The NF-κB Inhibitor LC-1 Has Single Agent Activity in Multiple Myeloma Cells and Synergizes with Bortezomib

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Abstract

Multiple myeloma remains incurable with conventional therapeutics. Thus, new treatments for this condition are clearly required. In this study we evaluated the novel NF-κB inhibitor LC-1 in multiple myeloma cell lines and plasma cells derived from multiple myeloma patients. LC-1 was cytotoxic to multiple myeloma cell lines H929, U266, and JJN3, and induced apoptosis in a dose-dependent manner with an overall LD50 of 3.6 μmol/L (±1.8) after 48 hours in culture. Primary multiple myeloma cells, identified by CD38 and CD138 positivity, had a mean LD50 for LC-1 of 4.9 μmol/L (±1.6); normal bone marrow cells were significantly less sensitive to the cytotoxic effects of LC-1 (P = 0.0002). Treatment of multiple myeloma cell lines with LC-1 resulted in decreased nuclear localization of the NF-κB subunit Rel A and the inhibition of NF-κB target genes. In addition, LC-1 showed synergy with melphalan, bortezomib, and doxorubicin (combination indices of 0.72, 0.61, and 0.78, respectively), and was more effective when cells were cultured on fibronectin. These data show that LC-1 has activity in multiple myeloma cell lines and primary multiple myeloma cells, and its ability to inhibit NF-κB seems important for its cytotoxic effects. Furthermore, LC-1–induced transcriptional suppression of survivin and MCL1 provides a potential explanation for its synergy with conventional agents.

Research Article

Mol Cancer Ther; 9(6) June 2010; DOI: 10.1158/1535-7163.MCT-10-0104

Introduction

Multiple myeloma is an incurable hematologic cancer characterized by the accumulation of malignant plasma cells in the bone marrow (1, 2). Amassing of plasma cells leads to anemia, skeletal destruction causing considerable bone pain, impaired renal function, and hypercalcemia (1, 3). The disease may be de novo or may progress from pre-existing monoclonal gammopathy of undetermined significance. Successful treatment of active disease leads to plateau phase, but relapse is inevitable. After a variable number of plateau phases resistant disease emerges (1). The bone marrow microenvironment and its interaction with the myeloma plasma cells are crucial for the maintenance of the disease; the malignant plasma cells and stromal cells interact through direct cell contact, adhesion molecules, and cytokine signaling to promote tumor cell survival (1).

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At present, the treatment of choice for multiple myeloma patients <65 years of age is induction chemotherapy followed by high-dose chemotherapy with autologous stem cell transplantation (4). In some patients, long-term disease-free survival has been achieved by allogeneic stem cell transplant, but this is associated with extremely high levels of treatment-related mortality (1, 4). Recent studies have identified many new agents, including bortezomib, thalidomide, and lenalidomide, which in combination with other drugs have increased response rates and improved progression-free survival and possibly even overall survival (5–7).

NF-κB is a family of transcription factor dimers made up of combinations of p50, p52, c-Rel, p65/Rel A, and Rel B. These dimers are inactive in the cytoplasm in most normal cells resulting from their interactions with inhibitor of NF-κB (IκB) proteins. Extracellular signals activate NF-κB by proteasome-mediated degradation of IκB and nuclear translocation of NF-κB (8). Multiple myeloma cell lines have been shown to exhibit constitutive activation of NF-κB, and this correlated with their sensitivity to IκB inhibition (9). The majority of multiple myeloma cases have also been shown to have high levels of NF-κB, due either to genetic mutation affecting the NF-κB pathway or to cell-cell contact and cytokine stimulation in the bone marrow microenvironment (9, 10). Consequently, the NF-κB pathway represents a promising target for the treatment of multiple myeloma.

Several NF-κB inhibitors, including parthenolide, increase the cytotoxicity of anticancer agents, and in...
multiple myeloma parthenolide has been shown to weaken the protective effects of the bone marrow microenvironment to the myeloma cells (11, 12). Furthermore, parthenolide seems to be preferentially cytotoxic to myeloma cells compared with normal bone marrow stem cells and peripheral blood mononuclear cells (12). This finding is in keeping with the results described in chronic lymphocytic leukemia (CLL) in which parthenolide induces apoptosis in CLL cells but not in normal T-lymphocytes or bone marrow hematopoietic progenitors (13, 14). LC-1 is a dimethylamino derivative of parthenolide which has >1,000-fold higher solubility than the parental molecule and hence has potential therapeutic activity (15). Studies in acute myeloid leukemia (AML) showed that AML progenitor cells were targeted by LC-1 but normal hematopoietic progenitor cells were spared (16). In primary CLL cells, LC-1-induced apoptosis was preceded by a reduction in NF-κB DNA binding, suggesting that inhibition of NF-κB is a primary regulator of LC-1 cytotoxicity (17). Given the importance of NF-κB in the maintenance of malignant plasma cells we set out to establish whether LC-1 would have potential therapeutic utility in this disease.

Materials and Methods

Cell culture

Multiple myeloma cell lines H929 and U266 were purchased from the American Tissue Culture Collection at the start of this study, and the JNJ3 cell line was obtained from Dr Guy Pratt (Institute for Cancer Research, University of Birmingham). All cell lines were certified mycoplasma free. Cultures were maintained at concentrations between 2 × 10^3 and 1 × 10^5 cells/mL in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Bone marrow samples were collected from newly diagnosed multiple myeloma patients with the patients’ informed consent. Primary cells extracted from bone marrow were drawn by density gradient centrifugation with Histopaque (Sigma) and were maintained in RPMI, with 10% fetal bovine serum and 1% penicillin/streptomycin. Bone marrow samples were resuspended in the supplied binding buffer containing calcium chloride and incubated with Annexin V–FITC in the dark for 10 minutes. Untreated samples were also prepared in this manner. Cells were then washed with PBS and resuspended in the supplied binding buffer, and 1 μg/mL propidium iodide (PI) was added. All data were acquired on a FACS Calibur flow cytometer (Becton Dickinson) and analyzed using WinMDI software. LD₅₀ values (the concentration of drug required to kill 50% of the cells) were calculated using CalcuSyn software (Biosoft). All experiments were done in triplicate.

Measurement of in vitro apoptosis in primary multiple myeloma cells

In this study changes in forward light scatter (FSC) and side light scatter (SSC) characteristics were used to quantify apoptotic and viable plasma cell populations. The quantitation of apoptosis using a FSC/SSC gating strategy in conjunction with back gating of phycoerythrin-labeled CD38⁺ (Caltag Medsystems) and allophycocyanin-labeled CD138⁺ (BD Biosciences) cells allowed simultaneous acquisition of data in viable and apoptotic plasma cells. All LD₅₀ values were derived from the dose-response curves.

Caspase-3 activation

Cell lines were incubated at 37°C in a humidified 5% carbon dioxide atmosphere in the presence of LC-1 at 5 μmol/L and 20 μmol/L for 48 hours. Cells were then harvested by centrifugation. Subsequently the cells were incubated for 1 hour at 37°C in the presence of the PhiPhiLux G1D2 substrate (Calbiochem). The substrate contained two fluorophores separated by a quenching linker sequence that was cleaved by active caspase-3. Once cleaved, the resulting products fluoresced green and were quantified using flow cytometry. Data on the caspase-3 activation of the cells were collected on a FACS Calibur flow cytometer and analyzed using WinMDI software.

NF-κB enzyme-linked immunosorbent assay

Cells were incubated for 4 hours with LC-1 (5, 10, 20, and 40 μmol/L), doxorubicin (0.125, 0.250, 0.500, 1, 2, and 4 μmol/L), melphalan (5, 10, 20, and 40 μmol/L), and bortezomib (2.5, 5, 10, 20, and 40 nmol/L). Subsequently, 5 × 10^6 cells were harvested, nuclear extracts were prepared, and Rel A NF-κB DNA binding was quantified using a previously described enzyme-linked immunosorbent assay method (17). An optical density reading at 450 nm (OD₄₅₀) was recorded for each sample on a microtitre plate reader (Biorad). OD₄₅₀ values were then converted into picograms Rel A/ng of nuclear extract for each sample tested from a standard curve constructed using known quantities of recombinant Rel A NF-κB protein.

Gene expression analysis by quantitative reverse transcriptase-PCR

H929, U266, and JNJ3 cells were treated for 6 hours with 10 μmol/L and 20 μmol/L LC-1 before being harvested for RNA extraction. Untreated cells were also
collected. Additionally, H929 cells were treated with melphalan (10 and 20 μmol/L) and bortezomib (20 and 40 nmol/L), as single agents and with the combinations of LC-1 and melphalan (ratio 1:1) and LC-1 and bortezomib (ratio 500:1). RNA was extracted using the RNAeasy kit, including the on column DNase I treatment, according to the manufacturer’s protocol (Qiagen). RNA (1 μg) was reverse transcribed into cDNA in 20 μL reactions in 5 mmol/L MgCl2, 1 nmol/L dGTP, 1 nmol/L dATP, 1 mmol/L dTTP, 1 mmol/L dCTP, 1 U/μL RNase inhibitor, 2.5 U/μL MuLV reverse transcriptase, 2.5 μmol/L random hexamers, and 1 X PCR buffer II (ABI). The reaction conditions for the reverse transcription were 25°C for 10 minutes, 42°C for 30 minutes, and 95°C for 5 minutes. cDNA was stored at -20°C until used in quantitative PCR. Quantitative PCR was done using a Light Cycler and FastStart DNA Master SYBR Green I (Roche Diagnostics Ltd.) according to the manufacturer’s instructions. cDNA (1 μL) was amplified per reaction using specific primers for survivin, MCL1, and BAX. The levels of expression of these genes were compared with the level of ABL expression from the same sample done concurrently. The primers used were:

- ABL: 5- TTACAGCGCCAGTACATCGACTT-3 (forward)
  5-CGGTTCAGCGTGATGATTGCTT-3 (reverse)
- Survivin: 5- TTTGGGAATCTGGAGATGA-3 (forward)
  5- CGGATGAACTCCTGTCCTTT-3 (reverse)
- MCL1: 5- GCCAGAACACAAAGCCCAAT-3 (forward)
  5- AACCTCAAAAAGCCAATCCA-3 (reverse)
- BAX: 5- TTGGTTCCAGGTTCATCC-3 (forward)
  5- TTGAAGTTGCGCTACAAAA-3 (reverse)

Synergy with standard treatments

Synergy between LC-1 and doxorubicin, melphalan, and bortezomib was assessed in the multiple myeloma cell line H929. The ratio of LC-1 to doxorubicin was 500:1, LC-1 to melphalan 1:1, and LC-1 to bortezomib 500:1. These ratios were determined experimentally and were kept constant throughout the subsequent experiments. Cells were incubated with each agent separately and in combination for 48 hours before assessment of apoptosis using Annexin V and PI positivity and for- ward and side scatter gating by flow cytometry. Calcusyn software was used to determine whether any synergy existed between the agents using the median effect method (18).

Cell adhesion-mediated drug resistance on drug sensitivity in multiple myeloma cell lines

Culture plates (48 wells) were coated with 200 μL 10 μg/mL fibronectin (Calbiochem) per well overnight. Nonspecific binding was prevented by incubating the plate with 100 μL 1% bovine serum albumin/well for 2 hours, followed by three washes with PBS. H929 cells were added at 1 × 10⁶ cells/ml (0.5 mL/well) and al- allowed to adhere for 2 hours. Nonadherent cells were removed by gentle washing. Cells were also added to a 48-well culture plate not coated with fibronectin. The cells were treated for 48 hours with the combination of LC-1 and melphalan (ratio 1:1), LC-1 and bortezomib (ratio 500:1), and LC-1 and doxorubicin (ratio 500:1). Adhered cells were resuspended by pipeting, and were analyzed by flow cytometry.

Statistical analysis

All statistical analyses were done using Graphpad Prism 4.0 software (Graphpad Software Inc.). Drug sensitivity for each individual drug and drug combination was evaluated using nonlinear regression and line of best fit dose-response curves. Curves were then analyzed using the median effect method to determine the degree of synergy (18). Combination indices and dose reduction indices were assessed using Calcusyn software (Biosoft).

Results

Toxicity of LC-1 in multiple myeloma cell lines

Multiple myeloma cell lines H929, U266, and JJN3 were treated with LC-1 at doubling concentrations between 0.625 μmol/L and 20 μmol/L for 48 hours. In addition, aliquots of each cell line were cultured without drug as untreated controls. The cells were then stained with Annexin V and PI and analyzed by flow cytometry. Figure 1A shows the dose-response of the three cell lines treated with LC-1. All three cell lines showed a concentration-dependent increase in apoptosis, as evidenced by increasing Annexin V positivity. Mean LD50 values for each cell line were calculated and are shown in Fig. 1B.

Caspase-3 activation

Caspase-3 activation was assessed by flow cytometry following treatment of the cell lines with 5 and 20 μmol/L LC-1 in addition to untreated control cultures. After 48 hours’ exposure to LC-1 all three cell lines showed marked evidence of concentration-dependent activated caspase-3 (Fig. 1C).

NF-κB (Rel A) DNA binding

We have previously shown that the activity of LC-1 in primary CLL cells is associated with the inhibition of NF-κB (17). In accordance with this, all three cell lines showed a dose-dependent decrease in the DNA binding of the NF-κB subunit Rel A (p65) following exposure to LC-1 for just 4 hours (Fig. 1D). This indicates that LC-1 inhibits NF-κB in myeloma cells in a dose-dependent manner, and that this inhibition precedes the induction of apoptosis. In contrast, none of the other drugs tested showed a significant reduction in Rel A DNA binding (Fig. 2A). Indeed, melphalan and doxorubicin both significantly induced Rel A DNA binding (P = 0.003 and P = 0.001, respectively), reinforcing the assertion that NF-κB activation plays a role in the development of drug resistance against these agents (19, 20).
Gene expression changes resulting from LC-1 treatment

Survivin (BIRC5) is a NF-κB regulated gene that has been shown to play an important role in multiple myeloma (21, 22). LC-1 treatment for 6 hours resulted in a decrease in survivin RNA expression relative to untreated controls in all three cell lines (Fig. 2B). MCL1 expression has also been implicated in the modulation of apoptosis in multiple myeloma (23, 24). MCL1 transcription was significantly reduced in all three cell lines following treatment with LC-1 for 6 hours (Fig. 2C). Furthermore, transcription of the proapoptotic gene BAX was shown to be significantly induced over the same timeframe (Fig. 2D).

LC-1 preferentially kills primary multiple myeloma cells

Given the promising profile of LC-1 in multiple myeloma cell lines, we investigated the effect of LC-1 on primary multiple myeloma cells. In all the cases tested (7 of 7) LC-1 preferentially targeted the CD38+/CD138+ multiple myeloma cells, as evidenced by the concentration-dependent disappearance of these cells (Fig. 3A). This was accompanied by a dose-dependent increase in apoptosis, as evidenced by Annexin V positivity and changes in forward and side scatter, which was consistent in all seven primary samples investigated. Furthermore, freshly isolated bone marrow cells were significantly less susceptible to the cell killing effects of LC-1 with a mean LD50 value of 21 μmol/L as compared with a mean LD50 of 4.9 μmol/L for CD38+/CD138+ multiple myeloma cells (Fig. 3B and C).

Synergy between LC-1 and standard treatments in multiple myeloma

We next investigated potential synergy between LC-1 and some of the standard treatments for multiple myeloma, i.e., melphalan, bortezomib, and doxorubicin, using the H929 cell line. We experimentally determined the optimal molar ratio for the various combinations. Subsequently, we measured the cytotoxic effects of each drug

Figure 1. LC-1 induces apoptosis in the multiple myeloma cell lines U266, H929, and JJN3. A, cell death in response to LC-1 was measured by increases in Annexin V/PI positivity compared with untreated cells. B, mean LD50 (μmol/L) values for the three multiple myeloma cell lines following 48-hour treatment with LC-1. C, caspase-3 activation was observed in a concentration-dependent manner in all three multiple myeloma cell lines when treated with LC-1 at 5 and 20 μmol/L for 48 hours. D, the level of the nuclear NF-κB Rel A subunit was measured by enzyme-linked immunosorbent assay in nuclear extracts prepared from the H929 cell line treated with LC-1 for 4 hours. The measurement showed a concentration-dependent decrease in nuclear localization of Rel A following treatment with LC-1 compared with untreated control cells.
and drug combination over a 48-hour culture period. The combination index values revealed synergistic effects for the combination of LC-1 with all three agents (Fig. 4A). In combination with melphalan, bortezomib, and doxorubicin, LC-1 gave mean combination index values of 0.72, 0.61, and 0.78, respectively, in the H929 cell line, with synergy seen over a wide range of concentrations. These data provide a good rationale for using LC-1 in combination with all three of these existing therapies in the treatment of multiple myeloma. To begin to understand the molecular mechanism for the observed synergy, we analyzed the transcription of survivin (BIRC5), MCL1, and BAX for each agent alone and for the combination of LC-1 with both melphalan and bortezomib following culture for 6 hours. H929 cells showed an increase in survivin expression as a result of treatment with melphalan, whereas bortezomib had little effect on the transcription of this gene. In contrast, LC-1 caused a reduction in survivin transcription, and the combination of LC-1 with melphalan (1:1), and LC-1 with bortezomib (500:1), resulted in decreased survivin expression compared with untreated controls (Fig. 4B). Melphalan and bortezomib treatment of H929 cells increased the expression of the antiapoptotic gene MCL1 whereas LC-1 caused a marked decrease in the transcription of this antiapoptotic gene. In combination, LC-1 with melphalan and LC-1 with bortezomib reduced the expression of MCL1 to levels below those found in the untreated control samples (Fig. 4C),

Figure 2. LC-1 significantly inhibits Rel A DNA binding and alters NF-κB target gene transcription. A, Rel A nuclear localization was also measured in multiple myeloma cell lines treated with bortezomib, melphalan, and doxorubicin after 4 hours. In contrast to the decrease in nuclear Rel A localization with increasing LC-1 concentration, bortezomib did not affect Rel A localization, whereas melphalan and bortezomib both resulted in significantly increased Rel A in the nucleus with increasing concentration of the drugs. Quantitative PCR was done on RNA extracted from multiple myeloma cell lines treated for 6 hours with LC-1. The relative expression of survivin, MCL1, and BAX was determined as a ratio to the expression of the housekeeping gene ABL and was compared with the ratios determined in untreated control samples. B, LC-1 induced significant decreases in survivin and MCL1 (C) expression, but caused a significant increase in BAX expressions (D).
thereby providing a rationale for the synergy seen between these agents in combination. All three agents, both alone and in combination, induced expression of the proapoptotic gene \( \text{BAX} \) (Fig. 4D).

**Cell adhesion–mediated drug resistance**

It has been shown that multiple myeloma cells in the bone marrow microenvironment are less sensitive to chemotherapeutic agents (25, 26). This type of drug resistance is termed cell adhesion–mediated drug resistance (CAM-DR). To assess whether LC-1 would be subject to CAM-DR in multiple myeloma cells, we cultured the H929 cell line on fibronectin-coated plates and compared the response to drug with standard liquid cultures. Contrary to our expectations, LC-1 was significantly more effective at killing H929 cells in the presence of fibronectin \( (P = 0.005) \). In contrast, melphalan \( (P = 0.04) \), doxorubicin \( (P < 0.0001) \), and bortezomib \( (P = 0.013) \) showed significantly reduced potency in H929 cells adhered to fibronectin when compared with standard liquid cultures (Fig. 5A and B). These data further strengthen the rationale for the use of LC-1 in early-phase clinical trials in multiple myeloma.

**Discussion**

Over the last two decades, progress in our understanding of the biology of multiple myeloma has enabled the development of new therapeutic agents with increased efficacy in this disease. However, despite the introduction...
of newer agents into clinical practice, survival remains poor with only moderate improvements in outcome for patients under 60 years of age and minimal improvement for older patients (27, 28).

It has become increasingly apparent that the pathogenesis of multiple myeloma is integrally linked to the relationship between malignant plasma cells and normal stromal cells (29). Both direct plasma cell:stromal cell contact and many differing soluble cytokines including interleukin-1β (IL-1β) and IL-6, insulin-like growth factor I (IGF-I), stromal cell derived factor 1 (SDF-1), B-cell activating factor of the tumor necrosis family (BAFF), and a proliferation-inducing ligand (APRIL) all play a role in malignant plasma cell survival, proliferation, and CAM-DR (30–33). Various intracellular proliferative/antiapoptotic signaling pathways are activated within myeloma cells, including phosphotidylinositol-kinase (PI-3K)/Akt, NF-κB, mitogen-activated protein kinase (MAPK), extracellular signaling related kinase (ERK), and Janus kinase 2 (JAK 2)/signal transducers and activators of transcription (STAT3; refs. 30, 34–36).

Pivotal in the progression and the development of CAM-DR in multiple myeloma are the NF-κB regulated genes IL-6, Cyclin D, VEGF, and MCL1. IL-6 is not only produced by normal bone marrow stromal cells but also by malignant plasma cells, giving rise to autocrine and paracrine survival pathways (30, 34). Indeed, autocrine IL-6 production is associated with more aggressive

Figure 4. LC-1 synergizes with melphalan, doxorubicin, and bortezomib. Synergy between LC-1 and melphalan (1:1), bortezomib (500:1), and doxorubicin (500:1) was investigated in the H929 cell line over a 48-hour period. A, all three existing agents tested in combination with LC-1 showed synergy, with combination values of <1. B, changes in the mRNA expression of survivin, MCL1, and BAX in response to treatment with LC-1, melphalan, bortezomib, and the combinations thereof revealed that survivin expression was decreased by LC-1 and by LC-1 in combination with melphalan and bortezomib. C, MCL1 expression was also decreased with LC-1 and with LC-1 in combination with both agents tested but only to the levels achieved by LC-1 alone. D, BAX expression was increased in response to treatment of the cell lines with all the drugs and also by LC-1 in combination with melphalan and bortezomib.
important drug resistance mechanism (19, 20). Bortezomib is a proteasome inhibitor that prevents the degradation of IκB proteins and hence blocks NF-κB activation (38). Indeed, the ability of bortezomib to kill dexamethasone-, melphalan-, and doxorubicin-resistant cells has been partially attributed to its NF-κB inhibitory properties (39). It is somewhat surprising, therefore, that bortezomib led to such a modest reduction in Rel A binding compared with LC-1, especially given the very low LD50 value of bortezomib. Intriguingly, LC-1 is synergistic with bortezomib in myeloma cell killing, suggesting that these two agents might be used to good effect in combination therapy. The fact that there is cytotoxic synergy between these agents suggests that the primary cellular targets of LC-1 and bortezomib are different. In this regard, our data confirm that although bortezomib inhibits NF-κB, the kinetics of this inhibition are much slower than those of LC-1. Therefore, bortezomib-mediated NF-κB inhibition is most likely the consequence of generalized proteasome inhibition rather than direct molecular targeting. LC-1 is also synergistic with melphalan and doxorubicin, and seems to reverse the effects of CAM-DR, at least in vitro. Contrary to the work of Yanamandra et al. (40), we found that bortezomib was less effective in the context of fibronectin-induced CAM-DR in myeloma cells. The differences between the results obtained may be technical in nature but may also reflect the inherently different properties of the cell lines employed in each setting.

Although the mechanism(s) that account for the cytotoxic synergy seen between LC-1 and the other agents tested in this study are likely to be multifactorial, the transcriptional suppression of survivin and MCL1 by LC-1 may be crucial. LC-1 in combination with melphalan and bortezomib caused a consistent transcriptional suppression of both survivin and MCL1. In contrast, both melphalan and bortezomib induced the transcription of survivin and MCL1 when tested as single agents. Given the known importance of these genes in the regulation of multiple myeloma cell apoptosis (21–24, 41, 42), LC-1-mediated suppression of their transcription may sensitize them to existing therapeutics and promote the synergy seen in this study. Certainly, the ability of LC-1 to kill both adherent and nonadherent plasma cells and its ability to synergize with agents already used for the treatment of this disease provide a strong rationale for the use of LC-1 in early-phase trials in multiple myeloma.

Disclosure of Potential Conflicts of Interest

P.A. Crooks holds stock in Leuchemix Inc. No other potential conflicts of interest were disclosed.

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Received 02/01/2010; accepted 03/09/2010; published OnlineFirst 06/01/2010.
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Published OnlineFirst June 1, 2010; DOI: 10.1158/1535-7163.MCT-10-0104

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2010;9:1574-1582. Published OnlineFirst June 1, 2010.

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