Telomerase Inhibition and Cell Growth Arrest After Telomestatin Treatment in Multiple Myeloma

Masood A. Shammas,[1,2,3] Robert J. Shmookler Reis,[4] Cheng Li,[2] Hemanta Koley,[1,3] Laurence H. Hurley,[5] Kenneth C. Anderson,[2,3] and Nikhil C. Munshi[1,2,3]

[1]Boston VA Health Care System, Boston, Massachusetts; [2]Dana Farber Cancer Institute, Boston, Massachusetts; [3]Harvard Medical School, Boston, Massachusetts; [4]Departments of Geriatrics, and Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, and Central Arkansas Veterans Health Care System, Little Rock, Arkansas; and [5]Arizona Cancer Center and College of Pharmacy, University of Arizona, Tucson, Arizona

ABSTRACT

Purpose: **The aim of this study was to test the efficacy of telomestatin, an intramolecular G-quadruplex intercalating drug with specificity for telomeric sequences, as a potential therapeutic agent for multiple myeloma.**

Experimental Design: **We treated ARD, ARP, and MM1S myeloma cells with various concentrations of telomestatin for 7 days and evaluated for telomerase activity. Myeloma cells were treated with the minimal effective telomestatin concentration for 3–5 weeks. Every 7th day the fraction of live cells was determined by trypan blue exclusion, aliquots of cells were removed for various molecular assays, and the remaining cells were replated at the same cell number and at the same concentration of telomestatin. Telomere length, apoptosis, and gene expression changes were monitored as described in detail in "Materials and Methods."**

Results: **Telomestatin treatment led to inhibition of telomerase activity, reduction in telomere length, and apoptotic cell death in ARD, MM1S, and ARP myeloma cells. Gene expression profile after 1 and 7 days of telomestatin treatment revealed ≥2-fold change in only 6 (0.027%) and 51 (0.23%) of 33,000 genes surveyed, respectively. No changes were seen in expression of genes involved in cell cycle, apoptosis, DNA repair, or recombination.**

Conclusions: **These results demonstrate that telomestatin exerts its antiproliferative and proapoptotic effects in myeloma cells via inhibition of telomerase and subsequent reduction in telomere length. We conclude that telomerase is an important potential therapeutic target for multiple myeloma therapy, and G-quadruplex interacting agents with specificity for binding to telomeric sequences can be important agents for additional evaluation.**

INTRODUCTION

Telomeres are DNA protein complexes at the ends of eukaryotic chromosomes that provide at least two essential functions, protecting of linear DNA ends from erosion over multiple replication cycles (1, 2), and protection of chromosome ends from recognition as double-strand DNA breaks and subsequent "repair" by exonucleolytic trimming and end-to-end fusion (3, 4). Telomeres also play a significant role in chromosomal replication during mitosis and meiosis (5), as alignment of homologous chromosomes in meiosis is initiated by telomeres (6). In all vertebrates, telomeric DNA consists of repeats of "TTAGGG" sequences (7–9). As DNA replication mechanisms are unable to replicate telomeric sequences distal to the last primase site, approximately 50–100 bp of telomeric DNA is therefore lost with each cell division (10). Telomerase, a ribonucleoprotein with reverse transcriptase activity, uses its RNA component as a template for the addition of G-rich repeats (TTAGGG) to the 3′ end of telomeric DNA (11), thereby compensating for the telomere loss associated with cell division.

Telomerase is expressed in primitive human hematopoietic cells and germ-line cells, but is undetectable in most other somatic tissues (12). In normal human diploid cells lacking telomerase, progressive telomere shortening leads to an early crisis in which short dysfunctional telomeres are recognized as DNA damage and may cause p53-mediated apoptosis. This may account for the limited proliferative potential of normal diploid cells (13); however, if p53 is mutated or lost, cells continue to divide and enter a late "crisis" with severe reduction in telomeres and chromosomal instability (14).

Because telomerase activity is present in most immortal and neoplastic cells (15–18) and is low or absent in somatic cells (12, 15, 19), inhibitors of telomerase activity hold promise as antiproliferative agents with specificity for tumor cells, while sparing most or all normal tissues. We have demonstrated previously that introduction of peptide nucleic acid complementary to telomerase RNA, an exogenous telomerase inhibitor, results in loss of telomerase activity, telomere shortening, and cell death in transformed human cells (20).

In this study we have examined the effects of telomestatin, a natural G-quadruplex-intercalating agent isolated from *Strep-*

Received 5/19/03; revised 10/3/03; accepted 10/7/03.

Grant Support: Multiple Myeloma Research Foundation (to M. A. S.), the Department of Veterans Affairs (Merit Review Award and REAP; R. J. S. R.), The VA Merit Review Grant, the National Institutes of Health Grant P50-100707 and P01-78378 and Leukemia and Lymphoma Society Scholar in Translational Research Award (to N. C. M.); as well as National Institutes of Health Grants (R01-50947; P50-100707; and P01-78378), Doris Duke Distinguished Clinical Research Scientist Award, and the Cure for Myeloma Fund (to K. C. A.).

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: Nikhil C. Munshi, Dana-Farber Cancer Institute, 44 Binney Street, M557, Boston, MA 02115. E-mail: Nikhil Munshi@DFCI.Harvard.edu. Phone: (617) 632-5607; Fax: (617) 632-4862; E-mail: nikhil_munshi@dfci.harvard.edu.

tomyces anulatus 3533-SV4, which specifically interacts and stabilizes intramolecular G-quadruplex structures, especially those formed by human telomeric DNA $(TTAGGG)_n$ (21). It is a more potent and specific telomerase inhibitor than previously described G-quadruplex intercalating agents (21). After treatment of multiple myeloma (MM) cells with telomestatin, we report inhibition of telomerase activity, reduction in telomere length, followed by cell growth inhibition, with minimal associated changes observed on gene microarray profiling.

MATERIALS AND METHODS

Chemicals. Telomestatin, isolated from *Streptomyces anulatus* 3533-SV4, was dissolved in DMSO and used at 1–10 μM concentrations (21).

Cell Lines. Myeloma cell lines ARD and ARP were kindly provided by Dr. Joshua Epstein (University of Arkansas for Medical Sciences, Little Rock, AR). The MM1S cell line was provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). All of the MM cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, South Logan, UT).

Experimental Design. MM cell lines (ARD, ARP, and MM1S) were treated with telomestatin at 1, 5, or 10 μM concentrations (in RPMI 1640 plus 10% fetal bovine serum) in a relatively dark room to reduce exposure of telomestatin to light. On day 7, cells were harvested and evaluated for telomerase activity to determine the minimal telomestatin concentration required for inhibition of >80% enzyme activity. This minimal effective telomestatin concentration was used in subsequent experiments. Constant numbers of cells were plated in multiple 100-mm dishes (5×10^5 cells/dish) and treated with telomestatin. The fraction of live cells was determined weekly by trypan blue exclusion, aliquots of cells were harvested for molecular assays, and the remaining cells were replated at the same cell number (5×10^5 cells/dish) and at the same concentration of the inhibitor. Cells aliquoted for telomere length assay were stored at −150°C, whereas those aliquoted for analyses of gene expression changes and apoptosis were processed immediately.

Assay of Telomerase Activity. Telomerase activity was assayed using a fluorescence-based TRAPeze^R XL telomerase detection kit (Intergen, Purchase, NY). Lysates (1000 cell-equivalents) were mixed with TRAPeze^R XL reaction mix containing Amplifuor primers and incubated at 30°C for 30 min. Amplified telomerase products were quantitated with a fluorescence plate reader. Telomerase activity (in total product generated units) was calculated by comparing the ratio of telomerase products to an internal standard for each lysate, as described by the manufacturer.

Estimation of Telomere Length. Genomic DNA was isolated from telomestatin-treated cells using "Puregene" DNA isolation kits (Gentra Systems, Minneapolis, MN), and telomere length was estimated using the TeloTAGGG Telomere Length Assay (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN). In brief, 6 μg of genomic DNA was digested with a 6-fold excess of restriction enzymes *Hinf*I and *Rsa*I. Digested DNA, along with a size standard (1-kb DNA ladder), was electrophoresed on 0.8% agarose gel and transferred to Hybond-*n* + nylon membrane (Amersham Biosciences Corp, Piscataway, NJ) as described by the manufacturer. Membranes were then hybridized to a digoxigenin-labeled telomere-specific probe, and telomeric DNA was detected by incubation with alkaline phosphatase coupled to antidigoxigenin antibody, followed by washing and reaction with a chromogenic substrate of alkaline phosphatase.

Apoptosis. Apoptotic MM cells were detected using an Annexin V-Biotin Apoptosis Detection kit (Oncogene Research Products, San Diego, CA). Telomestatin-treated myeloma cells (1×10^6 cells/ml) were mixed with annexin V-biotin and medium-binding reagent, and incubated in the dark for 15 min at room temperature. Cells were then centrifuged and medium was replaced with 1 × Binding Buffer (Oncogene Research Products) containing FITC-streptavidin (Amersham). Propidium iodide was added to discriminate early apoptotic cells from late apoptotic or necrotic cells. A portion of cell suspension (50 μl) was placed onto a glass slide, covered with a coverslip, and viewed immediately using a fluorescence microscope equipped with FITC (green) and propidium iodide (red) filters. Two hundred cells, representing at least five distinct microscopic fields, were analyzed to assess the fraction of FITC and propidium iodide-labeled cells for each sample.

Gene Expression Analysis. MM1S cells treated with 1 μM telomestatin were harvested on days 1 and 7, and total RNA was isolated using an RNeasy kit (Qiagen Inc., Valencia, CA) as described by the manufacturer. Total RNA (10–15 μg) was reverse transcribed to get cDNA using the Superscript II RT kit (Invitrogen Life Technologies, Inc., Carlsbad, CA). cDNA was used in an *in vitro* transcription reaction to synthesize biotin-labeled cRNA using ENZO RNA labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY). Labeled cRNA was purified with the RNeasy Mini-kit (Qiagen Inc.) and quantitated. Purified cRNA (15 μg) was hybridized to Human Genome U133 (HG-U133) GeneChip arrays (Affymetrix) according to the manufacturer's protocol. The HG-U133 set consists of two GeneChip arrays representing ~33,000 human genes. GeneChip arrays were scanned on a GeneArray Scanner (Affymetrix, Inc., Santa Clara, CA).

Microarray Data Analysis. Normalization of arrays and calculation of expression values was performed using the DNA-Chip Analyzer program (22). Arrays were normalized based on relative signal produced for an invariant subset of genes. This model-based method was used for probe selection and computing expression values (22, 23). By pooling hybridization information across multiple arrays, it is possible to assess standard errors for the expression level indexes. This approach also allows automatic probe selection in the analysis stage to reduce errors due to cross-hybridizing probes and image contamination. We also used several high-level analysis functions in DNA-Chip Analyzer for comparative analysis and hierarchical clustering.

RESULTS

Inhibition of Telomerase Activity in MM Cells by Telomestatin. Telomestatin is a relatively specific and more potent telomerase inhibitor than previously described G-quadruplex intercalators (21). We evaluated the effect of telomestatin at 1, 5, or 10 μM for 7 days on telomerase activity in human MM

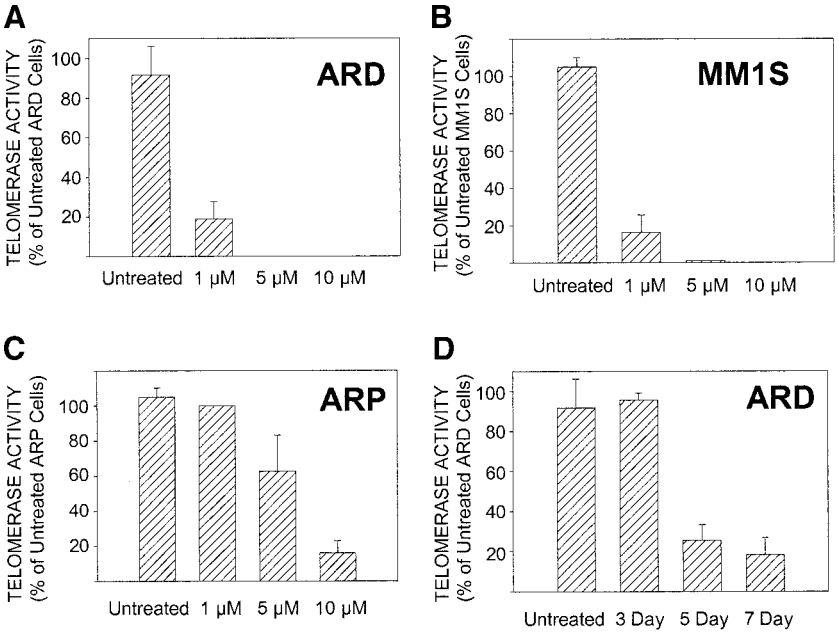

Fig. 1 Effect of telomestatin treatment on telomerase activity in multiple myeloma (MM) cells. Telomerase activity in telomestatin-treated ARD, MM1S, and ARP MM cells was determined by fluorometric detection of telomeric extension products. Lysates (1000 cell-equivalents) were mixed with TRAPeze^{R} XL reaction mix containing Amplifuor primers, and incubated for 30 min at 30°C. Telomerase products were quantitated using a fluorescence plate reader. Telomerase activity after 7 days of treatment with telomestatin is presented as percentage of activity in the untreated cells. (*A*) ARD cells; (*B*) MM1S cells; (*C*) ARP cells; (*D*) ARD cells were treated with telomestatin (1 μM) for 0, 3, 5, or 7 days. Telomerase activity was measured in the cell lysates and presented as percentage of activity in untreated cells.

cell lines, ARD, MM1S, and ARP. Telomestatin at 1 μM was sufficient to inhibit >90% of telomerase activity in ARD and MM1S cells (Fig. 1, *A* and *B*). With their higher telomerase activity, however, ARP cells required 10 μM telomestatin for inhibition of >80% telomerase activity (Fig. 1*C*). A time course (Fig. 1*D*) of telomestatin treatment (1 μM) in ARD MM cells showed no inhibition at 3 days, but similar levels of inhibition of telomerase activity on days 5 and 7.

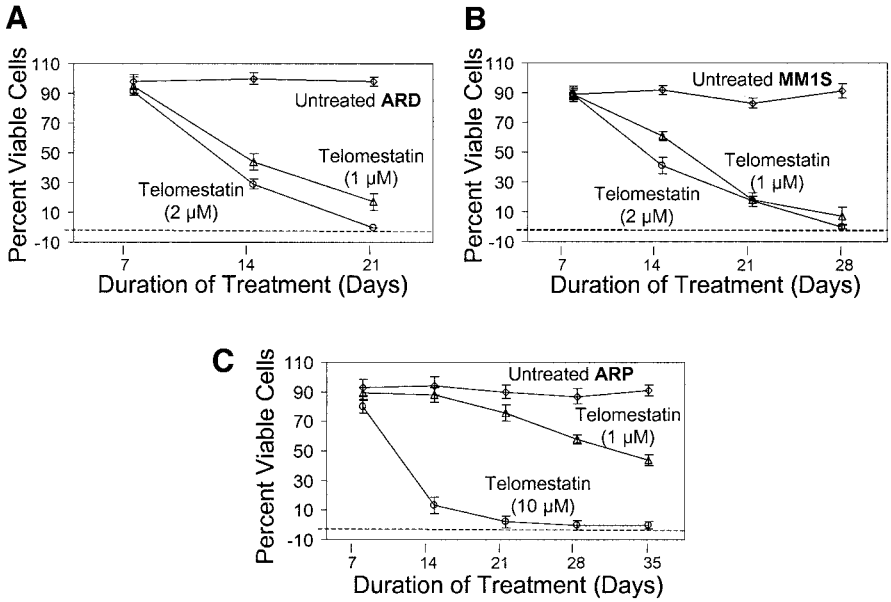

Fig. 2 Effect of telomestatin treatment on multiple myeloma (MM) cell survival. *A*, ARD cells were cultured in medium containing no telomestatin, 1.0 μM telomestatin, or 2.0 μM telomestatin. Cells were harvested weekly and the viable (trypan-blue excluding) cell number was counted. The growth curve shows the mean of three independent experiments, with SE *error bars* shown for points at 7, 14, and 21 days. *B*, MM1S cells were cultured in medium containing no telomestatin, 1.0 μM telomestatin, or 2.0 μM telomestatin. Cells were harvested weekly and the viable (trypan-blue excluding) cell number was counted. The growth curve shows the mean of three independent experiments, with SE *error bars* shown for points at 7, 14, and 21 days. *C*, ARP cells were cultured in medium containing no telomestatin, 1 μM telomestatin, or 10 μM telomestatin. Cells were harvested weekly and the number of viable (trypan-blue excluding) cells was counted. The growth curve shows the mean of three independent experiments, with SE *error bars* shown for points at 7, 14, and 21 days.

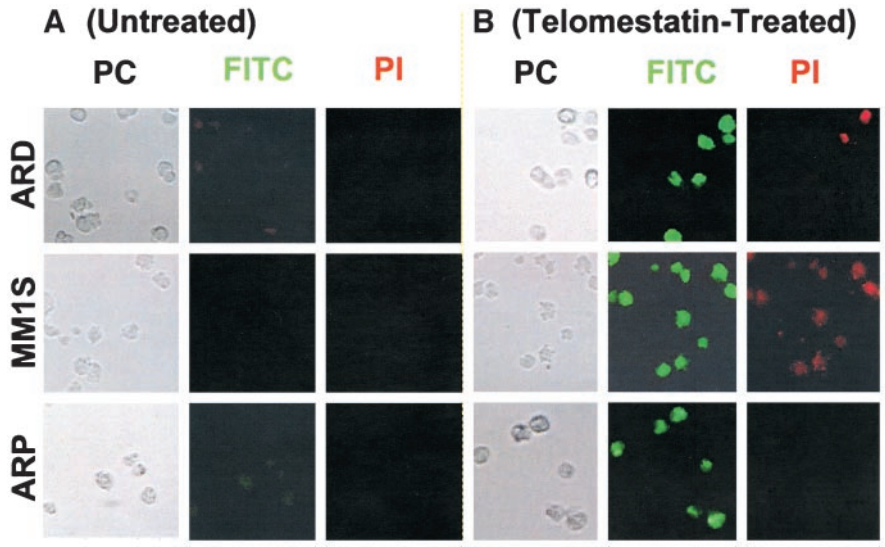

Fig. 3 Apoptosis after telomestatin treatment of multiple myeloma (MM) cells. MM cells were analyzed for apoptosis using a Annexin V-biotin Apoptosis Detection kit. Cells were mixed with annexin V-biotin, incubated for 15 min at room temperature, and treated sequentially with streptavidin conjugated to FITC and propidium iodide (*PI*). Apoptotic cells within the same microscopic field were viewed and photographed by phase contrast (*PC*), by fluorescence emitted at 518 nm (FITC filter), and at 620 nm (PI filter). Using the FITC filter, early apoptotic cells (positive for Annexin V-Biotin-FITC staining) appear bright green; and using PI filter, the late apoptotic or necrotic cells (positive for PI) appear reddish. (*A*) Untreated cells; (*B*) telomestatin-treated cells.

Growth Inhibition After Telomestatin Treatment of MM Cells. ARD, MM1S, and ARP cells were cultured in the presence or absence of telomestatin, and the viable cell fraction was determined by trypan blue exclusion weekly. Viable cell numbers declined over 3–5 weeks to 2–20% of the starting cell number. Treatment with telomestatin at 1 μM for 3 weeks resulted in cell death in 81% ± 6% ARD cells, 97% ± 3% MM1S cells, and 42% ± 2% ARP cells, respectively (Fig. 2, *A–C*). Complete cell death was observed after exposure to telomestatin at 2 μM (ARD and MM1S cells) and 10 μM (ARP cells) concentrations (Fig. 2).

The Nature of Cell Death After Telomerase Inhibition. We analyzed MM cells for apoptosis after telomestatin treatment. After 2-week exposure to telomestatin, 61% of ARD cells, 90% of MM1S cells, and 69% of ARP cells stained positive for annexin V (Fig. 3*B*), whereas untreated ARD, MM1S, and ARP cells were each <1% annexin V positive (Fig. 3*A*).

Effect of Telomerase Inhibitors on Telomere Length. Genomic DNA was isolated from MM cells treated with telomestatin for 2–3 weeks, and median (50th percentile) telomere length was estimated. Telomestatin treatment led to reduction in median telomere length by 1.6 kbp in ARD cells after 2 weeks (Fig. 4, *Lane 1*) but only by 0.4 or 0.5 kbp in MM1S cells after 2 or 3 weeks, respectively (Fig. 4, *Lanes 2* and *3*). In ARP cells, telomestatin treatment led to >3.1 kbp reduction in median telomere length (Fig. 4, *Lane 4*).

Gene Expression Profiles After Telomestatin Treatment. We analyzed gene expression profiles of MM1S myeloma cells after 1 and 7 days of telomestatin treatment (Table 1) using HG-U133 GeneChip arrays (Affymetrix) representing ~33,000 genes. However, no significant change was observed in the expression of genes involved in cell proliferation or cell cycle control, apoptosis, telomere maintenance, or DNA repair and recombination. Expression of nucleases and mismatch repair genes was also unaltered. Hypoxia-inducible factor 1-responsive gene (HIF-1), multiple endocrine neoplasia I, SEC63, and nuclear factor (erythroid-derived 2) -like 1 were among the genes with ≥2-fold up-regulation after both 1 and 7 days of exposure to

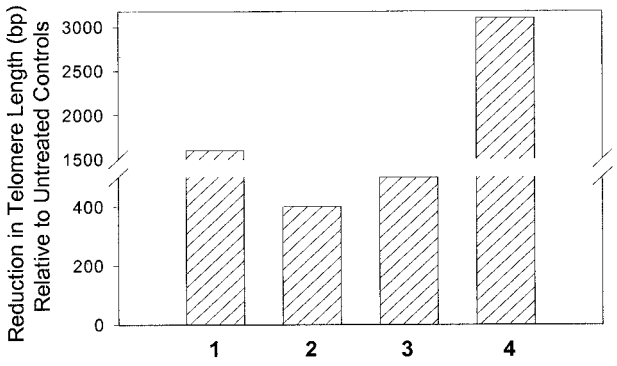

Fig. 4 Reduction in telomere length after telomestatin treatment of multiple myeloma (MM) cells. MM cells were treated with telomestatin as indicated. Telomere terminal restriction fragment length was measured as described. Median telomeric DNA restriction fragments (50th percentile lengths) were calculated from telomere fragment size distributions in scanned lanes. Reduction in telomere length is shown relative to untreated controls. Reduction in telomere length in (*1*) ARD cells after exposure to telomestatin for 14 days; (*2*) MM1S cells after exposure to telomestatin for 14 days; (*3*) MM1S cells after exposure to telomestatin for 21 days; (*4*) ARP cells after exposure to telomestatin for 14 days.

Table 1 Gene expression profiles of MMIS myeloma cells after 1 and 7 days of telomestatin treatment

Serial #	Gene name	Accession #	Day 1	Day 7
1	HIF-1 responsive RTP801	NM_019058.1	11.7	5.3
2	Multiple endocrine neoplasia I	AU134977	3	17.4
3	SEC63 protein	AK001465.1	2.1	6.2
4	Nuclear factor (erythroid-derived 2)-like 1	AI361227	2.1	5.1
5	Serine/arginine repetitive matrix 2	AI655799	NC[a]	4.2
6	KIAA0220 protein	BG256504	NC	8.1
7	Hypothetical protein DJ328E19.C1.1	NM_015383.1	NC	7.2
8	RNA helicase-related protein	NM_007372.1	NC	4.1
9	Nuclear receptor corepressor 2	NM_006312.1	NC	6.2
10	WW domain-containing adapter with a coiled-coil region	NM_016628.1	NC	3.4
11	Testis derived transcript (3 LIM domains)	NM_015641.1	NC	3.4
12	KIAA0449 protein	NM_017596.1	NC	4.1
13	Hypothetical protein MGC5306	BC001972.1	NC	5.2
14	Hypothetical protein FLJ20719	BE299495	NC	3.6
15	Delta sleep inducing peptide, immunoreactor	AL110191.1	NC	5.1
16	Protease, serine, 15	U02389.1	NC	3.1
17	Suppressor of Ty 6 homolog (*S. cerevisiae*)	D79984.1	NC	3.3
18	Golgin-67	AF204231.1	NC	8.6
19	ARF GTPase-activating protein	AI826075	NC	3.5
20	WW domain-containing adapter with a coiled-coil region	NM_018604.1	NC	2.8
21	Splicing factor, arginine/serine-rich 4	NM_005626.1	NC	2.7
22	ATPase, Class I, type 8B, member 1	BG252666	NC	4.7
23	Small nuclear ribonucleoprotein 70kDa polypeptide	NM_003089.1	NC	2.3
24	Splicing factor, arginine/serine-rich 11	NM_004768.1	NC	2.8
25	Karyopherin β 2b, transportin	BG258639	NC	2.2
26	Scaffold attachment factor B	NM_002967.1	NC	2.7
27	C9orf10 protein	BE963765	NC	2.3
28	Ewing sarcoma breakpoint region 1	BC004817.1	NC	2.4
29	Arsenate resistance protein ARS2	AI523895	NC	2.7
30	YY1 associated protein	NM_018253.1	NC	2.5
31	Intercellular adhesion molecule 2	AA126728	NC	3.4
32	Insulin receptor	AA485908	NC	3.8
33	Hypothetical protein FLJ10460	NM_018097.1	NC	2.8
34	KIAA0916 protein	AA488899	NC	2.8
35	Dihydrolipoamide branched chain transacylase	NM_001918.1	NC	2.5
36	Homo sapiens cDNA FLJ11999 fis,	AK022061.1	NC	2.6
37	Bromodomain containing 2	S78771.1	NC	3.1
38	Rag C protein	NM_022157.1	NC	2.5
39	G-2 and S phase expressed 1	BC006325.1	NC	3.1
40	Nuclear pore complex interacting protein	AC002045	NC	2.4
41	Minor histocompatibility antigen HA-1	BE349017	NC	2.9
42	Hypothetical protein FLJ14251	NM_024881.1	NC	2.6
43	Hypothetical protein FLJ20254	NM_017727.1	NC	3.1
44	SEC24 related gene family, member C (*S. cerevisiae*)	NM_004922.1	NC	2.5
45	Microtubule-actin cross-linking factor 1	AB029290.1	NC	4.3
46	ESTs, Weakly similar to hypothetical protein	AW516297	NC	2.9
47	Adaptor-related protein complex 3, δ 1 subunit	NM_003938.1	NC	2.2
48	KIAA1641 protein	NM_025190.1	NC	5.1
49	H4 histone family, member G	NM_003542.2	NC	3.1
50	Regulator of G-protein signalling 1	NM_002922.1	−3.4	4.1
51	Immunoglobulin heavy constant γ 3	M87789.1	−6.9	3.1

[a] NC, no change.

telomestatin (Table 1). Among those genes altered only after 7 days of treatment with telomestatin, the most noted were nuclear receptor corepressor (a transcriptional repressor), scaffold attachment factor B involved in binding of chromatin to the nuclear matrix or scaffold, RNA helicase-like protein involved in RNA processing and ribosome assembly, G_2 and S phase expressed 1 (a cell cycle associated protein), and Yin Yang 1 associated protein (a transcription factor). Majority of the genes with changed expression were of unknown function and importance.

DISCUSSION

Telomerase activation and subsequent maintenance of telomeres are required for survival of proliferating tumor cells. Higher levels of telomerase activity are detected in MM cells compared with normal plasma cells.[6] On the basis of earlier observations that oligonucleotides complementary to the RNA

[6] Unpublished observations.

component of telomerase inhibit telomerase activity and limit proliferative potential of transformed human cell lines (20), we have evaluated telomestatin, a novel telomerase inhibitor, as a potential therapeutic in MM. Telomestatin specifically interacts with and stabilizes intramolecular G-quadruplex structures $(TTAGGG)_n$ (21). Inhibition of telomerase activity is mediated by binding of two telomestatin molecules to each intramolecular G-quadruplex structure, especially telomere-related G-quadruplex structures, making it an attractive agent, compared with the less specific G-quadruplex-intercalating agents such as TMPyP4 (24, 25).

The effective dose of telomestatin appears to be associated with the level of telomerase activity. MM cells with low levels of telomerase activity (ARD and MM1S) were almost completely inhibited by 1 μM telomestatin, whereas the cells with high telomerase activity (ARP) required 10 μM telomestatin for a similar level of inhibition. These results also parallel our observations with other G-quadruplex inhibitors (26). Because telomeric structure is maintained by telomerase (27), higher levels of the enzyme may preserve telomere structure even in the presence of low doses of telomestatin. Although the concentration of telomestatin required for inhibition of telomerase activity *in vitro* is 5 nM only (28), the dose required *in vivo* is much higher and varies from cell line to cell line. Tauchi *et al.* (29) have observed that 1 μM telomestatin inhibited only ~25% and 45% of telomerase activity at 48 h in leukemia cell lines K562 and OM9;22, respectively, whereas a dose of 10 μM telomestatin was required to observe a nearly complete inhibition of telomerase activity in these cell lines at 48 h. Because telomeric DNA does not exist as G-quadruplex under physiological salt and temperature conditions (30), formation and stabilization of such a structure *in vivo* may depend on intracellular levels of appropriate cations, RecQ family of DNA helicases, and telomerase. Whereas certain cations promote formation of G-quadruplex structure, RecQ helicases unfold them into double-stranded DNA (31). These factors, which differ among various cancer cell lines, may contribute to the higher concentration of telomestatin required for telomerase inhibition *in vivo*.

We determined the minimal concentration of telomestatin required by incubating the cells in the presence of various doses of drug for 7 days. At the minimal concentrations used, telomestatin and other G-quadruplex interactive agents including TMPyP4 (26) and QQ98[7] required 5–7 days for complete inhibition of telomerase activity in human cells. Because G-rich sequences do not usually exist as G-quadruplex structures (30) and G-quadruplexes may be adversely affected by intracellular levels of telomerase and RecQ helicases, the delayed inhibition of telomerase by telomestatin in myeloma cells may reflect the time required for conversion of all of the telomeric DNA into G-quadruplexes and their subsequent stabilization by minimal amount of telomestatin used.

In all three of the cell lines, telomestatin inhibition of telomerase activity resulted in >80% cell death after a lag period of 7–14 days. In contrast, prolonged treatment of MM cell lines with low concentration of telomestatin (0.01 μM) was not associated with cell death (data not shown). A lag phase of 7–14 days followed by abrupt cell death suggests that gradual telomere shortening leads to a critical telomere length threshold followed by apoptotic cell death.

Telomestatin-mediated growth arrest of MM cells is associated with a marked reduction in telomere length. Telomere length in MM1S was reduced by only 400 bp. Because median telomere length of MM1S cells is only 2.2 kbp, which is close to the putative minimum telomere length for survival of human cells (10), the observed small reduction in telomeres can be a cell lethal event. The relative larger observed reduction in telomere length in ARP and ARD cells treated with telomestatin may both reflect their greater initial telomere length and a consequence of their higher proliferation rates. Effects of telomestatin on alternative mechanisms of telomere maintenance may also account for different levels of reduction in telomere length in these cell lines.

Although normal diploid cells (lacking telomerase) lose 50–100 bp of telomeric DNA per cell division, telomere loss after exogenous inhibition of telomerase in cancer and immortal cells does not seem to proceed with the usual rate, probably because of the existence of various genetic and epigenetic factors, including intracellular levels of nucleases, in these cells. Tauchi *et al.* (29) have also observed that telomestatin treatment of leukemia cells K562, with a heterogeneous population of telomeres and an average telomere length of 5.2 kbp, led to reduction in telomere length by ~ 2200 bp after only 10 population doublings. Average telomere length reduction in K562 and OM9;22 cell lines at 20 population doublings was 2800 bp and 500 bp, respectively. Consistent with our data, these leukemia cell lines also showed a large variation in the reduction of telomere length after exposure to telomestatin.

A number of other G-rich regions in the human genome have the potential to form four-stranded G-quadruplex structures. These sequences are widely dispersed in the genome, especially in the promoter regions of many genes and at recombination hotspots (25, 32). Although telomestatin is an intramolecular G-quadruplex-interactive compound and has relatively high specificity for telomeric G-quadruplex structures (20), it may affect expression of other genes, which can account for the antiproliferative activity of this agent. Therefore, we evaluated the effects of telomestatin treatment on the gene expression profile of MM cells using the HG-U133 gene array chips (Affymetrix) representing ~33,000 genes. At the drug concentrations used, at least 2-fold change in expression were observed in only 6 genes after 1 day, and 51 genes after 7 days. None of these genes plays any significant role in DNA recombination, repair, cell cycle control, and apoptosis. Importantly, change in expression of telomerase or associated genes were not observed.

These data confirm that telomestatin induces apoptotic MM cell death predominantly through its effect on telomerase function, impairing its ability to extend telomeres.

[7] M. A. Shammas, H. Koley, D. G. Beer, C. Li, R. K. Goyal, and N. C. Munshi. Growth arrest, apoptosis, and telomere shortening of Barrett's associated adenocarcinoma cells by a telomerase inhibitor. Gastroenterology, in revision, 2003.

REFERENCES

1. Olovnikov, A. M. Telomeres, telomerase, and aging: origin of the theory. Exp. Gerontol., *31:* 443–448, 1996.

2. Olovnikov, A. M. The role of incomplete terminal repair of chromosomal DNA in the aging of neurons and postmitotic organisms. Izv Akad Nauk. Ser. Biol., 504–507, 1995.

3. Blackburn, E. H. Telomere states and cell fates. Nature (Lond.), *408:* 53–56, 2000.

4. Blackburn, E. H. Switching and signaling at the telomere. Cell, *106:* 661–673, 2001.

5. Dandjinou, A. T., Dionne, I., Gravel, S., LeBel, C., Parenteau, J., and Wellinger, R. J. Cytological and functional aspects of telomere maintenance. Histol. Histopathol., *14:* 517–524, 1999.

6. Price, C. M. Telomeres and telomerase: broad effects on cell growth. Curr. Opin. Genet. Dev., *9:* 218–224, 1999.

7. Allshire, R. C., Gosden, J. R., Cross, S. H., Cranston, G., Rout, D., Sugawara, N., Szostak, J. W., Fantes, P. A., and Hastie, N. D. Telomeric repeat from T. thermophila cross hybridizes with human telomeres. Nature (Lond.), *332:* 656–659, 1988.

8. de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M., and Varmus, H. E. Structure and variability of human chromosome ends. Mol. Cell. Biol., *10:* 518–527, 1990.

9. Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven, L. L., Jones, M. D., Meyne, J., Ratliff, R. L., and Wu, J. R. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. USA, *85:* 6622–6626, 1988.

10. Harley, C. B., Futcher, A. B., and Greider, C. W. Telomeres shorten during ageing of human fibroblasts. Nature (Lond.), *345:* 458–460, 1990.

11. Blackburn, E. H. Telomerases. Annu. Rev. Biochem., *61:* 113–129, 1992.

12. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer [see comments]. Science (Wash. DC), *266:* 2011–2015, 1994.

13. Hayflick, L. The cellular basis for biological aging. *In:* C. E. Finch, and Hayflick, L. (ed.) Handbook of the Biology of Aging, pp. 159–186. New York: Van Nostrand Reinhold Co., 1977.

14. Chin, L., Artandi, S. E., Shen, Q., Tam, A., Lee, S. L., Gottlieb, G. J., Greider, C. W., and DePinho, R. A. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell, *97:* 527–538, 1999.

15. Shay, J. W., and Bacchetti, S. A survey of telomerase activity in human cancer. Eur. J. Cancer, *33:* 787–791, 1997.

16. Shay, J. W., and Wright, W. E. The reactivation of telomerase activity in cancer progression. Trends Genet., *12:* 129–131, 1996.

17. Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., and Greider, C. W. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. Cancer Res., *56:* 645–650, 1996.

18. Wu, K. D., Orme, L. M., Shaughnessy J Jr., Jacobson, J., Barlogie, B., Moore, M. A. Telomerase and telomere length in multiple myeloma: correlations with disease heterogeneity, cytogenetic status, and overall survival. Blood, *101:* 4982–4989, 2003.

19. Broccoli, D., Young, J. W., and de Lange, T. Telomerase activity in normal and malignant hematopoietic cells, Proc. Natl. Acad. Sci. USA, *92:* 9082–9086, 1995.

20. Shammas, M. A., Simmons, C. G., Corey, D. R., and Reis, R. J. Telomerase inhibition by peptide nucleic acids reverses 'immortality' of transformed human cells. Oncogene, *18:* 6191–200, 1999.

21. Kim, M. Y., Vankayalapati, H., Shin-Ya, K., Wierzba, K., and Hurley, L. H. Telomestatin, a potent telomerase inhibitor that interacts quite specifically with the human telomeric intramolecular g-quadruplex. J. Am. Chem. Soc., *124:* 2098–2099, 2002.

22. Li, C., and Hung Wong, W. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. Genome Biol., *2:* 2001.

23. Li, C., and Wong, W. H. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. USA, *98:* 31–36, 2001.

24. Grand, C. L., Han, H., Munoz, R. M., Weitman, S., Von Hoff, D. D., Hurley, L. H., and Bearss, D. J. The cationic porphyrin TMPyP4 down-regulates c-MYC and human telomerase reverse transcriptase expression and inhibits tumor growth *in vivo*. Mol. Cancer Ther., *1:* 565–573, 2002.

25. Izbicka, E., Wheelhouse, R. T., Raymond, E., Davidson, K. K., Lawrence, R. A., Sun, D., Windle, B. E., Hurley, L. H., and Von Hoff, D. D. Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. Cancer Res., *59:* 639–644, 1999.

26. Shammas, M. A., Shmookler Reis, R. J., Akiyama, M., Koley, H., Chauhan, D., Hideshima, T., Goyal, R. K., Hurley, L. H., Anderson, K. C., Munshi, N. C. telomerase inhibition and cell growth arrest by g-quadruplex interactive agent in multiple myeloma. Mol. Cancer Ther., *2:* 825–833, 2003.

27. Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A., and Hahn, W. C. Telomerase maintains telomere structure in normal human cells. Cell, *114:* 241–253, 2003 Jul 25.

28. Shin-ya, K., Wierzba, K., Matsuo, K., Ohtani, T., Yamada, Y., Furihata, K., Hayakawa, Y., and Seto, H. Telomestatin, a novel telomerase inhibitor from Streptomyces anulatus. J. Am. Chem. Soc., *123:* 1262–1263, 2001.

29. Tauchi, T., Shin-Ya, K., Sashida, G., Sumi, M., Nakajima, A., Shimamoto, T., Ohyashiki, J. H., and Ohyashiki, K. Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways. Oncogene, *22:* 5338–5347, 2003.

30. Phan, A. T., and Mergny, J. L. Human telomeric DNA: G-quadruplex, i-motif and Watson-Crick double helix. Nucleic Acids Res., *30:* 4618–4625, 2002.

31. Li, J. L., Harrison, R. J., Reszka, A. P., Brosh, R. M., Jr., Bohr, V. A., Neidle, S., and Hickson, I. D. Inhibition of the Bloom's and Werner's syndrome helicases by G-quadruplex interacting ligands. Biochemistry, *40:* 15194–15202, 2001.

32. Simonsson, T. G-quadruplex DNA structures–variations on a theme. Biol. Chem., *382:* 621–628, 2001.