Small-molecule c-Myc inhibitor, 10058-F4, inhibits proliferation, downregulates human telomerase reverse transcriptase and enhances chemosensitivity in human hepatocellular carcinoma cells

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c-Myc oncogene is critical for the development of hepatocellular carcinoma. Given the successful use of small-molecule inhibitors on cancers, targeting c-Myc with small-molecule inhibitors represents a promising approach. The potential of using small-molecule c-Myc inhibitor, 10058-F4, was evaluated on hepatocellular carcinoma cell lines, HepG2 and Hep3B cells. HepG2 cells were more sensitive to 10058-F4 than Hep3B cells, as demonstrated by reduced cell viability, marked morphological changes and decreased c-Myc levels. 10058-F4 arrested the cell cycle (at G_0/G_1 phase) and induced apoptosis upon extended treatment. These observations might be attributable to the increased cyclin-dependent kinase inhibitor, $p21^{WAF1}$, and decreased cyclin D3 levels. Besides, 10058-F4 also significantly decreased the α-fetoprotein levels, an indicator for hepatocellular carcinoma differentiation. We further found that 10058-F4 inhibited the transactivation of human telomerase reverse transcriptase, downregulated human telomerase reverse transcriptase expression and abrogated telomerase activity. In addition, pretreatment with 10058-F4 increased the chemosensitivity of HepG2 cells to low-dose doxorubicin, 5-fluorouracil and cisplatin. Therefore, small-molecule c-Myc inhibitors might represent a novel agent, alone or in combination with conventional chemotherapeutic agents, for anti-hepatocellular carcinoma therapy. *Anti-Cancer Drugs* **18:161–170 © 2007 Lippincott Williams & Wilkins.**

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, especially in the Asia-Pacific region, owing to a high prevalence of chronic hepatitis B virus (HBV) infection [1]. The oncogenic HBx in HBV plays an important role in hepatocarcinogenesis, as demonstrated by a high incidence of HCC in HBx-expressing transgenic mice [2]. HBx directly upregulates Myc expression and induces hepatocarcinogenesis [3]. In addition, chromosomal studies on premalignant lesions in human liver have shown that alterations in chromosome 1p, 6q, 8q and 13q occur exclusively in HCC, suggesting that these regions harbor genes critical for hepatic carcinogenesis. The fluorescence in-situ hybridization study further shows that chromosomal changes at 8q encompass the amplification of c-Myc [4]. The c-Myc amplification appears to be critical for hepatocarcinogenesis as it can be found only in cancer cells, not in noncancerous tissues [5]. Besides, c-Myc amplification, often found in younger patients with HCC, portends a poorer prognosis [6]. Therefore, c-Myc is a potential target for developing a novel anti-HCC therapy. The potential of targeting c-Myc for HCC has been demonstrated in several reports. Downregulation of c-Myc *in vitro* by antisense oligonucleotides or interference RNA or *in vivo* by conditional inactivation has been shown to inhibit the growth and induce differentiation of HCC [7–9]. The use of antisense oligonucleotides or interference RNA, however, is potentially limited by ineffective delivery into cancer cells.

c-Myc encodes a basic loop–helix–loop zipper (bHLHZip) transcription factor that contributes to tumorigenesis through promoting cell proliferation, inhibiting apoptosis and cell differentiation [10]. Blocking c-Myc activity using small-molecule compounds inhibits cell proliferation and Myc-induced transformation [11–13]. To transactivate its target genes, c-Myc needs to dimerize with Max, another bHLHZip protein, through their bHLHZip domains to bind to their DNA recognition site, the E-box element.

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Given the experience of using small-molecule inhibitors against oncogenic proteins in lung cancer and chronic myeloid leukemia (CML) (for a review, see [14,15]), we reasoned that such small-molecule c-Myc inhibitors might be useful for novel anti-HCC therapy. To test this hypothesis, we used a recently identified small-molecule inhibitor, 10058-F4, which has been shown to block the Myc/Max dimerization, to examine its effects on HCC cells [12]. Here we report that 10058-F4 decreased c-Myc protein levels, inhibited proliferation of HepG2 cells likely through upregulation of cyclin-dependent kinase (cdk) inhibitor, $p21^{WAF1}$ and lowered intracellular levels of α-fetoprotein (AFP). Treatment with 10058-F4 also downregulated human telomerase reverse transcriptase (hTERT) at the transcriptional level. In addition to inhibiting the proliferation of HepG2 cells, 10058-F4 enhanced sensitivity to conventional chemotherapeutic agents, doxorubicin, 5-fluorouracil (5-FU) and cisplatin. Therefore, small-molecule c-Myc inhibitors, either alone or in combination with conventional chemotherapeutic agents, might represent a novel anti-HCC therapy.

Materials and methods

Cells and reagents

HCC cell lines, HepG2 and Hep3B cells (American Type Culture Collection, Manassas, Virginia, USA) were maintained in minimum essential medium with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 10% fetal bovine serum (Invitrogen, Carlsbad, California, USA). Cells in exponential growth phase were used for experiments. c-Myc inhibitor, 10058-F4; (Z,E)-5-(4-ethylbenzylidine)-2-thioxothiazolidin-4-one (molecular weight 249.4; Calbiochem, San Diego, California, USA), was dissolved in dimethylsulfoxide (DMSO) at 40 mmol/l and further diluted to indicated concentrations in culture medium before use. Antibodies against c-Myc, $p21^{WAF1}$, $p27^{KIP1}$, p53 and cyclin D3 were obtained from Cell Signaling (Danvers, Massachusetts, USA), hTERT and β-actin from Novus Biologicals (Littleton, Colorado, USA), and cyclin D1 and cyclin D2 from Santa Cruz Biotechnology (Santa Cruz, California, USA). 5-FU (Costa Mesa, California, USA), doxorubicin (Pharmacia, New York, USA) and cisplatin (Bristol–Myers Squibb, New York, USA) were diluted to desired concentrations with culture medium before use.

Cytotoxicity assays

Cells were plated in quadruplicate at a density of 10 000 cells/well in 96-well plates and treated with indicated concentrations of 10058-F4. At indicated time points, the morphological changes of cells were photographed, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, Missouri, USA) at 50 μg/well was added for 4 h and the produced formazan was solubilized by DMSO. Optical density was measured at 570 nm with a microplate reader. Cells treated with equivalent amounts of DMSO were used as controls.

Electrophoretic mobility shift assay

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA) were carried out according to the instructions provided in the commercial Myc/Max EMSA kit (AY1020; Panomics, California, USA). For antibody supershifts, anti-Myc (Cell Signaling Technology) at a 1/100 dilution was added to the binding reactions. Electrophoresis gels were transferred to positively charged nylon membranes and detected by the streptavidin–horseradish peroxidase developing system provided in the kit.

Sequencing of the basic loop–helix–loop zipper dimerization domain

To investigate the differential effects of 10058-F4 between HepG2 and Hep3B cells, the bHLHZip region (amino acids 354–434) was sequenced after polymerase chain reaction (PCR) amplification using the primer pairs shown in Table 1 [11].

Cell-cycle analysis and apoptosis measurement by flow cytometry

Cells were washed once with phosphate-buffered saline, permeabilized with two volumes of ice-cold 95% alcohol for 30 min and stained with 50 μg/ml propidium iodide (PI; Sigma) for 1 h in the presence of 100 μg/ml RNase I for 30 min before data acquisition using FACSCalibur (Becton Dickinson, Franklin Lakes, New Jersey, USA). The results were analyzed by ModFit 2.0 (Verfity Software House, Topsham, Maine, USA). Apoptosis was determined using the annexin-V/PI detection assay. Cells (10^5) were incubated at room temperature in 200 μl buffer containing fluorescein-conjugated human annexin-V antibody (5 μl, Sigma) and 5 μg/ml PI in the dark for 20 min, followed by flow-cytometric analysis.

TRAPeze assay

Telomerase activity was measured with the TRAPeze gel-based telomerase detection kit (Chemicon, Temecula, California, USA) according to the manufacturer's instructions. Cells were lysed with 1 × (3-[3-cholamidopropyl-

Table 1 PCR primers used in this study

Genes	Sequences	Annealing temperature
c-Myc bHLHZip	Forward: 5′-TGCACCAGCCCCAGGTCCTC-3′ Reverse: 5′-TCCTTACTTTTCCTTACGCA-3′	58
hTERT promoter	Forward: 5′-AGTGGATTCGCGGGCACAGA-3′ Reverse: 5′-TTCCCACGTGCGCAGCAGGA-3′	58
hTERT	Forward: 5′-GGAGCAAGTTGCAAAGCATTG-3′ Reverse: 5′-TCCCACGACGTAGTCCATGTT-3′	55
β-actin	Forward: 5′-GGACTTCGAGCAAGAGATGG-3′ Reverse: 5′-AGCACTGTGTTGGCGTACAG-3′	55

BHLHZip, basic loop–helix–loop zipper; hTERT, human telomerase reverse transcriptase; PCR, polymerase chain reaction.

dimethylammonio]-1-propane-sulfonic acid) lysis buffer, and cell extracts were added to the reaction mixture and subjected to PCR reaction recommended in the kit. PCR products were separated by electrophoresis on a 12.5% nondenaturing polyacrylamide gel, stained with SYBR green and visualized under ultraviolet illumination.

Measurement of α-fetoprotein and immunoblotting

Cells were lysed in NETN lysis buffer (50 mmol/l Tris/HCl pH 7.5, 150 mmol/l NaCl, 1 mmol/l ethylene diamine tetra-acetate, 1% NP-40), supplemented with the commercial cocktail of protease inhibitors (Amresco, Solon, Ohio, USA) and cellular debris removed by centrifugation. Protein concentration in the supernatant was determined by using the Bradford reagent (Amresco) and bovine serum albumin (Sigma) as a standard. One hundred micrograms of protein supernatant resuspended in 200 µl phosphate-buffered saline was assayed for the amount of AFP using an Abbott AxSYM automated immunoassay analyzer (Abbott Laboratories, Abbott Park, Illinois, USA). The measured AFP levels (ng/ml) were then converted to ng/100 µg of total protein. The remaining protein supernatants were resolved in 6% sodium dodecyl sulphate–polyacrylamide gels, and transferred to polyvinylidine diflouride membrane and immunoblotted with appropriate antibody. Immunoblots were probed with appropriate horseradish peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminence kit (NEN, Wellesley, Massachusetts, USA).

Construction of human telomerase reverse transcriptase-luciferase reporter and luciferase assays

A 329-bp DNA fragment from the hTERT core promoter region encompassing −276 to +53 was amplified by PCR using genomic DNA from mononuclear cells of a healthy volunteer with primers shown in Table 1 [14]. The PCR product of the hTERT promoter was cloned into pGL3-enhancer firefly luciferase reporter plasmid (Promega, Madison, Wisconsin, USA) and confirmed by DNA sequencing. Transactivation studies of the hTERT promoter were performed by liposomally transfecting 10^5 HepG2 cells with 5 µg of the reporter plasmid and 0.5 µg of phRL-TK plasmid (Promega). Twenty-four hours after transfection, transiently transfected cells were treated with 100 µmol/l 10058-F4 for 24 h and cell lysates were assayed for luciferase activity using the dual luciferase reporter assay (Promega). The firefly luciferase activity was normalized against renilla luciferase activity and expressed as relative luciferase units. Results are representative of three independent sets of experiments.

Quantitative real-time polymerase chain reaction

Cells treated with solvent or 100 µmol/l 10058-F4 for 24 h were harvested, and total RNA was extracted with a RNA extraction kit (Protech, Taipei, Taiwan). One microgram of total RNA was first transcribed into cDNA in 25 µl reagent mix and 1/25 of the cDNA was subjected to quantitative real-time PCR for hTERT transcripts using a SYBR Green PCR kit (ABI, Foster City, California, USA) on a ABI 9700HT machine following the manufacturer's protocols. Primer sequences and annealing temperature for hTERT and β-actin control are shown in Table 1. Reactions were performed in triplicate for each sample. Control reactions lacking cDNA template were included to assess specificity, and showed no appreciable amplification (data not shown). The fluorescence intensity, which is related to the initial numbers of RNA copies, was determined by the threshold cycle (C_T). Relative expression of individual genes at indicated time points was calculated based on comparative computed tomography method ($\Delta\Delta C_T$). The serial changes of individual genes were plotted against the data derived from solvent-treated cells.

Statistical analysis

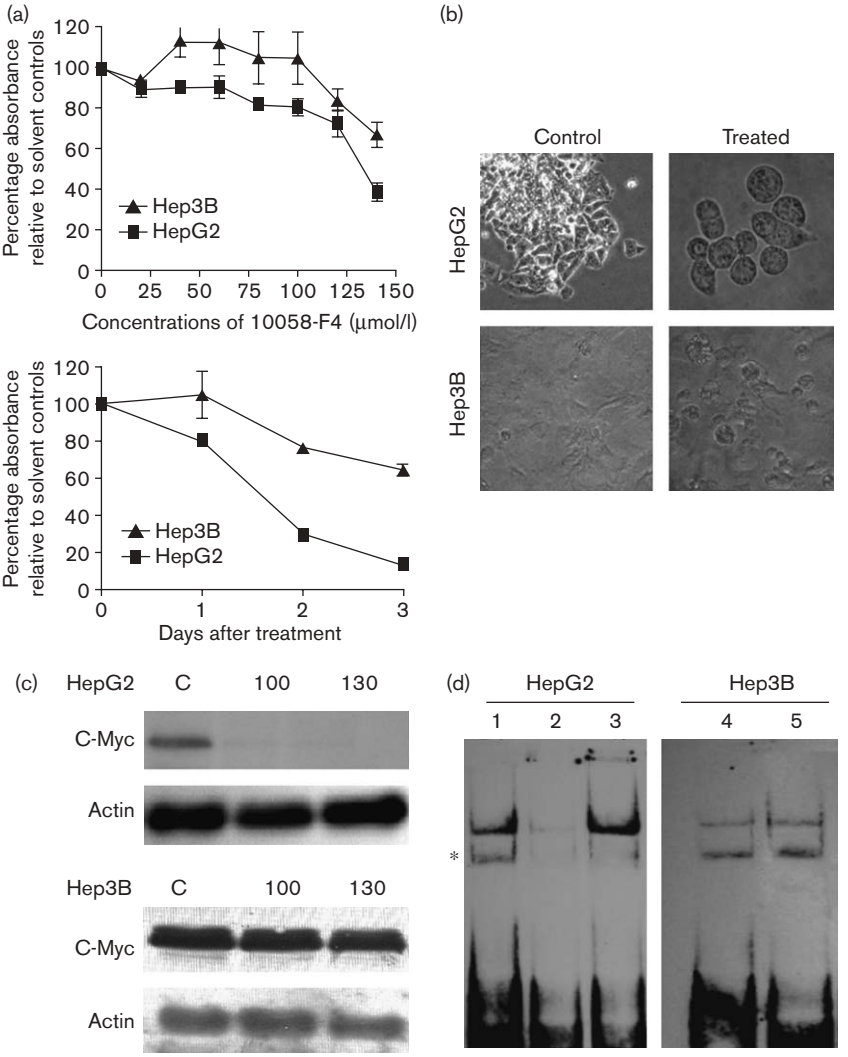
The results were reported as mean ± SEM and the statistical significance ($P < 0.05$) was determined by two-sided Student's *t*-tests.

Results

Small molecule c-Myc inhibitor, 10058-F4, inhibits the growth of HepG2 cells, lowers c-Myc proteins, leading to reduced binding to DNA targets

We first used MTT assays to evaluate the activity of 10058-F4 on HepG2 and Hep3B cells, and found that HepG2 cells were more sensitive to 10058-F4 than Hep3B cells (Fig. 1a, upper panel). The differences became more remarkable when treatment with100 µmol/l 10058-F4 was extended to 3 days (Fig. 1a, lower panel). All of HepG2 cells treated with 100 µmol/l 10058-F5 for 24 h exhibited marked morphological changes (Fig. 1b, upper panel). Treated cells were rounded up and more dispersed without aggregation. In contrast, there was no significant morphological change in treated Hep3B cells (Fig. 1b, lower panel). We next evaluated whether treatment with 10058-F4 affects the c-Myc protein levels or its binding to the E-box-containing oligonucleotides. c-Myc protein in HepG2 cells became almost undetectable after exposure to either 100 or 130 µmol/l 10058-F4 for 24 h (Fig. 1c, upper panel). In contrast, the level of c-Myc in treated Hep3B cells remained unchanged (Fig. 1c, lower panel). As 10058-F4 has been previously shown to inhibit c-Myc activity by blocking heterodimerization between c-Myc and Max, we used EMSAs to investigate whether 10058-F4 affects the binding of c-Myc proteins to its DNA targets. In HepG2 cells treated with10058-F4, there were no detectable binding of c-Myc protein to the E-box-containing oligonucleotides (Fig. 1d, lane 2). The absence of binding was further validated by the supershift assay using an untreated HepG2 cell lysate (Fig. 1d, lane 3). In 10058-F4-treated Hep3B cells, however, the binding of c-Myc to its target was not affected, showing no inhibition of Myc/Max dimerization

Fig. 1



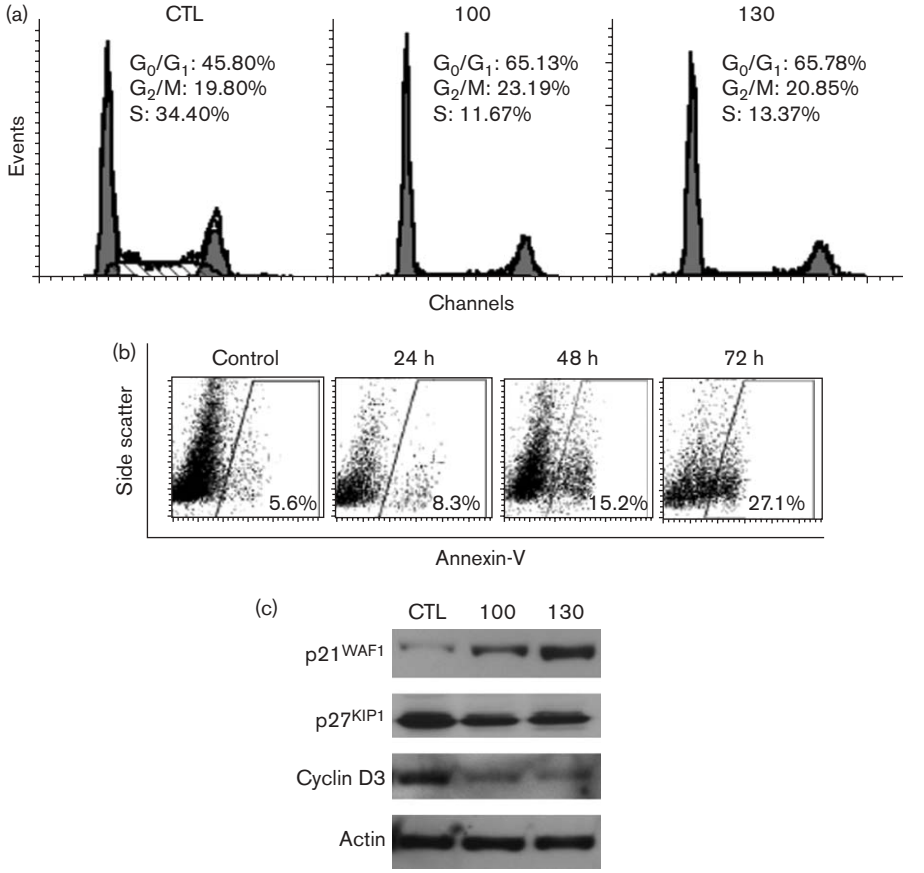
The effect of c-Myc inhibitor, 10058-F4, on different types of hepatocellular carcinoma cell lines. (a) Growth inhibition of HepG2 and Hep3B cells by 10058-F4 was assessed by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assays after exposure to indicated concentrations of 10058-F4 for 24 h (upper panel) and to 100 μmol/l of 10059-F4 for up to 3 days (lower panel). Data represent the mean ± SEM of experiments in quadruplicates. (b) Morphological changes of HepG2 and Hep3B cells after being treated with 100 μmol/l 10058-F4 for 24 h. (c) Changes in the levels of c-Myc protein in HepG2 and Hep3B after being treated with 100 and 130 μmol/l 10058-F4 for 24 h and detected by immunoblotting with anti-c-Myc antibody. Beta-actin was used as the protein loading control. (d) Electrophoretic mobility shift assay and supershift assay of HepG2 and Hep3B cells. Lane 1: untreated HepG2 cells; lane 2: HepG2 cells treated with 100 μmol/l 10058-F4 for 24 h; lane 3: untreated HepG2 cells preincubated with anti-c-Myc antibody; lane 4: untreated Hep3B cells; lane 5: Hep3B cells treated with 100 μmol/l 10058-F4 for 24 h. *Myc/Max dimers.

in treated Hep3B cells. These results also suggest that the activity of 10058-F4 correlates with the post-treatment levels of c-Myc protein (Fig. 1d, lanes 4 and 5).

c-Myc inhibitor arrests the cell cycle at G_0/G_1 phase and induces apoptosis of HepG2 cells

As MTT assays only evaluate the numbers of viable cells, and cannot distinguish the causes of growth inhibition, we next examined how 10058-F4 affects cell growth. HepG2 cells, but not Hep3B cells, exhibited significant cell-cycle arrest at G_0/G_1 phase when exposed to either 100 or 130 μmol/l 10058-F4 for 24 h (Fig. 2a, data for Hep3B not shown). We next investigated whether 10058-F4 also induced apoptosis in HepG2 cells. In contrast to the marked cell-cycle arrest, we could only detect a slight increase in the apoptotic cells in HepG2 cells after being treated with 100 μmol/l 10058-F4 for 24 h (Fig. 2b; 5.8% in controls vs. 8.3% in treated cells; left two panels). Apoptosis became more remarkable after 48 and 72 h of treatment (Fig. 3, right two panels). These results

Fig. 2



Changes of cell cycle, apoptosis and cell-cycle-related proteins in 10058-F4-treated HepG2 cells. (a) HepG2 cells were treated with either 100 or 130 μmol/l 10058-F4 for 24 h and analyzed for the changes in cell cycle by flow cytometry. (b) Annexin-V staining of HepG2 cells after being treated for indicated hours. Numbers in each panel represent the percentage of annexin-V^+ cells. (c) Western blotting of cell-cycle-related protein, $p21^{WAF1}$, $p27^{KIP1}$ and cyclin D3 after being treated with 100 or 130 μmol/l 10058-F4 for 24 h. Beta-actin was used as the protein loading control.

indicate that the 10058-F4-induced growth inhibition in HepG2 cells is mostly caused by cell-cycle arrest.

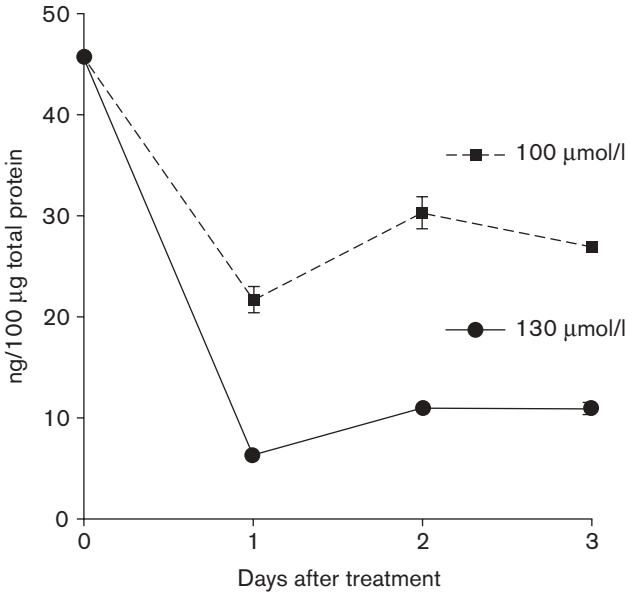
Recent studies have shown that the resistance to imatinib in CML is caused by point mutations in the BCR/ABL that affect the binding of imatinib to its target site [16]. To investigate whether the differential activity between HepG2 and Hep3B cells was due to mutations in the bHLHZip region, we sequenced the bHLHZip region, and found no mutations in either HepG2 or Hep3B cells.

Previous studies have shown that c-Myc could abrogate the p53-induced cell-cycle arrest, and that HepG2 cells, which contain wild-type p53, were more sensitive to chemotherapeutic agents than p53-deleted Hep3B cells. We next examined whether the differential activity of 10058-F4 between HepG2 and Hep3B was due to p53 status, but found no detectable p53 protein in both HepG2 and Hep3B cells before and after treatment with 10058-F4 (data not shown) [17,18]. Taken together, these results suggest that differential sensitivity to 10058-F4 in HepG2 and Hep3B cells is independent of mutations in the c-Myc bHLHZip region or p53.

To further elucidate mechanisms underlying 10058-F4-induced growth inhibition, we examined the levels of cyclin-dependent kinase inhibitors, $p21^{WAF1}$ and $p27^{KIP1}$, and found that 10058-F4 markedly increased the $p21^{WAF1}$, but only slightly reduced $p27^{KIP1}$ protein levels (Fig. 2c). This result also indicates that 10058-F4 activates $p21^{WAF1}$ in a p53-independent manner.

D-type cyclins play important roles in the G_1-phase entry and exit, and cyclin D2 has been shown to be a direct target of c-Myc activation [19,20]. As 10058-F4 arrested cell cycle at G_0/G_1 phase, we next measured the changes of D-type cyclins 24 h after treatment [20]. The expression of cyclin D1 or cyclin D2 in our batch of

Fig. 3



Reduction in intracellular levels of α-fetoprotein by 10058-F4. HepG2 cells treated with either 100 or 130 µmol/l 10058-F4 for up to 3 days were harvested daily to examine the changes of intracellular levels of AFP using an AxSYM automated immunoassay analyzer. The measured values were normalized to 100 µg total cellular protein. Error bars represent SEM.

HepG2 cells was barely detectable before and after treatment with 10058-F4. Instead, the cyclin D3 levels were markedly decreased on treatment (Fig. 2c, results for cyclin D1 and D2 not shown). Therefore, 10058-F4-induced growth inhibition is likely mediated by upregulation of p21 and indirect downregulation of cyclin D3.

10058-F4 reduces the α-fetoprotein levels in HepG2 cells

In the animal model of Myc-induced HCC, downregulation of c-Myc leads to differentiation of cancer cells to normal cellular lineages and tissue structures [9]. One of the early biomarkers for detecting differentiation and monitoring therapeutic effect of HCC is the reduction of AFP [21,22]. We next investigated whether 10058-F4 could lower intracellular levels of AFP. Treatment with 100 and 130 µmol/l 10058-F4 rapidly decreased intracellular AFP level after 24 h of treatment in a dose-dependent manner, indicating that 10058-F4 induces not only cell-cycle arrest but also hepatic differentiation of HepG2 cells (Fig. 3).

10058-F4 inhibits the human telomerase reverse transcriptase promoter activity and the human telomerase reverse transcriptase activity

c-Myc, directly or indirectly, activates multiple genes responsible for cell proliferation, survival and resistance to apoptosis [10]. One of the direct downstream target genes is hTERT [23]. Increased hTERT activity can be found only in cancerous, but not noncancerous hepatic tissues. We next examined whether 10058-F4 can directly inhibit the transactivation of hTERT by using a luciferase reporter construct containing the two E-box binding sites in the proximal region of hTERT core promoter. Although the transfection efficiency varied in separate sets of experiments, cells transfected with empty vector did not have significant changes in luciferase activity upon treatment. In contrast, treatment with 100 µmol/l 10058-F4 for 24 h, markedly and consistently reduced the luciferase activity to the levels similar to that observed in cells transfected with empty vectors (Fig. 4a). To further study whether the downregulation of hTERT promoter activity led to reduced mRNA transcription, we used real-time PCR to assess the changes of hTERT mRNA. Treatment with 10058-F4 decreased the levels of hTERT mRNA to 1/32 of the controls, shown by a significant increase in ΔC_T value between c-Myc and β-actin internal control from -1.16 ± 0.04 to 4.68 ± 0.32 (Fig. 4b). In agreement with the decrease in hTERT mRNA levels, exposure to 100 µmol/l 10058-F4 for 24 h lowered the hTERT protein in a time-dependent manner (Fig. 4c), resulting in undetectable hTERT activity after 48 h of treatment (Fig. 4d).

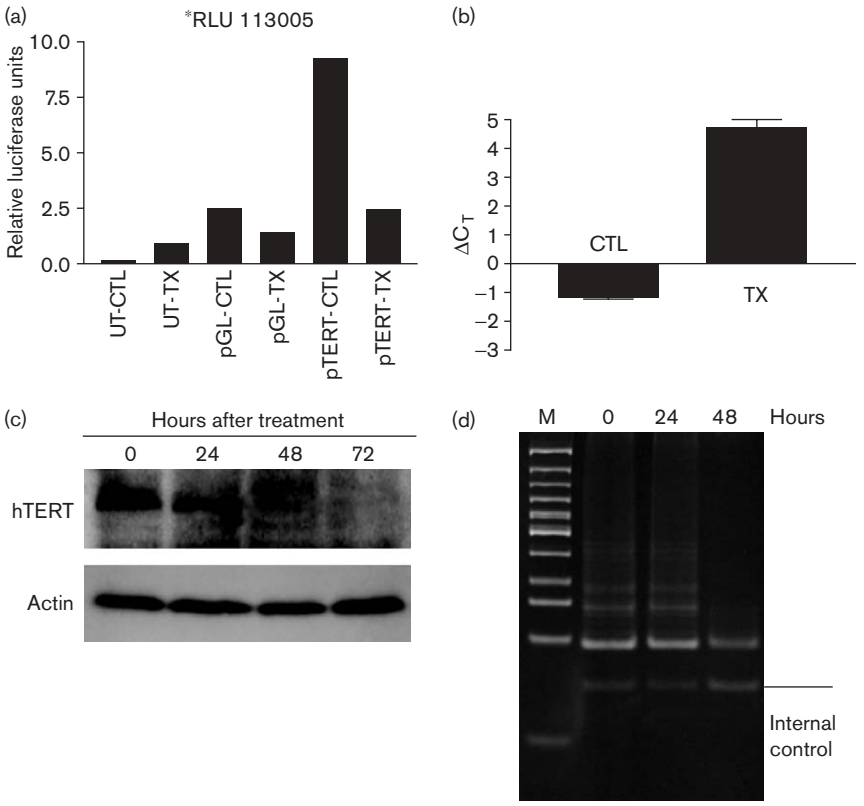
10058-F4 enhances chemosensitivity of hepatocellular carcinoma to conventional chemotherapeutic agents

Recently, inhibition of telomerase using different strategies has led to telomere shortening, growth arrest and apoptosis [24–26]. Furthermore, telomerase antagonists not only inhibit the growth of HCC, but also increase the chemosensitivity [24]. As patients with HCC often have impaired hepatic reserve because of advanced liver cirrhosis, making them unable to receive higher dose of chemotherapy, we investigated whether the 10058-F4-induced downregulation of hTERT could be used to sensitize HepG2 cells to conventional chemotherapeutic agents commonly used for HCC. HepG2 cells pretreated with 100 µmol/l 10058-F4 for 6 h were being treated with low doses of doxorubicin, 5-FU or cisplatin for 24 h before being subjected to MTT assays. The sensitivity of HepG2 cells to these agents is significantly enhanced by 10058-F4, indicating that small-molecule c-Myc inhibitors not only inhibit proliferation of HCC cells when used alone but can also be used as an adjunct agent to enhance conventional chemotherapy (Fig. 5).

Discussion

c-Myc plays an important role during hepatocarcinogenesis, and several studies have shown that downregulating c-Myc activity induces tumor shrinkage [9]. Given the promising experience in blocking oncogenic proteins by small molecule inhibitors for CML, we investigated the potential of using the small-molecule Myc inhibitor, 10058-

Fig. 4



10058-F4 inhibits activation of human telomerase reverse transcriptase (hTERT) in HepG2 cells. (a) HepG2 cells transiently cotransfected with renilla luciferase constructs and firefly luciferase reporter constructs containing two E-boxes in the proximal region in hTERT promoter were treated with 100 μmol/l 10058-F4 for 24 h before analyses. The firefly luciferase activity was expressed as relative luciferase unit (RLU) relative to the renilla luciferase activity. The results represent a representative data of three repeated experiments. UT-CTL, untransfected and solvent-treated cells; UT-TX, cells untransfected but treated with 10058-F4; pGL-CTL, transfected with promoterless firefly luciferase constructs and treated with solvent; pGL-TX, transfected with promoterless luciferase constructs and treated with 10058-F4; pTERT-CTL, transfected with hTERT promoter constructs and treated with solvent; pTERT-TX: transfected with hTERT promoter constructs and treated with 10058-F4. (b) Quantitative real-time polymerase chain reaction of the hTERT expression using methods described in Materials and methods. (c) Protein expression of hTERT in HepG2 cells was examined every 24 h after being treated with 100 μmol/l 10058-F4 for up to 72 h. β-Actin was used as the protein-loading control. (d) Measurement of hTERT activity by TRAPeze assays in HepG2 cells after being treated with 100 μmol/l 10058-F4 for up to 48 h.

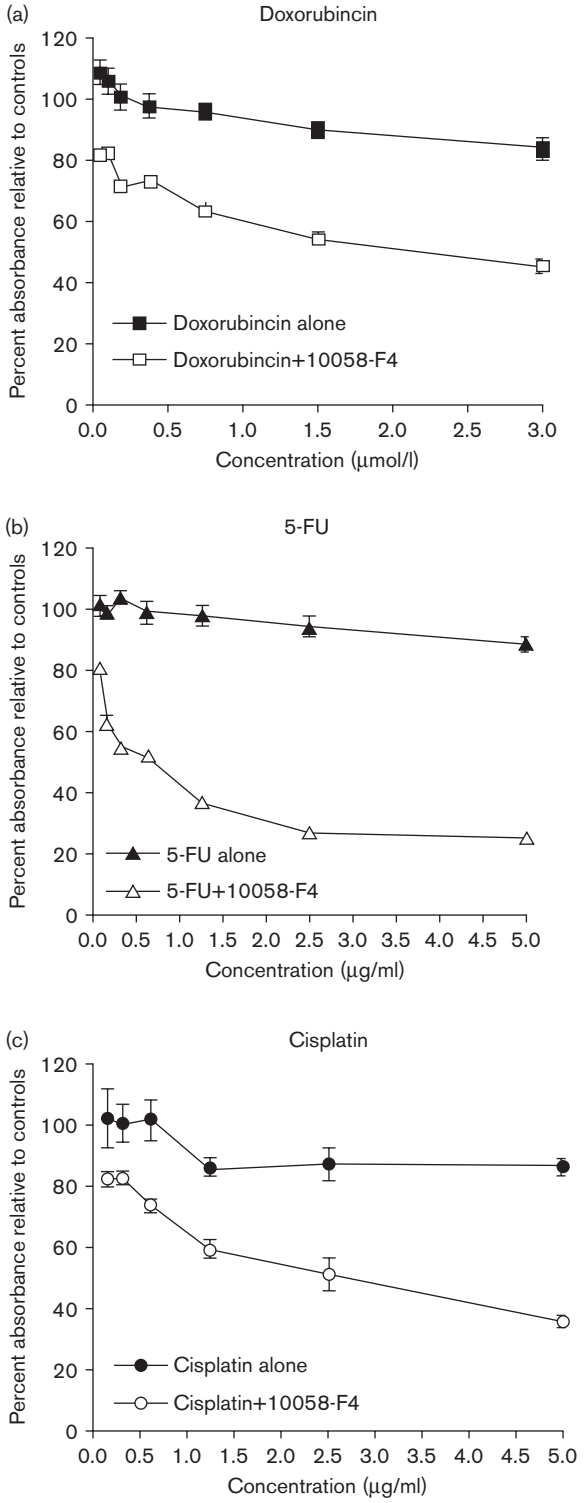
F4, on human HCC cells and found that 10058-F5 significantly inhibited proliferation of HepG2 cells and induced apoptosis over extended treatment.

Studies have reported that 10058-F4 inhibits c-Myc activity by blocking Myc/Max dimerization, but how it affects c-Myc protein has not been studied [12,13]. The lowered c-Myc protein after treatment could explain the decreased binding to E-box-containing oligonucleotides observed in EMSA studies. Our results suggest that 10058-F4 might not only block the Myc/Max dimerization, but also utilize other mechanisms in regulating c-Myc activity.

The reasons why HepG2 cells were more sensitive than Hep3B cells are still unclear. As with imatinib resistance in CML, multiple mechanisms, such as gene amplification, kinase domain mutations, decreased degradation or increased MDR1 protein levels, could lead to drug resistance [27,28]. Although we did not find any mutation in the bHLHZip region in Hep3B cells, mutations outside this region might contribute to the resistance to 10058-F4. For example, hotspot mutations, which are often found within or near the evolutionary conserved Myc box 1, enhance transforming activity by decreased proteasome-mediated turnover [29]. Alternatively, overexpression of the MDR1 gene also contributes to the resistance to imatinib in CML, but it is less likely in our situation as HepG2 cells express higher MDR1 protein than Hep3B cells [28,30].

The upregulation of $p21^{WAF1}$ could explain the 10058-F4-induced growth inhibition. c-Myc directly represses the transactivation of $p21^{WAF1}$ and induces S-phase entry, and

Fig. 5



10058-F4 enhances the cytotoxicity of conventional chemotherapeutic agents. HepG2 cells with or without pretreatment with 100 μmol/l 10058-F4 for 6 h were treated with indicated concentrations of (a) doxorubicin, (b) 5-fluorouracil (5-FU) or (c) cisplatin for 24 h before being subjected to 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assays. Data represent the mean ± SEM of experiments in quadruplicates.

repression of c-Myc is a necessary event in $p21^{WAF1}$ activation during differentiation of multiple cell types [31,32]. In contrast to the reports that $p27^{KIP1}$ is an important target of c-Myc in the induction of proliferation [20,33,34], there was no major changes in $p27^{KIP1}$ levels in 10058-F4-treated cells. As $p27^{KIP1}$ is sequestered with cyclin D2 after dissociation with cdk complex upon activation of Myc, the lack of $p27^{KIP1}$ changes could be explained by the absence of detectable cyclin D2 in our study [20].

Activation of c-Myc leads to upregulation of multiple cyclins in a cell-dependent manner [10,19]. For example, c-Myc has been shown to transactivate cyclin D2 directly [20]. Although there is evidence that activation of c-Myc correlates with induction of cyclin D3, deletion of two E-box-containing sequences in the cyclin D3 promoter does not affect its expression, suggesting that c-Myc uses an indirect mechanism to regulate cyclin D3 [35–37]. The relationship between c-Myc and cyclin D3, however, appears to be more complex. When tested on different cell types, c-Myc and cyclin D3 appear to function independently in regulating cell proliferation [38,39].

Although downregulating c-Myc is an effective way to inhibit tumor growth, the consequences of Myc inactivation vary in different types of cancers. Persistent c-Myc inactivation could repress the leukemia growth, but not for long [40]. For osteogenic sarcoma, however, even a brief inactivation could have a long-lasting effect [41]. For HCC, continuous inactivation of c-Myc is required to prevent the regrowth of tumors [9]. Thus, a c-Myc inhibitor alone might not be sufficient and the addition of an agent that acts in a different mechanism is needed to eliminate HCC. One practical approach is to add conventional chemotherapeutic agents. As patients with HCC are often unable to tolerate higher doses of chemotherapy because of advanced liver cirrhosis, it would be preferable to sensitize cancer cells to lower doses of chemotherapy with agents that specifically target cancer cells. One of the targets is hTERT. In the hTERT promoter, there are 29 putative c-Myc/Max binding sites, and at least five of them could be bound by c-Myc/Max dimers [23]. Although hTERT is not oncogenic per se, the acquisition of hTERT expression is essential for maintaining malignant transformation [42]. hTERT is an ideal target for several reasons: (1) overexpression of hTERT can only be identified in hepatocellular carcinoma cells but not in noncancerous cells [43]; (2) loss-of-function mutations of hTERT have hardly been identified in cancer cells and amplification of hTERT is not a common event in HCC [44]; (3) inhibition of hTERT by RNAi or hTERT antagonists has been shown to inhibit proliferation of HCC [24,26] and (4) $HBsAg^+$ HCC has higher hTERT expression than $HBsAg^-$ ones [45]. In fact, downregulation of hTERT using various strategies has

been shown to sensitize cancer cells to chemotherapeutic agents [24,46,47]. In agreement with these reports, we show that, in addition to the growth inhibitory activity, 10058-F4 could be used to downregulate hTERT and enhance the sensitivity to conventional chemotherapeutic agents.

Given the prevalence and the poor prognosis of HCC, our study proves the principle that small-molecule c-Myc inhibitors, either alone or in combination with conventional chemotherapeutic agents, are potential agents for novel anti-HCC therapy.

References

1 Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002; **31**:339–346.
2 Yu DY, Moon HB, Son JK, Jeong S, Yu SL, Yoon H, *et al.* Incidence of hepatocellular carcinoma in transgenic mice expressing the hepatitis B virus X-protein. *J Hepatol* 1999; **31**:123–132.
3 Balsano C, Avantaggiati ML, Natoli G, De Marzio E, Will H, Perricaudet M, *et al.* Full-length and truncated versions of the hepatitis B virus (HBV) X protein (pX) transactivate the c-*myc* protooncogene at transcriptional level. *Biochem Biophys Res Commun* 1991; **176**:985–992.
4 Raidl M, Pirker S, Schulte-Hermann R, Aubele M, Kandioler-Eckersberger D, Micksche M, *et al.* Multiple chromosomal abnormalities in human liver (pre)neoplasia. *J Hepatol* 2004; **40**:660–668.
5 Chan KL, Guan XY, Ng IO. High-throughput tissue microarray analysis of c-*myc* activation in chronic liver disease and hepatocellular carcinoma. *Hum Pathol* 2004; **35**:1324–1331.
6 Peng SY, Lai PL, Hsu HC. Amplification of the c-*myc* gene in human hepatocellular carcinoma: biologic significance. *J Formos Med Assoc* 1993; **92**:866–870.
7 Xu Y, Wang YH, Gao JD, Ye J, Zhu HX, Xu NZ, *et al.* Suppression of c-*myc* expression by interference RNA in HepG2 hepatocellular carcinoma cells. *Zhonghua Zhong Liu Za Zhi* 2004; **26**:458–460.
8 Ebinuma H, Saito H, Saito Y, Wakabayashi K, Nakamura M, Kurose I, *et al.* Antisense oligonucleotide against c-*myc* mRNA induces differentiation of human hepatocellular carcinoma cells. *Int J Oncol* 1999; **15**:991–999.
9 Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, *et al.* MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular carcinoma. *Nature* 2004; **431**:1112–1117.
10 Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol* 1999; **19**:1–11.
11 Berg T, Cohen SB, Desharnais J, Sonderegger C, Maslyar DJ, Goldberg J, *et al.* Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts. *Proc Natl Acad Sci USA* 2002; **99**:3830–3835.
12 Yin X, Giap C, Lazo JS, Prochownik EV. Low molecular weight inhibitors of Myc–Max interaction and function. *Oncogene* 2003; **22**:6151–6159.
13 Mo H, Henriksson M. Identification of small molecules that induce apoptosis in a Myc-dependent manner and inhibit Myc-driven transformation. *Proc Natl Acad Sci USA* 2006; **103**:6344–6349.
14 Sawyers CL. Making progress through molecular attacks on cancer. *Cold Spring Harb Symp Quant Biol* 2005; **70**:479–482.
15 Liu HE, Lim KH, Huang MJ, Huang BS. Targeting epidermal growth factor receptor in lung cancer: perspective from the Asia-Pacific region. *Asia Pac J Clin Oncol* 2006; **2**:22–31.
16 Shah NP. Loss of response to imatinib: mechanisms and management. *Hematol Am Soc Hematol Educ Program* 2005; 183–187.
17 Hermeking H, Funk JO, Reichert M, Eellwart JW, Eick D. Abrogation of p53-induced cell cycle arrest by c-Myc. evidence for a inhibitior of $p21^{WAF1/CIP1SDI1}$. *Oncogene* 1995; **11**:1409–1415.
18 Qin LF, Ng IO. Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells. *Cancer Lett* 2002; **175**:27–38.
19 Hunter T, Pines J. Cyclins and cancer II: cyclin D and CKD inhibitors come of age. *Cell* 1994; **79**:573–582.
20 Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, *et al.* Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J* 1999; **18**:5321–5333.
21 Yao X, Hu J-F, Daniel M, Yien H, Lu H, Sharan H, *et al.* A novel orthotopic tumor model to study growth factor and oncogenes in hepatocarcinogenesis. *Clin Cancer Res* 2003; **9**:2719–2726.
22 Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S, *et al.* Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. *Int J Oncol* 2003; **103**:572–576.
23 Wu K-J, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, *et al.* Direct activation of TERT transcription by c-Myc. *Nat Genet* 1999; **21**:220–224.
24 Djojosubroto MW, Chin AC, Go N, Schaetzlein S, Manns MP, Gryaznov S, *et al.* Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. *Hepatology* 2005; **42**:1127–1136.
25 Guo Q-L, Lin S-S, You Q-D, Gu H-Y, Yu J, Zhao L, *et al.* Inhibition of human telomerase reverse transcriptase gene expression by gambogic acid in human hepatoma SMMC-7721 cells. *Life Sci* 2006; **78**:1238–1245.
26 Zhang PH, Zou L, Tu ZG. RNAi-hTERT inhibition hepatocellular carcinoma cell proliferation via decreasing telomerase activity. *J Surg Res* 2006; **131**:143–149.
27 Mahon FX, Deininger MWN, Schultheis B, Chabrol J, Reiffers J, Goldman JM, *et al.* Selection and characterization of BCR–ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood* 2000; **96**:1070–1079.
28 Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J, *et al.* MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. *Blood* 2003; **101**:2368–2373.
29 Bahram F, von der Lehr N, Cetinkaya C, Larsson L-G. c-Myc hot spot mutations in lymphoma result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood* 2000; **95**:2104–2110.
30 Manov I, Bashenko Y, Hirsh M, Iancu TC. Involvement of the multidrug resistance P-glycoprotein in acetaminophen-induced toxicity in hepatoma-derived HepG2 and Hep3B cells. *Basic Clin Pharmacol Toxicol* 2006; **99**:213–224.
31 Nagl NG Jr, Zweitzig DR, Thimmapaya B, Beck GR Jr, Moran E. The c-*myc* gene is a direct target of mammalian SWI/SNF-related complexes during differentiation-associated cell cycle arrest. *Cancer Res* 2006; **66**:1289–1293.
32 Mitchell KO, El-Deiry WS. Overexpression of c-Myc inhibit $p21^{WAF1/CIP1}$ expression and induces S-phase entry in 12-o-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells. *Cell Growth Diff* 1999; **10**:223–230.
33 O'Hagan RC, Ohh M, David G, de Alboran IM, Alt FW, Kaelin WGJ, *et al.* Myc-induced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression. *Genes Dev* 2000; **14**:2185–2191.
34 Yang W, Shen J, Wu M, Arsura M, Fitzgerald M, Suldan Z, *et al.* Repression of transcription of the $p27^{KIP1}$ cyclin-dependent kinase inhibitor gene by c-Myc. *Oncogene* 2001; **20**:1688–1702.
35 Janicke RU, Lin X-F, Lee FH, Porter AG. Cyclin D3 sensitizes tumor cells to tumor necrosis factor-induced c-Myc-dependent apoptosis. *Mol Cell Biol* 1996; **16**:5245–5253.
36 Song S, Copperman J, Letting DL, Blobel GA, Choi JK. Identification of cyclin D3 as a direct target of E2A using DamID. *Mol Cell Biol* 2004; **24**:8790–8802.
37 Bernardin-Fried F, Kummalue T, Jeijen S, Collector MI, Ravid K, Friedman AD. AML1/RUNX1 increases during G_1 to S cell cycle progression independent of cytokine-dependent phosphorylation and induces cyclin D3 gene expression. *J Biol Chem* 2004; **279**:15678–15687.
38 Rhee K, Bresnahan W, Hirai A, Hirai M, Thompson EA. c-Myc and cyclin D3 (CcnD3) genes are independent targets for glucocorticoid inhibition of lymphoid cell proliferation. *Cancer Res* 1995; **15**:4188–4195.
39 Ausserlechner MJ, Obexer P, Bock G, Geley S, Kolfer R. Cyclin D3 and c-Myc control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. *Cell Death Diff* 2004; **11**:165–174.
40 Felsher DW, Bishop JM. Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol Cell* 1999; **4**:199–207.
41 Jain M, Arvanitis C, Chu K, Dewey W, Leonhardt E, Trinh M, *et al.* Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Nature* 2002; **297**:102–104.
42 Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, *et al.* Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; **279**:349–352.
43 Takahashi S, Kitamoto M, Takaishi H, Aikata H, Kawakami Y, Nakanishi T, *et al.* Expression of telomerase component genes in hepatocellular carcinoma. *Eur J Cancer* 2000; **36**:496–502.

44 Takuma Y, Nuoso K, Kobayahi Y, Nakamura S, Tanaka H, Matsumoto T, *et al.* Telomerase reverse trasncriptase gene amplification in hepatocellular carcinoma. *J Gastroenterol Hepatol* 2005; **19**:1300–1304.
45 Guo Y, Zhou X, Liu E, Li X, Liu J, Yang Z, *et al.* Difference in hTERT gene expressions between HBsAg-positve and HBsAg-negative hepatocellular carcinoma. *J Huazhong Univ Sci Technol Med Sci* 2005; **25**:303–306.
46 Misawa M, Tauchi T, Sashida G, Nakajima A, Abe K, Ohyashiki JH, *et al.* Inhibition of human telomerase enhances the effect of chemotherapeutic agents in lung cancer cells. *Int J Oncol* 2002; **21**:1087–1092.
47 Kondo Y, Kondo S, Tanaka Y, Haqqi T, Barna BP, Cowell JK. Inhibition of telomerase increases the susceptibility of human malignant glioblastoma cells to cisplatin-induced apoptosis. *Oncogene* 1998; **16**: 2243–2248.