A Small-Molecule E2F Inhibitor Blocks Growth in a Melanoma Culture Model

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Abstract

HLM006474 was identified using a computer-based virtual screen and the known crystal structure of the DNA-bound E2F4/DP2 heterodimer. Treatment of multiple cell lines with HLM006474 resulted in the loss of intracellular E2F4 DNA-binding activity as measured by electrophoretic mobility shift assay within hours. Overnight exposure to HLM006474 resulted in down-regulation of total E2F4 protein as well as known E2F targets. The effects of HLM006474 treatment on different cell lines varied but included a reduction in cell proliferation and an increase in apoptosis. HLM006474 induced apoptosis in a manner distinct from cisplatin and doxorubicin. E2F4-null mouse embryonic fibroblasts were less sensitive than wild-type counterparts to the apoptosis-inducing activity of the compound, revealing its biological specificity. A375 cells were extremely sensitive to the apoptosis-inducing activity of the compound in two-dimensional culture, and HLM006474 was a potent inhibitor of melanocytes proliferation and subsequent invasion in a three-dimensional tissue culture model system. Together, these results suggest that interference with E2F activity using small molecules may have clinical application in cancer therapy. [Cancer Res 2008;68(15):6292–9]

Introduction

The E2F/Rb pathway is central to the regulation of the mammalian cell cycle, and thus, it seems a reasonable target for the development of chemotherapeutic agents (1–3). The E2F family is composed of nine members with various biological roles (4–6). E2F1 is the best studied member of the family and has been shown to have numerous and even opposing roles in cell growth control depending on the context of experimentation (1–3). In the context of drug-induced apoptosis of highly transformed cells, E2F1 is a downstream target of the ataxia telangiectasia mutated/ataxia telangiectasia mutated and Rad3-related signaling pathway (7) and contributes significantly to the apoptotic activity of DNA-damaging drugs and cyclin-dependent kinase inhibitors (7). In contrast, E2F4, the most abundant member of the E2F family, contributes to survival in the context of treatment with chemotherapeutic drugs or cyclin-dependent kinase inhibitors (8–11).

Whereas individual members of the E2F family have specialized roles, a variety of complementary approaches have shown that down-regulation of total intracellular E2F activity can lead to apoptosis (12), growth arrest (13, 14), or both (15). These observations suggest that small molecules that would inhibit the DNA-binding activity of E2F might have a significant benefit in cancer therapy. We have used the known crystal structure of the DNA-bound E2F4/DP2 heterodimer to guide a computational screen for small molecules that might inhibit this interaction (16). One small molecule, HLM006474, emerged with in vivo activity. This article describes an initial characterization of HLM006474 biological activities in a number of commonly examined cancer cell lines. HLM006474 was particularly active against a melanocyte cell line, A375. In two-dimensional culture, A375 cells were extremely sensitive to the apoptosis-inducing activity of the compound, and in a three-dimensional tissue culture model system HLM006474 was a potent inhibitor of A375 proliferation and invasion.

Materials and Methods

Computer docking and chemical synthesis. Details of the virtual screen and chemical synthetic strategy are provided in Supplementary Materials and Methods.

Cell lines and drug treatments. An expanded description of the cell lines used and the drug treatments is provided in Supplementary Materials and Methods.

Biochemical assays. Electrophoretic mobility shift assays (EMSA) were done using 20 µg of whole-cell extract and an 32P-labeled oligonucleotide probe, as previously described (17). Antibodies used in supershift experiments were E2F4 (mouse monoclonal 2-12E8 (ref. 18); a gift from J. Lees, MIT, Cambridge, MA), Rb (Calbiochem), p107 (Santa Cruz Biotechnology), p130 (Santa Cruz Biotechnology), E2F1 (Santa Cruz Biotechnology), E2F2 (Santa Cruz Biotechnology), E2F2 (Neomarkers), E2F3 (Santa Cruz Biotechnology), and E2F3 (Santa Cruz Biotechnology). EMSA signals were captured with a Storm Phospholmager and band intensities quantified with ImageQuant Software. Quantitative EMSA assays were done in triplicate. Western blots used 50 µg of whole-cell extract per lane as previously described (8, 9, 19). Primary antibodies used in these studies consisted of E2F4 (Santa Cruz Biotechnology), E2F5 (Santa Cruz Biotechnology), β-actin (Sigma), poly(ADP-ribose) polymerase (PARP; Cell Signaling), cyclin D3 (BD PharMingen), cyclin A (monoclonal gift from E. Leof, Mayo Clinic Cancer Center, Rochester, MN; ref. 20), p53 (BD PharMingen), Bax (Santa Cruz Biotechnology), Mcl-1 (Santa Cruz Biotechnology), and p107 (Santa Cruz Biotechnology). Detection of proteins was accomplished using horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence purchased from Amersham.

Cell cycle, apoptosis, and viability analysis. Cells were detached from culture plates by trypsin treatment, washed twice with PBS, and fixed in 70% ethanol. Fixed cells were washed twice with PBS and treated with RNase A and propidium iodide. Propidium iodide staining was examined with a Becton-Dickinson FACSscan instrument and Cell Quest software. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay for apoptosis used a PharMingen Apo-BrdU Kit
(8, 9, 19). 3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays were conducted using a CellTiter 96 AQueous One Cell Proliferation Assay Kit (Promega). Further details are provided in Supplementary Materials and Methods.

**Three-dimensional skin reconstruction model.** Culture inserts of a differentiated, full-thickness, three-dimensional skin reconstruction model of A375 melanoma cells were purchased from MatTek. These were prepared by culturing mixed suspensions of normal human epidermal keratinocytes and A375 cells (1:10 ratio) on fibroblast-contracted collagen gels and allowing differentiation for ~1 wk in serum-free media to form a three-dimensional skin-like structure. These cultures were treated with 0, 40, or 80 μmol/L 6474 and harvested after 0, 2, 5, 8, 12, 16, and 20 d.

**Results**

Identification and synthesis of HLM006474. Grid-Based Ligand Docking from Energetics (GLIDE, Schrödinger) was used to screen a 20,000-compound three-dimensional chemical database (from ChemDiv, Inc.; refs. 21, 22) for putative interactions with the known crystal structure of the E2F4/DP2 heterodimer (16). Four hundred small molecules emerged from the docking studies with predicted free energies ranging from ~10.95 to ~6.35 kcal/mol. These four hundred high-scoring molecules were screened for the ability to inhibit E2F4 DNA binding at 20 μmol/L in standard E2F EMSAs (23). Signal transducer and activator of transcription-3 EMSAs were used as negative control to ensure that inhibition was E2F specific (24). Incubation of these compounds with NIH-3T3 cell extracts identified 10 compounds with potential E2F4-inhibitory activity. To measure activity against a human cancer cell line, MCF-7, cells were treated with these 10 compounds in culture and inhibition of E2F4 DNA-binding activity was determined by EMSA. Only one of the 10 compounds, HLM006474, showed measurable activity in vivo (data not shown). HLM006474 was synthesized at a large scale as a pure sample (as described in Materials and Methods). The chemical synthesis and structure of HLM006474 are shown in Fig. 1A. HLM006474 is not specific to E2F4 and seems to inhibit binding by all E2F complexes (see Supplementary Figs. S1 and S2). However, because E2F4 is the most often dysregulated in human cancers, this was the focus of further studies.

HLM006474 was quantified using ImageQuant and the results of these assays clearly indicate that HLM006474 decreases E2F4 activity through inhibition of its DNA-binding activity and down-regulation of its expression.

HLM006474 inhibits E2F4 activity in vivo. The E2F/Rb pathway is disrupted in virtually every case of melanoma, and thus, we sought to determine whether HLM006474 would have activity in a melanoma model. To this end, A375 cells, which represent a commonly used melanoma cell line, were treated for 9 hours with various concentrations of HLM006474 to determine if the compound would have activity in vivo. Whole-cell extracts of treated cells were prepared and the DNA-binding activity of E2F4 was measured by EMSA. Figure 1B shows that at 10 and 20 μmol/L concentrations, HLM006474 has little effect on E2F4 DNA-binding activity in A375 cells; however, at 40 μmol/L E2F4 inhibition is clearly apparent and increases at 60 and 80 μmol/L concentrations. Because the observed loss of E2F4 DNA-binding activity could be the result of down-regulation of E2F4 protein, we performed Western blots on the same samples used for EMSA. We find that 9 hours of treatment with HLM006474 does not significantly affect the expression of E2F4 or E2F1, showing that the diminished E2F4 signal observed by EMSA is not due to decreased protein expression. Likewise, the expression of E2F1 was not affected by the compound at 9 hours. Actin served as a loading control in these experiments and in those that follow.

Phospholipase signals from four independent experiments were quantified using ImageQuant and the results are graphed in Fig. 1C. The apparent IC50 (drug concentration required to reduce total E2F4 DNA-binding activity by 50% of untreated cells) was calculated using the Statistical Analysis System (Proc Probit). Data indicate an in vitro IC50 of 29.8 μmol/L (~7.6 μmol/L). In the experiments that follow, 40 μmol/L drug was used as standard HLM006474 concentration because that concentration of drug should reduce E2F4 activity by 50% to 75% and should limit off-target effects.

HLM006474 treatment leads to down-regulation of total E2F4 protein. To determine the activity of HLM006474 over time, A375 cells were treated with 40 μmol/L of compound and examined at 0, 3, 6, 9, 12, 18, and 24 hours by EMSA and Western blot (Fig. 2). With a single 40 μmol/L dose, inhibition of E2F4 DNA-binding activity became apparent 9 hours following treatment and persisted for up to 24 hours. By 24 hours, a decrease in total E2F4 protein became apparent, suggesting that inhibition of E2F4 DNA-binding may predispose E2F4 to degradation. The level of proapoptotic E2F1 increases at early time points, but is diminished at 24 hours. EMSA activities from A375 cells treated with HLM006474 were quantified using ImageQuant and the results are plotted in Fig. 2B. This analysis indicates that half of the E2F DNA-binding activity is lost between 9 and 12 hours after 40 μmol/L HLM006474 treatment. The observation that HLM006474 down-regulates total E2F4 protein was not anticipated; however, it may contribute to the lasting biological effect of the compound. Collectively, these data indicate that HLM006474 inhibits E2F4 activity through inhibition of its DNA-binding activity and down-regulation of its expression.

HLM006474 can induce apoptosis. The preliminary data described above strongly suggest that HLM006474 might serve as an effective chemotherapeutic agent. To examine its effect on a range of commonly studied cell lines, we used standard MTS assays to quantify cell viability following HLM006474 treatment. The results of these assays clearly indicate that HLM006474 decreases the number of viable cells over the experimental time course (see Supplementary Fig. S3). In no case does the compound seem to increase proliferation, as might occur if depression of E2F activity would be sufficient to induce cellular proliferation. To determine if treatment with HLM006474 contributes to apoptosis, A375, MDA-MB-231, MCF-7, and human foreskin fibroblasts cells were treated with 40 μmol/L HLM006474 for 24 hours and subjected to a fluorescence-activated cell sorting–based TUNEL assay (Apo-BrdU Kit from BD Pharmingen). Figure 3A reveals a dramatic induction of apoptosis in the A375 and 231 cell lines. In contrast, HLM006474 did not induce an obvious increase in apoptosis in human foreskin fibroblast or MCF-7 cells.

To further examine the timing of HLM006474-induced apoptosis, A375 cells were treated with 40 μmol/L HLM006474, harvested, and fixed at various time periods. Cells were then stained with propidium iodide to examine cell cycle status as estimated by flow cytometry. Whereas no other obvious cell cycle effects were observed (data not shown), Fig. 3B highlights the significant increase in sub-G1 DNA content of the cells beginning ~9 hours following HLM006474 treatment. Likewise, PARP cleavage (Fig. 3C) indicates significant apoptosis by 12 hours following HLM006474 treatment. Thus, HLM006474-induced apoptosis seems to temporally follow the down-regulation of E2F4 DNA binding and to be
largely coincident with E2F4 protein down-regulation (see Fig. 2B).

Experiments described in Supplementary Fig. S4 show that melanoma cell lines with multiple drug resistance are also sensitive to treatment with HLM006474. Taken together, these results show that HLM006474 is a potent inducer of apoptosis.

HLM006474 treatment leads to apoptosis in a manner distinct from traditional chemotherapeutic drugs. To compare the mechanism of HLM006474-induced apoptosis with that of several standard DNA-damaging drugs, A375 cells were treated for 24 hours with 40 μmol/L HLM006474, 10 μmol/L cisplatin, 10 nmol/L doxorubicin, 10 μmol/L etoposide (VP-16), or with two-drug combinations. We have previously shown that these chemotherapeutic drugs lead to a modest repression of E2F4 expression (in several cell lines) and that E2F4 deficiency leads to an increased susceptibility to the action of these drugs (8). As expected, Fig. 4 reveals that each of these drugs individually...
reduced E2F4 levels in A375 cells after 24 hours of treatment. However, every two-drug combination essentially eliminated E2F4 expression; suggesting that HLM006474 may synergize with these various drugs in the elimination of E2F4 activity.

We have previously shown that cyclin D3 promoter is up-regulated on serum stimulation dependent on an E2F site at position -143 to -135 (25). Figure 4 reveals that HLM006474 treatment significantly reduces cyclin D3 protein expression, thus supporting the hypothesis that HLM006474 is blocking at least a subset of E2F-regulated genes. Treatment with the traditional chemotherapeutics cisplatin, doxorubicin, and VP-16, in contrast, had little effect on cyclin D3 (Fig. 4) or other cell cycle factors (data not shown). Western blots for PARP and the cleaved/activated form of PARP revealed that HLM006474 is a potent inducer of PARP cleavage, with no synergy between HLM006474 and the other drugs observed at these concentrations.

A Western blot against p53 was also done to determine if p53 might play a role in HLM006474-induced apoptosis (Fig. 4). As expected, the traditional chemotherapeutic agents each induced p53 expression; however, HLM006474 did not—in fact, it may block p53 induction in A375 cells. Mcl-1, a prosurvival member of the Bcl-2 family, is known to be E2F regulated (26, 27). Western blots for Mcl-1 suggest that HLM006474 may slightly repress Mcl-1 in A375 cells. These results suggest that apoptosis induced by HLM006474 acts through a mechanism distinct from other traditional chemotherapies and may therefore be useful in malignancies that have become resistant to drugs that function through these pathways.

**HLM006474 activity is partially dependent on E2F4.** To determine if the effect of HLM006474 is dependent on E2F4, we compared the HLM006474 response of immortalized mouse embryonic fibroblasts (MEF) derived from E2F4-deficient mice with that of MEFs derived from wild-type siblings. Figure 5A shows that HLM006474 induces a 2-fold increase in the level of apoptosis in wild-type (WT) cells as compared with that in E2F4-deficient cells. We have found these same E2F4-deficient MEFs to be more sensitive to every other drug that we have ever tested (8), and thus the resistance to HLM006474 is even more convincing. The finding that E2F4-null MEFs are affected by HLM006474 suggests that E2F4 is not the sole target and that down-regulation of additional E2Fs likely contributes to cell death. This is consistent with the biochemical evidence (supplementary Figs. S1 and S2) that HLM006474 inhibits all E2F family members. Figure 5B shows that PARP cleavage is evident in E2F4-proficient MEFs even as low as 20 μmol/L HLM006474, whereas 60 μmol/L HLM006474 is required to detect PARP cleavage in E2F4-deficient MEFs. Collectively, these data
indicate that apoptosis induced by HLM006474 is, in part, dependent on E2F4.

**HLM006474 inhibits A375 proliferation in a three-dimensional model system.** Given the biological and biochemical response of A375 cells to HLM006474 in cell culture, we postulated that this compound may inhibit malignant growth in a three-dimensional skin model of A375 invasion. In this model, the highly metastatic A375 melanocytes were mixed with normal human keratinocytes and seeded on fibroblast-contracted collagen gels. The mixed cells were then induced to differentiate in serum-free media to form three-dimensional, highly differentiated, full-thickness skin-like tissues. After 7 days of differentiation, the cells were then treated with 0 (DMSO carrier alone), 40, or 80 μmol/L of HLM006474. The three-dimensional models were then cultured for 2, 5, 8, 12, 16, and 20 days. At the appointed time, three-dimensional cultures were fixed in formalin, paraffin embedded, sectioned, and either stained with H&E or processed for immunohistochemistry with antibodies against S-100 to measure expression of a melanocyte marker, E2F4, to determine if it were down-regulated as in two-dimensional culture; activated caspase-3 to measure apoptosis; and Ki-76 to measure the proliferative index.

Figure 6A highlights the H&E stain of tissues over time in the absence and presence of HLM006474. Due to space limitations, the results of 80 μmol/L HLM006474 treatment are not shown herein (there is essentially no melanocyte proliferation at 80 μmol/L). In this figure, the keratinocytes form the upper epidermal layer, with the second distinct layer of cells representing the melanocytes. In the early time points, this layer is only a few cells thick, and these cells are distinguished by their dark nuclear staining. The third distinct layer represents the fibroblast-contracted collagen that makes up the underlying dermal substrate. Over time, the metastatic melanocytes proliferate and form nodes, which grow and invade the underlying collagen substrate. This growth and invasion is clearly evident in the DMSO-treated samples.

To confirm the presence and growth of melanocytes, the tissues were subjected to immunohistochemistry with a melanocyte marker (S-100; see Fig. 6B). Figure 6B reveals strong expression of S-100 in control treated cells. In contrast, only a few S-100-positive cells are observed in the HLM006474-treated tissues, making it clear that HLM006474 significantly inhibited the proliferation and subsequent invasion of the melanocytes into the collagen layer. Because a reduction in S-100 expression is considered an excellent marker for the successful treatment of melanoma (28), these results suggest that HLM006474 is a highly effective inhibitor of malignant growth in this model system. The compound had no obvious deleterious effects on the other cells (fibroblasts and keratinocytes) making up the three-dimensional tissue.

Western blots of cells treated with HLM006474 in two-dimensional cultures indicated that the compound led to significant down-regulation of the E2F4 protein. To determine if E2F4 was reduced in three-dimensional culture, sections were subjected to E2F4 immunohistochemistry. Figure 6C reveals that E2F4 levels are clearly reduced in the treated tissues. Although there are less total cells in the HLM006474-treated tissue (which slightly complicates direct comparisons), it is clear that a lower fraction of HLM006474-treated cells stain positively for E2F4, and of those that are positive the staining is generally less intense. Finally, it is noted that in the treated tissues the remaining E2F4 is predominantly nuclear, whereas in the untreated cells a fraction of E2F4 is also located in the cytoplasm. Because E2F4 is known to shuttle between the cytoplasm (G1-S) and nucleus (G0-G1) during the cell cycle, this result may primarily reflect a quiescent state in the HLM006474-treated cells (29). These results indicate that HLM006474 is likely hitting its intended target in the three-dimensional culture.

To determine whether the inhibition of invasion and melanocyte proliferation was due to increased apoptosis, sections were stained with a marker for apoptosis (activated caspase-3). No difference was observed in samples at day 12, 16, or 20, and as such we stained at earlier time points reasoning that apoptosis might be an early event that would eliminate cells that would later invade the collagen substrate. Supplementary Fig. S5 reveals that no significant difference in activated caspase-3-positive cells was observed even in the day 2, 5, or 8 tissues. These data do not fully rule out a possibility for apoptosis in the three-dimensional culture model, however, because it is possible that these dying cells are simply hard to detect. To determine whether an inhibition of cell division might account for inhibition of melanocyte proliferation, tissue sections were stained with a proliferative marker (Ki-67; Fig. 6D). The Ki-67 staining clearly reveals a decrease in the number of proliferative cells when treated with HLM006474. Thus, the most obvious mode of action of HLM006474 in the three-dimensional model is in the inhibition of proliferation.

**Discussion**

Previous work suggested that down-regulation of total E2F activity within cancer cells would lead to either growth arrest or
apoptosis, and thus we predicted that a small-molecule inhibitor of E2F activity might have therapeutic efficacy in cancer. To test this hypothesis, we screened for compounds that might inhibit E2F DNA binding and identified one small molecule that clearly targets E2F in vivo. In vivo, this inhibitor leads to significant down-regulation of E2F4 protein. This unexpected activity may account for the primary biological activity and specificity of HLM006474 and provides an easy way to monitor its biological activity (E2F4 Western blotting or immunohistochemistry). The mechanism by which HLM006474 leads to down-regulation of E2F4 will be a topic for future research.

HLM006474 is predicted to make hydrogen bonds with three residues that are absolutely conserved within the E2F family, and thus, there is no reason to expect that HLM006474 is specific to E2F4 heterodimers. Indeed, Supplementary figures show that essentially all E2F complexes detectable by standard EMSA assay are inhibited by HLM006474. Whereas HLM006474 is not specific to the DNA-binding domain of E2F4, experiments with

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**Figure 6.** HLM006474 inhibits melanocyte proliferation in a three-dimensional skin model. A, H&E staining was done on thin sections of day 12, 16, and 20 tissues treated with either DMSO (a–c) or 40 μmol/L HLM006474 (d–f). Magnification, ×100. The top bright red layer represents the epidermis; the next layer of cells with dark blue nuclei represents the melanocyte layer; and the bottom largely unstained area represents the fibroblast-contracted collagen dermal substrate. Arrows in the DMSO only cells indicate cells and cell clusters that have invaded the dermal layer that are largely absent in the HLM006474-treated tissues. B, S100 immunohistochemistry was done as in A. Magnification, ×200. Arrows, S-100 positive cells, which are very rare in the HLM006474-treated tissue. C, E2F4 immunohistochemistry was done on thin sections as in A. Magnification, ×200. Arrows, darkly stained nuclei and lightly stained cytoplasm, which are rare in the HLM006474-treated tissues. D, Ki-67 immunohistochemistry was done on thin sections of day 2, 5, and 8 tissue treated with either DMSO (a–c) or 40 μmol/L HLM006474 (d–f). Magnification, ×200.
E2F4-knockout MEFs show that cells that have presumably adapted to the absence of E2F4 (which has prosurvival activity) are less sensitive to HLM006474 than similarly derived cells from littermate animals. This specificity is not trivial because we have repeatedly found E2F4-deficient MEFs to be more sensitive to every drug we have tested, including flavopiridol, SNS-032, roscovitine, cisplatin, and VP-16 (8). Although it does not formally rule out the possibility that other E2Fs are also important targets, this result strongly argues that E2F4 is an important target for HLM006474. This specificity may derive from the ability of HLM006474 to lead to the down-regulation of the E2F4 polypeptide. Future analysis will examine the specific contribution of other E2F family members to the activity of HLM006474.

It is known that a number of E2F-regulated promoters are primarily governed by transcriptional repression by E2F4/Rb family complexes during G1/S, followed by depression at the G1/S boundary. Given this mechanism, it would be predicted that blocking E2F DNA-binding activity should result in up-regulation of these genes, which might possibly result in increased cell growth. This is a very serious consideration because it may ultimately limit the therapeutic efficacy of E2F-targeted therapies. At this point, we have no direct evidence that HLM006474 can result in a net increase in cell growth in any cell line that we have tested. However, we note that only a subset of cell lines treated with HLM006474 are significantly growth inhibited at 40 μmol/L (see Supplementary Fig. S3). Higher HLM006474 concentrations are more effective; however, to limit off-target effects, we have primarily done experiments at 40 μmol/L because it is just above the IC50 of 29.8 μmol/L (+7.6 μmol/L). Future experiments will address the possibility that in some cell lines HLM006474 increases cell proliferation but simultaneously increases apoptosis such that a net increase in cell number is not observed in the MTS assay. Nonetheless, our results suggest that generating a net increase in tumor growth with E2F inhibitors is not likely, consistent with the literature (12–15).

HLM006474 clearly induces apoptosis in sensitive cell lines such as A375 and 231. Although the phenomenon is well described in the literature (12–15), the exact mechanism of “E2F deficiency–induced apoptosis” has not been adequately investigated. It has been shown that derepression can be an important mechanism of E2F regulation (30), and it is straightforward to speculate that inhibition of E2F might lead to derepression of cell death proteins. We have previously shown that E2F4 significantly contributes to survival during drug-induced apoptosis and that several standard chemotherapeutic drugs significantly reduce E2F4 expression (8). Figure 4 reveals that HLM006474 synergizes with cisplatin, doxorubicin, and VP-16 to reduce E2F4 levels. Based on our work (8), as well as work published by others (6, 10, 11), we hypothesize that E2F deficiency–induced apoptosis is primarily the result of down-regulation of the prosurvival role of E2F4. At this point, we have not identified the key E2F target that may mediate this effect. P53 does not seem to play a role (Fig. 4), and Mcl-1 is modestly down-regulated in A375 cells following HLM006474 treatment (Fig. 4), which could account for the cell sensitivity to the compound. Likewise, pRb and p107 are down-regulated following HLM006474 treatment (not shown). Because Rb family members are known to have prosurvival roles, it is possible that their down-regulation may contribute to cell death (31, 32). Future work will investigate this hypothesis further.

Although the biochemical mechanisms of HLM006474 action and specificity remain to be fully elucidated, we have clearly identified a compound with significant biological activity that targets E2F4. The biological activity of HLM006474 was shown most convincingly in a three-dimensional model of melanocyte proliferation and invasion. In this experiment, we highlight the ability of HLM006474 to inhibit the proliferation and subsequent invasion of A375 melanocytes into an underlying dermal substrate. This model further showed that HLM006474 reduced the levels of E2F4 in the treated melanocytes and that the reduction of E2F4 resulted in a significant decrease in cellular proliferation as measured by Ki-67 staining. Surprisingly, this model did not detect significant levels of apoptosis (as measured by activated caspase-3 immunohistochemistry) in the treated versus control melanocytes, although the compound induced significant apoptosis in two-dimensional culture. One explanation for this observation is that the apoptotic cells are simply not detected efficiently by the assay in three dimensions. Alternatively, it is possible that the apoptosis-inducing activity of HLM006474 is limited in three-dimensional culture due to survival contacts present there and that the main biological activity of HLM006474 is indeed the ability to inhibit cell cycle progression. We believe that these results provide proof of principle that E2F inhibitors may have therapeutic potential in the appropriate context.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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