Tyr15 in all the studied tumor cell lines, was consistent with a p53-independent block in S and G2 (Fig. 6). We matched the 24 h time point from FACS (Fig. 4) showing accumulation of S phase cells with Western blots in Fig. 6. The purpose of the long time point in cell cycle analysis was to document significant cell death (presence of significant sub-G1 population). Furthermore, a 6 h exposure to 100 nmol/L HKH40A followed by drug washout was sufficient to induce exactly the same result as continuous treatment with the drug (data not shown), indicating that the effects of HKH40A were irreversible.

Discussion

This study demonstrates that the antitumor agent HKH40A at low nanomolar concentrations (10–30 nmol/L) arrests proliferation of HCC cells in vitro in a p53-independent manner. At concentrations around 100 nmol/L and higher the agent induces apoptosis in all studied HCC lines after 120 h, although the induction of cell death is faster in cells with wt p53. The clonogenic assay and FACS experiment also showed that the cell growth inhibition and induction of apoptosis in vitro is irreversible after 6 h treatment with 100 nM of the agent. It is worth to note that the 100 nM concentrations of

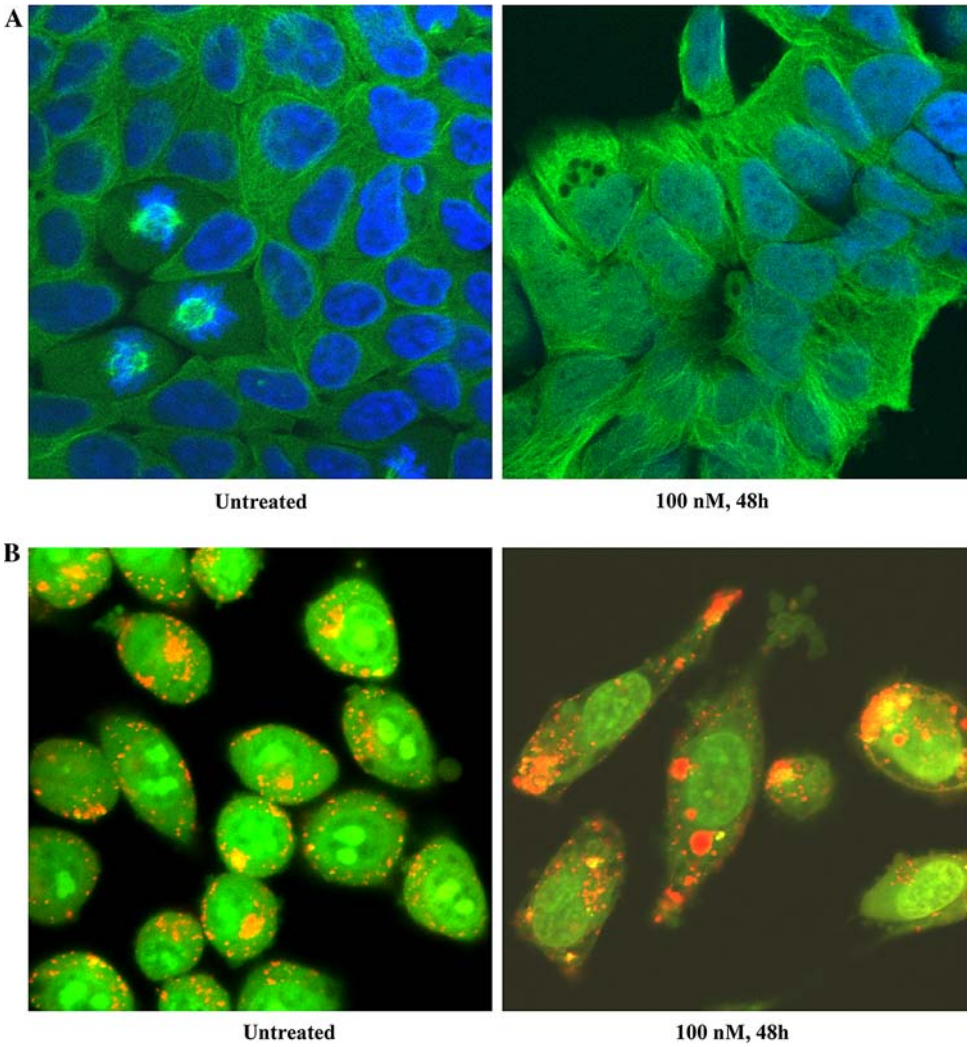


Fig. 5 Laser scanning confocal microscopy of HKH40 -treated Hep 3B cells demonstrated that nuclear envelope remains intact while chromatin condensation and mitotic spindle formation are inhibited. **a** Staining with an anti-tubulin antibody (*green*) and Hoechst 33342 dye (*blue*). **b** Staining with acridine orange (lysosome-*orange*, cytoplasm and nucleus-*green*)

HKH40A are achievable in the blood plasma of drug-treated animals and can be maintained for at least 6 h [1]. The exact molecular mechanisms for HKH40A-mediated cell growth inhibition are not yet established. HKH40A, like its desmethoxy analog WMC-79, can also bind to DNA and interfere with the action of DNA-operating proteins. We recently found that HKH40A has multiple effects on DNA topoisomerases [4]. This in part may account for the early (24 h), p53-independent accumulation of drug-treated cells in S phase (Fig. 4). It is well established that inhibition of topoisomerase catalytic activity impairs replication of DNA and leads to S phase block [11]. However, our experiments with topoisomerase-I deficient cells also showed that inhibition of this enzyme is not exclusively responsible for drug activity on cell growth, and other cellular targets besides topoisomerases are also affected [4].

The rapid arrest of drug-treated cells in S-phase clearly indicates an interference with DNA replication. The process of DNA replication involves many chromatin and DNA-modifying and DNA-synthesizing proteins, which potentially can be targeted by the agent [12, 13]. Replication of DNA is tightly regulated on many different levels because it must occur at the right time and only once per cell cycle. The timing is carefully choreographed during the G1 and S phases of the cell cycle [14, 15]. Disruption in the timing of replication leads to premature entry into S phase that cannot be completed, which results in mitotic crisis and cell death [16, 17].

A number of factors are known to play crucial roles in the initiation and elongation stages of DNA and these include the licensing kinase Cdc6, and Cdc7 kinase. Cdc6 kinase was shown to mediate loading of MCM proteins onto chromatin. Thus, Cdc6 is an essential factor for generation of pre-replicative complexes [18, 19]. Cdc6 gene is not expressed in most normal tissues, in contrast with its marked expression in proliferating cancer cells [18], which makes this kinase an excellent cancer-selective chemotherapeutic target. Cdc7 kinase is required for continuous DNA replication in mammalian cells, and its inactivation leads to arrest of replication forks and to almost immediate replication block (S-phase arrest), which generates DNA structure checkpoint signals (inactivation of Cdk1 kinase by phosphorylation at Tyr15),

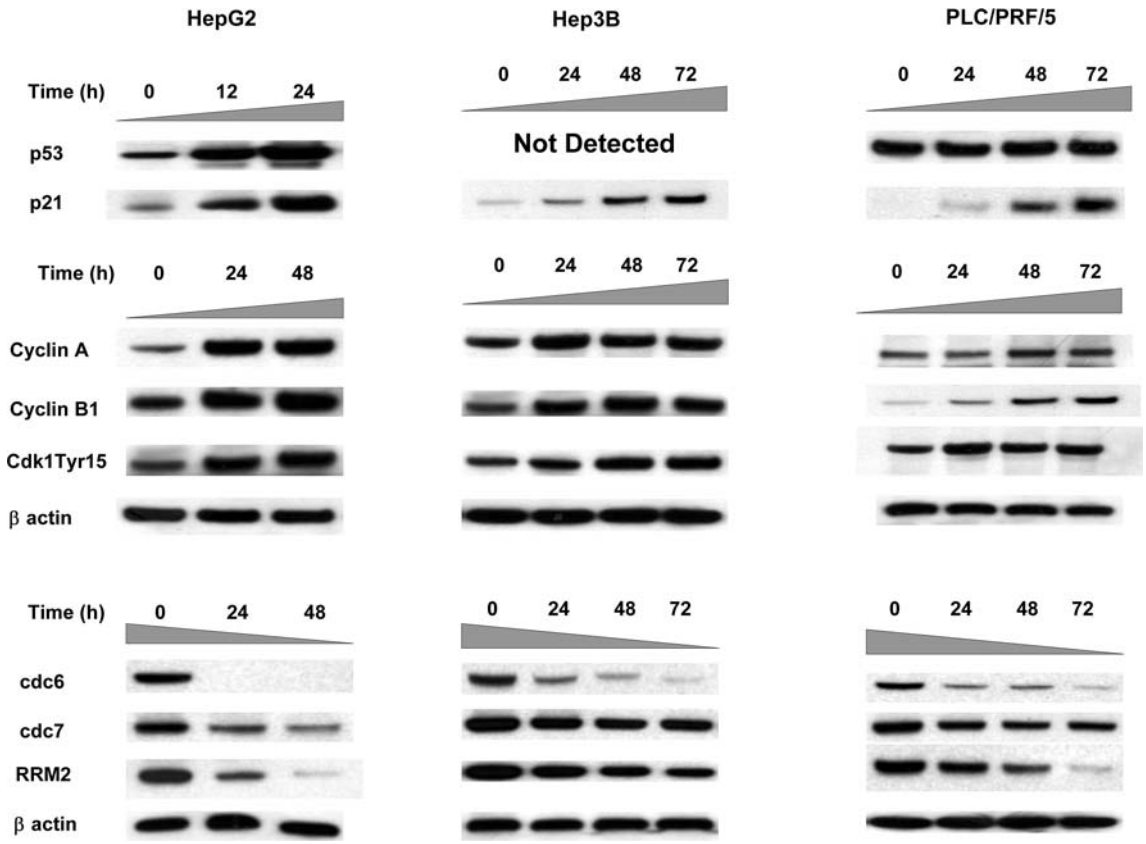


Fig. 6 Western blot analysis of HCC cell lines which were exposed to HKH40A. Cells were grown in the absence or present of 100 nmol/L HKH40A. After indicated times, cells were lysed and total protein was extracted, separated by PAGE, electro-transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting with the indicated primary antibodies. Actin was used as a loading control

resulting in recombinational repair and eventual cell death by either p53-independent apoptosis or aberrant mitosis [16, 20]. By contrast, in the absence of adequate Cdc7, normal cells appear to be protected from progression through the lethal drug-induced S-phase by a p53-dependent pathway [17].

As shown by our Western blot analysis (Fig. 6), HKH40A triggers significant reduction in the levels of Cdc6 and Cdc7 kinases, and also caused down-regulation of RRM2. RRM2 plays a key role in DNA synthesis and repair, and its activity is crucial for cell growth [21]. We previously found, that in colon tumor cells treated with HKH40A, the down-regulation of the RRM2 protein was a consequence of a very dramatic (140-fold at 48 h time-point) reduction in the expression level of the gene encoding the M2 subunit of ribonucleotide reductase [22, 23]. However, at this stage we do not know if this is also true for the HCC cells and whether the same mechanism, involving prior changes in gene expression is responsible for the reduced protein levels of Cdc6 and Cdc7 kinases.

HKH40A treatment also caused inactivation of Cdk1 kinase, as well as elevation of cyclin A and Cyclin B1 levels, consistent with a G2/M block. Cyclin B1 is regulated both temporally throughout the cell cycle, and spatially between the cytoplasm and the nucleus. Protein levels of cyclin B1 begin to accumulate late in S-phase, peak at the G2/M transition, and decline considerably by the onset of anaphase [see Fig. 4 in ref. 1]. Thus, decreased cyclin B1 expression can be found in growth inhibition, or increased levels can be found, as in our experiments, in cell cycle block at G2/M [24, 25].

The data presented here strongly suggest that the potent growth-inhibitory activity of HKH40A is mediated by apoptosis, in association with a significant reduction of Cdc6 and Cdc7 kinase and RRM2 levels. These critical elements of replication may be excellent targets for chemotherapeutic intervention with agents like HKH40A.

Furthermore, HKH40A displayed potent activity in an orthotopic rat HCC model in vivo. It caused almost complete disappearance of growing HCC cells and not just growth inhibition, without apparent toxicity, as judged by physical appearance and weight gain at the doses used. HKH40A thus represents a new class of anticancer drug with potential for further development as an agent against HCC.

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References

1. Hariprakasha HK, Kosakowska-Cholody T, Meyer C et al (2007) Optimization of naphthylimido-imidazoacridone with potent antitumor activity leading to clinical candidate HKH40A (RTA 502). J Med Chem 50:5557–5560
2. Cholody WM, Kosakowska-Cholody T, Hollingshead MG, Hariprakasha HK, Michejda CJ (2005) A new synthetic agent with potent but selective growth-inhibitory activity against cancer. J Med Chem 48:4474–4481
3. Treason SG, Casas-Finet JR, Cholody WM, Kosakowska-Cholody T, Gryczynski ZK, Michejda CJ (2003) Bisimidazoacridones: 2. Steady-state and time-resolved fluorescence studies of their diverse interactions with DNA. Photochem Photobiol 78:313–322
4. Kosakowska-Cholody T, Cholody WM, Hariprakasha HK et al (2003) WMC79 and HKH40A—potent agent against gastrointestinal cancers: molecular targets and mechanism of action. In: Proceedings of the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, November 17–21, Boston, MA. p 58. (abstract A36)
5. Kosakowska-Cholody T, Cholody WM, Monks A, Woynarowska BA, Michejda CJ (2005) WMC-79, a potent agent against colon cancers, induces apoptosis through a p53-dependent pathway. Mol Cancer Ther 4:1617–1627
6. Kosakowska-Cholody T, Cholody WM, Kar S, Carr BI, Hollingshead MG, Michejda CJ (2003) Mechanism of action of WMC79, a potent anticancer agent, in cells where the p53 tumor suppressor gene is mutated or not expressed. Proc Amer Assoc Cancer Res 44:725 (abstract 3640)
7. Thomas MB, Zhu AX (2005) Hepatocellular carcinoma: the need for progress. J Clin Oncol 23(13):2892–2899
8. Monks A, Scudiero D, Skehan P et al (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 83:757–766
9. Hernandez L, Cholody WM, Hudson EA, Resau JH, Pauly G, Michejda CJ (1995) Mechanism of action of bisimidazoacridones, new drugs with potent, selective activity against colon cancer. Cancer Res 55:2338–2345
10. Novicki DL, Jirtle RL, Michalopoulos G (1983) Establishment of two rat hepatoma cell strains produced by a carcinogen initiation, phenobarbital promotion protocol. In Vitro 19:193–202
11. Etievant C, Kruczynski A, Barret JM et al (2000) F 11782, a dual inhibitor of topoisomerases I and II with an original mechanism of action in vitro, and markedly superior in vivo antitumour activity, relative to three other dual topoisomerase inhibitors, intoplicin, aclarubicin and TAS-103. Cancer Chemother Pharmacol 46(2):101–113
12. Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. Ann Rev Biochem 71:333–374
13. Kelly TJ, Brown GW (2000) Regulation of chromosome replication. Ann Rev Biochem 69:829–880
14. Stillman B (1996) Cell cycle control of DNA replication. Science 274:1659–1664
15. Woo RA, Poon RY (2003) Cyclin-dependent kinases and S phase control in mammalian cells. Cell Cycle 2:316–324
16. Kim JM, Nakao K, Nakamura K et al (2002) Inactivation of Cdc7 kinase in mouse ES cells results in S-phase arrest and p53-dependent cell death. EMBO J 21:2168–2179
17. Montagnoli A, Tenca A, Sola F et al (2004) Cdc7 inhibition reveals a p53-dependent replication checkpoint that is defective in cancer cells. Cancer Res 64:7110–7116
18. Luo F, Yee JK, Huang SH, Wu LT, Jong AY (2006) Downregulation of human Cdc6 protein using a lentivirus RNA interference expression vector. Methods Mol Biol 342:287–293
19. Lau E, Abraham RT, Jiang W (2006) The functional role of Cdc6 in S-G2/M in mammalian cells. EMBO Rep 7:425–430
20. Kim JM, Yamada M, Masai H (2003) Functions of mammalian Cdc7 kinase in initiation/monitoring of DNA replication and development. Mutat Res 532:29–40
21. Kolberg M, Strand KR, Graff P, Andersson KK (2004) Structure, function, and mechanism of ribonucleotide reductases. Biochim Biophys Acta 1699:1–34
22. Kosakowska-Cholody T, Meyer CJ, Wick MJ et al (2006) HKH40A (RTA 502) potently down-regulates ribonucleotide reductase and produces tumor regression in vivo. Proc Amer Assoc Cancer Res 47:2157
23. Kosakowska-Cholody T, Cholody WM, Hariprakasha HK, Meyer CJ, Michejda CJ (2007) Gene expression profiles in HCT116 and HT29 cells exposed to RTA 502 lead to insights into the mechanism of action. Proc Amer Assoc Cancer Res 48:1158 (abstract 4895)
24. Cappelletti V, Fioravanti L, Miodini P, Di Gronzo G (2000) Genistein blocks breast cancer cells in the G(2)M phase of the cell cycle. J Cell Biochem 79:594–600
25. Sakashita F, Osada S, Takemura M, Imai H, Tomita H, Nonaka K, Takahashi T, Seishama M (2008) The effect of p53 gene expression on the inhibition of cell proliferation by paclitaxel. Cancer Chemother Pharmacol 62:379–385