Bortezomib, Dexamethasone, and Fibroblast Growth Factor Receptor 3–Specific Tyrosine Kinase Inhibitor in t(4;14) Myeloma

Guido Bisping, Doris Wenning, Martin Kropff, Dirk Gustavus, Carsten Müller-Tidow, Matthias Stelljes, Gerd Munzert, Frank Hilberg, Gerald J. Roth, Martin Stefanic, Sarah Volpert, Rolf M. Mesters, Wolfgang E. Berdel, and Joachim Kienast

Abstract

Purpose: Novel drugs including targeted approaches have changed treatment paradigms for multiple myeloma (MM) and may also have therapeutic potential in the poor-prognosis t(4;14) subset; t(4;14) results in overexpressed and activated fibroblast growth factor receptor 3 (FGFR3). Blocking this receptor tyrosine kinase (RTK) induces apoptosis in t(4;14)+ MM cells and decreases adhesion to bone marrow stromal cells (BMSC). Using combinations of novel drugs, we investigated potential enhancement of single-agent activities within the tumor cells, targeting of the marrow micromilieu, or circumvention of drug resistance in t(4;14)+ MM.

Experimental Design: We tested effects on apoptosis and related signaling pathways in the t(4;14)+ MM subset, applying drug combinations including a FGFR3 tyrosine kinase inhibitor (RTKI), the proteasome inhibitor bortezomib, and dexamethasone.

Results: RTKI, bortezomib, and dexamethasone were active as single agents in t(4;14)+ MM. RTK inhibition triggered complementary proapoptotic pathways (e.g., decrease of Mcl-1, down-regulation of p44/42 mitogen-activated protein kinase, and activation of proapoptotic stress-activated protein/c-Jun NH2-terminal kinases). Synergistic or additive effects were found by combinations of RTKI with dexamethasone or bortezomib. In selected cases of t(4;14)+ MM, triple combinations were superior to dual combinations tested. Prevention from MM cell apoptosis by BMSC or exogenous interleukin-6 was circumvented by drug combinations. In t(4;14)+, N-ras–mutated NCI-H929 cells, resistance to RTKI was overcome by addition of dexamethasone. Notably, the combination of RTKI and dexamethasone showed additive proapoptotic effects in bortezomib-insensitive t(4;14)+ MM.

Conclusions: Combining novel drugs in poor-prognosis t(4;14)+ MM should take into account at least bortezomib sensitivity and probably Ras mutational status.

Recently, the introduction of novel agents, especially the proteasome inhibitor bortezomib (Velcade), thalidomide, and its structural analogue lenalidomide (Revlimid), has changed treatment paradigms for newly diagnosed multiple myeloma (MM), for therapeutic approaches at the time of MM relapse, as well as for maintenance therapy after stem cell transplantation (1–11).

Novel combinations implementing targeted drugs are currently under evaluation in clinical phase I to III trials or have already proven to be highly effective for MM treatment (1, 5–7, 9–12). Nonetheless, there is evidence that clinical outcome can still be determined by cytogenetically defined risk factors. MM harboring a translocation (4;14) or (14;16) is characterized by poor prognosis (13, 14).

The t(4;14) involves the partner region fibroblast growth factor receptor 3 (FGFR3)/MMSET at 4p16.3 and is related to overexpressed and constitutively activated FGFR3 (15). We and others have shown anti-myeloma effects of selective small compound receptor tyrosine kinase inhibitors (RTKI) selectively targeting FGFR3 in t(4;14)+ MM. Blocking the kinase domain of FGFR3 has been shown to reduce proliferation and induce apoptosis in t(4;14) myeloma cells, to decrease adhesion to bone marrow stromal cells (BMSC), and to abrogate secretion of interleukin-6 (IL-6) from BMSC (16, 17).

Here, we addressed rationales for the combination of targeted drugs in poor-prognosis t(4;14)+ MM (18) with or without mutated Ras or primary bortezomib insensitivity. This approach included the application of a selective RTKI.
Translational Relevance
The success of novel therapeutic approaches in multiple myeloma (MM) suggests that treatment strategies will be tested, which comprise combinations of bortezomib and other targeted drugs. With focus on the poor-prognosis t(4;14)+ MM subgroup (over)expressing fibroblast growth factor receptor 3 (FGFR3), we tested effects combining bortezomib and a FGFR3-specific tyrosine kinase inhibitor (RTKI) supplemented by dexamethasone. Synergistic or additive proapoptotic effects were found by combinations of RTKI with dexamethasone or bortezomib activating complementary proapoptotic pathways and thereby overcoming microenvironmental mediated drug resistance. We found that combining novel drugs in poor-prognosis t(4;14)+ MM needs to take into account Ras mutational status and bortezomib sensitivity. Ras-mediated drug resistance seems to be overcome by dexamethasone, whereas bortezomib insensitivity might be circumvented by combination of RTKI and dexamethasone. Here, we provide rationales for the use of novel drug combinations with respect to the poor-prognosis t(4;14)+ MM subgroup that need to be validated in clinical trials.

Materials and Methods

Patients. Bone marrow samples from patients with active MM were studied. Patients gave written informed consent before the sampling procedure. The bone marrow aspirate study protocol was approved by the Institutional Review Board of the University of Muenster.

Reagents. A 0.2 mol/L stock solution of the investigational small-molecule inhibitor BIBF 1000 synthesized in a medicinal chemistry program was prepared as previously described (16). Dexamethasone was purchased from Sigma-Aldrich. Bortezomib (Velcade) was delivered by Ortho Biotech (Division of Janssen Cilag, Neuss, Germany). Recombinant human IL-6 was obtained from R&D Systems. Annexin V-FITC and propidium iodide (PI) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies raised against phospho-mitogen-activated protein kinase (MAPK; p44/42), phospho-stress-activated protein kinase (MAPK; p44/42), phospho-c-Jun NH2-terminal kinase (SAPK/JNK; Thr 183/Tyr185), caspase-3, caspase-8, caspase-9 and poly(ADP-ribose) polymerase (PARP) as well as the corresponding goat anti-rabbit or goat anti-mouse antibodies were obtained from Cell Signaling Technology. Mcl-1 antibody was purchased from Cell Signaling Technology. Fluorescently labeled chicken-anti-rabbit IgG (Jackson Immunoresearch Laboratories) was used as secondary antibody.

Cell cultures. The human myeloma-derived cell lines RPMI-8226, U-266, OPM-2, NCI-H929, L-363, and LP-1 were obtained from the German Collection of Microorganisms and Cell Cultures The KMS-11 cell line was kindly provided by Takeki Otsuki (Kawasaki Medical School, Okayama, Japan) and UTMC-2 by Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). Cell lines and CD38high/CD138+-sorted marrow myeloma cells from patients were cultured in RPMI 1640 (Life Technologies).

Cultures of BMSCs from MM patients were established from the mononuclear cell fraction of marrow aspirates according to the method described previously (19) and maintained in MEM+s medium (Life Technologies). Contact MM-BMSC cocultures were established by directly seeding myeloma cells (2 × 107/mL) onto confluent BMSCs. All culture media were supplemented with 10% FCS (Invitrogen), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (PAA). For stimulation or inhibition experiments, cells were cultured in serum-free RPMI 1640. Cultures were maintained at 37°C and 5% CO2.

RNA preparation and cDNA synthesis. Total RNA was extracted with the QiAamp RNA Blood Mini kit (Qiagen) according to the manufacturer's protocol. CDNA was synthesized for 1 h at 37°C using 1 μg total RNA, 40 units/μL RNA guard (Amerham Pharmacia), 100 pmol/μL random hexamers (Amerham Pharmacia), 200 units Moloney murine leukemia virus reverse transcriptase (Life Technologies), 5× first-strand buffer (250 mmol/L Tris-HCl, 375 mmol/L KCl, 15 mmol/L MgCl2, 0.1 μmol/L DTT), 80 mmol/L deoxynucleotide triphosphates (Amerham Pharmacia), and 1 μg/mL bovine serum albumin (Serva). Analysis of N-ras and K-ras mutations. The cDNA from myeloma cells enriched by CD138+ magnetic bead cell sorting was amplified by PCR with primers spanning the mutation hotspots of codons 12, 13, and 61. The following primers were used: N-ras, TTCCCCGGTCTGTTGGCTAAAT (forward) and CCTTGGGGCGTCTCAGTATTGG (reverse); K-ras, CCGGCTCGCCGAGCTACCTC (forward) and TTCTTTAAGCTGCGAACC (reverse). PCR products were verified by agarose gel electrophoresis and purified. Direct cycle sequencing was done for both strands using the same primers as for PCR.

Quantitative Mcl-1 reverse transcription-PCR analysis. Quantitative PCR analysis of Mcl-1 RNA levels was done with the ABI Prism 7700 sequence detector (Perkin-Elmer). To amplify Mcl-1 cDNA, the following primer sequences were used: forward, 5′-GGTCCGATCACGAAATGT-3′; reverse, 5′-ATCAATTGCGAGAGT-3′ (primer design by Primer Express 2.0, blasted by BlastN5). SYBR Green (Applied Biosystems) was used as probe. 18S RNA was used as housekeeping gene (PCR kit by Applied Biosystems). The ratio of Mcl-1 to 18S RNA was calculated from the samples studied.

Fluorescence in situ hybridization analysis. Fluorescence in situ hybridization was used to examine the samples for deletions of the chromosome region 13q14.3 or monosomy 13 as well as for rearrangements of the IgH gene locus. Fluorescence in situ hybridization was done according to the manufacturer's instructions using commercially available probes. All probes were purchased from Abbott. The following probes have been used: LSI D13S319 (13q14.3) SpectrumOrange TM Probe, LSI 13q34 SpectrumGreen TM Probe, LSI IGH Dual Color Break Apart Rearrangement Probe, and LSI IGH/FGFR3 Dual Color Dual Fusion Translocation Probe.

Quantification of apoptotic cells. Using 24-well culture plates, 2 × 105 myeloma cells/mL were exposed to the novel indolomine BIBF 1000 (25–1,000 nmol/L), dexamethasone (0.5–10.0 μmol/L), and bortezomib (0.1–4.0 nmol/L), either alone or in combinations in the presence or absence of IL-6 (10 ng/mL) for 24 to 48 h. Subsequently, cells were collected, pelleted, and resuspended in Annexin binding buffer containing 10 nmol/L HEPEs/NaOH, 140 mmol/L NaCl, and 2.5 mmol/L CaCl2 (pH 7.4) and stained with FITC-conjugated Annexin V and PI. The quantification of apoptotic

cells was done by flow cytometry using a Becton Dickinson FACSCalibur (BD Biosciences). CellQuest Pro software (BD Biosciences) was applied for flow cytometric analyses. Data are presented as dot plots of at least 10,000 counted events per sample. All experiments were done under serum-free conditions.

Determination of additive or synergistic proapoptotic effects of drug combinations. To determine additive or synergistic proapoptotic effects when BIBF 1000 was combined with dexamethasone or bortezomib, OPM-2, NCI-H929, or U-266 cells were exposed to increasing concentrations of BIBF 1000 (25-1,000 nmol/L) and dexamethasone.
The CI indicates synergism for results below 0.8 and additivity for values between 1 and 1.2 (25, 26). The CI was defined as follows:

$$\text{CI}_{A,B} = \frac{[\Delta A_{A,B}] / D_A + [\Delta B_{A,B}] / D_B + [\alpha (D_{A,B} \times D_{B,A,B})] / D_A D_B]}{\text{control}}$$

where $\text{CI}_{A,B}$ is CI for fixed apoptotic effect (F) for the combination of substance A and substance B, $D_{A,B}$ = concentration of substance A in the combination of substance A and B giving the effect F; $D_{B,A}$ = concentration of substance B in the combination of substance A and B giving the effect F; $D_A = \text{concentration of substance A alone giving the effect F; }$ and $\alpha = \text{parameter set 0 when A and B are mutually exclusive and 1 when A and B are mutually nonexclusive.}$ The CI indicates synergy for results below 0.8 and additivity for values between 0.8 and 1.2. Subadditivity (a nonadditive effect) is given for values higher than 1.2 (25, 26).

**Table 1.** Quantification of apoptosis induction in MM cell lines exposed to RTKI, dexamethasone, bortezomib, or their respective combinations

<table>
<thead>
<tr>
<th>MM cell line</th>
<th>Chromosome 14 or 16 translocations</th>
<th>FGFR3</th>
<th>Ras mutation</th>
<th>Apoptotic cells (%), mean ± SE</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>OPM-2</td>
<td>t(4;14)</td>
<td>+ (K650E)</td>
<td>—</td>
<td>11.6 ± 1.4</td>
</tr>
<tr>
<td>KMS-11</td>
<td>t(4;14), t(14;16)</td>
<td>+ (Y373C)</td>
<td>—</td>
<td>11.4 ± 1.4</td>
</tr>
<tr>
<td>UTMC-2</td>
<td>t(4;14)</td>
<td>+ (wt)</td>
<td>—</td>
<td>27.7 ± 2.6</td>
</tr>
<tr>
<td>NCI-H929</td>
<td>t(4;14)</td>
<td>+ (wt)</td>
<td>+ (N13)</td>
<td>14.9 ± 2.3</td>
</tr>
<tr>
<td>LP-1 Complex</td>
<td>t(4;14)</td>
<td>+ (wt)</td>
<td>—</td>
<td>12.8 ± 1.0</td>
</tr>
<tr>
<td>L-363</td>
<td>Complex</td>
<td>+ (wt)</td>
<td>—</td>
<td>8.8 ± 1.6</td>
</tr>
<tr>
<td>RPMI-8226</td>
<td>t(16;22)</td>
<td>—</td>
<td>—</td>
<td>17.0 ± 1.7</td>
</tr>
<tr>
<td>U-266</td>
<td></td>
<td>—</td>
<td>—</td>
<td>17.6 ± 3.8</td>
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</table>

**NOTE:** Data represent at least five independent experiments for each MM cell line tested. Control DMSO vehicle control, BIBF 1000 (500 nmol/L); dexamethasone (10 μmol/L); bortezomib (2 nmol/L). P < 0.05 was considered significant (Mann-Whitney test). P1, BIBF 1000 versus control; P2, dexamethasone versus control; P3, bortezomib versus control; P4, BIBF 1000 + dexamethasone versus BIBF 1000; P5, BIBF 1000 + dexamethasone versus dexamethasone; P6, bortezomib + dexamethasone versus dexamethasone; P7, bortezomib + dexamethasone versus bortezomib; P8, bortezomib + BIBF 1000 versus BIBF 1000; P9, bortezomib + BIBF 1000 versus bortezomib; P10, bortezomib + dexamethasone + BIBF 1000 versus BIBF 1000 + dexamethasone; P11, bortezomib + dexamethasone + BIBF 1000 versus bortezomib + dexamethasone; P12, bortezomib + dexamethasone + BIBF 1000 versus BIBF 1000. Abbreviation: n.s., not significant.

(0.5-10.0 μmol/L) on the one hand and BIBF 1000 and bortezomib (0.1-4.0 nmol/L) on the other. Subsequently, the cell fractions undergoing apoptosis induced by the respective drug combinations were quantified by flow cytometric testing of increasing or decreasing concentrations, vice versa. Finally, data were depicted as isobologram plots (isoeffect curves for the same percentage of induced apoptosis, e.g., 50% apoptosis) as described by Tallarida (24). Apoptosis-inducing effects obtained with different combinations of BIBF 1000 and dexamethasone or bortezomib were additionally evaluated according to the method of Chou and Talalay (25, 26). Interactions between the double combinations were assessed by a combination index (CI). CI was defined as follows:

$$\text{CI}_{A,B} = \frac{[\Delta A_{A,B}] / D_A + [\Delta B_{A,B}] / D_B + [\alpha (D_{A,B} \times D_{B,A,B})] / D_A D_B]}{\text{control}}$$

where $\text{CI}_{A,B}$ is CI for fixed apoptotic effect (F) for the combination of substance A and substance B, $D_{A,B}$ = concentration of substance A in the combination of substance A and B giving the effect F; $D_{B,A}$ = concentration of substance B in the combination of substance A and B giving the effect F; $D_A = \text{concentration of substance A alone giving the effect F; }$ and $\alpha = \text{parameter set 0 when A and B are mutually exclusive and 1 when A and B are mutually nonexclusive.}$ The CI indicates synergy for results below 0.8 and additivity for values between 0.8 and 1.2. Subadditivity (a nonadditive effect) is given for values higher than 1.2 (25, 26).

**Immunoblotting of signaling molecules, caspases, and PARP.** For caspase cleavage assays, 1 × 10^6 myeloma cells/mL were subconfluently cultured on six-well culture plates and exposed to BIBF 1000, dexamethasone, and bortezomib either alone or in combinations for 12 to 24 h in accordance to the experimental setups of the assays for quantification of apoptosis by flow cytometry. Phospho-MAPK and phospho-SAPK/JNK immunoblotting of OPM-2 cells were done after a 4-h starvation period and subsequent exposure to BIBF 1000, dexamethasone, bortezomib, or their respective combinations for either 10 min or 24 h.

Proteins were extracted using radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris base (pH 8.0)] containing a protease inhibitor cocktail (Boehringer Mannheim) and the phosphatase inhibitors NaF (25 mmol/L; Sigma) and NaVO4 (2.5 mmol/L; Sigma). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore) and subsequently blotted using 0.8 mA/cm² current for 1.5 h. Membranes were blocked with 2% milk powder (Roth) and hybridized overnight at 4°C in the presence of the respective primary antibodies. Anti-rabbit IgG and anti-mouse IgG horseradish peroxidase–linked antibody (Cell Signaling Technology) were used as secondary antibodies dependent on the source of the first antibody. Finally, membranes were soaked in Luminol reagent (Santa Cruz Biotechnology) and light emission was detected on high-performance chemiluminescence films (Amersham).

**Statistics.** Data other than immunoblotting results are presented as individual data plots or as mean ± SE. Statistical analyses were done with Statistical Package for the Social Sciences package, version 14.0 (SPSS). Statistical significance of overall differences between multiple groups was analyzed by the Kruskal-Wallis one-way ANOVA. If the test was significant, pairwise comparisons were done by the multiple-comparisons’ criterion. Differences between two independent groups were analyzed by the Mann-Whitney rank sum test (27). A P value of ≤0.05 was considered significant.

**Results**

**Enhanced proapoptotic effects by drug combinations in t(4;14) MM.** As recently shown by our group and other investigators (16, 17, 28–30), targeted small molecules such as selective RTKIs are active in t(4;14) MM. Accordingly, we studied whether combinations of RTKIs with either bortezomib and/ or dexamethasone are capable of additively or synergistically enhancing apoptosis in t(4;14) MM. In t(4;14)+ OPM-2 cells, overexpressing constitutively activated mutant FGFR3 (15), the selective RTKI BIBF 1000, dexamethasone, and bortezomib significantly induced apoptosis when used as single agents (Fig. 1A; Table 1). To a similar extent, the KMS-11 cell line, carrying translocations t(4;14) and t(14;16), was sensitive to induction of apoptosis by BIBF 1000, dexamethasone, and bortezomib (Table 1). Another t(4;14)+ cell line tested, UTMIC-2, overexpressing wild-type FGFR3 (15), was also susceptible to BIBF 1000 and dexamethasone but largely resistant to...
To further investigate proapoptotic pathways in t(4;14)+ MM.

Dexamethasone compared with the respective controls (Fig. 2). Of note, the addition of neutralizing anti-IL-6 antibodies (3 µg/mL) to contact coculture retrieved a significantly augmented proportion of apoptosis by the combination of BIBF 1000 and dexamethasone, whereas bortezomib was not active when given alone, nor did it add markedly to the activity of dual-drug combinations (Table 1).

In OPM-2 and KMS-11 cells, no significant increase in apoptosis was found when bortezomib was combined with dexamethasone in comparison with single-agent proapoptotic effects of bortezomib (Fig. 1A; Table 1).

Circumvention of antiapoptotic bone marrow microenvironmental survival signals by drug combinations in t(4;14)+ MM. We also studied whether induction of apoptosis in t(4;14)+ MM was reverted by exogenous IL-6, known to promote proliferation, to protect against apoptosis, and to mediate drug resistance in MM (31–34). Therefore, OPM-2 cells were exposed to either BIBF 1000, dexamethasone, bortezomib, or the respective combinations in the presence of exogenous IL-6 (10 ng/mL). As depicted in Fig. 1A, proapoptotic effects of BIBF 1000 and dexamethasone were partly reverted by IL-6, most pronounced for the combination of BIBF 1000 and dexamethasone, whereas bortezomib-induced apoptosis was not or only marginally altered by IL-6.

In addition, we applied selected drug combinations to a contact coculture system consisting of human BMSCs seeded at the bottom of the culture dishes and OPM-2 cells directly grown on the adherent BMSCs. A significantly increased apoptosis of OPM-2 cells was preserved under contact coculture conditions when OPM-2 cells were exposed either to the combination of BIBF 1000 and bortezomib or to the triple combination of BIBF 1000, dexamethasone, and bortezomib (Fig. 2). Of note, the addition of neutralizing anti-IL-6 antibodies (3 µg/mL) to contact cocultures retrieved a significantly augmented proportion of apoptosis by the combination of BIBF 1000 and dexamethasone compared with the respective controls (Fig. 2).

**Fig. 2.** Circumvention of antiapoptotic bone marrow microenvironmental survival signals by drug combinations in t(4;14) MM. In t(4;14)+ OPM-2 cells, apoptosis was induced by either BIBF 1000 (500 nmol/L) or selected combinations of the RTKI, dexamethasone (10 µmol/L), and/or bortezomib (2 nmol/L) using a contact coculture setup with human BMSCs. A significantly increased apoptosis of OPM-2 cells was preserved under contact coculture conditions when OPM-2 cells were exposed either to the combination of BIBF 1000 and bortezomib or to the triple combination of BIBF 1000, dexamethasone, and bortezomib (P < 0.05, compared with untreated contact coculture controls). Of note, the addition of neutralizing anti-IL-6 antibodies (3 µg/mL) to contact cocultures significantly augmented the proportion of apoptosis induced by the combination of BIBF 1000 and dexamethasone compared with the respective control (P < 0.05). BMSCs (5 × 10⁴) were seeded into 24-well culture dishes. When BMSCs had grown to confluency, they were cocultured with 2 × 10⁵ myeloma cells. Experiments were done for 24 h under serum-free conditions. Columns, mean of four independent experiments; bars, SE. *, P < 0.05.

**Table 1.** Quantification of apoptosis induction in MM cell lines exposed to RTKI, dexamethasone, bortezomib, or their respective combinations (Cont’d)

<table>
<thead>
<tr>
<th>Apoptotic cells (% mean ± SE)</th>
<th>BIBF1000 + dexamethasone</th>
<th>Bortezomib + dexamethasone</th>
<th>Bortezomib + BIBF 1000</th>
<th>Bortezomib + BIBF 1000 + dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>83.9 ± 5.3</td>
<td>40.1 ± 6.2</td>
<td>56.9 ± 7.4</td>
<td>75.7 ± 11.9</td>
</tr>
<tr>
<td>P5</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>n.s</td>
</tr>
<tr>
<td>P6</td>
<td>61.4 ± 6.3</td>
<td>50.4 ± 3.9</td>
<td>54.9 ± 5.6</td>
<td>81.4 ± 3.6</td>
</tr>
<tr>
<td>P7</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>P8</td>
<td>55.3 ± 2.2</td>
<td>49.5 ± 2.2</td>
<td>35.6 ± 4.4</td>
<td>54.7 ± 2.1</td>
</tr>
<tr>
<td>P9</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.05</td>
<td>n.s</td>
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<tr>
<td>P10</td>
<td>35.7 ± 4.4</td>
<td>50.0 ± 4.4</td>
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<td>56.9 ± 6.3</td>
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<td>P11</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.05</td>
<td>n.s</td>
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<tr>
<td>P12</td>
<td>11.0 ± 2.6</td>
<td>27.1 ± 10.9</td>
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<td>28.6 ± 9.2</td>
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<tr>
<td></td>
<td>n.s</td>
<td>&lt; 0.05</td>
<td>n.s</td>
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<tr>
<td></td>
<td>18.1 ± 1.8</td>
<td>67.3 ± 9.1</td>
<td>63.6 ± 11.2</td>
<td>78.8 ± 8.3</td>
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<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
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<td></td>
<td>17.3 ± 2.6</td>
<td>58.4 ± 5.5</td>
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<tr>
<td></td>
<td>20.5 ± 6.2</td>
<td>53.1 ± 4.2</td>
<td>65.3 ± 2.2</td>
<td>61.7 ± 3.8</td>
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<tr>
<td></td>
<td>n.s</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
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</tbody>
</table>

Apoptotic cells (%, mean ± SE)

- **BIBF1000 + dexamethasone**
- **Bortezomib + dexamethasone**
- **Bortezomib + BIBF 1000**
- **Bortezomib + BIBF 1000 + dexamethasone**
dexamethasone used the caspase/PARP pathways with cleavage of the initiator caspase-8 and caspase-9 followed by activation of effector caspase-3 and terminal cleavage of PARP (Fig. 1B). Corresponding to the data illustrated in Fig. 1A, the highest degree of caspase-3 and PARP activation in t(4;14)+ OPM-2 cells was observed in the presence of BIBF 1000 plus dexamethasone (Fig. 1B). However, apoptosis of myeloma cells by bortezomib rather weakened activation of the caspase/PARP pathways in drug combinations despite additive proapoptotic effects (Fig. 1B).

Reverse regulation of phosphorylated MAPK and SAPK/JNK by drug combinations. It has been previously shown that selective antiapoptotic Mcl-1 protein expression in OPM-2 cells decreased on exposure to BIBF 1000 (500 nmol/L) or dexamethasone (10 μmol/L) either alone or in combination. As expected, exposure to bortezomib (2 nmol/L), either alone or in combination with the aforementioned drugs, resulted in intracellular preservation of Mcl-1 protein. In A and B, immunoblots after 6 h of drug exposure are shown that are representative of at least three experiments. C, despite down-regulation of Mcl-1 protein, counterregulatory increase of Mcl-1 transcripts was evident in OPM-2 cells on exposure to either of the drugs but particularly on exposure to drug combinations. Ratios of Mcl-1 over 18S RNA expression are shown as fold increase over the unstimulated controls (1.0). PCRs were done in duplicates. Points, mean of two independent experiments; bars, SE. *, P < 0.05; **, P = 0.001 versus untreated control.

Consequently, we next studied effects on p44/42 MAPK phosphorylation patterns by combinations of BIBF 1000, dexamethasone, and bortezomib exposed to myeloma cells. As shown in Fig. 1C, down-regulation of p44/42 MAPK was exclusive to BIBF 1000 and any of its combinations. This effect was observed after 20 minutes and sustained, though weakening, throughout a 24-hour period of drug exposure. It was paralleled by phosphorylation of the proapoptotic SAPK/JNK (35), underscoring the specific molecular mode of action of RTKIs interfering with constitutive FGFR3 signaling. As shown for short-term exposure (20 minutes), phosphorylation of MAPK was not altered by dexamethasone or bortezomib alone, whereas after 24 hours both agents slightly decreased MAPK phosphorylation, an effect that was most pronounced in the presence of BIBF 1000 (Fig. 1C), thus reflecting its effects on apoptosis.

Effects on Mcl-1 by drug combinations. Next, we studied the modulation of antiapoptotic Mcl-1 on induction of apoptosis by targeted drugs in t(4;14)+ and t(4;14)– MM (36). Immunoblots and quantitative PCR analyses on Mcl-1 expression were done. As shown in Fig. 3A, Mcl-1 protein was decreased by BIBF 1000 or dexamethasone either alone or, to a higher extent, by their combination. As expected, protein expression was not altered in U-266 cells by exposure either to BIBF 1000, dexamethasone, or their combination (Fig. 3B). Coexposure to bortezomib and BIBF 1000 or BIBF 1000 plus dexamethasone significantly enhanced Mcl-1 transcription (Fig. 3C). As previously reported and shown here, the presence of bortezomib led to intracellular preservation of Mcl-1 protein (Fig. 3A; refs. 37, 38).

Combination with dexamethasone overcomes ras-mediated resistance to RTKI. N- and K-ras mutations occur in up to 30% to 40% of MM and have been shown to correlate with an aggressive course of myeloma disease (39, 40). One of the mechanisms recently described is induction of cyclooxygenase-2 and consecutively enhanced binding of myeloma cells to extracellular matrix proteins conferring resistance to conventional chemotherapy (41).

We found a significantly marked proportion of apoptosis in t(4;14)+, N-ras–mutated NCI-H929 myeloma cells when dexamethasone was added to BIBF 1000 (Fig. 4A; Table 1). Note, BIBF 1000 or dexamethasone alone did not induce apoptosis in NCI-H929 cells to a significant degree over controls (Fig. 4A; Table 1). A distinct cleavage of caspase-8, caspase-9, caspase-3, and PARP was correlated to induction of apoptosis by RTKI and dexamethasone. In contrast, treatment with BIBF 1000 alone did not exhibit any cleavage of caspases or PARP, whereas dexamethasone, however, to a lesser extent, generated cleavage of caspase-8 and caspase-3. In this poor-prognosis MM subgroup, bortezomib also showed significant activity (Table 1). Its dual combination with dexamethasone tended to be superior to single-agent proapoptotic effects. U-266 cells, known to be resistant to dexamethasone and used as controls, did not show apoptosis or marked cleavage of caspases exposed to either BIBF 1000, dexamethasone, or their combinations (Fig. 4B).

Synergistic and additive proapoptotic effects by combination of RTKI and dexamethasone or bortezomib in t(4;14)+ MM. Next, proapoptotic activities of targeted drug combinations were tested pairwise versus at defined subtherapeutic concentrations according to the method described by Tallarida (24).
Fig. 4. The combination of BIBF 1000 and dexamethasone overcomes ras-mediated resistance in t(4;14)+ myeloma cells. A, t(4;14)+, N-ras – mutated NCI-H929 cells were relatively resistant to induction of apoptosis by BIBF 1000 alone, whereas adding dexamethasone to BIBF 1000 had significant additive proapoptotic effects (P < 0.005 versus untreated control). Apoptosis induction by dexamethasone or dexamethasone/BIBF 1000 was associated with cleavage of caspase-8, caspase-9, caspase-3, and PARP. B, control experiments with U-266 cells, known to be dexamethasone resistant, showed no effects of BIBF 1000 or its combination with dexamethasone. C, induction of apoptosis in dexamethasone-resistant, ras wild-type U-266 cells by targeted drug combinations. Apoptosis of U-266 cells increased significantly on exposure to bortezomib (P < 0.005). Although U-266 cells had been shown to be resistant to BIBF 1000 alone (see B), a further marginal but significant increase of apoptosis was observed when U-266 cells were exposed to bortezomib plus BIBF 1000 (P < 0.05). The addition of BIBF 1000 was associated with activation of the caspase-8, caspase-9, caspase-3, and PARP pathways. Addition of dexamethasone had no effect and might even have weakened the effects of bortezomib plus/minus BIBF 1000. However, these latter differences were not statistically significant.
Accordingly, MM cells were exposed to combinations of RTKI plus dexamethasone or RTKI plus bortezomib beginning from subtherapeutic concentrations of BIBF 1000 (range, 25-1,000 nmol/L), dexamethasone (range, 0.1-10.0 μmol/L), or bortezomib (range, 0.1-4.0 nmol/L) to map synergistic (supraadditive), additive, or subadditive (nonadditive) apoptotic effects. Subsequently, isobologram curves showing isoeffect apoptotic apoptosis was plotted for (t(4;14)+ OPM-2 cells (Fig. 5A), t(4;14)+, N-ras–mutated NCI-H929 cells (Fig. 5B), or t(4;14)+, dexamethasone-resistant U-266 cells (Fig. 5C).

The combination of BIBF 1000 and dexamethasone gave synergistic effects for OPM-2 (Fig. 5A) and N-ras–mutated NCI-H929 (Fig. 5B), both being (t(4;14)+. Additive proapoptotic effects were shown in OPM-2 cells for the combination of BIBF 1000 and bortezomib (Fig. 5A). Notably, no additive effects were found by the respective drug combinations in U-266 cells (Fig. 5C).

These findings were confirmed by calculations of CIs for selected myeloma cell lines. CIs were calculated as described in Materials and Methods (25, 26). Synergistic effects about induced apoptosis (CI lower than 0.8) were validated for the drug combination of BIBF 1000 and dexamethasone in t(4;14)+ OPM-2 cells (CI at 50% = 0.41, CI at 75% = 0.21, CI at 90% = 0.12; Fig. 5A, ●). Additive proapoptotic effects (CI between 0.8 and 1.2) were found in OPM-2 cells for the combination of BIBF 1000 and bortezomib (CI at 50% = 1.17, CI at 75% = 0.87, CI at 90% was not achievable; Fig. 5A, □). Likewise, synergistic effects (CI lower than 0.8) were shown in t(4;14)+, N-ras–mutated NCI-H929 cells for the combination of BIBF 1000 and dexamethasone (CI at 20% effect = 0.36, CI at 25% = 0.13, CI at 50%, 75%, and 90% not achievable; Fig. 5B, ●). However, the combination of BIBF 1000 and bortezomib had a subadditive effect in Ras-mutated NCI-H929 cells (calculated CI above 1.2; CI at 10% effect calculated 1.67, CI at 25% calculated 2.3, CI at 40% calculated 2.6, CI at 75% and 90% effect not achievable; Fig. 5B, □). For U-266 cells, none of the combinations showed additivity or synergism about drug-induced apoptosis (CI at 3.0; see Fig. 5C, ● and □).

In case of poor-prognosis MM, which in addition to a t(4;14) surprisingly tended to augment apoptosis induction in patients harboring a t(4;14) (patients 2 and 3) can be exactly correlated to the findings in the bortezomib-insensitive UTMCC-2 MM cell line (Table 1). In case of patient 2, addition of bortezomib was able to further increase the proportion of apoptosis when the cells were exposed to the combination of RTKI and dexamethasone. Data obtained in myeloma cells of patient 3 from subtherapeutic drug titrations of BIBF 1000 and dexamethasone or BIBF 1000 and bortezomib further indicate such an additive mechanism of action by these combinations (data not shown). In line with the data obtained from N-ras–mutated NCI-H929 and N-ras–mutated L-363 cells, we found similar proapoptotic effects by the combination of dexamethasone and BIBF 1000 or bortezomib in a patient with complex chromosomal aberrations and additional Ras mutation (patient 4; Table 2).

A series of CD138+ marrow myeloma cells obtained from patients with or without deletion 13 were predominantly sensitive to bortezomib-induced apoptosis. Addition of dexamethasone led to enhanced apoptosis, although to a variable degree (patients 5-7; Table 2). As shown for the respective cell lines above, addition of RTKI to such a dual combination surprisingly tended to augment apoptosis induction in patients 6 and 7 who did not carry a t(4;14).

Discussion

From previous reports, several lines of evidence suggest a rationale for combining different targeted drugs and adding dexamethasone in poor-prognosis t(4;14)+ MM.
Translocations of the IgH locus leading to dysregulation of the oncogenic partner regions FGFR3/MMSET at 4p16.3 are present in t(4;14)+ MM and are associated with poor prognosis (13, 15, 18).

Preclinical and clinical studies have been launched targeting FGFR3 by RTKIs, such as CHIR-258, PD173074, PKC412, and SU5402, or by the anti-FGFR3 antibody PRO 001 in the t(4;14)+ MM subgroup (17, 28–30, 42). We have previously shown proapoptotic effects of BIBF 1000, a novel selective RTKI, in t(4;14)+ MM (16). Furthermore, enhanced proapoptotic effects in t(4;14)+ MM by the combination of RTKIs and dexamethasone have recently been reported by our group and others (16, 17, 43).

Very remarkable anti-myeloma activity has also been shown for the proteasome inhibitor bortezomib, targeting the ubiquitin-proteasome pathway as well as the narrow microenvironment either alone or combined with other anti-myeloma agents (21, 22, 44–49).

In addition, dexamethasone is widely used as a partner for novel targeted strategies. Induction of apoptosis by dexamethasone leads to consecutive down-regulation of MAPKs and p70S6K (23). Its effects can be reverted by IL-6 that seems to be involved in the development of secondary dexamethasone resistance (23, 31). Conversely, it has been shown that bortezomib either alone or in combination can overcome dexamethasone resistance by abrogation of IL-6 signaling (45).

In our present series of t(4;14)+ MM cell lines and CD138+ isolated marrow myeloma cells from t(4;14)+ MM patients, a significantly increased induction of apoptosis was consistently found for the combinations of RTKI (BIBF 1000) with...
dexamethasone or bortezomib. We used concentrations of the novel RTKI BIBF 1000 (25-1,000 nmol/L) that were pharmacodynamically comparable with effects reached in vivo in mice and rats administrating 25 to 100 mg/kg orally daily, which were shown to be safe and well tolerable. For bortezomib, concentrations from 0.1 to 4.0 nmol/L were applied to cell cultures reflecting drug effects achievable in vivo. In addition, constitutive MAPK phosphorylation in t(4;14)+ MM was not significantly altered by bortezomib, whereas Mcl-1 was preserved rather independent of dexamethasone or bortezomib treatment (23). Down-regulation of antiapoptotic Mcl-1 protein was also observed in the presence of RTKI, whereas Mcl-1 was preserved under exposure to bortezomib in t(4;14)+ MM (36–38). Taken together, the data clearly favor the implementation of selective targeting of FGFR3 into combinations with dexamethasone or bortezomib for treatment of t(4;14)+ MM (3).

Another major concern, recently studied by Hoang and coworkers (41), is that Ras mutations have been associated with enhanced adhesion to fibronectin and chemoresistance through the induction of cyclooxygenase-2. We could show that resistance to RTKI (BIBF 1000) in t(4;14)+ NCI-H929 cells that may be due to mutated Ras can be overcome by the addition of dexamethasone (40). Of note, bortezomib alone was markedly active in this t(4;14)+ MM subgroup. Thus, it seems to be important that t(4;14)+ MM with mutated Ras be treated with bortezomib or in combination with dexamethasone when RTKis are to be used. However, isogenic myeloma cell lines with and without mutated Ras, as these exist for the colorectal HCT116 cell line, are not available to explicitly attribute these observed in vitro effects to Ras mutational status.

In case of bortezomib-insensitive t(4;14)+ MM, we also found additive effects for the combination of RTKI and dexamethasone. Finally, in dexamethasone- and RTKI-insensitive t(4;14)- MM harboring Ras wild-type, a significant, although limited, increase of apoptosis occurred by the addition of RTKI to bortezomib, although the FGFR3 target of the kinase inhibitor was absent. This effect was associated with additional activation of caspases and PARP and could be meaningful, although additive mechanisms were not confirmed by subtherapeutic dose titrations (44, 46, 47).

In summary, from our data, potential treatment perspectives can be derived for t(4;14)+ MM with or without Ras mutations and for selected t(4;14)+ MM that show primary exposure. In contrast, MAPK was markedly down-regulated when exposed to RTKI. These findings suggest that RTK inhibition is essential in disrupting MAPK proliferation and survival signals in t(4;14)+ MM. Further substantiating these observations, up-regulation of proapoptotic JNK/SAPK phosphorylation occurred in the presence of RTKI, whereas it was rather independent of dexamethasone or bortezomib treatment (23). Down-regulation of antiapoptotic Mcl-1 protein was also observed in the presence of RTKI, whereas Mcl-1 was preserved under exposure to bortezomib in t(4;14)+ MM (36–38). Taken together, the data clearly favor the implementation of selective targeting of FGFR3 into combinations with dexamethasone or bortezomib for treatment of t(4;14)+ MM (3).

### Table 2. Quantification of apoptosis induction in CD138+ sorted marrow myeloma cells obtained from MM patients exposed to RTKI, dexamethasone, bortezomib, or their respective combinations

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
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Abbreviations: ND, not determined due to limitations of the number of cells that have been isolated; Dex, dexamethasone; Bzb, bortezomib.

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6 Unpublished data.
insensitivity to bortezomib as well as for dexamethasone-resistant MM with or without t(4;14). It can be inferred that direct proapoptotic effects on t(4;14)+ MM cells as well as specific multitargeting of myeloma-marrow stromal cell interactions can be significantly enhanced by combinations of the novel drugs studied. Furthermore, Ras-mediated drug resistance might potentially be overcome by addition of dexamethasone, whereas bortezomib insensitivity could be circumvented by the combination of RTKI and dexamethasone in t(4;14)+ MM. Our in vitro data provide the rationale for the use of targeted drug combinations with respect to the t(4;14)+ MM subgroup and, in turn, warrant validation in t(4;14)+ subgroups in clinical trials (1–3).

Disclosure of Potential Conflicts of Interest

J. Kienast: commercial research grant for myeloma research. Boehringer Ingelheim Austria GmbH, Vienna, Austria.

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Bortezomib, Dexamethasone, and Fibroblast Growth Factor Receptor 3–Specific Tyrosine Kinase Inhibitor in t(4;14) Myeloma

Guido Bisping, Doris Wenning, Martin Kropff, et al.


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