Potent Antimyeloma Activity of a Novel ERK5/CDK Inhibitor

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Abstract

Purpose: To analyze the antimyeloma potential of TG02, an ERK5/CDK inhibitory drug.

Experimental Design: Utilizing different multiple myeloma cell lines we determined the effect of TG02 over viability by MTT assays. The apoptotic effect over multiple myeloma patient samples was studied ex vivo by cytometry. The mechanism of action of TG02 was analyzed in the cell line MM1S, studying its effect on the cell cycle, the induction of apoptosis, and the loss of mitochondrial membrane potential by cytometry and Western blot. Two models of multiple myeloma xenograft were utilized to study the in vivo action of TG02.

Results: TG02 potently inhibited proliferation and survival of multiple myeloma cell lines, even under protective bone marrow niche conditions, and selectively induced apoptosis of primary patient-derived malignant plasma cells. TG02 displayed significant single-agent activity in two multiple myeloma xenograft models, and enhanced the in vivo activity of bortezomib and lenalidomide. Signaling analyses revealed that the drug simultaneously blocked the activity of CDKs 1, 2, and 9 as well as the MAP kinase ERK5 in MM1S cells, leading to cell-cycle arrest and rapid commitment to apoptosis. TG02 induced robust activation of both the intrinsic and extrinsic pathways of apoptosis, and depletion of XIAP and the key multiple myeloma survival protein Mcl-1.

Conclusions: TG02 is a promising new antimyeloma agent that is currently in phase I clinical trials in leukemia and multiple myeloma patients. Clin Cancer Res; 19(10); 2677–87. ©2013 AACR.

Introduction

Multiple myeloma is a clinically heterogeneous disease characterized by the clonal expansion of malignant plasma cells in the bone marrow (1). Despite improvements in treatment and the clinical development of new drugs, the disease remains incurable. Therefore, new therapies are needed.

Deregulation of D-type cyclins is a common pathogenic occurrence in multiple myeloma and is considered an initiating event of the disease (2). Cyclins bind and activate members of the CDK family. CDKs are essential regulators of the cell cycle and transcription and are being explored as therapeutic targets in multiple myeloma (3). Several CDK inhibitors have shown preclinical antitumor activity (3–6) and the first phase I study with one such compound in a cohort of multiple myeloma patients has recently been published (7).

TG02 is a novel orally bioavailable multikinase inhibitor (8) that inhibits the cell cycle regulators CDK1 and CDK2 along with the transcriptional regulators CDK7 and CDK9 at low nanomolar concentrations (9). In addition, TG02 is also a potent inhibitor of the MAPK ERK5. In multiple myeloma, ERK5 can be activated by cytokines, such as IL-6, produced by the stromal compartment, and which support multiple myeloma proliferation and survival (10). Expression of a dominant negative form of ERK5 results in impaired proliferation of multiple myeloma cells, and augments the antimyeloma action of drugs used in the multiple myeloma clinic. Therefore, it is possible that drugs acting on the ERK5 route may be beneficial for the treatment of multiple myeloma. Moreover, in other cellular systems ERK5 has been shown to regulate the expression of cyclins, linking ERK5 activity to cell-cycle regulation (11).

The unique kinase inhibitory spectrum of TG02 suggested that this drug could have activity against multiple myeloma. Here we report that TG02 blocked signaling by CDKs 1, 2, 7, and 9 and ERK5, leading to potent and highly consistent antimyeloma activity against cell lines (even under bone marrow niche conditions) and ex vivo patient-derived malignant plasma cells. The drug induced a modest cell-cycle arrest and robust apoptosis in multiple myeloma cells by multiple mechanisms including depletion and/or cleavage of the antiapoptotic proteins XIAP and Mcl-1. TG02...
synergized with several antimyeloma drugs in vitro, significantly inhibited tumor growth in 2 multiple myeloma xenograft models and enhanced the activity of bortezomib and lenalidomide in vivo.

Materials and Methods

**Reagents and immunochemicals**

Cell culture media was purchased from LONZA. Sera and penicillin-streptomycin were purchased from Invitrogen. Protein A-Sepharose was from GE Healthcare. Dexamethasone, melphalan, and MIT were from Sigma Chemical. Bortezomib and lenalidomide were from LC laboratories (Woburn, MA). Z-VAD-FMK was from BD Biosciences.

Antibodies used for Western blotting against GAPDH, PARP, CDK1, CDK2, CDK7, AIF, Bcl-2, Mcl-1, and ERK1/2 (Santa Cruz Biotechnology); caspase-3, active-caspase-3, caspase-8, Bcl-X, XIAP, cytochrome C, SMAC/Diablo, cyclin B, and Rb (BD Biosciences); caspase-7, caspase-9, pRNA polymerase II (Ser2/5), pRb (Ser807/811), COX IV, and CDK9 (Cell Signalling Technology); RNA polymerase II (Covance); Endo G (Serotec), and phospho-histone H3 (Millipore) were used. Anti-HA and anti-ERK5 antibodies have been described (12, 13). Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Annexin V-FITC, anti–CD38-APC, anti–CD45-PerCP/Cy5, and anti–CD34-PE antibodies used for flow cytometry were from BD Biosciences.

**Cell lines, cell culture, and cell proliferation assays**

All cell lines were cultured as described (10). The multiple myeloma cell lines used were: MM1S, MM1R (from Dr. S. T. Rosen, Chicago), MM144, OPM2 (from Dr. S. Rudikoff, Bethesda, MD), U266, RPMI8226, and their chemoresistant derivatives generated by selection for resistance to specific drugs, such as doxorubicin (U266DOX4) or melphalan (U266LR7, RPMILR5; ref. 14), were obtained from Dr. W. Dalton (Tampa, FL), NCIC929 (from Dr. J. Teixido, Madrid, Spain), SJR and MGG. The last 2 were established in our laboratory from 2 patients with multiple myeloma. The origin of the MM1S-Luc cell line has been previously described (15).

**Materials and Methods**

**Cell viability** was measured by MTT assay (16). To evaluate the action of TG02 on multiple myeloma cells adherent to bone marrow stromal cells (BMSC), MM1S-Luc cells were cocultured with the HS-5 human stromal cell line as described (15).

**Ex vivo experiments with freshly isolated patient cells**

Patients provided informed consent following the policies of the research ethics committee of the University Hospital of Salamanca and the Declaration of Helsinki. Bone marrow samples were treated with ammonium chloride to remove erythrocytes. A total of 600,000 cells were seeded in 6-well plates and TG02 was added at different concentrations. Cells were incubated for 18 hours at 37°C in RPMI + 20% FBS. This incubation time was selected as culturing for longer times resulted in significant decrease in PC viability. For the coculture experiments 10⁶ primary cells were cultured over HS-5 cells in 100-mm dishes. Multiparametric analyses of apoptosis were done as described (15).

**Studies of synergism with other anti–multiple myeloma agents**

MM1S cells were treated for 48 hours with different doses of TG02 and dexamethasone, melphalan, bortezomib, and lenalidomide. The potency of the combination was analyzed with the CalcuSyn software (Biosoft), which is based on the Chou and Talalay method (17).

**Cell cycle and apoptosis studies**

To analyze the effect of TG02 on the cell cycle, MM1S cells were incubated with low concentrations of TG02 for 24 and 48 hours. Apoptosis was determined after incubating with 100 nmol/L TG02 for 0, 3, 6, 9, 12, and 24 hours. The cell-cycle profile and apoptosis were analyzed by cytometry (15). To detect DNA laddering, 15 × 10⁶ MM1S cells were seeded and incubated for 0, 12, and 24 hours with 100 nmol/L TG02. After that time cells were lysed and DNA was isolated and analyzed by agarose gel electrophoresis.

**Assessment of mitochondrial membrane potential**

Cells were stained with 0.5 μmol/L tetramethylrhodamine ethyl ester (TMRE, Invitrogen) for 30 minutes at 37°C. TMRE fluorescence was acquired on a FACScalibur flow cytometer. The data were analyzed with the CellQuest program (BD Biosciences).

**Subcellular fractionation, Western blotting, and immunoprecipitation**

A total of 30 μL of cytosolic and mitochondrial fractions, obtained using a previously described digitonin-based technique (18), were used for Western blot assays. Western blotting and immunoprecipitation were done as described (19).
**In vitro kinase assay**

MM1S cells treated with or without IL-6 (10 nmol/L; 15 minutes) were collected and lysed in ice-cold lysis buffer. ERK5 was immunoprecipitated with the anti-ERK5-PRO1 antibody at 4°C for at least 2 hours, and the immune complexes were washed with 1 mL of cold lysis buffer and 2 washes with 1 mL of kinase buffer (20 mmol/L HEPES, pH 7.6; 20 mmol/L MgCl2; 25 mmol/L β-glycerophosphate). The immunoprecipitates were then incubated with different concentrations of TG02 (10–10,000 nmol/L) for 30 minutes. Samples were analyzed by Western blotting with the anti-ERK5 (C-terminal) antibody.

**Multiple myeloma xenograft model**

For the human subcutaneous plasmacytoma models, CB17-SCID female mice (The Jackson Laboratory, Bar Harbor, ME) were subcutaneously inoculated into the right flank with \(2 \times 10^5\) (MM1S) or \(1 \times 10^5\) (OPM2) cells in 100 μL of RPMI-1640 medium and 100 μL of Matrigel (BD Biosciences). When tumors reached 130 to 200 mm³, mice were randomized and treatments started: TG02 60 mg/kg orally every 4 days or 30 mg/kg/day (OPM2); TG02 40 mg/kg orally every 4 days (MM1S); lenalidomide 20 mg/kg i.p. (intraperitoneal) daily (MM1S); bortezomib 0.5 mg/kg i.p. daily (MM1S) or 0.5 mg/kg i.p. twice weekly (OPM2); and the combination of TG02 with lenalidomide and TG02 with bortezomib at the same doses and schedule as in the single-drug groups. The control group received the vehicle alone. Caliper measurements of the tumor diameters were conducted twice a week. Animals were euthanized when their tumors reached 1.5 to 2 cm. Differences in tumor volumes between groups were evaluated using the nonparametric Mann–Whitney U test. Statistical analyses were done with the SPSS-17.0 (SPSS Inc.), and statistical significance was defined as \(P < 0.05\). All MM1S animal experiments were done according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Salamanca (Spain) in accordance with European Community guidelines on ethical animal research, established by the European Community (86/609/EEC). OPM2 animal experiments were done according to the protocols approved by the Charles River IACUC. Time to endpoint was defined as the time from the day of initiation of treatment to death as a result of toxicity, tumor growth, or any other cause. Survival data were plotted using Kaplan–Meier curves.

**Results**

**Expression of TG02 targets in multiple myeloma cells**

TG02 is a synthetic small molecule macrocycle (8) manufactured as the salt form, TG02 citrate, for improved pharmaceutical properties and bioavailability (Fig. 1A). Analysis of the kinase inhibitory profile indicated that TG02 targeted various kinases (9) implicated in multiple myeloma biology with IC₅₀ values <50 nmol/L (Supplementary Table S1). We tested the expression of the most sensitive targets in a panel of 12 multiple myeloma cell lines representative of distinct molecular alterations and drug-resistance status (Fig. 1B). CDK1, CDK2, CDK7, CDK9, and ERK5 were uniformly expressed. Therefore, several of the kinases targeted by TG02 were expressed in myeloma cell lines, opening the possibility that their aggregate inhibition could confer unique antimalmyeloma properties upon the drug.

**In vitro and ex vivo effect of TG02 against multiple myeloma cells**

Multiple myeloma cell lines were treated with increasing concentrations of the compound (1 nmol/L to 10 μmol/L) for 24, 48, and 72 hours and viability assessed by MTT assays. TG02 caused a dose-dependent decrease in MTT metabolism in all the cell lines (Fig. 2A). After 24 hours of drug exposure, the effect of TG02 was quite heterogeneous across the panel (IC₅₀ ~50 nmol/L to >10 μmol/L). By contrast, after ≥48 hours almost all lines were highly sensitive (IC₅₀ ~50 to 150 nmol/L), although a fraction of NCIH929 and U266LR7 cells remained resistant. Of note,
the 2 U266-derived chemoresistant cell lines were more resistant to TG02 than the parental cell line, suggesting that mechanisms of resistance to doxorubicin or melphalan raised by these cells also confer some degree of resistance to TG02.

The effect of TG02 was further evaluated ex vivo in the plasma cell population from bone marrow aspirates of 8 multiple myeloma patients. The toxicity of the compound on bone marrow progenitor cells (CD34+) was also evaluated in 7 of these patients (one patient lacked a discernible CD34+ compartment). After 18 hours, TG02 induced cell death in the plasma cell population in a dose-dependent manner, with a potency similar to the most sensitive multiple myeloma cell lines (Fig. 2B and Supplementary Fig. S1). There was also a dose-dependent cytotoxic effect of TG02 on the hematopoietic progenitor population, but to a lesser extent, suggesting that potential cytopenias induced by the drug might be reversible.

TG02 overcomes the survival advantage induced by cytokines and stromal cells

The adhesion of multiple myeloma cells to BMSCs triggers transcription and secretion of cytokines such as IL-6, which confer several advantages to the malignant plasma cell, including augmented proliferation, survival, and increased drug resistance (20). To in vitro evaluate if TG02 was able to inhibit this protective effect, luciferase-expressing MM1S-cells (MM1S-Luc) were cultured in the absence or presence of IL-6 (10 nmol/L), and treated with different doses of TG02 for 48 hours. Addition of IL-6 resulted in higher proliferation compared to untreated controls (Fig. 3A). TG02 overcame the proliferative/protective effect.
advantage conferred by IL-6. A second model employed MM1S-Luc cells cocultured with the luciferase-negative human stromal cell line HS-5 (21). Analogously to the results obtained with IL-6, TG02 overcame the proliferative and protective effect of the bone marrow cells on MM1S-Luc (Fig. 3B), even though the presence of HS-5 cells caused a slight shift to the right of the dose–response curve. Moreover, >80% of MM1S-Luc cells cocultured with HS-5 were killed under conditions where HS-5 cells themselves were unaffected (Fig. 3C), indicating that the lack of protective effects of bone marrow cells was not because of depletion of the HS-5 population. Finally, we tested whether HS-5 cells were able to protect primary myeloma cells obtained from patients from the action of TG02. As shown in Fig. 3D, freshly isolated BM cells from 3 myeloma patients were cocultured with HS-5 cells and treated ex vivo with TG02 (250 nmol/L) for 18 hours before staining as described earlier. Viability of untreated multiple myeloma patient cells was considered as 100%, and values of TG02-treated samples referred to their respective controls.

TG02 potentiates the action of antimyeloma agents

As most of the treatment regimens for myeloma patients are based on drug combinations, we studied the effect of TG02 in 2-drug combinations with agents normally used in the clinical management of myeloma, such as dexamethasone, melphalan, bortezomib, and lenalidomide. TG02 potentiated the effects of these drugs (Fig. 4A).

In vivo antitumor activity of TG02

The in vivo effect of TG02 was studied in 2 human multiple myeloma plasma cytoma xenograft models in CB17-SCID mice, the bortezomib-sensitive MM1S model and the more bortezomib-resistant OPM2 model. As shown in Fig. 4B, TG02 delayed tumor growth in the MM1S model and the difference with the control group started to be significant after 21 days of treatment. In the OPM2 model, TG02 inhibited tumor growth when given daily or intermittently, although the effect was significantly higher with the intermittent administration (Supplementary Fig. S2). We also treated mice with combinations of TG02 and lenalidomide or bortezomib (Fig. 4C). TG02 augmented the antitumor activity of these agents when compared to the effect of the agents administered alone. This delay in tumor growth correlated with an increase in time to endpoint of treated mice compared...
with the controls (Supplementary Fig. S3A and S3B). Weight analyses done on these mice indicated that TG02 or its combinations did not affect weight of the mice, suggesting that these treatments are well tolerated (Supplementary Fig. S3C).

**Action of TG02 on the cell cycle**

As kinases targeted by TG02 play important roles in cell-cycle progression, we explored whether cell-cycle effects of the drug could contribute to its antimyeloma action. In MM1S cells, TG02 induced a slight increase in the percentage of cells in the G2/M phases and decreased cells in the S phase (Fig. 5A). In addition, the drug also increased the amount of cells in the subG0 region, suggestive of induction of cell death. These effects of TG02 on the cell-cycle profile were already detectable as soon as 3 hours after treatment with TG02 (Supplementary Fig. S4). These data are compatible with an effect of TG02 on CDK2 and CDK1, blocking progression through G1/S and G2/M phases, respectively. In fact, treatment with TG02 decreased the levels of CDK1 and its phosphorylated form (Fig. 5B). Moreover, treatment with the drug also caused a decrease in cyclin B. The CDK1–cyclin B complex is important for G2/M transition of the cell cycle, so this decrease could contribute to the accumulation of cells at these phases. Phosphorylation of histone H3, which is considered a marker of mitosis, was upregulated by TG02, further supporting that the drug halted progression through mitosis.

Rb phosphorylation was used as a marker of CDK2 activity: TG02 induced the rapid (6–9 hours) dephosphorylation of Rb at serines 807 and 811 and also induced a decrease in the total expression of the Rb protein after 24 hours. Rb phosphorylation by CDK2 is necessary for entry into S phase of the cell cycle.

An important kinase targeted by TG02, and which controls proliferation of multiple myeloma cells is ERK5 (10). Under resting conditions, ERK5 migrates as a 120 kDa protein, and upon activation ERK5 undergoes a mobility shift easily detected by Western blotting (12). In MM1S treatment with IL-6 caused a shift in the mobility of ERK5 (Fig. 5C), indicative of activation. Preincubation with TG02 inhibited IL-6–induced ERK5 mobility shift in a dose-dependent manner (Fig. 5C), showing that TG02 can inhibit ERK5 in live cells.

To verify that TG02 directly inhibited ERK5 activity we established a novel in vitro assay based on the kinase activity of ERK5. In this assay, ERK5 is used as both the enzyme and the substrate. As shown earlier, IL-6–activated ERK5 in intact MM1S cells, and that caused a shift in its mobility (Fig. 5C and D, compare lanes 1 and 2). When active ERK5 immunoprecipitated from MM1S cells treated with IL-6 was subjected to the in vitro kinase assay, an additional shift in its
mobility was observed, indicative of hyperphosphorylation (Fig. 5D, lane 4). This supershift of ERK5 is probably because of self-phosphorylation (22). TG02 prevented the in vitro autophosphorylation of ERK5.

**TG02 interferes with protein synthesis**

The decrease in the amount of several proteins involved in cell-cycle progression upon treatment with TG02 suggested that this drug could be affecting their steady-state levels. Importantly, the TG02 targets CDK7 and CDK9 act as regulators of RNA polymerase II, an enzyme critical in protein synthesis. RNA polymerase II is recruited to gene promoters by transcription factors, and is phosphorylated in its C-terminal domain heptad repeats (23). At these heptad repeats, CDK7 phosphorylates RNA polymerase II on serine 5 during initiation of transcription, and CDK9 phosphorylates serine 2 during elongation of the transcripts. We used an antibody, which detects RNA polymerase II phosphorylated at these serines to evaluate the action of TG02 on RNA polymerase II–mediated transcription. Under resting conditions this antibody detected 2 bands (Fig. 5B). TG02 caused a decrease in the amount of the slower migrating band at the earliest time analyzed (3 hours). At this time, treatment with TG02 provoked an increase in the faster migrating form of RNA polymerase II, indicative of dephosphorylation of the upper phospho-RNA polymerase II form, which was converted into a hypophosphorylated RNA polymerase form. At later incubation times (12 and 24 hours), treatment with TG02 decreased the total amount of RNA polymerase II. At these times, the decrease in phosphorylated RNA polymerase II could be secondary to the reduction of its total amount.

**TG02 triggers apoptosis through caspase-dependent and -independent routes**

To analyze whether TG02 treatment caused apoptosis, MM1S cells were treated with 100 nmol/L TG02 for different times. There was a time-dependent increase of annexin A. MM1S cells were incubated with TG02 (10–100 nmol/L) for 24 hours, and the cell-cycle profile was examined by flow cytometry after propidium iodide staining. The bars represent the mean ± SD of the percentage of cells in each phase of the cell cycle from 2 different experiments. B, MM1S cells were treated with TG02 (100 nmol/L) for different times, and expression of target kinases and their substrates was analyzed by Western blotting. Equal loading was confirmed with an anti-GAPDH antibody. C, effect of TG02 on ERK5 activation. MM1S cells were preincubated for 30 minutes with the indicated concentrations of TG02, and then IL-6 (10 nmol/L) added where indicated. After immunoprecipitation of ERK5 from the cell lysates it was detected by Western as described in the Materials and Methods section. D, in vitro kinase of ERK5. MM1S cells were treated with IL-6 as earlier, where indicated, and ERK5 immunoprecipitated. TG02 was then added to the immunoprecipitates at the indicated concentrations and then the in vitro kinase reaction started by addition of ATP. ERK5, pERK5, and hyperphosphorylated ppERK5 were detected by Western blotting.
V-positive cells: the increase started after 6 hours of treatment with TG02 and after 24 hours almost all the cells were positive (Fig. 6A). In addition, internucleosomal DNA fragmentation was observed after treatment with TG02 (Supplementary Fig. S5A).

Because mitochondria play a central role in the regulation of apoptosis, we investigated the kinetics of changes in mitochondrial membrane potential (Δψm) during TG02 exposure using TMRE fluorescence. There was a time-dependent decrease in TMRE fluorescence after treatment with 100 nmol/L TG02 (Fig. 6A). The intermembrane space of mitochondria contains several proteins including cytochrome C, AIF, and SMAC/Diablo, which may act as mediators of apoptotic responses (24). Subcellular fractionation and Western blot analysis showed that TG02 induced release of cytochrome C from the mitochondria into the cytosol. We also detected an increase of AIF in the cytosol and a decrease of SMAC/Diablo in the mitochondria after treatment with TG02 (Fig. 6B).

Treatment with TG02 induced the cleavage of initiator caspases 7, 8, and 9 as early as 6 hours (Fig. 6C), leading to activation of the effector caspase 3 and cleavage of PARP, a caspase 3 substrate. In addition, TG02 treatment depleted the inhibitor of caspases XIAP in MM1S and some other myeloma cell lines (Fig. 6C and Supplementary Fig. S5B). The apoptosis induced by TG02 was partially mediated by caspases, as their inhibition by a pretreatment with the pan-caspase inhibitor Z-VAD-FMK reduced the percentage of apoptotic cells induced by the drug (Supplementary Fig. S5C). However, the inhibition was incomplete, even...
though caspase activation was still blocked at this point in cells treated with Z-VAD-FMK (Supplementary Fig. S5D).

**Downregulation of Mcl-1 is linked to TG02-induced cell death**

The effect of TG02 on Bcl-2 family members, antiapoptotic proteins that play important roles in the regulation of the integrity of the mitochondrial outer membrane, was studied. The antiapoptotic protein Bcl-2 was not affected by the compound and Bcl-X only modestly so. In contrast, Mcl-1 was downregulated after 6 hours of treatment (Fig. 6C and Supplementary Fig. S5B).

We hypothesized that if Mcl-1 was linked to the mechanism of cell death caused by TG02, then increasing its expression may provoke resistance to the action of the drug. HA-tagged Mcl-1 was overexpressed in MM1S cells by retroviral infection with HA-Mcl-1-IRES-GFP vector (Fig. 6D). In this experiment, GFP⁺ cells, expected to also over-express Mcl-1, were more resistant to TG02 than GFP⁻ cells (Fig. 6D), indicating that drug-induced effects on Mcl-1 contribute to the proapoptotic action of TG02.

**Discussion**

We report the antimyeloma action of TG02, a compound with a unique kinase inhibitory spectrum encompassing CDKs 1, 2, 7, and 9, ERK5, and JAK2/TYK2. TG02 was cytotoxic in all the multiple myeloma cell lines studied with IC₅₀ values commonly in the low nanomolar range, including lines sensitive and resistant to dexamethasone, melphalan, or doxorubicin. TG02 also killed patient malignant PCs but had a less pronounced effect on normal CD34⁺ hematopoietic progenitors. This is important because the maintenance of a therapeutic index may allow killing of the malignant PC population while sparing normal blood constituents.

CDK inhibition is an attractive therapeutic strategy for multiple myeloma. Several CDK inhibitors have shown antimyeloma activity in preclinical models (3–5), and the combination of inhibition of CDKs 1, 2, and 9 has been shown to be particularly effective at triggering apoptosis of malignant cells (25). Despite this, a recent clinical study with the broad-spectrum CDK inhibitor SNS-032 was negative (7). TG02 differs from SNS-032 in several important respects: first, TG02 also blocks several non-CDK targets relevant in myeloma biology; second, TG02 is considerably more potent than SNS-032 against CDK1 (9 nmol/L vs. 480 nmol/L; ref. 4); and third, TG02 has physico-chemical properties that permit flexible, oral dosing, whereas most CDK inhibitors, including SNS-032, are intravenous drugs (26, 27).

Treatment of most neoplasias is based on combinations of drugs, and TG02 was able to enhance the antimyeloma action of various compounds that are used regularly in the myeloma clinic. The in vivo studies, in addition to showing an effect of TG02 alone on the growth of multiple myeloma xenografts, also evidenced the increased antitumor effect of those combinations, especially that of TG02 and lenalidomide or bortezomib, opening the possibility of clinical trials of TG02 in combination with these agents.

Mechanistically, TG02 inhibited signaling mediated by several of its kinase targets, leading to cell-cycle arrest and apoptosis in multiple myeloma cells. Treatment with TG02 decreased the levels of CDK1 and its phosphorylated form, and also decreased cyclin B. These effects could be responsible for the accumulation of cells at the G₂/M boundary. In addition, inhibition of RNA polymerase II activity likely contributed to cell-cycle perturbation via depletion of various cell-cycle regulatory proteins (28), such as CDK2, and Rb. Nonetheless, cell-cycle arrest was modest in MM1S cells, probably obscured by a rapid commitment to apoptosis induced by even low concentrations of TG02 in this and other multiple myeloma cell lines.

Although there are chemotherapeutics and molecular targeted drugs in clinical use in multiple myeloma, the disease remains incurable because of the propensity of multiple myeloma cells for innate and acquired drug resistance. Thus, multitargeted drugs such as TG02 may offer an important new therapeutic approach. For instance, multiple myeloma cells nurtured by cytokines derived from bone marrow stroma show increased proliferation and resistance (29), but inhibition of the TG02 target kinases can reverse this process. Indeed, cytokine-activated ERK5 was affected in MM1S cells by TG02 suggesting that inhibition of ERK5 contributes to the ability of TG02 to overcome the protective effects of the bone marrow niche in multiple myeloma. ERK5 also mediates resistance to dexamethasone and bortezomib (10), and TG02 augmented the antimyeloma action of these agents in this study, indicating that blockade of ERK5 might enhance the combinatorial potential of TG02 in the clinic.

TG02 caused apoptosis of multiple myeloma cells, as indicated by annexin V staining, DNA laddering, and loss of mitochondrial membrane potential. The latter was also accompanied by the release of apoptotic mediators from the intermembrane space, most prominently cytochrome C that, together with APAF-1 and caspase 9, constitutes the apoptosome that triggers activation of effector caspases. TG02 treatment led to activation of multiple initiator and effector caspases, including caspase 3. A role of caspases in the action of TG02 was also supported by the partial inhibition of cell death caused by the caspase inhibitors. The release of proapoptotic proteins, such as AIF and endonuclease G, by the mitochondria that act through mechanisms independent of caspases (30, 31) may explain apoptosis induced by TG02 in the presence of the pan-caspase inhibitor Z-VAD-FMK. CDK7 and CDK9 inhibition probably contributed to induction of apoptosis via depletion of short-lived survival factors, such as Mcl-1 (28). In fact, increased expression of Mcl-1 through retroviral transduction provoked partial resistance to the action of TG02. Caspase-dependent cleavage and depletion of Mcl-1 is also thought to be important in bortezomib-induced cell death in multiple myeloma cells (32).
In summary, TG02 is a novel multikinase inhibitor that displays potent and consistent antimyeloma activity in vitro and in vivo, both as a single agent and in combination with approved drugs. Interestingly, in our hands other multi–tyrosine kinase inhibitors such as sorafenib or sunitinib present poor antimyeloma activity (unpublished data), indicating that the particular kinase spectrum of TG02 is well matched to the key oncogenic pathways in multiple myeloma. A phase I clinical trial in relapsed refractory multiple myeloma patients was recently initiated at 5 U.S. sites.

Disclosure of Potential Conflicts of Interest

F. J. Burrows and T. Parrott are current employees and S. Zaknoen has been a former employee of Tragara Pharmaceuticals. A. Pandiella received funding from TG Therapeutics to partially cover research costs. The other authors disclosed no potential conflicts of interest.

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