Dihydroorotate dehydrogenase inhibitor A771726 (leflunomide) induces apoptosis and diminishes proliferation of multiple myeloma cells

Philipp Baumann, Sonja Mandl-Weber, Andreas Völkl, Christian Adam, Irmgard Bumeder, Fuat Oduncu, and Ralf Schmidmaier

Department of Hematology and Oncology, Medizinische Klinik Innenstadt, Klinikum der Universität München, München, Germany

Abstract
Multiple myeloma is still an incurable disease; therefore, new therapeutics are urgently needed. A771726 is the active metabolite of the immunosuppressive drug leflunomide, which is currently applied in the treatment of rheumatoid arthritis, BK virus nephropathy, and cytomegalovirus viremia. Here, we show that dihydroorotate dehydrogenase (DHODH) is commonly expressed in multiple myeloma cells and primary multiple myeloma cells. The DHODH inhibitor A771726 inhibits cell growth in common myeloma cell lines at clinically achievable concentrations in a time- and dose-dependent manner. Annexin V-FITC/propidium iodide staining revealed induction of apoptosis of multiple myeloma cell lines and primary multiple myeloma cells. The 5-bromo-2′-deoxyuridine cell proliferation assay showed that inhibition of cell growth was partly due to inhibition of multiple myeloma cell proliferation. A771726 induced G1 cell cycle arrest via modulation of cyclin D2 and pRb expression. A771726 decreased phosphorylation of protein kinase B (Akt), p70S6K, and eukaryotic translation initiation factor 4E-binding protein-1 as shown by Western blotting experiments. Furthermore, we show that the stimulatory effect of conditioned medium of HS-5 bone marrow stromal cells on multiple myeloma cell growth is completely abrogated by A771726. In addition, synergism studies revealed synergistic and additive activity of A771726 together with the genotoxic agents melphalan, treosulphan, and doxorubicin as well as with dexamethasone and bortezomib. Taken together, we show that inhibition of DHODH by A771726/leflunomide is effective in multiple myeloma. Considering the favorable toxicity profile and the great clinical experience with leflunomide in rheumatoid arthritis, this drug represents a potential new candidate for targeted therapy in multiple myeloma. [Mol Cancer Ther 2009;8(2):366–75]

Introduction
Multiple myeloma is an incurable disease accounting for 1% to 2% of all neoplastic diseases (1) and it is characterized by the accumulation of monoclonal plasma cells in the bone marrow that produce a monoclonal immunoglobulin. In the last 15 years, treatment of multiple myeloma has changed rapidly; despite high-dose chemotherapy accompanied by autologous stem cell support as well as the introduction of new substances such as bortezomib, thalidomide, and lenalidomide, multiple myeloma cells become resistant to cytotoxic drugs and patients eventually die of tumor progression (2–6). Deregulation of several signaling pathways, such as the Akt/phosphatidylinositol 3-kinase, JAK/STAT pathway, and Ras/Raf/extracellular signal-regulated kinase pathways, has been described, contributing to drug resistance and proliferation (7, 8). These findings prompt the search for new targets.

Dihydroorotate dehydrogenase (DHODH) is the fourth and rate-limiting enzyme in the synthesis pathway of pyrimidines (9). Unlike other enzymes of this pathway, this enzyme is not localized in the cytoplasm but in the inner mitochondrial membrane. Besides catalyzing the reaction of dihydroorotate to orotate, it reduces ubiquinone in the inner mitochondrial membrane, linking the pyrimidine pathway to the mitochondrial respiratory chain (10). On stimulation with mitogens, proliferating lymphocytes expand their pyrimidine pool by 8-fold and are dependent on DHODH (11). Because mitogens drive cell growth survival and cell proliferation in multiple myeloma cells, this enzyme provides an attractive target in multiple myeloma. Leflunomide (Arava) is a well-known immunosuppressive drug that has been approved for the treatment of rheumatoid arthritis and has been used in >300,000 patients worldwide (10). Besides rheumatoid arthritis, leflunomide is also used as a virostatic drug because its activity against cytomegalovirus and the BK virus has been shown (12–16). Leflunomide is rapidly converted in the gastrointestinal tract into the active metabolite A771726 (17), which inhibits DHODH (18–21). The high level of protein binding leads to a plasma half-life of ~15 days (17). The drug is eventually

Received 7/16/09; revised 11/17/09; accepted 11/19/09; published OnlineFirst 01/27/2009.

Grant support: European Hematology Association fellowship 2005/28 (R. Schmidmaier) and Förderung von Forschung und Lehre of the LMU University of Munich (P. Baumann).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Philipp Baumann, Department of Hematology and Oncology, Medizinische Klinik Innenstadt, Klinikum der Universität München, Ziemssenstr. 1, 80336 München, Germany. Phone: 49-89-5160-2237; Fax: 49-89-5160-4412; E-mail: Philipp.Baumann@med.uni-muenchen.de

Copyright © 2009 American Association for Cancer Research. doi:10.1158/1535-7163.MCT-08-0664

Mol Cancer Ther 2009;8(2). February 2009
excreted into the urine as trifluromethanilone-oxyanillic acid. High drug concentrations up to 300 μmol/L can be reached and side effects are rare even at these concentrations (16).

Inhibition of DHODH and tyrosine kinases using leflunomide has never been elucidated in multiple myeloma. In this study, we show that DHODH is expressed in multiple myeloma cells and the active metabolite of leflunomide A771726 shows strong anti-multiple myeloma activity at clinically achievable concentrations.

Materials and Methods

Cells

OPM-2, RPMI-8226, NCI-H929, and U266 cell lines and the human bone marrow stromal cell line HS-5 were obtained from the American Type Culture Collection, grown in RPMI 1640 (Boehringer) containing 10% heat-inactivated FCS (Boehringer) in a humidified atmosphere (37°C; 5% CO2), and seeded at a concentration of 1 × 105 cells/mL. Cells have been regularly tested for Mycoplasma and were free of this contamination.

Purification of Primary Multiple Myeloma Cells

After informed consent was obtained from patients, mononuclear cells from bone marrow aspirates were isolated using CD138 MACS beads (Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, mononuclear cells were washed twice with chilled buffer (containing Dulbecco’s PBS, 0.5% FCS, and 2 mmol/L EDTA) and resuspended in buffer. CD138 MACS beads were added to the cells and incubated for 15 min at 4°C. After that, cells were washed again, resuspended in 1 mL buffer, and subjected to separation columns. After unlabelled cells passed through the column, the separation columns were removed from the separator and the labeled cells were flushed out of the separation columns. The separation procedure was repeated twice. After separation, the purity of CD138 cells was examined by flow cytometry using a CD138 PE-labeled antibody (BD Sciences). A purity of >90% CD138+ cells was accepted for further experiments. The Ethics Committee of the University of Munich approved the study.

Reagents

Melphalan, doxorubicin, and propidium iodide were purchased from Calbiochem and WST-1 was purchased from Roche. A771726 was bought from Calbiochem. Polyclonal primary antibodies against pAkt1/2 (Thr308 and Ser473), mcl-1, bim-EL, p21, cyclin A, cyclin D1, cyclin D2, cyclin E, caspase-3, bcl-2, cdc25a, cdk2, cdk4, cdk6, bax, DHODH, and actin were obtained from Santa Cruz Biotechnology and phospho-p70S6K (Thr421/422), phospho-mammalian target of rapamycin (Ser2448), phospho-4E-binding protein-1 (Thr389), p70S6K, cleaved caspase-3, pRb, bcl-xL, and mammalian target of rapamycin were obtained from Cell Signaling. Secondary antibodies raised against goat or rabbit epitopes were purchased from GE Healthcare. Bortezomib was purchased from Millennium Pharmaceuticals, and melphalan, treosulfan, dexamethasone, and doxorubicin were purchased from Sigma-Aldrich. Annexin V-FITC was purchased from Becton Dickinson. Conjugated antibodies for flow cytometry were obtained against CD11a, CD49d, CD54, CD126, and CD221 were obtained from Immunotech.

Cell Growth Assay

The WST-1 growth assay protocol was used as recommended by the manufacturer (Roche). Briefly, 4 × 105 cells per 100 μL were seeded to 96-well plates and incubated with the test compound for indicated periods. For the last 2 h of culture, cells were incubated with 10 μL WST-1. Absorbance at 440 nm was measured using a microplate ELISA reader to detect metabolically intact cells (with a reference wavelength of 680 nm).

Analysis of Apoptosis and Cell Death

Cells were stained with propidium iodide and Annexin V-FITC. Briefly, after two treatments with washing buffer [8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, and 1 L H2O (pH 7.2)], cells were resuspended in 400 μL Dulbecco’s PBS, and 100 μL of this cell suspension were incubated with 10 μL of 50 μg/mL propidium iodide and 5 μL Annexin V-FITC for 15 min at room temperature in the dark. Cells were analyzed by flow cytometry (Coulter EPICS XL-MCL; System II).

Cell Cycle Analysis

Cellular DNA content was determined by flow cytometry. Multiple myeloma cells were harvested and washed twice with ice-cold PBS. After that, cells were resuspended in 1 mM of 70% ethanol and fixed for 2 h at −20°C. Fixed cells were washed once with ice-cold PBS and resuspended in 400 μL PBS and 40 μL of 50 μg/mL propidium iodide were added to the cells. Cells were acquired by flow cytometry.

Western Blot Analysis

Cells were washed three times in ice-cold PBS and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.6), 157 mmol/L NaCl, 1 mmol/L Na2VO4, 10 mmol/L NaF, 10 mmol/L EDTA, and 1% (v/v) Igepal CA-630 (NP-40) with the addition of 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Extracted protein concentration was adjusted using a colorimetric assay (Bio-Rad Protein Assay). Proteins were subjected to SDS-PAGE and transferred to a polyvinyldene fluoride membrane (Millipore). The transfer buffer contained 25 mmol/L Tris-HCl, 192 mmol/L glycine, and 20% (v/v) methanol. The membranes were blocked with TBS containing 5% dried milk and 0.05% Tween 20. After washing four times with TBS with Tween 20, the membranes were incubated with appropriate primary and secondary antibodies and visualized by autoradiography using the ECL Western blotting detection system (GE Healthcare).

Cell Proliferation Assay Using 5-Bromo-2′-Deoxyuridine

Cell proliferation was determined using an ELISA-based colorimetric assay [Cell Proliferation ELISA; 5-bromo-2′-deoxyuridine (BrdUrd)] from Roche Diagnostics following the manufacturer’s instructions. Briefly, cells were seeded in 96-well plates at a concentration of 1.5 × 104 per well in RPMI 1640 supplemented with 10% fetal bovine serum

Mol Cancer Ther 2009;8(2). February 2009

Published OnlineFirst January 27, 2009; DOI: 10.1158/1535-7163.MCT-08-0664
with or without the substances to be tested. After ~54 h, BrdUrd labeling solution was added and the cells were cultured for another 18 h in a humidified atmosphere (37°C; 5% CO₂). Then, the cells were centrifuged and the supernatants were discarded. The cells were dried at 60°C for 1 h. After fixation and DNA denaturation with FixDenat for 30 min at room temperature, the cells were incubated with anti-BrdUrd-POD working solution for 90 min at room temperature. The anti-POD solution was removed and the cells were washed three times with washing buffer. Then, the tetramethylbenzidine substrate solution was added until color development was sufficient for detection. The reaction was stopped with 1 mol/L H₂SO₄ and the added until color development was sufficient for detection. and the cells were washed three times with washing buffer.

**Isobologram Analysis**

Isobologram analysis was done using the CalcuSyn software program (Biosoft, Ferguson). A combination index (CI) < 0.9 indicates synergism, whereas CI = 0.9 to 1.1 indicates additive effects.

**Statistics**

Mean and SD from representative experiments are shown in the figures. Data were confirmed by at least two independent experiments. The Wilcoxon test was used to compare different groups. P values < 0.05 were considered statistically significant.

**Results**

**DHODH Is Expressed in Multiple Myeloma Cell Lines and Primary Multiple Myeloma Cells**

Because no data regarding DHODH expression in multiple myeloma are available, we wanted to examine whether this enzyme is expressed in multiple myeloma cell lines and primary multiple myeloma cells. We therefore performed Western blotting experiments and blotted DHODH. Figure 1 shows that DHODH is expressed in all four multiple myeloma cell lines and primary cells obtained from bone marrow aspirates from multiple myeloma patients.

**DHODH Inhibitor A771726 Inhibits Cell Growth and Induces Apoptosis in Multiple Myeloma Cells**

We questioned whether A771726 affects cell growth in myeloma cells. We therefore incubated the human multiple myeloma cell lines OPM-2, RPMI-8226, NCI-H929, and U266 with clinically achievable concentrations of A771726 for 48 and 96 h and determined cell growth by WST-1 assay. The tetrazolium salt (WST-1) is changed to formazan by the succinate-tetrazolium reductase. The quantity of formazan dye is related directly with the number of metabolically active cells and was quantified by a multiwell photometer. Our experiments show that cell growth was totally abrogated on incubation with 200 μmol/L A771726 in all tested cells. The IC₅₀ was quite lower in NCI-H929 and RPMI-8226 cells in comparison with OPM-2 and U266 (Fig. 2A). Furthermore, we explored whether inhibition of cell growth was due to induction of apoptosis in multiple myeloma cells and therefore incubated the four multiple myeloma cell lines with increasing concentrations of A771726 for 72 h. After the incubation period, cells were stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. Our experiments show that apoptosis is moderately induced on incubation with 100 and 200 μmol/L (Fig. 2B). To confirm that apoptosis is induced by A7721726, we performed Western blotting experiments and blotted caspase-3 and cleaved caspase-3. We observed that caspase-3 was cleaved after a 48 h incubation period at 200 μmol/L A771726 (Fig. 2C). Furthermore, we analyzed primary multiple myeloma cells obtained from bone marrow aspirates from three patients. After purification with CD138 MACS, multiple myeloma cells were incubated with 0 to 300 μmol/L A771726 for 48 h and induction of apoptosis was determined by flow cytometry after staining with Annexin-V FITC and propidium iodide (Fig. 2D). As expected, apoptosis was induced in primary myeloma cells (75% at 200 μmol/L).

We conclude that A771726 strongly inhibits cell growth of multiple myeloma cells and induces apoptosis at higher concentrations.

**A771726 Inhibits Multiple Myeloma Cell Proliferation and Induces a G₁ Cell Cycle Arrest**

Because induction of apoptosis could not fully explain the reduction of cell growth, we asked whether A771726 leads to inhibition of cell proliferation in multiple myeloma cells and performed a BrdUrd assay. We incubated OPM-2, NCI-H929, RPMI-8226, and U266 myeloma cells with increasing concentrations of A771726 for 72 h. BrdUrd was added 18 h before harvesting the cells. Figure 3A shows that myeloma cell proliferation is strongly reduced by the active metabolite of leflunomide. Strong (up to 94% reduction) and quite similar effects were seen in all four tested cell lines with an IC₅₀ between 50 and 100 μmol/L. Because A771726 blocks proliferation of myeloma cells, we assumed a cell cycle arrest. OPM-2, NCI-H929, RPMI-8226, and U266 myeloma cells were incubated with increasing concentrations of leflunomide for 48 h. After the incubation period, cells were harvested and further prepared for cell cycle analysis. Figure 3B clearly shows that myeloma cells accumulate in the G₁ phase of the cell cycle.

We further asked why A771726 induces a G₁ cell cycle arrest. NCI-H929 and OPM-2 cells were incubated with 200 μmol/L leflunomide for up to 48 h and subjected to...
Western blots. It became evident that decreased cell proliferation and G1 cell cycle arrest was accompanied by a decrease of cdc25a, cyclin D2, cyclin E, cyclin A, and pRb. p21 protein was up-regulated, whereas the cyclin-dependent kinases as well as cyclin D1 seemed not to be affected (Fig. 3C). These findings were more obvious in the NCI-H929 than in the OPM-2 cell line.

In summary, inhibition of cell cycle progression and induction of a G1 cell cycle arrest represent further characteristics of A771726.

We therefore performed Western blotting experiments and incubated multiple myeloma cells with 200 μmol/L A771726 for up to 48 h and blotted proteins of the Akt signaling pathway and proapoptotic and antiapoptotic mitochondrial proteins. We revealed that A771726 strongly inhibits phosphorylation of Akt, p70S6K, and 4E-binding protein-1 (Fig. 4A). Interestingly, phospho-mammalian target of rapamycin slightly increased on incubation with A771726. Also, proapoptotic and antiapoptotic mitochondrial proteins were deregulated; the expression of the antiapoptotic bcl-xL protein was decreased and the expression of the proapoptotic proteins bim and bax was induced (Fig. 4B).

DHODH is the rate-limiting enzyme in the synthesis chain of uridine, a nucleotide needed for many cellular functions. We asked whether A771726 exerts its effects on multiple myeloma cells through inhibition of DHODH. We therefore incubated NCI-H929 and RPMI-8226 multiple myeloma cells with increasing concentrations of A771726

Figure 2. A771726 inhibits cell growth and induces apoptosis in multiple myeloma cells. A, multiple myeloma cell lines NCI-H929, OPM-2, RPMI-8226, and U266 were incubated with 0, 5, 10, 50, 100, and 200 μmol/L A771726 for 96 h and cell growth was determined by the WST-1 assay every 48 h. Mean and SD. Results from two independent experiments. B, NCI-H929, OPM-2, RPMI-8226, and U266 myeloma cells were incubated with 0, 5, 10, 50, 100, and 200 μmol/L A771726 for 72 h and induction of apoptosis was determined after Annexin V FITC/propidium iodide staining by flow cytometry. Results from three independent experiments. *, P < 0.05 versus control. C, NCI-H929, OPM-2, RPMI-8226, and U266 myeloma cells were incubated with 0, 50, 100, or 200 μmol/L A771726 for 48 h, harvested, and further processed for Western blotting. Cells were then lysed and subjected to SDS-PAGE, transferred to membranes, and blotted with the antibody indicated. D, primary myeloma cells from three myeloma patients were purified using CD138 MACS as described in Materials and Methods. Cells were then incubated with 0, 50, 100, 200, and 300 μmol/L A771726 for 48 h and induction of apoptosis was determined after staining with Annexin V-FITC and propidium iodide. Mean and SD.
together with 100 μmol/L uridine for 48 h and determined apoptosis after staining with Annexin V-FITC and propidium iodide. A771726 led to apoptosis at 50 and 100 μmol/L, which was completely reversed by 100 μmol/L uridine in the NCI-H929 cell line and almost completely reversed in the RPMI-8226 cell line. At 200 μmol/L A771726, uridine did not reverse induction of apoptosis (Fig. 4C).

Because we observed a modulation of the Akt pathway as well as a modulation of mitochondrial and cell cycle proteins, we asked whether these changes are dependent on DHODH. NCI-H929 myeloma cells were incubated with or without 200 μmol/L A771726 and with or without 100 μmol/L uridine for 48 h and blotted proteins, which were shown to be modulated by A771726. In this setting, uridine did not completely reverse any of the changes of protein expression/phosphorylation described above. Only a slight reversal of protein modulation was seen for pAkt (Thr308) and cyclin D2 (Fig. 4D).

Our findings suggest that (a) multiple myeloma cells have a high need of uridine and (b) induction of apoptosis, as well as cell growth and cell cycle arrest induced by A771726, is not exclusively due to inhibition of DHODH. Additional mechanisms, such as inhibition of the Akt pathway and modulation of proapoptotic and antiapoptotic proteins, contribute to the effect seen in multiple myeloma cells treated with A771726.

A771726 Inhibits Stromal Cell Medium-Induced Cell Growth and Leads to a Down-regulation of Adhesion Molecules and Cytokine Receptors

In multiple myeloma, the malignant plasma cell clone is localized in the bone marrow microenvironment, which has been shown to support survival, drug resistance, and cell proliferation of multiple myeloma cells. This is mediated by both cellular adhesion to bone marrow stromal cells and growth factors such as insulin-like growth factor-I and cytokines such as interleukin (IL)-6 through activating the Akt pathway.

Figure 3. A771726 inhibits multiple myeloma cell proliferation and induces a G1 cell cycle arrest. A, NCI-H929, OPM-2, RPMI-8226, and U266 cells were incubated with 0, 5, 50, 100, and 200 μmol/L A771726 for 72 h. After the incubation period, cell proliferation was determined using the BrdUrd proliferation assay. Results from two independent experiments. *, *P < 0.05 versus control. B, OPM-2, NCI-H929, RPMI-8226, and U266 myeloma cells were incubated with increasing concentrations of A771726 as indicated. DNA content was determined by flow cytometry after fixation with ethanol and staining the cells with propidium iodide. Results from two independent experiments done in triplicates. C, NCI-H929 and OPM-2 myeloma cells were incubated with 200 μmol/L for 4, 24, and 48 h. Cells were then lysed and directly subjected to SDS-PAGE, transferred to membranes, and blotted with the indicated antibodies.
Figure 4. Inhibition of DHODH leads to cellular uridine depletion, decreased phosphorylation of Akt, p70S6K, and 4E-binding protein-1, and modulation of bcl-2 family proteins in myeloma cells. **A** and **B**, NCI-H929 and OPM-2 myeloma cells were incubated with 200 μmol/L A771726 for 4, 24, and 48 h. Cells were then lysed and directly subjected to SDS-PAGE, transferred to membranes, and blotted with the indicated antibodies. **C**, NCI-H929 and RPMI-8226 myeloma cells were incubated with or without 100 μmol/L uridine and with increasing concentrations of A771726 for 48 h as indicated. Cells were then analyzed by flow cytometry after staining with Annexin V-FITC and propidium iodide. Results from two independent experiments. *, *P* < 0.05 versus control. In all figures, the used cell line is indicated in the control experiment. **D**, NCI-H929 cells were incubated for 48 h with or without 100 μmol/L uridine and with or without 200 μmol/L A771726. After that, cells were lysed and directly subjected to SDS-PAGE, transferred to membranes, and blotted with the indicated antibodies.
We therefore asked whether A771726 inhibits conditioned medium-induced increase of cell growth and incubated NCI-H929, U266, and RPMI-8226 multiple myeloma cells with 25% conditioned medium obtained from a 48 h HS-5 stromal cell culture together with 50 or 100 μmol/L A771726. HS-5 conditioned medium is known to contain several stimulating cytokines, including vast amounts of IL-6 (22, 23). After 48 h, cell growth was determined using the WST-1 growth assay. Figure 5A shows that, in all three cell lines, the stimulation with HS-5 stromal cell conditioned medium leads to an increase in myeloma cell growth (NCI-H929: 54%, RPMI-8226: 198%, and U266: 28%), which was nearly completely overcome by A771726. Because A771726 seemed to effectively inhibit the stimulation of HS-5 medium, we further analyzed the effect of A771726 on myeloma cell surface molecules and incubated NCI-H929, OPM-2, and U266 myeloma cells with increasing concentrations of A771726 and determined the surface expression of the integrins CD11a, CD49d, and CD54 and the cytokine/growth factor receptors CD126 (IL-6 receptor) and CD221 (insulin-like growth factor-I receptor) by flow cytometry. For primary multiple myeloma cells, only the surface expression of CD49d and CD126 was analyzed. We found that A771726 inhibits expression of CD126 and CD49d (Fig. 5B) in multiple myeloma cell lines as well as in primary multiple myeloma cells. The expression of CD11a, CD54, and CD221 was not changed (data not shown).

Because proliferating lymphocytes need increased amounts of pyrimidines and proliferation of multiple myeloma cells is induced by conditioned HS-5 medium as well as cytokines such as IL-6 (23), we asked whether HS-5 medium or IL-6 increases the expression of DHODH in myeloma cells. We therefore incubated NCI-H929 myeloma cells with medium obtained from a 48-h-old culture of HS-5 stromal cell or 15 ng/mL recombinant human IL-6 for 24 or 48 h and analyzed the expression of DHODH using Western blotting. Figure 5C clearly shows that stimulated multiple myeloma cells express increased amounts of DHODH.

**A771726 Synergizes with Common and New Anti-Myeloma Agents**

Drug combinations often lead to synergistic effects and reduce side effects during cytostatic treatment. Therefore, we next asked whether the incubation with A771726 might synergize with commonly used anti-myeloma agents and preincubated OPM-2, NCI-H929, and U266 myeloma cells with 50 or 100 μmol/L A771726 for 1 h. After the preincubation period, either 300 or 600 nmol/L doxorubicin, 5 or 10 μmol/L melphan, 0.5 or 5 μmol/L dexamethasone, 2 or 4 nmol/L bortezomib, or 30 or 60 μmol/L treosulfan was added to the culture. A sample of primary multiple myeloma cells was incubated with 50 and 100 μmol/L A771726 and with or without 5 or 10 μmol/L melphan. After the incubation period of 48 h, cell growth was determined by the WST-1 assay. Figure 6 shows that A771726 reduces cell growth by ~50% at the used concentrations. Maximum increase of cell growth inhibition was seen for the combinations with treosulfan and bortezomib. Analysis using the CalcuSyn software showed CI values < 0.9 for combinations with bortezomib and treosulfan, indicating that the combination with A771726 leads to synergistic effects. Combinations with melphalan and doxorubicin lead to CI values of 0.9 to 1.1, indicating additive activity in multiple myeloma cells. Addition of dexamethasone did not lead to additive or synergistic activity (Table 1).

**Discussion**

Leflunomide and its active metabolite A771726 show both immunosuppressive and virostatic characteristics. Leflunomide is rapidly taken up in the gastrointestinal tract and is rapidly and completely converted to its active metabolite A771726. In the therapy of rheumatoid arthritis, the drug is established in common treatment regimens (24). Usually, after an initial loading dose of 100 mg/d for 3 days, treatment is then continued with 20 mg/d, which can lead to concentrations of 63 μg/mL, corresponding to 233 μmol/L (25). In the treatment of the BK virus or cyto-megalovirus, higher doses are needed and a plasma level of 50 to 100 μg/mL, corresponding to 150 to 300 μmol/L, should be achieved. Even this dosage (usually 40 mg/d) is well tolerated, as described in a previously published report (16).

Our study shows that the clinically available DHODH inhibitor A771726 also has strong anti-myeloma effects. Up to 95% inhibition of multiple myeloma cell growth was observed at clinically achievable drug concentrations. Apoptosis was induced in multiple myeloma cell lines as well as in primary multiple myeloma cells obtained from three patients. A771726 also strongly inhibited multiple myeloma cell proliferation at lower concentrations. We observed a G1 cell cycle arrest with corresponding changes in the expression of cell cycle regulatory proteins. These results stand in line with results from others, who have seen an induction of a G1 arrest in mitogen-activated T lymphocytes (26) and B lymphocytes from patients with chronic lymphocytic leukemia (27). Cyclin D proteins are expressed at low levels in quiescent cells. In response to growth factors, they are up-regulated in proliferating cells (28). Cyclin D is dysregulated in virtually all multiple myeloma tumors. Whereas IgH translocations directly led to a modulation of cyclin D1 and cyclin D3 expression, most other multiple myeloma tumors, which show different chromosomal aberrations, show overexpression of cyclin D2. Fifty-four percent of all multiple myeloma tumors overexpress cyclin D1, 48% overexpress cyclin D2, 3% overexpress cyclin D3, and 8% overexpress both cyclin D1 and cyclin D2 (29). Therefore, the finding that A771726 decreases cyclin D2 expression is important and suggests an additional mechanism of action for A771726.

We suspected inhibition of changes in cellular signaling on incubation with higher concentrations of A771726 and found that 200 μmol/L A771726 strongly inhibited the Akt pathway. This pathway confers to cell survival and...
proliferation and therefore is crucial in multiple myeloma. These results are consistent with the findings from others (30), who have shown A771726-induced Akt inhibition in mast cells. In addition, we saw changes in the regulation of bcl-2 family proteins. Proapoptotic bax and bim-EL were up-regulated, whereas antiapoptotic bcl-xL was down-regulated. These findings suggest that apoptosis induced by A771726 is induced through inhibition of the Akt pathway and deregulation of bcl-2 family members.

The apoptotic effect of lower concentration of A771726 on multiple myeloma cells was partly reversed by 100 μmol/L uridine, the final product of the pyrimidine synthesis chain. Apoptosis induced by higher concentrations of A771726 was not overcome by uridine. Western blotting experiments revealed that changes in cellular signaling induced by A771726 are only partly reversed by the coincubation with uridine. This shows that effects mediated by A771726 are not exclusively due to inhibition of DHODH. Additional mechanisms such as inhibition of the Akt pathway and modulation of proapoptotic and antiapoptotic proteins contribute to the effect seen in multiple myeloma cells treated with A771726 and seem to be independent of DHODH.

Primary multiple myeloma cells are mostly localized in the bone marrow and are embedded in the bone marrow microenvironment. Integrins such as VLA-4 and LFA-1 provide cellular adhesion of multiple myeloma cells to bone marrow stromal cells. This has been shown to lead to
increased survival in multiple myeloma cells (31–33). Furthermore, paracrine and autocrine secretion of cytokines and growth factors is provided by the bone marrow milieu, which results again in the stimulation of cell growth, proliferation, and survival. IL-6 and insulin-like growth factor-I are the major soluble factors in multiple myeloma that lead to phosphorylation and activation of the signaling pathways JAK/STAT, Raf/MEK/extracellular signal-regulated kinase, and Akt/mammalian target of rapamycin/p70S6K.

We therefore asked whether A771726 may diminish the survival signals of soluble factors and incubated multiple myeloma cells with conditioned medium obtained from HS-5 stromal cells with and without A771726. We found that, besides inhibition of basal cell growth, incubation with A771726 inhibited the stimulatory effect of HS-5 medium. To clarify this, we asked whether A771726 modulates the expression of integrins and growth factor/cytokine receptor and found that incubation with A771726 decreased expression of VLA-4 and IL-6 receptor on the multiple myeloma cell surface.

There are several reports suggesting that a high pyrimidine pool is necessary for proliferating lymphocytes (11). To increase the pool of pyrimidines, lymphocytes require both the nucleoside uptake transporters and the de novo synthesis of pyrimidines (34). We therefore asked whether the expression of DHODH is modulated after stimulation with conditioned stromal cell medium or recombinant human IL-6 and found that DHODH is increasingly expressed on cytokine stimulation.

Additionally we asked whether A771726 acts synergistically with melphalan, doxorubicin, dexamethasone, or bortezomib. Because treosulfan, which is becoming increasingly in use in multiple myeloma therapy, has shown to be active in myeloma (35, 36), we also tested this promising compound in combination. In our experiments, additive (melphalan and doxorubicin) and synergistic (bortezomib and treosulfan) effects were seen when A771726 was combined with established myeloma drugs. These results suggest potential clinical activity of combination therapies.

In summary, we show that the DHODH inhibitor A771726/leflunomide is strongly active in myeloma cells. Therefore, our results provide the framework for clinical studies in myeloma patients to improve myeloma patients’ prognosis.

Table 1. A771726 exerts synergistic activity against myeloma cells when combined with common and new anti-myeloma agents

<table>
<thead>
<tr>
<th>A771726</th>
<th>Doxorubicin</th>
<th>FA CI</th>
<th>Melphalan</th>
<th>FA CI</th>
<th>Dexamethasone</th>
<th>FA CI</th>
<th>Bortezomib</th>
<th>FA CI</th>
<th>Treosulfan</th>
<th>FA CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.3</td>
<td>0.240923 0.963</td>
<td>5</td>
<td>0.267689 0.996</td>
<td>50</td>
<td>0.419247 1.068</td>
<td>0.002</td>
<td>0.341808 0.593</td>
<td>0.004</td>
<td>0.173886 0.284</td>
</tr>
<tr>
<td>100</td>
<td>0.6</td>
<td>0.152991 1.297</td>
<td>10</td>
<td>0.118814 0.795</td>
<td>50</td>
<td>0.30704 1.585</td>
<td>0.002</td>
<td>0.173886 0.284</td>
<td>0.004</td>
<td>0.173886 0.284</td>
</tr>
<tr>
<td>A771726</td>
<td>Melphalan</td>
<td>FA CI</td>
<td>Dexamethasone</td>
<td>FA CI</td>
<td>Bortezomib</td>
<td>FA CI</td>
<td>Treosulfan</td>
<td>FA CI</td>
<td>A771726</td>
<td>Treosulfan</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>0.267689 0.996</td>
<td>10</td>
<td>0.118814 0.795</td>
<td>0.002</td>
<td>0.341808 0.593</td>
<td>0.004</td>
<td>0.173886 0.284</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>0.118814 0.795</td>
<td>50</td>
<td>0.30704 1.585</td>
<td>0.173886 0.284</td>
<td>0.173886 0.284</td>
<td>60</td>
<td>0.156365 0.586</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data obtained from the experiments shown in Fig. 6 were analyzed using the CalcuSyn software to show additive/synergistic effects. In this experiment, NCI-H929 cells were preincubated with/without 50 or 100 μmol/L A771726 for 1 h. After the incubation period, 300 nmol/L doxorubicin, 5 μmol/L melphalan, 2 nmol/L bortezomib, 30 μmol/L treosulfan, or 5 μmol/L dexamethasone was added to the culture and cells were given to 96-well plates. After the incubation period for 48 h, cell growth was determined by the WST-1 assay. Values obtained from the NCI-H929 cell line were analyzed. CI = 1.1 to 0.9 indicates additive effects, whereas CI < 0.9 indicates synergism.

Figure 6. A771726 exerts synergistic activity on multiple myeloma cells when it is combined with common and new anti-myeloma agents. NCI-H929, OPM-2, and U266 myeloma cells were preincubated with/without 50 or 100 μmol/L A771726 for 1 h. After the incubation period, 300 nmol/L doxorubicin, 5 μmol/L melphalan, 2 nmol/L bortezomib, 30 μmol/L treosulfan, or 5 μmol/L dexamethasone was added to the culture and cells were given to 96-well plates. Additionally, primary multiple myeloma cells were preincubated with 50 or 100 μmol/L A771726 and 5 or 10 μmol/L melphalan was added after 1 h. After the incubation period for 48 h, cell growth was determined by the WST-1 assay. Results from two independent experiments. *, P < 0.05 versus control.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

P. Baumann designed the research, supervised the laboratory work, analyzed the data, and wrote the article; S. Mandl-Weber performed the experiments, analyzed the data, and approved the article; A. Volki, C. Adam, I. Burneder, and F. Oduncu contributed to the laboratory work and approved the article; and R. Schmidmaier designed the research, supervised the group, analyzed the data, and significantly contributed to the article.

References

Molecular Cancer Therapeutics

Dihydroorotate dehydrogenase inhibitor A771726 (leflunomide) induces apoptosis and diminishes proliferation of multiple myeloma cells

Philipp Baumann, Sonja Mandl-Weber, Andreas Völkl, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-08-0664

Cited articles
This article cites 36 articles, 13 of which you can access for free at:
http://mct.aacrjournals.org/content/8/2/366.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/8/2/366.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.